BIO490 Independent Research

Senior Honors Thesis
Thai Q. Dao ’15
Pablo D. Jenik
Franklin & Marshall College – Biology Department
Spring 2015

Submitted: 05/15/2015
Seed development in *Arabidopsis thaliana*

Characterizing ASIL1 and ASIL2 transcription factors and their role in repressing the maturation program during early embryogenesis

ABSTRACT ........................................................................................................................................ 2

I. INTRODUCTION .......................................................................................................................... 3
   A. Arabidopsis embryogenesis: an Overview
   B. Positive Regulators of Maturation
   C. Negative Regulators of Maturation
      a. After maturation
      b. Early embryogenesis
   D. Project Aim

II. RESULTS .................................................................................................................................... 14
   A. Promoter analysis of ASIL1 and ASIL2
   B. Effects of ASIL1 and ASIL2 on maturation genes

III. DISCUSSION ............................................................................................................................ 18
   A. Regulation of ASIL1 during embryogenesis
   B. ASIL1 and ASIL2 as negative regulators of maturation genes

IV. CONCLUSIONS AND FUTURE DIRECTIONS ........................................................................... 25

V. MATERIALS AND METHODS .................................................................................................. 27

VI. FIGURE ...................................................................................................................................... 30

ACKNOWLEDGEMENT ............................................................................................................... 50

REFERENCES ............................................................................................................................... 51
ABSTRACT

The evolutionary success of seed plants can be attributed in part to their ability to produce a resilient structure, the seed, which allows the embryo to suspend development and only resume its life cycle once suitable environmental conditions are met. Seed formation not only significantly increases embryo survivability, but also allows the embryo to cross extraordinary distances, all while lying dormant. Seed plants have thus been able to occupy and adapt to almost every terrestrial habitat on the planet.

Mature seeds are desiccated, and contain storage products that are to be used by the seedling after germination. These properties are the outcome of the embryo maturation program. A complex network is involved in timing maturation during embryo development. Several “master regulators” (the “LEC genes”) are required for the induction of the maturation program in mid-embryogenesis, but the mechanisms that repress this program during early embryogenesis are less clear. Previous research in Dr. Jenik’s lab identified several genes in *Arabidopsis thaliana* (*DCL1, ASIL1, ASIL2, HDA6*) that are required to prevent the early onset of the maturation program (Willmann et al., 2011).

The genes that are the focus of this project are *ASIL1* (*ARABIDOPSIS 6B-INTERACTING PROTEIN1-LIKE1*) and its closest paralog *ASIL2*, which encode proteins of the plant-specific trihelix transcription factor family. *ASIL1* has been shown to repress the maturation program both in the seedling and in the embryo (Gao et al., 2009, 2011). I seek to characterize the roles of *ASIL1* and *ASIL2* during early seed development, first by characterizing the expression of two of the “LEC genes” (*LEAFY COTYLEDON2* [*LEC2*] and *FUSCA3* [*FUS3*]) in wild type and mutant embryos using reporter genes. Early analyses indicate that the seeds of *asil1-1, asil2-1, and asil1-1 asil2-1* double mutants show early expression of *LEC2*. I also study the regulation of *ASIL1* and *ASIL2* throughout embryogenesis by generating deletions in the promoter regions of both genes and fusing them to reporter genes. After studying five such constructs of *ASIL1*, I have identified several putative regulatory sequences both in the promoter region, as well as in the 5’ UTR of the gene.
I. INTRODUCTION

A. *Arabidopsis* embryogenesis: an Overview

The embryogenesis of wild-type *Arabidopsis thaliana* can be subdivided into a series of stages, based on the shape of the embryo: preglobular, globular, transition, heart, torpedo (linear cotyledon), bent green cotyledon, and mature. These stages make up two major phases of development. The first, embryo morphogenesis, which extends until the heart stage, establishes the spatial distribution of cells and determines tissue types (Fig. 1 and Jenik et al., 2007). During this phase, the zygote divides into a small, apical cell, and a large, lower basal cell, which will give rise to the embryo proper and the suspensor, respectively. The suspensor consists of relatively few cells, and serves to attach the embryo proper to the inside of the seed while passing nutrients from maternal tissues into the developing proembryo (Golderg *et al.*, 1994).

The second phase is embryonic maturation, which begins at the heart stage. It is during this phase that the embryo accumulates storage products, including seed storage proteins (SSP) and lipids (Baud *et al.*, 2008), and proplastids mature into chloroplasts (Mansfield and Briarty, 1991). Once the embryo fills the seed, i.e. reaches the mature stage, it acquires the ability to withstand desiccation, desiccates, and enters dormancy (Vicente-Carbajosa and Carbonero, 2005). Optimal seed development requires precise timing of the maturation program, which is achieved by a complex network that contains positive regulators that activate the program during mid-embryogenesis. Negative regulators are also required to repress the program both during early embryogenesis and after germination, to prevent expression of maturation traits before morphogenesis has completed, or in vegetative tissues. However, it is not known whether the same genes are involved at these different developmental stages.
B. Positive regulators of maturation

Efforts to gain insight into the regulatory network of embryo maturation have revealed DNA sequence motifs that are overrepresented in the promoter region of genes that are expressed in the embryo specifically during maturation. These cis-acting elements include the ABRE (which include the CAAT-box), ABRE-like, DPBF1, DPBF2, and RY motifs. Transcriptional factors that bind, or can potentially bind these motifs have also been identified, including ENHANCED EM LEVEL (EEL) and the bZIP transcription factors (TFs) (Belmonte et al., 2013).

In addition, in Arabidopsis, four genes serve as the central master positive regulators of maturation: LEAFY COTYLEDON1 (LEC1), which encodes a HEME-ACTIVATED PROTEIN3 (HAP3) subunit of CCAAT box-binding factors (CBF), along with LEC2, FUSCA3 (FUS3), and ABA INSENSITIVE3 (ABI3), all of which are B3 domain transcription factors (Fig. 2) (Giraudat et al., 1992; Lotan et al., 1998; Luerßen et al., 1998; Stone et al., 2001). LEC1, LEC2, and FUS3 belong to the LEC family, which is defined by their ability to induce leafy traits to cotyledon when overexpressed, while ABI3 does not (Meinke et al., 1994).

LEC1 is a central regulator that controls many aspects of both early and late embryo development. The LEC1 gene was first described by Meinke (1992), who reported that lec1 mutant embryos were intolerant of desiccation, and shows characteristics of postgerminative seedling development (leaf-like cotyledons). During embryogenesis, LEC1 transcript level is present in the embryo proper at high level from the pre-globular stage, peaking at the heart stage, and declining afterwards up to the green mature stage (Lotan et al., 1998; Belmonte et al., 2013). lec1 mutant cotyledons develop trichomes, which typically only forms on leaves of stems of Arabidopsis, suggesting that LEC1 plays a role in maintaining embryonic organ identity. Ectopic
expression of LEC1 is sufficient to induce embryonic programs in vegetative cells, as indicated by the formation of embryo-like structure of leaves, the arrest of vegetative development, and the activation of genes encoding SSPs such as cruciferin A, and 2S (Lotan et al., 1998). Further analysis shows that expression of LEC1 in vegetative tissues leads to activation of FUS3 and ABI3 transcript, followed by upregulation of SSP genes (Kagaya et al., 2005b). In addition, the levels of FUS3 and ABI3, but not LEC2 transcripts are drastically diminished in the matured embryos of lec1 mutant (To et al., 2006). Together, these data suggest that LEC1 is involved in a complex hierarchical network of genes that controls the activation of the embryonic maturation program, and that FUS3 and ABI3 act downstream of LEC1 to turn on expression of SSP genes.

