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ORIGINAL RESEARCH

A reevaluation of the role of the *ASIL* trihelix transcription factors as repressors of the seed maturation program

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Abstract

Developmental transitions are typically tightly controlled at the transcriptional level. Two of these transitions involve the induction of the embryo maturation program midway through seed development and its repression during the vegetative phase of plant growth. Very little is known about the factors responsible for this regulation during early embryogenesis, and only a couple of transcription factors have been characterized as repressors during the postgerminative phase. *Arabidopsis* 6b-INTERACTING PROTEIN-LIKE1 (ASIL1), a trihelix transcription factor, has been proposed to repress maturation both embryonically and postembryonically. Preliminary data also suggested that its closest paralog, ASIL2, might play a role as well. We used a transcriptomic approach, coupled with phenotypical observations, to test the hypothesis that ASIL1 and ASIL2 redundantly turn off maturation during both phases of growth. Our results indicate that, contrary to what was previously published, neither of the *ASIL* genes plays a role in the regulation of maturation, at any point during plant development. Analyses of gene ontology (GO)-enriched terms and published transcriptomic datasets suggest that these genes might be involved in responses during the vegetative phase to certain biotic and abiotic stresses.

KEYWORDS
Arabidopsis, embryo, embryonic maturation program, trihelix factor

1 | INTRODUCTION

Desiccated seeds are the main form of propagation of flowering plants. As one botanist said, they are "a baby plant, in a box, with its lunch" (Hanson, 2015). The "lunch" is composed of a set of storage products (oils, proteins, and/or starch, depending on the species) that will feed the germinating seedling until it can grow autonomously. Those storage products also make seeds the main source of food for humanity. Our model organism, *Arabidopsis thaliana*, belongs to the *Brassicaceae* family, whose seeds are commercially important, being the source of rapeseed, canola and mustard oil, and the condiment mustard. The accumulation of storage products (seed filling) depends on the activation of the maturation program during seed development (Baud et al., 2008). This program starts during mid-embryogenesis, following (and sometimes partly overlapping) the patterning phase, which is when the tissue types and axes are laid out. In *Arabidopsis*, the first indication of maturation, seed greening, starts at the heart stage of development, and seed filling commences at the late heart stage (O'Neill et al., 2019). The main positive regulators that induce the seed maturation program are a small set of transcription factors belonging to two different protein families: the B3 domain-containing LEAFY COTYLEDONS2 (LEC2), ABA INSENSITIVE3 (ABI3), and FUSCA3 (FUS3), and the NF-YB factors LEC1 and LEC1-LIKE (L1L). From their initials, they are collectively called the *LAFL* factors. The genes encoding these proteins are not entirely redundant: the phenotypes of the corresponding *lafl* mutants are only partially overlapping (reviewed by Lepiniec et al., 2018). These positive regulators have complicated, organ-specific patterns of cross- and self-regulation (To et al., 2006). Several lines of evidence have also identified transcription factors of the bZIP, MYB, and MADS-box families as probable positive regulators of maturation (Alonso et al., 2009; Bensmihen et al., 2002; Wang et al., 2009; Yamamoto et al., 2009; Zhang et al., 2009; Zheng et al., 2009). This gene regulatory network involved in seed maturation is at least partially conserved in all seed plants (Verdier & Thompson, 2008; Vicente-Carbajosa & Carbonero, 2005).

The seed maturation program, which involves dramatic changes to the embryonic transcriptome (Belmonte et al., 2013), is tightly regulated. This program needs to be repressed during the first part of embryo development, as well as during and after germination. This second stage corresponds to another major developmental switch, the embryo-to-vegetative transition. A number of factors are known to be involved in this latter process. Many were isolated in genetic screens that searched for the expression of embryonic traits (such as seed storage proteins, SSPs) in seedlings. This ectopic expression can lead, in some cases, to growth arrest (Zhang & Ogas, 2009). Most of these repressors are chromatin and nucleosome modifiers, including Polycomb Group (PcG) proteins, members of the SWI/SNF2 complex, and histone deacetylases and methyltransferases (Bouyer et al., 2011; Kim et al., 2012; Molitor et al., 2014; Tanaka et al., 2008; Tang et al., 2008, 2012; Wang et al., 2016; Zhang et al., 2012). However, none of the proteins encoded by these genes are DNA sequence-specific, and they are presumably recruited to the promoters of the embryonic genes by transcriptional regulators. Only a handful of transcription factors are known to target and regulate maturation genes postembryonically. The best studied are the redundant B3-domain proteins VP1/ABSCISIC ACID INSENSITIVE 3-LIKE/HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE 2 (VAL1/HSI2) and VAL2/HSL1. These proteins indirectly repress the SSP genes, *LEC1*, *LEC2*, and *FUS3*, by recruiting the histone deacetylase HDA19 and PcG proteins and preventing the expression of the activator *AGAMOUS-LIKE15* (*AGL15*) (Chen et al., 2018; Chhun et al., 2016; Gao et al., 2009; Jia et al., 2013; Suzuki et al., 2007; Tsukagoshi et al., 2007; Yang et al., 2013; Zhou et al., 2013). The brassinosteroid signaling pathway acts together with VAL1 to repress of *AGL15* (Ruan et al., 2021). Another repressive transcription factor is SCARECROW-LIKE15 (SCL15), which acts through its interaction with the histone deacetylase HDA19 (Gao et al., 2015).

At least one more transcriptional regulator, the focus of the present study, was proposed to repress maturation genes during vegetative development: *Arabidopsis* 6b-INTERACTING PROTEIN-LIKE1 (ASIL1, encoded by *At1g54060*). This factor was first found as a protein that bound, in a yeast assay, the promoter of the SSP *At2S3*. The researchers then isolated a putative null allele, *asil1-1*; 14-day-old *asil1-1* seedlings ectopically expressed several of the maturation-related genes, SSPs, and storage triacylglycerols (TAGs) (Gao et al., 2009). ASIL1 belongs to the plant-specific trihelix family of transcription factors (also known as GT-factors), which are characterized by a DNA-binding domain containing three helices and a long alpha-helical domain at the C-terminus that is likely involved in protein dimerization (Kaplan-Levy et al., 2012). In *Arabidopsis*, there are 28–30 genes belonging to this family, grouped into five clades. *ASIL1* is in the SIP1 clade, along with 10 other genes of mostly unknown function (Kaplan-Levy et al., 2012; Yasmeen et al., 2016). *ASIL1*'s closest homologue, in sequence conservation and gene structure, is *ASIL2* (*At3g14180*). The encoded proteins share 52% sequence identity and 65% similarity (Figure S1), and neither gene contains introns. There are *ASIL2* orthologues throughout the land plants (including nonseed plants), suggesting an ancestral role unrelated to seed maturation (Barr et al., 2012). In contrast, *ASIL1* arose from *ASIL2* by gene duplication in the *Brassicaceae* (Barr et al., 2012). There are some sequence differences between the trihelix domains of ASIL1 and ASIL2, but these fall outside of the predicted DNA-binding helix, helix 3 (Barr et al., 2012; Nagata et al., 2010) (Figure S1). Therefore, it is possible that ASIL2 binds to the same DNA sequence as ASIL1 (the GT-box [GTGAA/CT/C], Gao et al., 2009) and that these genes have redundant functions. Both genes are expressed throughout the plant, as can be seen by examining the transcriptomic datasets available through the eFP Browser (Winter et al., 2007).

Much less is understood about the pathways involved in keeping off the maturation program during the first part of embryogenesis and how much these pathways have in common with those repressing embryonic traits after germination. The chromatin remodeler CHR5 is involved in opening the chromatin of the maturation genes (Shen et al., 2015). MicroRNAs are also known to have a role: mutations in *DICER-LIKE1* (*DCL1*) that reduce or eliminate their biosynthesis induce

precocious expression of all the maturation genes, as early as the early globular stage (Nodine & Bartel, 2010; Willmann et al., 2011). It is not known which miRNA targets are responsible for this regulation, but members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* family (in particular *SPL10* and *11*) have been proposed (Nodine & Bartel, 2010). Recent research suggests that the *VAL* genes are not involved in regulating maturation in embryos (Jia et al., 2013; Schneider et al., 2016) but that they instead regulate the setting of the vernalization program (Tao et al., 2019). However, there is evidence for a role in embryo maturation for *ASIL1* and *ASIL2*. Gao et al. (2011) extended their work on *asil1-1* by looking at mutant siliques. They found elevated levels of *LAFL* and SSP genes at 4–6 days after pollination (DAP) (about heart to early torpedo stages), earlier than what was detected in the wild type, and concluded that *ASIL1* prevented their precocious expression. We found that *ASIL1* and *ASIL2* were downregulated in *dcl1-15* torpedo stage embryos and presented data that indicated that *asil1* and *asil1 asil2* mutants accumulated chlorophyll and *At2S3* too early (Willmann et al., 2011). These studies suggested that *ASIL1*, and possibly *ASIL2*, may repress maturation both during and after embryogenesis.

The hypothesis driving the present study, based on what was known at the time (Barr et al., 2012; Gao et al., 2009, 2011; Willmann et al., 2011), was that *ASIL1* and *ASIL2* redundantly repress the maturation program during early embryogenesis, as well as after germination. To test this hypothesis, we performed transcriptomic analyses of single and double mutant embryos, and of single and double mutant seedlings. We validated our results with other expression and phenotypic data. Contrary to our expectations, and to previously published research (Gao et al., 2009, 2011; Willmann et al., 2011), we found no evidence that the *ASIL* genes regulate the embryonic maturation program. Subsequent analyses suggested that the *ASIL* genes might have a role in the response to biotic and abiotic stresses. Further research will be required to confirm and expand on these latter findings.

2 | EXPERIMENTAL PROCEDURES

2.1 | Plant material and growth conditions

Plants were grown at 22°C with 16 h of light in a growth chamber (Conviron). Seeds were planted directly in soil (Fafard-2, SunGro Horticulture) supplemented with Osmocote Plus 15-9-12 fertilizer. When necessary, seeds were germinated on plates with 1/2× Murashige–Skoog (MS) medium (2.2 g/L MS salts, 1× Gamborg's vitamins, 0.5 g/L MES, 10 g/L sucrose, and 7.5 g/L tissue culture agar, pH 5.7) (all reagents from Sigma unless specified otherwise) and grown at 22°C in a lighted incubator (Percival Scientific).

Several of the mutants and reporters used have been described previously: *asil1-1* (Gao et al., 2009), *asil2-1* (Willmann et al., 2011), *asil2-2* (Koryachko et al., 2015), *hsl1-1* (Tsukagoshi et al., 2007), *val1-1* and *val1-2* (Suzuki et al., 2007), and *At2S3p:GFP* (Kroj et al., 2003). All mutants are in a Columbia background, except for *asil2*-2, which is in a Landsberg *erecta* background. Seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) (*Ler* [CS28445], Col [CS28166], *asil1-1* [SALK_124095C], *asil2-1* [SAIL_258_F06.V2], *hsl1-1* [SALK_059568C], *val1-2* [SALK_088606C]), Rob Martienssen (Cold Spring Harbor Labs) (*asil2-2* [ET8777]), Masaharu Suzuki (U. of Florida) (*val1-2 val2-1*), and François Parcy (Biosciences and Biotechnology Institute of Grenoble) (*At2S3p:GFP*). Plants were PCR-genotyped as needed, using the primers listed on Table S1.

2.2 | Construction of *35S* lines

The coding sequences for *ASIL1* or *ASIL2* were PCR-amplified from UNI clone plasmids (Yamada et al., 2003) obtained from the ABRC (*U09441* for *ASIL1* and *U19643* for *ASIL2*) and then inserted into p*ENTR/D-TOPO* (Thermo Fisher Scientific) (see primer sequences in Table S1). These plasmids were then recombined into p*GWB2* (Nakagawa et al., 2007) using LR Clonase II (Thermo Fisher Scientific) to generate p*35S:ASIL1* and p*35S:ASIL2*. The plasmids were transformed into *Agrobacterium tumefaciens* GV3101. *Arabidopsis* plants (Columbia background) were then transformed using the floral dip method (Clough & Bent, 1998). The selection of transgenic plants was done on the MS plates described above, with the addition of 50 μg/ml kanamycin and 100 μg/ml ampicillin.

2.3 | Microscopy and histochemistry

All our observations were carried out on a Leica DMRB microscope equipped with ProgRes MFcool and ProgRes C5 cameras (Jenoptik). Images were acquired with the ProgRes software and processed as necessary (overall brightness, contrast). Figures were assembled using Adobe Photoshop CC 2018.

For the clearing of whole seeds, siliques were opened with fine tweezers and the seeds placed on a slide with Hoyer's solution, as described in O'Neill et al. (2019). Cleared seeds were observed using differential interference contrast (DIC) optics. Embryos were staged according to Jürgens and Mayer (1994).

To analyze GFP expression, embryos were dissected out of the seeds with needles directly onto a slide with a drop of 5% sucrose (Fisher Scientific) and observed immediately. For GFP and chlorophyll fluorescence, the excitation/emission wavelengths were 480/535 and 560/645 nm, respectively.

For the detection of lipids, seedlings were grown on MS plates for 12 days at 22°C and then transferred to plates supplemented with 50 μM ABA for 2 days. The seedlings were then stained overnight at room temperature in a Fat Red solution (0.1% Sudan Red 7B) (Brundrett et al., 1991) and rinsed twice with water before observation.

Seedlings were imaged on a Leica M80 dissecting microscope equipped with a Leica IC80 HD camera.

2.4 | Total RNA extraction for RNAseq

For the RNAseq experiment, seeds were germinated and grown for 14 days on MS plates without sucrose; 100 mg of tissue (in triplicates per genotype) were frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Minikit (QIAGEN), per the manufacturer's directions. The eluted RNA was treated with DNase I (QIAGEN) for 25 min at room temperature. The treated RNA was precipitated overnight with ethanol and resuspended in 21.725 μl of water plus 0.225 μl of RNase Out (Thermo Fisher Scientific). The average yield was 1.2 μg/μl (range: 0.6–1.9 μg/μl).

In the case of embryos, we hand-pollinated flowers and collected siliques at 5 DAP. We hand-dissected the embryos from the seeds under a Leica M165C dissecting microscope using tungsten needles (Electron Microscopy Sciences) in 20 μl of 5% sucrose in RNase-free water. We collected ~200 late heart embryos per replicate (three replicates per genotype) in 400 μl of 5% sucrose on ice. We then washed and concentrated the embryos using a modification of the protocol by Perry and Wang (2003). We layered the embryo suspension on a 1-ml cushion of 9:1 5% sucrose: Percoll (Amersham) and centrifuged for 10 min at 1,000 × ***g***. We resuspended the embryo pellet in 200 μl of 5% sucrose and ran it through a cushion again. We washed the Percoll by resuspending the embryo pellet in 200 μl of 5% sucrose, then centrifuged for 2 min at 1,000 × ***g***, and froze the pellet in liquid nitrogen. Total RNA was purified using PureLink RNA reagent (Thermo Fisher Scientific), according to the manufacturer's directions, and resuspended in 10 μl of RNase-free water. The average yield was 51 ng/μl (range: 8–88 ng/μl).

In all cases, RNA purity and integrity were confirmed using an Agilent 2100 Bioanalyzer (Eukaryote Total RNA Pico assay).

2.5 | Construction of libraries and RNAseq

Embryo RNA-Seq libraries were prepared using Nugen Ovation RNA-Seq Systems for Model Organisms starting with 70–90 ng of total RNA. Seedling RNA-Seq libraries were constructed from 5 μg of total RNA, using the library preparation protocol described by Kumar et al. (2012), with the exception of the RNA and mRNA isolations (polyA RNA was isolated from total RNA as described in the Supplementary Methods 2 of Kumar et al. (2012). The NEXTflex ChIP-Seq Barcodes (BioScientific) were used as Illumina-compatible adapters. Libraries were quantified using Quant-iT PicoGreen dsDNA Reagent (Grand Island, NY) and a Nanodrop ND-3300 instrument (Thermo Fisher Scientific) and sequenced on a HiSeq 4000 sequencer (Illumina). The sequencing was carried by the DNA Technologies and Expression Analysis Core at the UC Davis Genome Center.

Sequenced reads were demultiplexed, quality-filtered, and reads corresponding to rRNA sequences were removed. The resulting filtered reads were mapped to *Arabidopsis* primary transcripts (TAIR10) using bowtie v0.12.7 with parameters -v 2 -5 10 -3 40 -m 1 –best --strata.

We used the EdgeR package (v3.10.5) to obtain normalized expression values using the Trimmed Mean of M-values (TMM) method and to identify differentially expressed genes (DEGs) between the different genotypes (FDR < 0.05, Robinson et al., 2010).