LEC2, FUS3, and ABI3 all accumulate primarily during seed development. These three genes are closely related phylogenetically and belong to one class of B3 domain transcriptional factor, which is unique to Planta (Hershkovitz and Leipe, 1998). However, their specific functions have diverged. Like lec1, loss-of-function lec2 and fus3 mutants show defects in cotyledon identity, namely the formation of trichomes on the adaxial surface, suggesting that LEC2 and FUS3 also function during early embryogenesis to maintain cotyledon identity (Meinke et al., 1994). Also like LEC1, ectopic expression of LEC2 in seedlings is sufficient to induce somatic embryogenesis, while this phenotype is milder in ectopically expressed FUS3 mutants (Stone et al., 2001, Gazzarrini et al., 2004). LEC2 and FUS3 have been found to bind the RY (CATGCA) elements in the promoter region of 2S albumin At2S3, an SSP gene, and redundantly activate its transcription (Kroj et al., 2003). Expression analysis reveals that LEC2 directly activates genes involved in maturation, including hallmark SSP genes encoding 2S and 12S storage proteins, oleosin, and sterolosin, as well as EEL, a regulator of reserve accumulation (Baybrook et al., 2006). Meanwhile, FUS3 has been shown to directly up-regulate maturation
genes, including genes involved in fatty acid biosynthesis and oil-body biogenesis (Yamamoto et al., 2010). Among these genes, some are also under direct, or potentially direct regulation of LEC2, ABI3, or both (Wang and Perry, 2013). In addition, FUS3 directly activates expression of two genes encoding transcriptional factor AGAMOUS-LIKE15 (AGL15), and BABY BOOM (BBM), both of which are primarily expressed in developing embryos, and can induce somatic embryogenesis (Boutilier et al., 2002; Thakare et al., 2008, Wang and Perry, 2013).

Unlike the mutants of the LEC genes, loss-of-function mutation in ABI3 does not confer leafy traits to cotyledons (Nambara et al., 1995). Nevertheless, abi3 mutants do show reduced level of SSP expression, as well as defect in desiccation tolerance (Parcy et al., 1994). ABI3, while possessing the B3 domain capable of interacting with the RY motif, has also been shown to associate with bZIP transcription factors such as bZIP10 and bZIP25, which can bind the G-box in the promoter of At2S1, also an SSP gene, and induce its expression (Lara et al., 2003). Genome-wide analysis reveals a set of target genes for ABI3, most of which require the hormone abscisic acid to be activated, and are expressed specifically during the maturation phase (Mönke et al., 2012).

These master regulators LEC1, LEC2, FUS3, and ABI3 interact in a complex and redundant regulatory network during seed development. LEC1 and LEC2 both up-regulate expression of FUS3 and ABI3, but it is unknown whether the former two interact with each other. Furthermore, ABI3 and FUS3 positively regulate themselves and each other, creating a positive feedback loop that leads to sustained expression of these genes in the embryo (To et al., 2006).

Plant hormones play an important role in controlling this gene network. Auxin has been reported to be a key factor triggering somatic embryogenesis in Arabidopsis, and application of auxin led to an increase in LEC2 level (Gaj, 2004; Ledwoń and Gaj, 2009). The expression of
three *YUCCA* genes, which are involved in tryptophan-dependent auxin biosynthesis, were also found to be controlled by LEC2, suggesting that LEC2 plays a role in promoting endogenous auxin level (Wójcikowska *et al.*, 2013). The role of auxin regarding maturation in normal embryo is less clear, as mutants of the *YUCCA* genes show defects in cell fate identity, but not in maturation (Cheng *et al.*, 2007). Absisic acid (ABA) and gibberellic acid (GA) also interact with this gene network, as high ABA/GA ratio has been established to promote maturation (Santos-Mendoza *et al.*, 2008). ABA is required for the induction of several SSP genes by FUS3 and ABI3 (Kagaya *et al.*, 2005b; Parcy *et al.*, 1994). Meanwhile, FUS3 has been shown to repress GA biosynthetic genes, and the ABA/GA ratio in *fus3* mutant is lower than in wild-type seeds. Inversely, at the point of germination, high level of GA inhibits FUS3 and ABA in order to reverse maturation-induced dormancy (Fig. 3) (Gazzarrini *et al.*, 2004).

**C. Negative regulator of maturation**

   *a. After maturation*

Several elements are known to act upstream of and repress these master regulators after germination (Fig. 4). One of these mechanisms of regulation is Histone H3 Lys27 trimethylation (H3K27me3) deposition by Polycomb-group (PcG) proteins, such as Polycomb repressive complex 2 (PRC2). Loss of PRC2 function results in mutant seeds with defects in dormancy and germination that are indicative of a failure to exit the embryonic phase, as well as seedlings with neoplastic, callus-like structures typical of somatic embryogenesis (Bouyer *et al.*, 2011). It has been shown that histone trimethylation by PRC2 negatively regulates the transcription level of *LEC1, LEC2, FUS3*, and *ABI3* in *Arabidopsis* vegetative tissues (Berger *et al.*, 2011; Bouyer *et al.*, 2011). Another chromatin-modifying factor, histone deacetylase (HDAC) also plays an important role in transcriptional repression and phase transition. Inhibition of HDA6 and HDA19
activity in post-germinative *Arabidopsis* results in growth arrest, and ectopic formation of embryo-like structures, coupled with elevated level of *LEC1*, *FUS3*, and *ABI3* (Tanaka *et al.*, 2008). Furthermore, PcG proteins and HDAC are not sequence-specific, and must act in conjunction with other transcriptional factors to repress their target genes.

Two other B3 domain proteins, VP1/ABI3-LIKE or HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE2 (VAL1/HSI2), and VAL2/HSI2-Like1 (HSL1) act as transcriptional repressors that inhibit sugar-inducible expression of maturation genes during seedling development (Tsukagoshi *et al.*, 2007). Disruption of HSI1 and HSI2 results in seedlings that go into developmental arrest a few days after germination, with swollen hypocotyls and calluses that accumulate SSP and oil bodies. It has also been reported that VAL1/HSI2 and VAL2/HSL1 acts downstream of HDA19 and PRC2 to repress maturation genes (Veerappan *et al.*, 2014; Zhou *et al.*, 2013). The repressive effect of chromatin modification and these TFs, however, has not been investigated in early embryos.

ARABIDOPSIS 6B-INTERACTING PROTEIN1-LIKE1 (ASIL1), a nuclear trihelix transcription factor, has also been reported to prevent expression of the genes encoding storage products in *Arabidopsis* seedlings. The trihelix DNA-binding proteins are a family of transcriptional factor unique to plants, suggesting their involvement in plant-specific regulatory pathways (Ayadi *et al.*, 2004). The trihelix family is distinguished by a conserved trihelix (helix-loop-helix-loop-helix) structure in their DNA-binding domain, which specifically binds GT elements (Nagano *et al.*, 2001; Ni *et al.*, 1996). The trihelix family can be divided into five subfamilies: ASIL1, GT-1, GT-2, GT-3, and At1h21200, all of which contain an N-terminal trihelix I domain, and a C-terminal α-helical region (Fig. 5A,B) (Gao *et al.*, 2009). Members of the trihelix family show extensive functional divergence, such as embryo development, organ
morphogenesis, stress resistance (both biotic and abiotic), and various other metabolic pathways. However, the functional relationship among these TFs is poorly understood, and it’s unknown whether they share a common underlying mechanism (Qin et al., 2014). ASIL1 has been shown to repress the transcription of At2S3 by binding the GT element near the RY repeats in the promoter of this gene (Gao et al., 2009). Since other SSP genes like CRC and Oleo, as well as the LEC genes also contain GT-boxes in their promoter, it has been presumed that ASIL1 bind these to repress them as well. Seedlings of asil1 mutant show significant elevation in the levels of LEC1, LEC2, FUS3, ABI3, suggesting indirect suppressive effect of ASIL1 on maturation through these master regulators (Gao et al., 2009). It appears, therefore, that ASIL1 acts at two levels, affecting the master regulators and the SSP genes themselves.