2.6 | Reverse transcriptase-quantitative polymerase chain reaction

To measure the levels of mRNAs using RT-qPCR, total RNA was extracted from seedlings as described above. For embryos, flowers were tagged at anthesis and then siliques of the appropriate genotype and age were collected and frozen in liquid nitrogen. Biological triplicates were used in all cases. Total RNA was extracted from the siliques using the PureLink RNA reagent; 0.5–1 μg of total RNA were converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's directions, in a 20-μl volume. The resulting cDNA was diluted with 100 μl of water, and 2 μl were used as template for the qPCR reactions. These 20-μl reactions also contained 0.5 μM of each primer (see Table S1 for primer list) and 10 μl of iTaq Universal SYBR Green Supermix (Bio-Rad). Reactions were carried out in a Bio-Rad CFX96 thermocycler, with three technical replicates per reaction. Relative mRNA levels were calculated using the $2^{-\Delta Cq}$ method, with *EIF4A1* (*At3g13920*) as the normalizing control. Graphs were generated in Microsoft Excel. Statistical analyses were done in SPSS v25 (IBM).

2.7 | Measurement of GSLs

The plants were grown in long day conditions in a chamber with 16-h light at 100- to 120-mE light intensity, with two complete independent replicates. Eight plants were measured per genotype and replicate. Four weeks after sowing, one of fully mature leaves of each plant was harvested and stored in 90% (v/v) methanol at −20°C to inhibit enzymatic breakdown of glucosinolates (GSLs) before extraction. The GSLs were extracted and analyzed by HPLC according to previously described methods (Kliebenstein et al., 2001).

2.8 | Accession numbers

Accession numbers (gene identification numbers) for all genes analyzed in the paper are listed in Tables 2 and 3.

RNAseq sequence reads were deposited in the Gene Expression Omnibus (GEO) repository with accession numbers GSE163006 (seedlings) and GSE163007 (embryos) (GSE163009 superseries).

3 | RESULTS

3.1 | Expression of the *ASIL* genes during embryogenesis and characterization of alleles used in this work

Part of our initial hypothesis was that the *ASIL* genes repress the maturation program during early embryogenesis, before the heart stage. A possible mechanism for this action would be that the *ASIL* genes are expressed at higher levels during early development and decrease later. Previous reports about the levels of *ASIL1* suggested minor oscillations during embryogenesis, with lowest levels at 4–6 DAP (around the start of maturation) (Gao et al., 2009). To further assess the levels of *ASIL1* and *ASIL2*, we looked at the data generated by two experiments that analyzed the transcriptomes at several embryonic stages (Belmonte et al., 2013; Hofmann et al., 2019). In both cases, it can be seen that *ASIL1* and *ASIL2* are expressed at average levels throughout embryogenesis and that their transcripts are somewhat lower later in development (Figure 1a,b; Figure S1A,B). But there is no clear anticorrelation, as it would be expected, with the expression of *LAFL* genes (like *FUS3* and *LEC2*) or SSPs (*At2S3*) (Figure 1a,b; Figure S1A,B). While the *ASIL* transcripts do not behave in the ways we anticipated, and most of the regulation of gene expression is at the transcriptional level (Li & Biggin, 2015), there are some known cases where posttranscriptional regulation is crucial. In an example relevant for the process of maturation, *FUS3* mRNA increases during

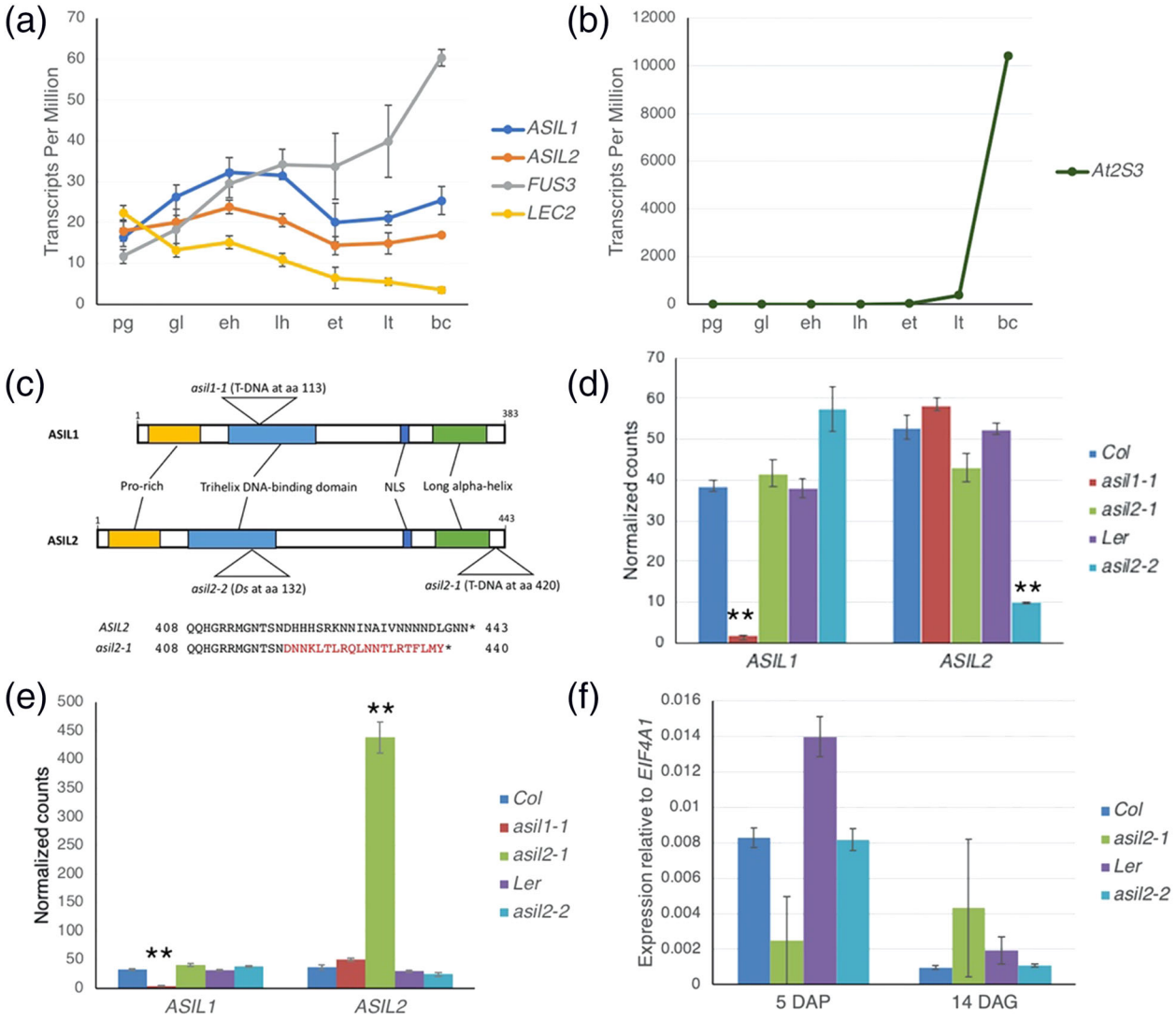

FIGURE 1 Characterization of the *asil* alleles. (a) Levels of expression of the *LAFL* genes in the embryo proper at different developmental stages. (b) Expression of a seed storage protein gene in the embryo proper at different developmental stages. Data from Hofmann et al. (2019). Stages: pg: preglobular, gl: globular, eh: early heart, lh: late heart, et: early torpedo, lt: late torpedo, bc: bent cotyledon. (c) Schematic of the ASIL1 and ASIL2 proteins with the location of the insertions in the mutant alleles and sequence of the C-terminal portion of the *ASIL2* and *asil2-1* alleles. (d) Expression of *ASIL1* and *ASIL2* in wild type and mutant 5 DAP seeds. (e) Expression of *ASIL1* and *ASIL2* in wild type and mutant 14 days after germination (DAG) seedlings. (d) and (e) represent data from the RNAseq experiments (three biological replicates). (f) Expression of *ASIL2* in wild type and mutant 5 DAP seeds and 14 DAG seedlings, measured by RT-qPCR (three biological replicates). Analysis of variance (ANOVA) with Tukey's post hoc test $^{*}p < .05$, $^{**}p < .01$. Error bars: in a,b,f: ±SEM; in d,e: ±SD

embryonic development (Figure 1a; Figure S1A), but the protein is degraded after the early torpedo stage (Lu et al., 2010). Therefore, it is possible that there is a layer of regulation of the ASIL proteins that we cannot see in these data. Yet, another possible mechanism would be that, while the level of the *ASIL* mRNAs does not change, the regulation could be exerted by an unknown interacting partner of varying expression.