In addition to chromosomal modification and regulation by TFs, miRNA-mediated repression of maturation has also been reported in vegetative tissues. miRNAs are ~21-nucleotide RNAs that recognize complementary sequences in target mRNAs and mediate post-transcriptional regulation (Bartel, 2004). Plants possess a very high degree of complementarity among miRNAs and their targets, the latter of which are often key regulators of development, including transcription factors (Rhoades et al., 2002). In addition, complementary sites are highly conserved in flowering plants. As a result, miRNA targets in plants are readily identifiable. Mutants for ARGONAUTE1 (AGO1), which encode a protein involved in the biosynthesis of miRNAs, show ectopic expression of seed maturation genes. Further analysis shows that a decrease in miR166 level is partly responsible for this phenotype. Overexpression of miR166 targets such as type III HD-ZIP transcriptional factors PHB, which binds directly to the LEC2 promoter, is sufficient to induce seed maturation genes in seedlings (Tang et al., 2012).
b. Early embryogenesis

Less is known about the mechanism that inhibits the maturation program during early embryogenesis (Fig. 6). The RNase III DICER-LIKE1 (DCL1) is part of a complex that generates microRNAs (miRNAs), which inhibit translation either by targeting mRNAs for degradation or blocking recruitment of ribosomes (Willman et al., 2011). The dcl1-15 loss-of-function mutant shows multiple defects that are associated with early entry into the maturation phase, including early accumulation of chlorophyll, abnormal cell patterning, up-regulation of genes involved in storage products biosynthesis, accompanied by down-regulation of genes involved in cell cycle and development during the early globular stage (Willmann et al., 2011). miRNAs are therefore important negative regulators of maturation.

Two miRNA targets, SPL10 and SPL11 are severely de-repressed in dcl1 mutants, resulting in premature transition to the maturation phase (Nodine and Bartel, 2010). ASIL1, and its closest paralog ASIL2, have also been reported to act downstream of miRNA to repress maturation early in embryogenesis. Both genes are down-regulated in dcl1 torpedo-stage embryos, while both FUS3 and LEC2 are up-regulated (Willman et al., 2011). However, neither ASIL1, ASIL2, LEC2, nor FUS3 are direct targets of miRNAs, as their sequences are not complimentary to any known Arabidopsis miRNAs, suggesting that miRNA-mediated regulation of these genes is indirect. Embryos of asil1 and asil2 mutants show early chlorophyll fluorescence, as well as early expression of At2S3, a phenotype that is similar to, but milder than that of dcl1 embryos (Willman et al., 2011). This phenotype is accentuated in double mutants, suggesting a concerted mode of action, or at least partial redundancy between ASIL1 and ASIL2. Furthermore, asil1 mutants also show an early increase in the level of LEC2, FUS3, and ABI3 transcripts at early-embryogenesis, as well as greater accumulation of CRC, proposing a single
mode of action for *ASIL1* both before and after maturation (Gao et al., 2011). *ASIL1* has also been found to be expressed in the chalazal seed coat and the micropylar endosperm very early in development (during the pre-globular and globular stages), but its function in these regions is unknown (Le et al., 2010).

Due to the large number of interactors involved, the big picture of the regulatory machinery of maturation remains elusive. Namely, it is unknown whether maturation is turned off during early seed development and after germination by the same mechanisms. Certain components, such as the miRNA-mediated pathway, as well as transcription factors like *ASIL1*, have been reported to be present during both of these developmental phases, making the case that at least a part of the mechanism governing the repression of seed maturation is recycled from early embryogenesis to vegetative development.

**D. Project Aims**

Here, I attempt to confirm the role of *ASIL1* and *ASIL2* in repressing the maturation program during embryo morphogenesis. Two methods are conventionally used to study the expression pattern of a gene or promoter sequence. One method, reverse transcription polymerase chain reaction (RT-PCR), involves the isolation of mRNA from cells or tissues, followed by reverse transcription into cDNA and subsequent amplification of the genes of interest. However, my research poses a challenge to this approach, as it is difficult to manually isolate enough embryos from seeds to purify mRNA. Alternatively, the transcriptome of whole siliques (which are *Arabidopsis* fruits that contain the seeds) could be extracted but at the cost of introducing expression signal non-specific to the embryo.

The second method is to fuse a reporter gene to the promoter sequence of interest, and transform it into the system under study. The promoter sequence drives transcription and
translation of the reporter protein, which can easily be identified. The reporter gene chosen for this project is \( \beta \)-glucuronidase (GUS), which encodes a hydrolase capable of transforming the colorless X-Gluc into a clear blue product (Fig. 7) (Jefferson et al., 1987). Fusion of LEC2 and FUS3 promoters to GUS therefore allows the spatiotemporal expression pattern of these genes to be observed and compared among wild-type, single, and double mutant embryos (Kroj et al., 2003). The two mutants under study are asil1-1, which contains a T-DNA insertion in the coding region 346 bp downstream of the translation start site, and asil2-1, which contains a T-DNA insertion 1262 bp downstream of the start codon, near the 3’ end of the coding region (Fig. 8) (Gao et al., 2009; McElver et al., 2001). One drawback of this method is that the level of gene expression cannot be precisely quantified based on GUS staining intensity. However, the percentage of embryos expressing GUS activity in the embryo proper can be measured and used as an indicator of transcription level. For instance, in plants containing the pLEC2:GUS transgene, most of the embryos will show GUS staining at stages where LEC2 expression is high, while only a few embryos will exhibit visible staining at stages where pLEC2:GUS transcription is low. This methodology is similar to that used by Willmann et al. (2011) to study premature chlorophyll accumulation in asil1-1 and asil2-1 mutants. My hypothesis is that LEC2:GUS and FUS3:GUS show higher activity during early embryo development in all asil1-1, asil2-1, and double mutants than in wild-type embryos.

I am also interested in seeing how ASIL1 and ASIL2 are expressed and regulated throughout embryogenesis. Previous studies using microarray on whole siliques or seedlings suggest that ASIL1 transcript is elevated following germination, but is maintained at low level during seed development (Gao et al., 2009; Le et al., 2010). In this study, I use GUS as a reporter to closely examine the expression pattern of the two ASIL genes in early embryos. In
addition, deletions in the promoter region of each gene are generated in order to define regulatory elements within these promoters, and ultimately to identify the factors that regulate the ASIL genes via binding to these elements. Past experiments by Bethany Johnson ’13 and Jon Marks ’14 showed that translational fusions (promoter region with the entire coding sequence, up to +1445 bp for ASIL1 and +1558 bp for ASIL2) are expressed in the embryo. However, the transcriptional fusions that do not include the coding sequence (up to +50 bp for ASIL1 and +30 bp for ASIL2) are not, suggesting that an important regulatory region may lie within the coding sequence or the 5’. As a result, new translational fusions, and transcriptional fusions containing the 5’ untranslated region (UTR) up to +316 bp for ASIL1, and +286 for ASIL2 are being generated and analyzed.
II. RESULTS

A. Promoter analysis of ASIL1 and ASIL2

In order to survey for putative regulatory sequences in the promoter regions of ASIL1 and ASIL2, several fusion constructs containing different lengths of promoters and/or coding sequences fused to the GUS coding sequence are being made, a list of which can be found in Fig. 9, Fig. 10, and Table I. So far, five transcriptional fusion constructs containing the 5’ UTR of ASIL1 have been transformed into plants, whose GUS expression profiles have been analyzed, while one transcriptional fusion and five translational fusion are now in destination vectors in Agrobacteria and ready to be transformed into Arabidopsis. Six transcriptional fusion constructs containing the 5’ UTR of ASIL2 have been cloned into entry vector, while the PCR products for six other translational fusions have been successfully purified.

Analysis of the five plant lines containing different ASIL1 transcriptional fusion constructs focuses on the period of development from the pre-globular stage (8-cell) to the mid-heart stage, as I am particularly interested in the expression of ASIL1 from the early stages of embryo morphogenesis until the maturation program is induced. Representative images of the embryo at each stage of development from each plant line are shown in Fig. 11, and the summary of the data is presented in Fig. 12. It is important to point out that apart from only a few exceptions, GUS staining of all constructs at all described stages is not restricted to the embryo, but is present throughout the endosperm in a non-nuclear-specific manner, making it difficult to tell whether the construct is expressed in the embryo proper or not. The pTD1U construct, which contains the sequence from -1324 to +316 base pairs (bp), shows high level of expression at the 8-cell stage, gradually decreasing up to the early-heart stage with the exception of a small local peak at the late-globular stage, and then begins to increase starting at the mid-heart stage. The
pTD2U construct (-1113 +316 bp) shows consistently high level of expression throughout development, even at the late globular stage. The pTD3U (-677 +316), and the pTD5U (-437 +316) constructs share the same expression profile, with GUS signal being present in all pre-globular embryos, and decreasing steadily until the late globular stage, where it is maintained at low level throughout the rest of embryogenesis. Compared to pTD3U and pTD5U, the expression level of the pTD6U (-299 +316) construct is low at the pre-globular stage, and briefly recovers at the late-globular stage before decreasing even further starting at the heart stage.

Due to strong GUS staining in the endosperm obscuring the embryos, dissection was performed to manually isolate embryos from their seeds in staining solution. While the number of embryos retrieved via this method is low, and thus precluding efforts of statistical analysis, preliminary data suggest that in most of the constructs, even the ones with low late expression, GUS staining does not occur in the embryo proper until the mid-globular, from which point it is maintained throughout the rest of embryogenesis (Fig. 13). One exception is pTD1U, for which only a few embryos from the globular stage were collected, and no staining was observed.

Meanwhile, analysis was also performed on plant lines generated by previous students, specifically, the translational fusion from -437 to +1445 bp of the ASIL1 gene fused to GUS (pBJ37). GUS staining of seeds from this line shows expression of pBJ37 in the embryo proper beginning at the mid globular stage, continued throughout the rest of embryogenesis (Fig. 14A). Expression is also seen in the chalazal seed coat and the funiculus, which attaches the seed to the silique, as early as the 16-cell stage, but not in the embryo proper at this stage (Fig. 14B). In contrast, staining in the chalazal seed coat and the funiculus cannot be distinguished in the 5’ UTR fusion due to the entire endosperm being stained. pBJ37 also displays staining in the endosperm nuclei starting at the globular stage, but not in every seed (Fig. 14C).
B. Effect of ASIL1 and ASIL2 on maturation genes

To determine whether ASIL1 and ASIL2 act during early seed development to repress the master regulators of maturation, I studied the expression of GUS driven by the promoter of *LEC2* or *FUS3* on wild-type, *asil1-1, asil2-1*, and *asil1-1 asil2-1* double mutant background. Representative images of the embryo from each genotype at the stages of interest are shown in Fig. 15, and a summary of the data is presented in Fig. 16. GUS staining in wild-type embryos reveals expression of *LEC2* in the embryo proper initiating at the mid-globular stage. However, staining at this stage, if at all, is weak and localized around the hypophysis. In fact, most wild-type embryos do not stain until the late-globular-to-early-heart transition (Fig. 15). In *asil1-1, asil2-1*, and double mutants, on the other hand, a significantly larger portion of embryos show staining for *pLEC2:GUS* in the embryo proper at the mid-globular stage compared to wild-type (Fisher’s exact test: *asil1-1, p < 0.001; asil2-1, p < 0.001; asil1-1 asil2-1, p < 0.001*). At the early globular stage, the *asil2-1* mutant, but not the *asil1-1* or the double mutant, shows significantly higher number of embryo with *pLEC2:GUS* expression in the embryo proper than the wild-type (Fisher’s exact test: *asil1-1, p = 1, asil2-1, p = 0.01, asil1-1 asil2-1, p = 0.6*). By the late-globular stage, *LEC2* expression was seen in almost all of mutant embryos regardless of genotype (Fisher’s exact test: *asil1-1, p = 0.018, asil2-1, p < 0.001, asil1-1 asil2-1, p = 0.003*). Apart from the early globular stage, no statistical significance was observed among the mutant phenotypes.

Preliminary analysis of *pFUS3:GUS* expression shows that *FUS3* transcript is not present in the wild-type embryo proper until the early heart stage, at which point staining is very weak. *FUS3* expression is clearly upregulated soon afterwards, as evident by the strong staining at the mid-heart stage in all embryos (Fig. 17). The current generations of *asil1-1, asil2-1*, and double mutants containing the *pFUS3:GUS* transgene, however, fails to produce staining signal at any
stage of development, suggesting that the transgene has been silenced in these plant lines. The spontaneous silencing of transgene either immediately after transformation or after several generations is a phenomenon that has been reported (Eissenberg, 1989). This phenomenon is typically caused by unexpected interactions of the transgene with its neighboring environment in the host genome, as well as epigenetic instability, and can be bypassed by restarting from previous generations.
III. DISCUSSION

The ability to develop seeds provides one of the keys to the evolutionary success of land plants. The maturation program allows the seed to accumulate storage products, acquire desiccation tolerance, and enter dormancy prior to germination. However, due to the high energy expense involved in the major switch in the transcriptome upon induction of maturation, as well as the interference by enhanced storage products accumulation on the cell cycle, this program is tightly controlled by a complex network of positive and negative regulators, allowing the embryo to turn the maturation phase on and off with high efficiency (Belmonte et al., 2013). While many members of this network have been worked out (Baud et al., 2008), the complete picture has yet to be revealed, especially during early seed development. Here, we characterize the activity of ASIL1 and ASIL2, and their role in repressing the maturation program in early embryogenesis.

A. Regulation of ASIL1 during embryogenesis

Microarray analysis of micro-dissected tissues shows that in the peripheral endosperm, ASIL1 expression begins at a relatively high level during the pre-globular stage, reaching its peak at the globular stage, then diminishing at the heart stage, and then increasing again from the torpedo stage (Fig. 17) (Belmonte et al., 2013). Regarding expression in the endosperm, of all the constructs analyzed, pTDIU (-1324 +316 bp) shows a pattern that correlates the most with the expression pattern of ASIL1 from the microarray data of Le et al. (2010), as the former is up-regulated during the pre-globular and early-globular stages, down-regulated at the mid-globular and early-heart stage, and up-regulated again starting at the mid-heart stage (Fig. 10). The trend of up-down-up regulation is similar to that of the microarray data, but the stages at which the changes occur are shifted slightly earlier in pTDIU. In addition, pTDIU expression also reaches a local maximum at the late-globular stage that is absent from the ASIL1 microarray data. It can
therefore be proposed that there lie one or more elements upstream of -1324 bp that are necessary to repress ASIL1 transcription at the late-globular stage in the endosperm, as well as to ensure that dynamic changes in ASIL1 activity happen at the exact right stages (Fig. 17).