To study the effects of the *ASIL* genes during embryogenesis, we used several alleles that had been isolated previously but not necessarily fully characterized (Koryachko et al., 2015; Willmann et al., 2011). For *ASIL1*, we worked with *asil1-1*, the same allele studied by Gao et al. (2009). This allele has a T-DNA insertion between helices 1 and 2 of the DNA-binding domain (Figure 1c, Figure S2). The insertion leads to a significant reduction in transcript levels (Gao et al., 2009) (confirmed in the RNAseq experiment described in detail in a later section; see Figure 1d,e) and to a truncated transcript (Figure S3A; (Gao et al., 2009). This indicates that it is likely a null allele. For *ASIL2* we isolated two alleles: *asil2-1* and *asil2-2*. *asil1-1* and *asil2-1* are in the Columbia (Col) background, whereas *asil2-2* is in the Landsberg *erecta* (L*er*) background. *asil2-1* has a T-DNA insertion at the very end of the coding sequence. The insertion would lead to a replacement of the C-terminal 23 aminoacids (Figure 1c, Figure S2). This region of the protein has been shown, in other members of the trihelix family, to mediate dimerization (Ayadia et al., 2004; Hiratsuka et al., 1994; Lam, 1995). The *ASIL2* mRNA levels in *asil2-1* are either the same or higher than the wild type (Figure 1d–f), and the transcript appears to be longer (Figure S3B). These facts, together with the transcriptomic data discussed in detail later, suggest *asil2-1* is a peculiar, possibly dominant-negative, allele. *asil2-2* has an insertion of a modified *Ds* element (Sundaresan et al., 1995) between helices 2 and 3 of the trihelix domain (Figure 1c, Figure S2). The levels of its mRNA are either similar to the wild type or reduced (Figure 1d–f). Like for *asil1-1*, the position of the insertion suggests that *asil2-2* is a null allele. This is supported by the distribution of RNAseq reads that indicate a truncated transcript (Figure S3B). Neither *ASIL1* nor *ASIL2* contains introns, making it unlikely that the insertions are removed from the mRNA by splicing (Figure S3A,B). We did not observe changes in the amount of *ASIL1* mRNA in *asil2* mutants, and vice versa, indicating that these genes do not regulate each other (Figure 1d–f).

3.2 | Changes in the levels of activity of the *ASIL* genes do not alter embryonic development

We and others previously reported that patterning did not appear to be affected in embryos with mutations in *asil1-1*, *asil2-1*, or *asil1-1 asil2-1* (Gao et al., 2009; Willmann et al., 2011). This is also the case for embryos with mutations in *asil2-2* or *asil1-1 asil2-2*. We also evaluated the rate of development in these lines. *Arabidopsis* embryos within a silique develop fairly synchronously, and each silique shows a narrow range of stages typical of the number of days that have elapsed since pollination (DAP) (Jürgens & Mayer, 1994; O'Neill et al., 2019). Our previous qualitative observations implied that there were no differences in rate between the wild type and the different mutant embryo combinations (Willmann et al., 2011). To confirm that, we quantitated the percentage of embryos at each stage at 5 DAP and saw almost no discrepancies between genotypes. The distribution of stages was only different for *asil1-1 asil2-1* embryos (χ^2 test, $p = .00004$), which develop slightly faster (Figure 2a). In embryos overexpressing *ASIL1* or *ASIL2*, we did not see differences in

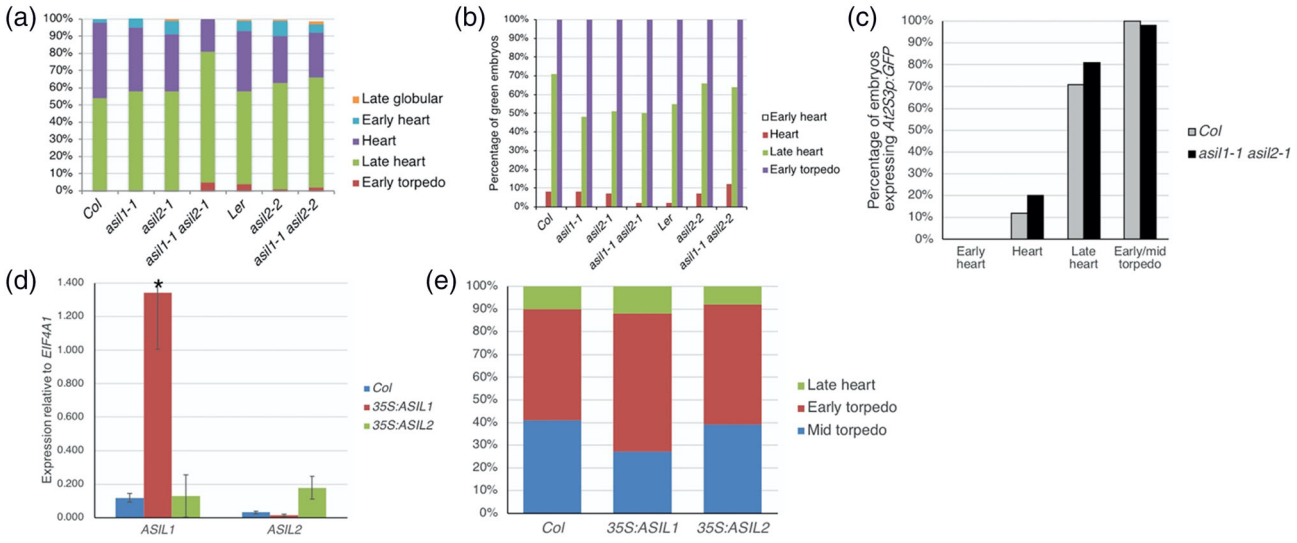

FIGURE 2 Embryonic phenotypes of *asil* mutants and overexpressors. (a) Percentages of embryos at different stages in 5 DAP siliques for the different genotypes ($n = 120–160$ embryos per genotype). (b) Percentage of embryos at different stages that are green for the different genotypes ($n = 60–250$ embryos per stage per genotype). (b,c) Percentage of embryos at different stages that express *At2S3p:GFP* for Col and *asil1-1 asil2-1* ($n = 35–88$ embryos per stage per genotype). For (b) and (c), there were no significant differences between wild type and mutants (Fisher's exact test). (d) Expression of *ASIL1* and *ASIL2* in overexpressor lines, measured by RT-qPCR (biological triplicates). Error bars: ±SEM. *$p < .05$ (*t* test). For *ASIL2* in *35S:ASIL2*, $p = .08$ (*t* test). (e) Percentages of embryos at different stages in 6 DAP siliques for overexpressor lines ($n = 62–132$ embryos per genotype, two plants per genotype)

morphology either, and only *35S:ASIL1* embryos developed a little bit slower (χ^2 test, $p = .017$) (Figure 2e). The combination of these data indicates that lower or higher levels of *ASIL* gene activity have no or very minor effects on the progression of embryogenesis or the patterning of the embryo. This is not entirely surprising, because we have shown that it is possible for embryos to have an earlier or later onset of the maturation program without a significant effect of embryonic morphology, as is the case with certain combinations of *dcl1* alleles or some *lafl* alleles (O'Neill et al., 2019; Willmann et al., 2011).

3.3 | Mutations in the *ASIL* genes do not affect the embryonic maturation program

To test our hypothesis that the *ASIL* genes repressed maturation in the embryo, we took a comprehensive look at the transcriptome of *asil1-1* and *asil2* single and double mutant embryos. The predictions were that there would be an earlier onset of the maturation program, as described by Gao et al. (2011) in *asil1-1*, and an even more marked increase in the corresponding transcripts in the double mutants, indicating redundancy. To evaluate the embryonic transcriptomes, we decided to use late heart stage embryos at which point the maturation program is just beginning (O'Neill et al., 2019). We reasoned that, if maturation started earlier in the mutants, we would see significantly higher levels of maturation-related transcripts at this stage. We performed RNAseq on manually isolated embryos from 5 DAP siliques, which are enriched in the late heart stage (Figure 2a). We collected three samples each of embryos from *asil1-1*, *asil2-1*, *asil2-2*, *asil1-1 asil2-1*, *asil1-1 asil2-2*, and the corresponding wild types (Col and L*er*). Each biological replicate consisted of ~200 embryos. After data processing, one of the *asil1-1 asil2-1* and one of the *asil1-1 asil2-2* replicates were removed, because they had a high percentage of rRNA and a low percentage of reads mapping to the genome. The Pearson correlation coefficients between pairs of replicates was .96 or higher. For the complete list of genes and their expression, see Dataset S1.