The decrease in activity in the endosperm around the transition from globular to heart stage is eliminated in the pTD2U construct, suggesting the presence of a DNA sequence that negatively regulates ASIL1 transcription from mid-glob to early-heart between 1324 bp and 1113 bp upstream of the transcription start site. In fact, judging from the small magnitude of the fall in activity in pTD1U, and the local peak at the late-globular stage, it might be proposed that this negative regulatory element extends even beyond -1324 bp, and that the full disruptive effect of this element has been lost in the pTD1U fragment. Deletion from -1113 to -677 bp in pTD3U, and to -437 bp in pTD5U results in the same loss of expression starting at the late-globular stage, lasting until the mid-heart stage and potentially later in development, thus providing evidence for a positive regulatory element in these 400 base pairs that is specific to mid-, and possibly late-embryogenesis. The pTD6U construct, which extends only up to -299 bp upstream of the transcription start site, seems to lose the ability to up-regulate expression during the early stages of development, but gain some activity at the transition stage before being extinguished altogether by the mid-heart stage. It thus appears that the ~100 base pairs between -437 and -299 bp contain some regulatory sequence that allow for positive control of ASIL1 expression in the pre- to early-globular stages, and negative control at the late-globular stage. Collectively, these data suggest that in the peripheral endosperm, ASIL1 activity is regulated by multiple factors at different stages of embryo development: initial activation and maintenance of expression during early embryogenesis, repression around the late-globular-early-heart stage, and reactivation at later stages.
Another factor to consider is the expression pattern of the fusion constructs compared to that of the microarray data in the embryo proper. As the embryo proper is what directly undergoes embryogenesis to form the mature embryo, control of ASIL1 activity in this region might be more important than in the endosperm. Previous analysis using qRT-PCR on whole siliques by Gao et al. (2009) suggests dynamic expression of ASIL1 throughout seed development, with the level of transcript being up-regulated during early (pre-globular to early-globular) and late embryogenesis (heart to cotyledon stage), and down-regulated in mid-embryogenesis (transition between the globular and the heart stage). Microarray analysis of micro-dissected tissues reveals a similar pattern in the embryo itself, with the addition of strong expression in the chalazal seed coat during the pre-globular stage, and in the micropylar endosperm during the globular stage (Fig. 18) (Belmonte et al., 2013; Le et al., 2010). While data collection of isolated embryos is at a very preliminary stage, none of the transcriptional fusions analyzed appear to have similar expression pattern. In fact, activity of pTD1U-pTD6U in the embryo proper is reminiscent of that of the translational fusion pBJ37, whose expression is abolished from the embryo until the mid globular stage (Fig. 13, and 14). One or more elements upstream of the current longest promoter fragment (-1324 bp) might be required to activate ASIL1 transcription during the pre-, early-, and mid-globular stages in the embryo proper.

That the promoter region of a gene contains several regulatory elements, each driving a specific spatio-temporal aspect of the overall expression pattern is a phenomenon that has been widely reported (Howard and Davidson, 2004; Kulkarni and Arnosti, 2005; Tadaka and Jürgens, 2007). The ability to divide the promoter into separate sequences of distinct functions allows the cell to fine-tune the activity of the gene, and make room for dynamic control of transcriptional potential. It is also important to note that the promoter region of a plant gene typically extends to
2-2.5 kilobases (kbs) upstream of the open reading frame, and therefore it is likely that the longest promoter fragment in this study (pTD1U) is not representative of native expression pattern of ASIL1, and more cis-acting elements remain to be discover beyond -1324 bp.

On the other hand, regulatory elements can also be found downstream of the transcription start site: in the 5’ UTR, in introns, or 3’ UTR (Deyholos and Sieburth, 2000; Larkin et al., 1993; Riechmann, 2002). For example, the second intron of the AGAMOUS (AG) coding sequence contains elements that interact with transcriptional repressors and are required for activation of stamen-specific and carpel-specific expression of the gene (Deyholos and Sieburth, 2000). While the ASIL1 coding region contains no intron, it is possible that one or more cis-acting elements lie in the 5’ or 3’ UTR. Studies conducted by previous students showed that the ASIL1 gene sequence up to +50 bp and ASIL2 gene sequence up to +30 bp from the transcription start site are not expressed in the seed at any stage. In contrast, my transcriptional 5’ UTR fusions containing the sequence from up to +316 bp of ASIL1 were expressed even at very early stages, suggesting that either the short sequences previously generated suffered defects in transcriptional or translational potential, or there exists a cis-acting element required for activation of ASIL1 transcription in the 5’ UTR of the gene, between +50 and +316 bp. A summary of all putative regulatory sequences can be found in Fig. 17.

In addition, pBJ37 (-437 +1445 bp) staining has also been observed in the endosperm nuclei starting at the globular stage and is maintained throughout the rest of embryogenesis. Whether ASIL1 expression in the endosperm has any role in seed development is still unknown, but it does correlate with the whole-seed staining pattern as observed in the pTD5U fusion constructs (-437 +316 bp). pTD5U shows GUS expression in the endosperm from the pre-globular stage throughout seed development, but unlike pBJ37, the signal is diffused throughout
the cytoplasm instead of being localized in the nuclei. Since a transcriptional fusion does not contain the coding sequence of the gene, the resulting protein is not expected to localize to the nucleus as would a translational fusion, and thus producing GUS signal across the entire endosperm layer. Other than the fact the pTD5U staining signal is unlocalized compared to pBJ37, their expression patterns are almost identical, suggesting that there is no regulatory element of transcription between +316 and +1445 bp.

Interestingly, while pBJ37:GUS (ASIL1 -437 +1445) does not show expression in the micropylar endosperm at any stage (in contrast to microarray data by Le et al., 2010, which might be indicative of an upstream positive element for this particular region), there is visible staining in the chalazal seed coat and the funiculus as early as the pre-globular stage, and is maintained throughout the heart stage. The chazalal endosperm joins the maternal vascular tissue with the seed integuments, and has been implicated in the transfer of nutrients and minerals to developing seeds (Nguyen et al., 2000; Otegui et al., 2002; Le Jean et al., 2005). Moreover, microarray data indicates that the chalazal endosperm experiences up-regulation of LEC1, LEC2, and FUS3 transcript beginning at the heart or torpedo stage, which is later than other tissues whose same maturation genes level starts to increase at the globular to transition stage (Belmonte et al., 2013). Comparative studies of the biochemical composition and the transcriptome of the chalazal endosperm in wild-type and asill-1 mutants may elucidate the role of ASIL1, if at all, in preventing seed loading via these pathways during early seed development.
B. ASIL1 and ASIL2 as negative regulators of maturation genes

LEC2 and FUS3 are two of the master regulators of the maturation program, and their presence activates the transcription of SSP-encoding genes (Kroj et al., 2003). We observed that in the wild type, LEC2 expression is present in a small number of embryos at the mid-globular stage. However, it is not until the late-globular-early-heart transition, just before the onset of maturation, that most of the embryos show staining for LEC2:GUS. In all asil1, asil2, and double mutants, a much greater percent of mid-globular and late-globular embryo demonstrates LEC2 expression (Fig. 14). The increase in the fraction of embryos with LEC2 expression from the mid-globular to late-globular stage in the mutants resembles that from the late-globular to early-heart stage in wild-type seeds, suggesting a shift forward in the onset of maturation program towards early embryogenesis. No statistical differences have been observed among the single and double mutants at the mid- and late-globular stage, suggesting that ASIL1 and ASIL2 act in a linear pathway or as part of a complex to regulate LEC2 expression during these stages. The fact that pLEC2:GUS transcription is up-regulated at the early-globular stage in the asil2 mutant compared to asil1 and double mutant might be an artifact of small sample size.

In the wild type, FUS3 shows an expression pattern that is slightly later than that of LEC2, being excluded entirely from the embryo proper until the early heart stage (Fig. 15), lending support to the model that FUS3 is upregulated in part by LEC2 (To et al., 2003). More data is being generated to compare the expression of FUS3:GUS in the asil mutants to that of wild-types.