To our surprise, there were very few DEGs between the mutants and the corresponding wild types (summarized in Table 1; for lists, see Datasets S2A-L). There were actually more differences between the Col and L*er* wild types than with any of the mutants. The *asil1-1* and *asil2* mutants seem to have very little effect on embryo development, at least at this stage. We focused our attention on the genes related to maturation (*LAFL* genes and *WRINKLED1* [*WRI1*; the main activator of the lipid metabolism], storage proteins, oleosins), which, according to Gao et al. (2011), were expected to be significantly upregulated at this timepoint. Surprisingly, none of these genes was expressed at higher levels than in the wild type (except for, marginally, *OLEOSIN2* [OLEO2] in *asil1-1*) (Table 2). These results appear to indicate that the *ASIL* genes are not repressors (or activators) of the maturation program in embryos.

We validated the RNAseq data using RT-qPCR. We decided to focus only on the single and double mutants for the presumed null alleles (*asil1-1* and *asil2*-2), because they are more likely to uncover the wild type function of the genes. We measured the master regulators (the *LAFL* genes: *FUS3*, *LEC2*, *LEC1*, and *ABI3*) and three of the genes encoding products of maturation (*At2S3*, *CRUCIFERIN A1* [CRA1], and *OLEO1*). Because the RT-qPCR data from Gao et al. (2011) was from a pool of 4–6 DAP siliques, we decided to test not just the 5 DAP timepoint (heart and late heart embryos), when maturation is just starting, but also 3 DAP (globular stages) when we expect little to no expression of most of these genes and 6 DAP (late heart to mid-torpedo stages), when maturation products begin to accumulate. We also measured the mRNAs of maturation products at 10 DAP (bent cotyledon stage), mid-maturation. We opted to extract RNA from pools of siliques (three independent pools per genotype), because of the difficulty of isolating the number of embryos required for this technique, especially for earlier stages. All the genes analyzed are only expressed in the seed, so the silique tissue is not expected to interfere with the measurements. It led, however, to more variable, noisier data, as can be appreciated in Figure 3.

Early in development (3 DAP), we only tested the *LAFL* genes, because the maturation products are not expected to be expressed, even in *asil1-1*. At the globular stages, these genes were barely expressed, as expected (Gao et al., 2011) (see the *y*-axis scale), but there was statistically significant upregulation for all four of them in *asil1-1*, and for *ABI3* in *asil1-1 asil2-2* (Figure 3a). However, even in *asil1-1*, the overall levels of expression were very low at this stage.

The results at 5 DAP were mostly consistent with those of the RNAseq, with almost no genes expressed at significantly different levels between the mutants and the wild type siliques (the exceptions were *ABI3* and *OLEO1* in *asil2-2* and *FUS3* in *asil-1 asil2-2*) (Figure 3b, d). There were some genes in *asil2-2* (*FUS3*, *LEC2*, *CRA1*) or *asil1-1 asil2-2* (*LEC1*, *LEC2*, *CRA1*) backgrounds that showed a higher mean of expression, but the difference did not reach the cutoff for statistical significance (analysis of variance [ANOVA] followed by Tukey's post hoc test).

At 6 DAP (Figure 3c,e) there were, again, mostly no differences in expression between wild type and mutants, with only *At2S3 and OLEO1* being elevated in the double *asil1-1 asil2-2*. Just like at 5 DAP, there were genes with higher means, but that did not meet the statistical criteria for difference (*FUS3* in *asil1-1*, *CRA1*, *OLEO1* and *At2S3* in *asil2-2*, *FUS3*, *LEC1*, *ABI3*, *CRA1* in *asil1-1 asil2-2*).

Finally, at mid-maturation (10 DAP), when the amounts of transcript for storage products are very high (as reflected in the *y*-axis scale), we found no differences between the wild type and the mutants (Figure 3f). This was consistent with the results of Gao et al. (2011) for *asil1-1* versus wild type in their 7–9 or 10–12 DAP silique pools.

Taken together, the mRNA expression data indicate that mutations in one or both of the *ASIL* genes do not lead to an early onset of the maturation program in embryos. Any minor differences in expression observed in the mutants very early (at 3 DAP), if real, have no impact on the expression of maturation genes later on or in the accumulation of transcripts for storage products mid-maturation. And given that the RT-qPCR was done on RNA extracted from whole siliques, rather than isolated embryos, it is not possible to know

TABLE 1 Number of DEGs in mutant late heart embryos and in mutant 14 DAG seedlings

Number of DEGs in late heart embryos				
Comparison		**Nuclear-encoded genes**	**Mitochondria-encoded genes**	**Chloroplast-encoded genes**
Col vs. L*er*	Up	1,447	9	1
	Down	990	14	0
asil1-1 vs. Col	Up	107	3	0
	Down	44	0	0
asil2-1 vs. Col	Up	2	0	0
	Down	21	0	0
asil2-2 vs. L*er*	Up	49	0	0
	Down	43	0	0
asil1-1 asil2-1 vs. Col	Up	255	0	1
	Down	117	1	0
asil1-1 asil2-2 vs. (Col + L*er*)	Up	123	0	0
	Down	38	0	0
Number of DEGs in 14 DAG seedlings				
Comparison		**Nuclear-encoded genes**	**Mitochondria-encoded genes**	**Chloroplast-encoded genes**
Col vs. L*er*	Up	3,537	0	9
	Down	3,560	11	2
asil1-1 vs. Col	Up	1,808	0	0
	Down	1,884	17	0
asil2-1 vs. Col	Up	4,168	12	36
	Down	3,781	1	1
asil2-2 vs. L*er*	Up	549	9	4
	Down	832	0	0
asil1-1 asil2-1 vs. Col	Up	3,920	0	24
	Down	3,587	12	1
asil1-1 asil2-2 vs. (Col + L*er*)	Up	1,289	0	0
	Down	761	5	0

Note: The *asil1-1 asil2-2* numbers reflect only genes that were misregulated when compared with both Col and L*er*, to avoid genes that changed because of the accession differences.
Abbreviations: DAG, days after germination; DEGs, differentially expressed genes.

whether the small differences we observed were coming from the embryo or from other seed compartments, which also express some of the maturation genes, as well as *ASIL1* and *ASIL2* (Figure S1C-E) (Belmonte et al., 2013).

The expression data, however, contradict our previous findings that suggested precocious maturation in the mutants (Willmann et al., 2011). In that work, we showed that *asil1-1*, *asil2-1*, and *asil1-1 asil2-1* accumulated chlorophyl earlier than wild type (although the penetrance of the phenotype was not very high) and that *asil1-1 asil2-1* embryos showed *At2S3p:GFP* expression at early heart or heart stage, as opposed to late heart stage in the wild type. In light of the conflicting results, we decided to redo those experiments more thoroughly.

In Willmann et al. (2011), we observed the chlorophyll in the embryos by imaging cleared whole seeds for red fluorescence. Our subsequent, and much more extensive, work on the greening of embryos (O'Neill et al., 2019) taught us that that was not a trustworthy technique, because of the difficulty of distinguishing fluorescence in the embryo versus the endosperm and variation due to the intactness of the seed and other factors. This led to very inconsistent results from batch to batch, except in cases where the early accumulation of chlorophyll was dramatically high (like in the *dcl1* embryos). We found instead that looking at the actual greening of the embryo under DIC optics was a much more reliable and robust indicator of chlorophyll levels. Thus, we reanalyzed the progression of embryo greening in all the genotypes used for RNAseq with this technique and found it not to differ from the corresponding wild types (Figure 2b). These newer findings are consistent with the transcriptomic data.

Regarding the expression of the reporter *At2S3p:GFP*, in Willmann et al. (2011), we had only analyzed a handful of embryos per stage, from a segregating population in an *asil1-1 ASIL2/asil2-1*

TABLE 2 Expression of maturation genes in late heart embryos

AGI ID	Gene symbol	Col	*asil1-1*	*asil2-1*	*asil1-1 asil2-1*	Ler	*asil2-2*	*asil1-1 asil2-2*
Master regulators of maturation								
At3g24650	***ABI3***	265 ± 34	232 ± 33	258 ± 18	213 ± 24	287 ± 32	295 ± 12	232 ± 29
At3g26790	***FUS3***	38 ± 2	43 ± 7	46 ± 4	42 ± 6	54 ± 2	51 ± 11	44 ± 7
At1g21970	*LEC1*	119 ± 13	140 ± 7	149 ± 22	144 ± 19	129 ± 19	138 ± 26	128 ± 7
At1g28300	***LEC2***	19 ± 2	20 ± 2	20 ± 5	20 ± 0.4	18 ± 1	21 ± 4	19 ± 4
At5g47670	*L1L*	30 ± 15	32 ± 10	46 ± 11	26 ± 3	24 ± 3	29 ± 6	26 ± 2
At3g54320	*WRI1*	115 ± 20	126 ± 11	134 ± 14	114 ± 2	124 ± 19	133 ± 20	129 ± 14
Seed storage proteins								
At5g44120	*CRA1*	10 ± 0.7	11 ± 3	5 ± 2	9 ± 2	4 ± 1	5 ± 2	7 ± 2
At1g03880	*CRB*	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0 ± 0	0.3 ± 0.5
At4g28520	***CRC***	0.8 ± 0.6	0.5 ± 0.4	0.1 ± 0.1	0.2 ± 0.3	0.4 ± 0.5	0.3 ± 0.3	0.6 ± 0.6
At4g27140	*At2S1*	5 ± 1	5 ± 1	4 ± 1	3 ± 0.8	3 ± 0.6	3 ± 1	5 ± 1
At4g27150	*At2S2*	2 ± 1	1 ± 0.9	0.6 ± 0.3	0.3 ± 0.2	0.3 ± 0.4	0.4 ± 0.3	0.4 ± 0.6
At4g27160	*At2S3*	0.5 ± 0.4	0.6 ± 0.6	0.6 ± 0.7	0.1 ± 0.1	0.5 ± 0.4	0.3 ± 0.3	0.2 ± 0.2
At4g27170	*At2S4*	0.1 ± 0.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
At5g54740	*At2S5/At2S-like*	0 ± 0	0.6 ± 0.6	0.5 ± 0.4	0.1 ± 0.1	0.3 ± 0.4	0.6 ± 0.3	0.2 ± 0.0
Oleosins								
At3g01570	*S1/OLEO5*	9 ± 4	11 ± 1	12 ± 1	7 ± 0.5	8 ± 1	6 ± 1	13 ± 2
At3g27660	*S2/OLEO4*	24 ± 1	14 ± 3*	25 ± 4	16 ± 1	17 ± 4	18 ± 5	18 ± 10
At4g25140	*S3/OLEO1*	26 ± 13	18 ± 8	30 ± 11	9 ± 0.2**	16 ± 3	22 ± 4	16 ± 4^^
At5g40420	***S4/OLEO2***	46 ± 13	48 ± 11*	64 ± 16	34 ± 3	36 ± 6	38 ± 8	42 ± 9
At5g51210	*S5/OLEO3*	161 ± 32	169 ± 33	207 ± 41	120 ± 16**	99 ± 14	96 ± 5	99 ± 15^^
At2g25890	*SM3*	3 ± 0.6	4 ± 1	4 ± 1	2 ± 0.1	3 ± 1	4 ± 1	3 ± 1

Note: Expression as normalized counts ± SD (rounded up to the nearest unit, except for those <1). Genes in bold: genes reported upregulated at 4–6 DAP in Gao et al. (2011). *FUS3* is down ($p < .01$) in Col compared with L*er*; *CRA1*, *S5/OLEO3*, and *S3/OLEO1* are upregulated ($p < .01$) in Col compared with L*er*. *$p < .05$ compared with corresponding WT. **$p < .01$ compared with corresponding WT. ^^$p < .01$ different from Col but not from L*er*.

background. Our more recent work (O'Neill et al., 2019) demonstrated that even in the wild type *At2S3p:GFP* can be detected as early as the heart stage, and even occasionally at the early heart stage, weakening our original claims. In our new experiment, not only we observed many more embryos (40–50 per stage), but we also used a homozygous *asil1-1 asil2-1* background. In this case, we found no significant differences between the percentages of embryos expressing *At2S3p:GFP* in wild type and mutant embryos (Figure 2c).

In conclusion, our expression, greening, and reporter analyses of *asil* single and double mutant combinations do not support a role for these genes as repressors of the maturation program in early embryogenesis, in contrast to what was proposed by Gao et al. (2011) and ourselves (Willmann et al., 2011).

3.4 | The overexpression of the *ASIL* genes does not repress maturation in embryos

Another way of testing whether a transcription factor has a repressive function is to increase its levels and study whether the presumed targets are reduced in their expression. The promoter of choice for overexpression in plants has been that of the Cauliflower Mosaic Virus *35S* gene (Benfey & Chua, 1990). This promoter becomes active in embryos around the heart stage of development (Odell et al., 1994). Therefore, *35S:ASIL1* and *35S:ASIL2* transgenes may not have a large effect in the expression of the *LAFL* genes, which starts before this stage, but they should lead to the repression of the genes encoding storage products, which are activated at the late heart stage or later. *35S:ASIL1* and *35S:ASIL2* plants were indistinguishable from the untransformed Col plants, and their embryos developed at a similar rate and were morphological identical to those of the wild type (Figure 2e), even though *ASIL1* was overexpressed 11-fold and *ASIL2* 6-fold in siliques at 7 DAP (Figure 2d). We studied the expression of *At2S3*, *CRA1*, and OLEO1in pooled siliques at early (7 DAP) and mid-maturation (10 DAP). The only differences we observed with the wild type were at 7 DAP, when there were lower levels of *CRA1*, in *35S:ASIL1*, and higher levels (the opposite of what was expected) of *At2S3* in *35S:ASIL2* (Figure 3g,h).

In sum, we do not see any firm evidence, based on the overexpression lines, that *ASIL1* or *ASIL2* act as repressors of maturation. This confirms the previous observations from the single and double mutant combinations.

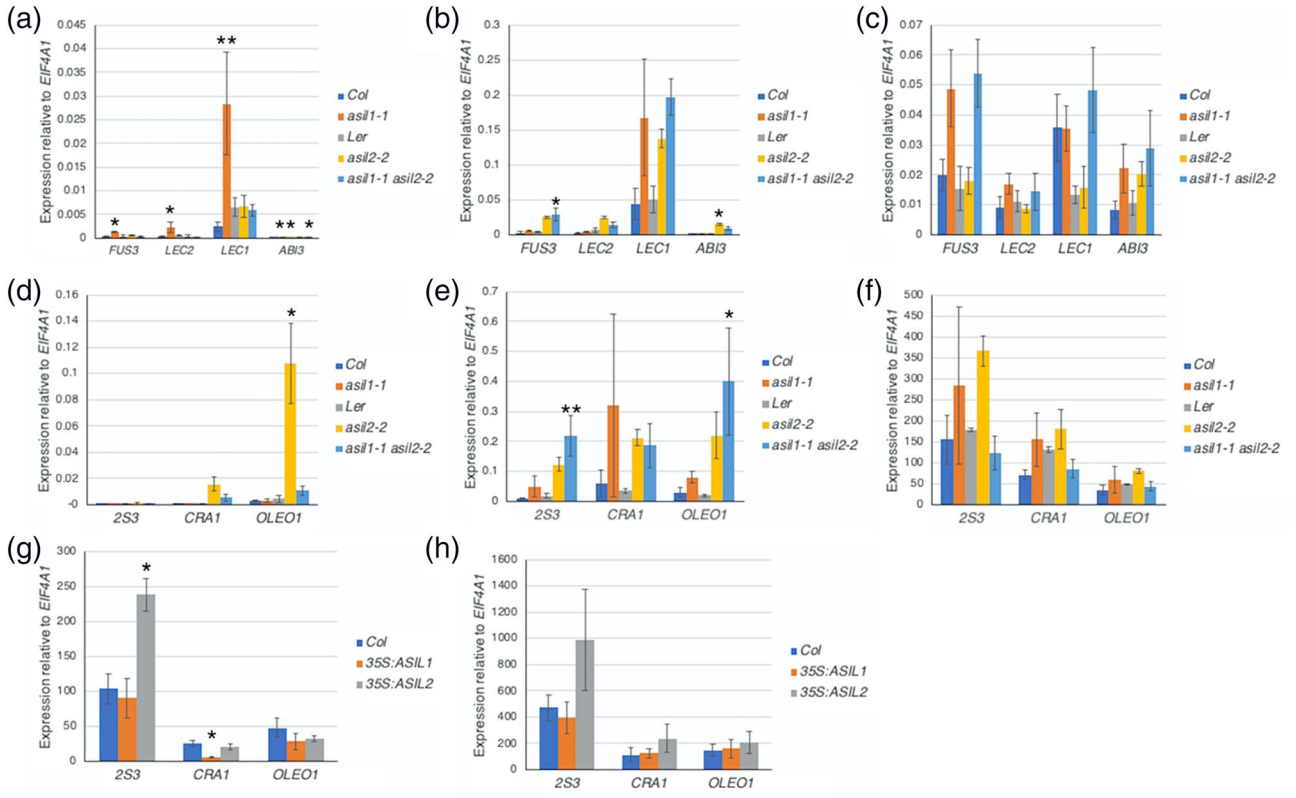

FIGURE 3 Gene expression in *asil* mutant and overexpressing siliques measured by RT-qPCR. (a–c) Expression of *LAFL* genes in mutant siliques at (a) 3 DAP, (b) 5 DAP, (c) 6 DAP. (d–f) Expression of genes encoding maturation products in mutant siliques at (d) 5 DAP, (e) 6 DAP, and (f) 10 DAP. (g,h) Expression of genes encoding maturation products in siliques of overexpressors at (g) 7 DAP, (h) 10 DAP. $^{*}p < .05$, $^{**}p < .01$ (analysis of variance [ANOVA] followed by Tukey's post-hoc test). Error bars: ±SEM of three biological replicates