So far, several factors have been revealed to regulate the maturation program during early embryogenesis. FUS3 and ABI3 upregulate their own and each other’s expression during maturation, with both being upregulated by LEC1 and LEC2. These four genes are the master
regulators of the maturation program (To et al., 2003). Nodine and Bartel (2010) and Willman et al. (2011) have proposed that miRNAs are key negative regulators of the maturation program. Mutation of the DCL1 gene, which encodes a member of the complex that generates miRNAs, results in early chlorophyll fluorescence, and accumulation of starch grains, lipid bodies, and storage proteins. The SPL genes, including SPL10 and SPL11, have been shown to be targets of miRNAs, and are de-repressed in dcl1 mutants, leading to early transition to maturation (Nodine and Bartel, 2010). It has been proposed that these SPL genes redundantly regulate the same target, and an increase in their transcription level beyond a threshold allows the maturation program to occur. So far, no study has been conducted to confirm this. In addition, microarray analysis of dcl1 mutants shows a down-regulation in the level of ASIL1 and ASIL2 transcript, accompanied by an up-regulation of LEC2, FUS3, and other maturation genes (Willman et al., 2011). Since miRNAs regulate their targets exclusively by silencing complementary mRNAs (as reviewed by Jones-Rhoades et al., 2006), and ASIL1 and ASIL2 are not direct targets of miRNAs as their sequences are not complementary to any known miRNA, it can be inferred that ASIL1 and ASIL2 act downstream of one or more repressors that are themselves targeted by miRNAs. It is possible that ASIL1 and ASIL2 act downstream of the SPL genes and upstream of the LEC genes, repressing the later while being repressed by the former (Fig. 4). Both SPL10 and SPL11 are predicted to contain transcriptional repression domains (Mitsuda and Ohme-Takagi, 2009), and SPL9, a close homolog of SPL10, has been shown to repress other genes by interfering with a transcriptional activation complex (Gou et al., 2011).
IV. CONCLUSIONS AND FUTURE DIRECTIONS

Several conclusions can be drawn from this work. Firstly, the promoter region of \textit{ASIL1} extending up to 1324 bp upstream of the open reading frame contains multiple regulatory elements that control different facets of \textit{ASIL1} expression pattern. In the 5’ UTR, the sequence between +50 and +316 bp also is potentially essential for activation of \textit{ASIL1} transcription in the seed. Moreover, there is evidence that the full-length promoter of \textit{ASIL1} extends beyond -1324 bp, and consists of more regulatory elements that might interact in a complex pathway to drive the spatio-temporal expression of the gene in the endosperm and the embryo proper.

Naturally, the generation of longer promoter fragments is in order for further characterization of ASIL1 transcription regulation. I have made several attempts to design primers for \textit{ASIL1} that are up to ~2.5 kb upstream of the transcription start site, but they have failed to yield PCR products. Moreover, it will be helpful to look at native \textit{ASIL1} and \textit{ASIL2} expression in their genomic context. To this end, transgenic plant lines containing the entire chromosomal regions where \textit{ASIL1} and \textit{ASIL2} are located fused to yellow fluorescence protein (YFP – another reporter gene) will be generated. Presumably, these transgenic lines will provide expression patterns that are equivalent to the full-length promoters of the two \textit{ASIL} genes. Meanwhile, the rest of the fusion constructs for \textit{ASIL1} and \textit{ASIL2} will be transformed into plants, and will be analyzed. Once the regulatory elements are better defined, the next step is to test for protein interactors with these sequences using the yeast-one-hybrid screen, in order to identify the direct regulators of the \textit{ASIL} genes. Candidate proteins will include the aforementioned SPL genes, SPL10 and SPL11, as they possess transcription repression domain, and are known to act downstream of miRNAs to regulate maturation (Nodine and Bartel, 2010).
Secondly, loss-of-function *asil1* and *asil2* single and double mutant results in premature expression of *LEC2* compared to that in wild-type. These results support my hypothesis that ASIL1 and ASIL2 function in repressing the master regulators of maturation during early embryogenesis. While the expression profile of *FUS3:GUS* in mutant embryos remain to be seen, it is expected that FUS3 will be expressed earlier in all single and double mutants compared to wild-type in a similar manner to *LEC2*. Currently, mutant plant lines containing the FUS3:GUS transgene fail to stain, despite staining being observed in earlier generations. Alternatively, RNAseq can be employed to look at the entire transcriptome of the *asil* mutants instead of analyzing one gene at a time. Laser capture microdissection can be utilized to isolate embryos from each developmental stage of each genotype, and their mRNA library can subsequently be sequenced and analyzed for differential expression (Espina et al., 2007). Whole transcriptome analysis may provide a more precise and efficient method of studying expression of maturation genes in the *asil* mutants, as well as a better picture of direct targets of the two *ASIL* genes and their involvement in the maturation program.
V. MATERIALS AND METHODS

**Plant Care:** Plants were grown at 20-24°C, approximately 70% relative humidity, in continuous light (100-150 µmol/m²). All mutants and reporter lines are on Col background and have been described before: pLEC2:GUS, and pFUS3:GUS (Kroj, et al., 2003), asil1-1 (Gao et al., 2009), and asil2-1 (Willmann et al., 2011).

**Polymerase Chain Reaction:** Promoter and coding sequences for ASIL1 and ASIL2 were amplified via PCR using Phusion polymerase (NEB). Template DNA was purified from wild-type Columbia Arabidopsis thaliana plants. A list of primers used can be found in Table I. The PCR parameters were: 30 seconds at 98°C, 35 cycles of 10 seconds at 98°C, 30 seconds at 60 or 65°C, and 120 seconds at 72°C, followed by a final extension of 120 seconds at 72°C. PCR products were then gel-purified using the GeneJET™ Gel Extraction Kit (Fermentas).

**TOPO Cloning, E. coli Transformation and LR Recombination:** TOPO cloning into pENTR-D/TOPO and E. coli transformation were performed using the protocol in accordance with the pENTR™ Directional TOPO® Cloning Kit by Invitrogen™ for the Gateway® System. Transformed E. coli were grown overnight at 37°C on LB-agar plates containing 50 µg/mL kanamycin. Individual colonies were then grown overnight at 37°C in 3 mL of LB media containing 50 µg/mL kanamycin.

Plasmid DNA was then recovered from the transformed E. coli using the GeneJET™ Plasmid Miniprep Kit from Fermentas. Purified plasmid DNA was then sent to Pennslyvania State University for sequencing in order to confirm the insertion.

Following confirmation, LR Recombination Reactions were performed following the protocol of the Gateway® LR Clonase™ II Enzyme Mix kit by Invitrogen™ for the Gateway® System. The destination vectors pGWB3 (containing the GUS reporter gene) were used in these
reactions (Nakagawa et al., 2007). E. coli were transformed and plasmids purified using the same procedures mentioned previously. Purified plasmid DNA was then cut using the enzyme HindIII for 1 hour at 37°C. Products were then analyzed using gel electrophoresis to confirm proper orientation and identity of the insert.

**Agrobacterium-mediated Transformation:** Transgenic plants were generated via Agrobacterium-mediated transformation using the following protocol. 25 µl of competent Agrobacterium tumefaciens were thawed per transformation. 1 µl of miniprepped plasmid was added. The mixture was then placed in a chilled electroporation cuvette (eppendorf) and electroporated at 2.4 kV with an eppendorf Electroporator 2510. 1 ml of LB media was then added, and the mixture was incubated for 4 hours at 28°C with gentle agitation. 150 µl of the mixture was then plated on LB agar plates containing 50 µg/mL kanamycin and 10 µg/mL of gentamycin and incubated for 4 days at 28°C.