3.5 | *asil* seedlings do not ectopically express the embryonic maturation program

Our hypothesis for the vegetative (postgermination) phase was the same as for the early embryonic phase: that the *ASIL* genes worked as redundant repressors of the embryonic program (in particular maturation). This prediction was based largely on the data presented by Gao et al. (2009). To test this idea, we first intended to confirm the transcriptomic phenotype of *asil1-1* seedlings using RNAseq and then expand those analyses to *asil2* mutants and double mutants. The first step was to establish the best timepoint to carry out the RNAseq experiment. Gao et al. (2009) chose 14 days after germination (DAG) (including a 2-day treatment with 50 μM ABA) to analyze the transcriptome and then individual mRNAs, even though the levels of *ASIL1* appeared to peak at 1 h after planting, which is also when derepression of genes in the mutant is first observed (germination itself takes about 40 h [Dekkers et al., 2013]). We first measured the levels of expression of the *LAFL* genes, *CRC* and *At2S3* in *asil1-1*, *asil2-1* and the double mutant *asil1-1 asil2-1* at different timepoints. We used RT-PCR to test seedlings at 4, 7 (plus/minus 50 μM ABA), 9, and 14 (±50 μM ABA) DAG. When we failed to amplify any of the genes using the RT-PCR primers used by Gao and colleagues (Gao et al., 2009), we switched to primers that had been validated in JJH's lab (Table S1, we then used these primers for the rest of our experiments, including those shown in Figure 3). We also added a positive control, 14 DAG seedlings homozygous for *val1-2* and segregating *hsl1-1*, which should moderately overexpress the genes in question (Suzuki et al., 2007; Tsukagoshi et al., 2007). To our surprise, we were unable to detect any of the maturation genes in any of our samples, other than in the positive control (data not shown). Given this situation, we decided to use 14 DAG seedlings, in triplicate, for our experiment, without ABA treatment. We reasoned that, like in other mutants that fail to repress maturation after germination (e.g., Tsukagoshi et al., 2007), *asil* mutants may take several days to accumulate the corresponding transcripts. We did not add ABA to avoid confounding effects due to potential differential responses to the hormone. In any case, the overexpression of maturation genes in *asil1-1* at 14 DAG was robust even without ABA in Gao et al.'s hands (Gao et al., 2009).

Our RNAseq analyses revealed that the mutant seedlings had large numbers of misexpressed transcripts compared to the wild type: 5% (*asil2-2*) to 27% (*asil1-1 asil2-1*) of the genome, with similar numbers of up- and downregulated transcripts in each case (Table 1; Datasets S3 and S4A-L). However, we could not find a single gene related to maturation among the lists of misregulated transcripts. As can be seen on Table 3, none of these genes were expressed in wild

TABLE 3 Expression of maturation genes in 14 DAG seedlings

AGI ID	Gene symbol	Col	*asil1-1*	*asil2-1*	*asil1-1 asil2-1*	Ler	*asil2-2*	*asil1-1 asil2-2*
Master regulators of maturation								
At3g24650	***ABI3***	0 ± 0	0 ± 0	0.03 ± 0.05	0.03 ± 0.05	0.02 ± 0.04	0.08 ± 0.07	0 ± 0
At3g26790	***FUS3***	0 ± 0	0 ± 0	0.03 ± 0.07	0 ± 0	0.02 ± 0.04	0.02 ± 0.03	0.02 ± 0.04
At1g21970	***LEC1***	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
At1g28300	*LEC2*	0.27 ± 0.37	0.05 ± 0.08	0 ± 0	0.1 ± 0.1	0.07 ± 0.07	0.13 ± 0.09	0.03 ± 0.04
At5g47670	*L1L*	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
At3g54320	*WRI1*	5 ± 1	5 ± 0.8	5 ± 0.3	4 ± 2	5 ± 0.8	5 ± 0.8	5 ± 1
At5g13790	*AGL15*	1.5 ± 0.3	0.9 ± 0.2	1.2 ± 0.3	1.5 ± 0.2	1.1 ± 0.03	1.0 ± 0.4	1.3 ± 0.7
Seed storage proteins								
At5g44120	***CRA1***	0.06 ± 0.1	0.04 ± 0.07	0.07 ± 0.06	0.09 ± 0.1	0 ± 0	0 ± 0	0.02 ± 0.04
At1g03880	*CRB*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
At4g28520	***CRC***	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.04	0 ± 0	0 ± 0	0 ± 0
At4g27140	*At2S1*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
At4g27150	*At2S2*	0 ± 0	0 ± 0	0 ± 0	0.02 ± 0.04	0 ± 0	0 ± 0	0 ± 0
At4g27160	***At2S3***	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
At4g27170	*At2S4*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
At5g54740	*At2S5/At2S-like*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Oleosins								
At3g01570	*S1/OLEO5*	1 ± 0.4	1 ± 0.2	0.6 ± 0.05	0.7 ± 0.2	0.6 ± 0.3	0.5 ± 0.07	0.3 ± 0.3*
At3g27660	*S2/OLEO4*	2 ± 0.5	1 ± 0.4	1 ± 0.3	0.6 ± 0.2**	2 ± 0.7	3 ± 0.4	1 ± 0.2*
At4g25140	***S3/OLEO1***	0.1 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0.01 ± 0.02	0 ± 0	0 ± 0
At5g40420	***S4/OLEO2***	0 ± 0	0.04 ± 0.07	0 ± 0	0.02 ± 0.04	0.01 ± 0.02	0 ± 0	0 ± 0
At5g51210	*S5/OLEO3*	1 ± 0.2	0.07 ± 0.1	0.06 ± 0.06	0.05 ± 0.04	0.2 ± 0.08	0.2 ± 0.2	0.2 ± 0.2
At2g25890	*SM3*	0.07 ± 0.06	0 ± 0	0.03 ± 0.05	0.03 ± 0.05	0.02 ± 0.04	0.08 ± 0.07	0 ± 0

Note: Expression as normalized counts ± SD (rounded up to the nearest unit, except for those <1). Genes in bold: genes reported upregulated at 14 DAG in Gao et al. (2009).

Abbreviations: DAG, days after germination.

*$p < .05$ compared with corresponding WT. **$p < .01$ compared with corresponding WT.

type (as expected) or mutant seedlings. This was consistent with the exploratory RT-PCR experiments described above.

We further evaluated the RNAseq data using RT-qPCR on seedlings grown on the same conditions that were used by Gao et al. (2009) (12 days on plain MS plates followed by 2 days on plates with 50 μM ABA), to make sure we had not missed anything by skipping the ABA treatment. We measured the *LAFL* genes and the same maturation products as we did in the embryo (*OLEO1*, *At2S3*, *CRA1*) in *asil1-1*. We also looked at a subset of them (*FUS3*, *LEC2*, *CRA1*) in all the mutant genotypes. As a positive control, we used 14 DAG *val1-2 val2-1* seedlings, which robustly overexpress most of these genes (except for *LEC2*) (Suzuki et al., 2007). Again, biological triplicates were used. As expected from the literature, and confirming the RNAseq data, the expression for all of these genes in the wild type ranged from undetectable to extremely low (Figure 4a,b). More importantly, there were no significant differences in expression in any of the mutants, other than in *val1-2 val2-1* (Figure 4a–d, notice the difference in scales in the *y*-axes). These experiments validate our transcriptomic data and suggest that the addition of ABA dos not affect the expression of this subset of genes in these genotypes.

In terms of visible phenotypes, there was nothing obvious to indicate ectopic expression of embryonic traits, such as swollen hypocotyls or growth arrest (Zhang & Ogas, 2009). *asil1-1* seedlings have minor differences with Col ones in the length of petioles, and *asil1-1* plants are slightly shorter and later flowering (Gao et al., 2009). We did not notice any apparent differences between *asil2* single and *asil1-1 asil2* double mutant seedlings and plants and their wild type counterparts (Figures S4 and 4e–k).