One colony from each plate was then grown overnight at 28°C with gentle agitation in 20 mL of LB media containing 50 µg/mL kanamycin and 10 µg/mL of gentamycin (glycerol stocks were made at this point, using 0.3 mL of glycerol and 0.65 mL of Agrobacterium culture). 30 mL of LB media containing 50 µg/mL kanamycin and 10 µg/mL of gentamycin was then added to each sample and grown overnight at 28°C with gentle agitation. Agrobacterium cells were then pelleted at 2700 xg for 15 minutes, and resuspended in 5% sucrose solution to OD600 = 0.8. Silwett was then added to a concentration of 0.05%.

Plants were prepared for transformation by manual removal of siliques. Plants were then dipped into Agrobacterium solution for approximately 90 seconds, then placed under plastic lids and remained covered in the greenhouse for 2 days before covers were removed and plants were staked.
Transgenic Plant Selection: Before plating, seeds were sterilized by first soaking them in 70% ethanol for 2 minutes and pouring off the excess liquid. Seeds were then soaked in 20% bleach, 0.5% SDS solution for 15 minutes, after which the excess liquid was pipetted out. Seeds were then washed three times using sterile water.

Following sterilization, seeds were plated via pipetting on plates containing 2.15 g/L of Murashige and Skoog basal salt mixture, 0.5 g/L MES, 10 g/L of sucrose, 1X of Gamborg’s B5 vitamins, 7.5 g/L TC agar, pH 5.7, with 50 µg/mL of kanamycin and 100 µg/mL of ampicillin. Seeds grew for 7-10 days before resistant seedlings were removed from the plates and planted in soil. Seedlings were kept covered in the greenhouse for 2-3 days following transplantation.

Visualization of Transgenic Embryos – GUS: Siliques of plants transgenic for constructs containing GUS were manually dissected on microscope slides under a bright field microscope using forceps. Siliques were incubated first in 600 µl of cold 90% acetone, washed three times with 1 mL of 100 mM phosphate buffer pH 7, and then incubated in 300 µl of staining solution containing 100 mM phosphate buffer, pH 7, 1 mM EDTA, 1% Tween 20, 2.5 mM potassium ferrocyanide/ferricyanide, and 1 mg/mL X-Gluc (Sigma-Aldrich), overnight at 37°C in a 24-well plate. Seeds were collected, mounted in Hoyer’s clearing solution (chloral hydrate, glycerol and gum Arabic in water), then visualized using bright field on a Leica DMRB microscope, and pictures were taken using a Jenoptik C5 camera. Alternatively, embryos were manually dissected out of seeds already immersed in 60 µl of the staining solution on a microscope slide, and were left to stain at 37°C overnight a closed plastic container containing wet paper towels to maintain humidity. The embryos were visualized using bright field on a Leica DMRB microscope, and pictures were taken using a Jenoptik C5 camera.
VI. FIGURES

Figure 1. Arabidopsis embryogenesis. Schematic representation of the different stages of Arabidopsis seed development. Images from Le et al., 2010.
Figure 2. Master regulators of maturation. Schematic description of the four master regulators of the maturation program. Black arrows indicate positive regulation among the master regulators.
Figure 3: Positive Regulators of maturation. Schematic description of the maturation program and regulators.

Black arrows indicate positive regulation among the master regulators. Broken arrows indicate positive regulation that are not between two master regulators. Grey arrows indicate general positive regulation between a factor and maturation.

**Regulators**:
- AB13
- FUS3
- LEC2
- LEC1
- AGL15
- ABA
- GA
- YUC1
- auxin
- BZI25
- BZI10
- desiccation tolerance
- chloroplastic maturation
- BBM

**Program**:
- maturation Program
Figure 4. Repression of maturation after germination. Schematic description of the four master regulators and other negative regulators of the maturation program after germination. Black arrows indicate positive regulation among the master regulators. Broken blunted line indicates negative regulation.
Figure 5. Trihelix transcription factor.
(A) A phylogenetic tree of the trihelix transcription factor family. ASIL1 and ASIL2 are highlighted by the red box. Figure adapted from Gao et al., 2009.
(B) Structure and motifs of the ASIL1 sub-family. The conserved trihelix is in red, the α-helical region is in purple, and the green oval is the putative nuclear localization sequence.
Figure 6. Repression of maturation in early embryogenesis. Proposed schematic description of the four master regulators and other negative regulators of the maturation program during early seed development. Black arrows indicate positive regulation among the master regulators. Broken blunted line indicates negative regulation. The diagram depicts interactions and regulatory relationships among the key players in the maturation program.
Figure 7. Principle of β-glucuronidase staining. X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), a substrate of β-glucuronidase is cleaved to produce glucuronic acid and chloro-bromoindigo. When oxidized, chloro-bromoindigo dimerizes to produce the insoluble blue precipitate dichloro-bromoindigo.
Figure 8. Schematic representation of the T-DNA insertion alleles of *asil1-1* and *asil2-1* in *Arabidopsis*. The ASIL1 and ASIL2 genes have no intron. Numbers indicate positions of T-DNA insertions with respect to the A of the translational start codon.
Figure 9. ASIL1 Fusion Constructs. Transcriptional and translational fusions generated using the different lengths of the promoter region, along with the entire coding sequence (A) or the 5'UTR (B) of ASIL1 fused to GUS coding sequence.
Figure 10. ASIL2 Fusion Constructs. Transcriptional and translational fusions generated using the different lengths of the promoter region, along with the entire coding sequence (A) or the 5'UTR (B) of ASIL2 fused to GUS coding sequence.
Table I. Transcriptional and translational fusions generated using the presumed full-length or partial promoter region, along with the entire coding sequence or the 5’UTR of *ASIL1* and *ASIL2*.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>PCR reaction worked?</th>
<th>In entry vector?</th>
<th>Sequence checked?</th>
<th>In destination vector?</th>
<th>In Agro?</th>
<th>In plants?</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1324 +316</td>
<td>Y</td>
<td>pTD1</td>
<td>Y</td>
<td>pTD1U</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>-1113 +316</td>
<td>Y</td>
<td>pTD2</td>
<td>Y</td>
<td>pTD2U</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>-1028 +316</td>
<td>Y</td>
<td>pTD14</td>
<td>Failed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-677 +316</td>
<td>Y</td>
<td>pTD3</td>
<td>Y</td>
<td>pTD3U</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>-457 +316</td>
<td>Y</td>
<td>pTD4</td>
<td>Y</td>
<td>pTD4U</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>-437 +316</td>
<td>Y</td>
<td>pTD5</td>
<td>Y</td>
<td>pTD5U</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>-299 +316</td>
<td>Y</td>
<td>pTD6</td>
<td>Y</td>
<td>pTD6U</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>-175 +316</td>
<td>Y</td>
<td>pTD7</td>
<td>Y</td>
<td>pTD7U</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>-94 +316</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1028 +1445</td>
<td>Y</td>
<td>pTD15</td>
<td>Y</td>
<td>pTD15U</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>-457 +1445</td>
<td>Y</td>
<td>pTD9</td>
<td>Y</td>
<td>pTD9U</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>-437 +1445</td>
<td>Y</td>
<td></td>
<td>Failed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-299 +1445</td>
<td>Y</td>
<td>pTD11</td>
<td>Y</td>
<td>pTD11U</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>-175 +1445</td>
<td>Y</td>
<td>pTD12</td>
<td>Y</td>
<td>pTD12U</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>-94 +1445</td>
<td>Y</td>
<td>pTD13</td>
<td>Y</td>
<td>pTD13U</td>
<td>Y</td>
<td>N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fragment</th>
<th>PCR reaction worked?</th>
<th>In entry vector?</th>
<th>Sequence checked?</th>
<th>In destination vector?</th>
<th>In Agro?</th>
<th>In plants?</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2472 +286</td>
<td>Y</td>
<td>pTD16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1912 +286</td>
<td>Y</td>
<td>pTD17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1552 +286</td>
<td>Y</td>
<td>pTD18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1172 +286</td>
<td>Y</td>
<td>pTD19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1150 +286</td>
<td>Y</td>
<td>pTD20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-512 +286</td>
<td>Y</td>
<td>pTD21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2472 +1558</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1912 +1558</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1552 +1558</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1172 +1558</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1150 +1558</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-512 +1558</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 11. Expression of different ASIL1 fusion constructs with GUS. Representative images are taken from the early to middle stages of embryo development (pre-globular to mid-heart) of each construct from pTD1U to pTD6U. Whole siliques were stained in wells containing 300 µL staining solution overnight.
Figure 12: Expression of different ASIL1 fusion constructs with GUS in embryos.

Fractions of embryos that express pTD1U-pTD6U in the embryo proper during the 8-cell, 16-cell, early globular, and mid-globular, etc.

Data were collected using GUS staining in wells.

<table>
<thead>
<tr>
<th>Construct</th>
<th>% staining in the endosperm/Ep</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTD1U(1324+316)</td>
<td>0.00%</td>
</tr>
<tr>
<td>pTD2U(1179+316)</td>
<td>20.00%</td>
</tr>
<tr>
<td>pTD3U(877+316)</td>
<td>40.00%</td>
</tr>
<tr>
<td>pTD4U(677+316)</td>
<td>60.00%</td>
</tr>
<tr>
<td>pTD5U(437+316)</td>
<td>80.00%</td>
</tr>
<tr>
<td>pTD6U(299+316)</td>
<td>100.00%</td>
</tr>
</tbody>
</table>
Figure 13. Expression of different ASIL1 fusion constructs with GUS in isolated embryos. Representative images are taken from the early to middle stages of embryo development (peri-globular to mid-heart) of each construct from pTD1U to pTD6U. Seeds were manually broken open to expose embryos to the staining solution overnight.
Figure 14. Expression pattern of pBJ37 in embryos as visualized by GUS staining.
(A) Isolated embryos from the pre-globular to the late heart stage, showing GUS expression beginning at the mid-globular stage.
(B) Staining of pBJ37 in the chalazal seed coat (thin arrow) and funiculus (thick arrow) during the pre-globular stage.
(C) Staining of endosperm nuclei at the heart stage. A total of 134 embryos were counted.
Figure 15. Expression pattern of pLEC2:GUS in asil1-1, asil2-1, asil1-1 asil2-1 and wild-type embryos as visualized by GUS staining. Wild-type embryos do not show staining in the embryo proper until the late-globular stage, while mutant embryos begins expressing pLEC2:GUS at the mid-globular stage. Whole siliques were stained in wells containing 300 µL staining solution overnight.
Figure 16. Expression of different ASIL1 fusion constructs with GUS in isolated embryos. Fractions of asil1, asil2, double mutant, and wild-type embryo embryos that express LEC2:GUS in the embryo proper during the early, mid, and late globular stage. Data were collected using GUS staining in wells. Fisher's exact test was performed for each mutant genotype at early, mid, and late-globular stage. Asterisks indicate significant deviation from wild-type (Fisher's exact test, p < 0.05).
**pFUS3:GUS**

![Expression pattern of pFUS3:GUS in wild-type embryos as visualized by GUS staining.](image)

Wild-type embryos do not show staining in the embryo proper until the early heart stage. Whole siliques were stained in wells containing 300 µL staining solution overnight.
Figure 18. *ASIL1* expression in the seed by microarray data.
(A) Change in expression level of ASIL1 in the embryo proper and the peripheral endosperm throughout embryogenesis. Data reported in Belmonte *et al.*, 2013.
(B) Visual representation of the expression patterns of *ASIL1* in *Arabidopsis thaliana* seeds in five different stages of development. Data from Le *et al.* 2010, as represented in the eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Seed).
Figure 19. A map of the putative regulatory sequences in the promoter region and 5' UTR of ASIL1. These sequences were determined by comparing the expression pattern among different transcriptional, translational fusion constructs and microarray data. The question mark represents the undetermined full-length promoter region of ASIL1.
Table II. Primers generated for transcriptional and translational fusions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASIL1</td>
<td>ASIL1-1324</td>
<td>CACCCCCAGTGCTTTAGGAGGTATC</td>
</tr>
<tr>
<td></td>
<td>ASIL1-1113</td>
<td>CACCCCAGCGATTTGAACAGTCTTTAGG</td>
</tr>
<tr>
<td></td>
<td>ASIL1-1028</td>
<td>CACCCGTTACAGAAGCCATATCAGC</td>
</tr>
<tr>
<td></td>
<td>ASIL1-677</td>
<td>CACCTATTGCTACTTTCTCTTTCCACGAC</td>
</tr>
<tr>
<td></td>
<td>ASIL1 F2 (-437)</td>
<td>CACCTTTCAAATGGATCTTCCTCT</td>
</tr>
<tr>
<td></td>
<td>ASIL1-299</td>
<td>CACCTTTTTCTAAAGCTTTCCACG</td>
</tr>
<tr>
<td></td>
<td>ASIL1-175</td>
<td>CACCTCTGTTTTTATTGGGCCTTG</td>
</tr>
<tr>
<td></td>
<td>ASIL1-94</td>
<td>CACCTTTTGAGACTTGAGACAACC</td>
</tr>
<tr>
<td></td>
<td>ASIL1+316</td>
<td>CTGAATCTCGTCGTCGTCCTCTC</td>
</tr>
<tr>
<td></td>
<td>A1TL1R (+1445)</td>
<td>GCTACTTTACATTGGCCGTAT</td>
</tr>
<tr>
<td>ASIL2</td>
<td>ASIL2-2472</td>
<td>CACCATGAGATCAGCAGAGTAGTG</td>
</tr>
<tr>
<td></td>
<td>A2TL 2F (-1912)</td>
<td>CACCACAGACCCCAGAAACC</td>
</tr>
<tr>
<td></td>
<td>A2TL 4F (-1552)</td>
<td>CACCTCAAGAATTTCGCCAGGTACAGGCC</td>
</tr>
<tr>
<td></td>
<td>ASIL2-1172</td>
<td>CACCGGTCCAACGGCCGAAAATGG</td>
</tr>
<tr>
<td></td>
<td>ASIL1-1150</td>
<td>CACCTCAGGAAGTGGAGAAACCAC</td>
</tr>
<tr>
<td></td>
<td>ASIL2+286</td>
<td>GGAGGAAGACGGATCGGTTGAATC</td>
</tr>
<tr>
<td></td>
<td>A2TL 2R (+1558)</td>
<td>ATTACCCAAATCGTTGTGTGT</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENT

I would like to thank Dr. Pablo Jenik for his guidance in the lab, his constant support throughout this project, the amazing Thanksgiving dinner with his lovely family, as well as previous students in the lab whose work led up to this project. I also want to acknowledge the Biology Department of Franklin & Marshall College for being the most awesome bunch of people I have ever had the chance to work with. This research was funded by Franklin & Marshall College, and I was the recipient of the Hackman Summer Scholarship for the year 2013 and 2014.

Figure 20. A dinosaur in its natural habitat. Photo courtesy by Dr. Pablo Jenik.
REFERENCES


Regulation of gene expression programs during Arabidopsis seed development: Roles of

Evolutionary history of trihelix family and their functional diversification. *DNA Res.*


e0085.

Deciphering gene regulatory networks that control seed development and maturation in

Stone, S., Kwong, L., Yee, K., Pelletier, J., Lepiniec, L., Fischer, R., Goldberg, R., Harada, J.
2001 LEAFY COTYLEDON2 encodes a B3 domain transcriptional factor that induces


Tanaka, M., Kikuchi, A., Kamada, H. 2008. The Arabidopsis histone deacetylases HDA6 and
HDA19 contribute to the repression of embryonic properties after germination. *Plant
Physiol.*: 146(1): 149-161.