To obtain independent confirmation of the lack of embryonic traits in *asil* mutant seedlings, we studied the accumulation of TAGs in the seedlings. Mutants that express the maturation program during the seedling stage contain high levels of TAGs, whereas the wild type has little (Bouyer et al., 2011; Chhun et al., 2016; Tang et al., 2012; Tsukagoshi et al., 2007). We tested the accumulation of TAGs by staining with Fat Red, a method that has been used often for this phenotype (Bouyer et al., 2011; Chhun et al., 2016; Henderson et al., 2004; Tang et al., 2012; Tsukagoshi et al., 2007). We tested

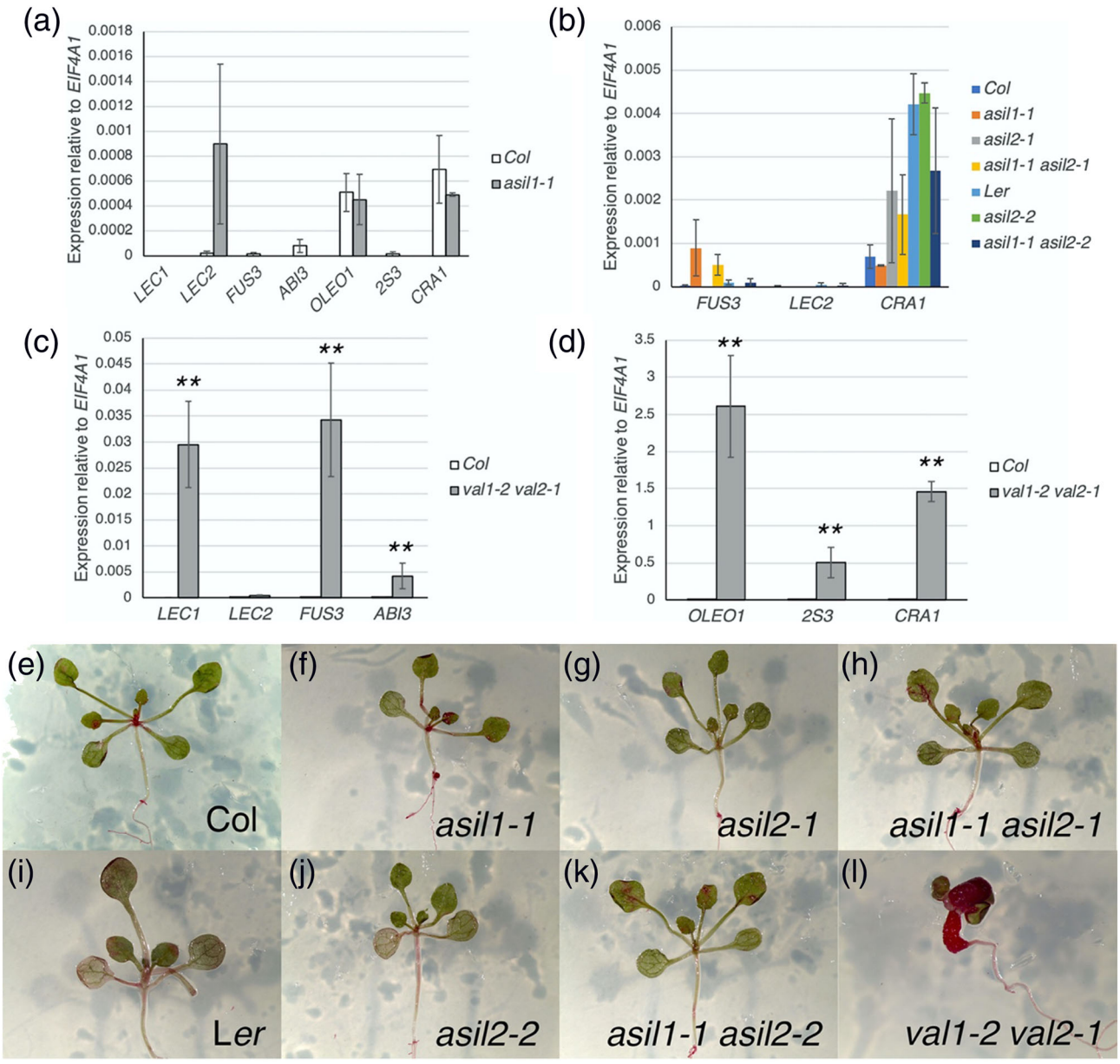

FIGURE 4 Characterization of *asil* mutant seedlings. (a–d) Expression of selected maturation genes in 14 days after germination (DAG) seedlings (a) *asil1-1* versus Col, (b) all *asil* mutants versus their corresponding wild types, (c,d) *val1-2 val2-1* versus Col. *$p < .05$, **$p < .01$ (analysis of variance [ANOVA] followed by Tukey's post hoc test). Error bars: ±SEM of three biological replicates. (e–l) 14 DAG seedlings (the last 2 days exposed to 50 μM ABA) stained with Fat Red. Magnification: (e–k) 0.75×, (l) 1.25×

14 DAG ABA-treated seedlings, grown in the same conditions as the ones used for RT-qPCR. For the first attempt, we stained the wild type and all mutants for 2 h. None of the seedlings stained. Because we had been expecting to see signal in *asil1-1*, we extended the incubation period to overnight. We also added *val1-2 val2-1* as a positive control. The *val1-2 val2-1* seedlings, as previously described, showed a deep red color all over, indicating high level of TAG accumulation (Tsukagoshi et al., 2007) (Figure 4l). In contrast, both Col and L*er* and the various *asil* mutant combinations had the same pattern of staining: light red roots and meristems and pale red hypocotyls and petioles (Figure 4e–k). This pattern is similar to what has been reported before for the wild type (Bouyer et al., 2011; Chhun et al., 2016; Henderson et al., 2004). There was no obvious difference in staining between mutants and wild types suggesting that, at least within the sensitivity of this method, the accumulation of TAGs in all these genotypes is minimal.

The overall conclusion from our work (transcriptomic and lipid analyses) is that the embryonic maturation program is not ectopically expressed in *asil* mutant seedlings, indicating that the *ASIL* genes are not vegetative repressors of maturation.

3.6 | *asil2-1* might be a dominant-negative allele

One issue that we could address with the RNAseq data was the nature of the *asil2-1* allele. While *asil2-2* has an insertion that would clearly render the encoded product nonfunctional, the insertion in *asil2-1* is predicted to change only the last 23 aminoacids of the protein (Figure 1c). Given this relatively small change, and the fact that the *asil2-1* mRNA was still present (Figure 1d–f; Figure S3), it was hard to guess the nature of this mutant allele and whether the mutation leads to a loss- or a gain-of-function.

RNAseq of *asil2-1* produced the highest number of DEGs out of all the mutant genotypes studied (7949; Table 1). This is 33% of the 24,100 transcripts detected in the experiment and twice the number of genes regulated by *asil1-1* (3692; Table 1). When the list of *asil2-1* DEGs is compared with those of *asil2-2*, it is clear that they are mostly different sets (Figure 5a). This lack of similarity to the putative null allele suggests that the *asil2-1* is not a loss-of-function allele. We wondered whether *asil2-1* is an overactive, hypermorphic allele. If that were the case, we would predict a phenotype that is the opposite to that of *asil2-2*; that is, genes upregulated in *asil2-2* would be downregulated in *asil2-1* and vice versa. The corresponding comparison (Figure 5b) suggests that this not quite the case either (although half of the genes that were down in *asl2-2* were up in *asil2-1*) (see Dataset S4 for the lists of genes for each mutant).

The comparison of DEGs between *asil2-1* and *asil1-1*, however, is quite interesting. There is a lot of overlap between the genes affected by these two alleles of different genes; 66% of the genes upregulated in *asil1-1* are also upregulated in *asil2-1*, whereas 28% of the genes upregulated in *asil2-1* are also upregulated in *asil1-1* (Figure 5c). For the downregulated DEGs, the percentages are 64% and 32%, respectively (Figure 5c). The *asil1-1 asil2-1* double mutant is mostly an addition of both single mutant lists, and only 19% of genes are unique to the double mutant (both for the up- and downregulated sets; Figure 5c). These remarkable results suggest that the ASIL2-1 protein might be interfering with the action of the ASIL1 protein, in a dominant-negative manner, leading to an *asil1-1*-like phenotype in *asil2-1* mutants. Trihelix factors are known to function as dimers, and the C-terminal sequences have been shown to be involved in dimerization (the last 28 aminoacids for GT-3a, 24 for GT-1, and 46 for GT-1a) (Ayadia et al., 2004; Hiratsuka et al., 1994; Lam, 1995). These are precisely the sequences that are changed in ASIL2-1. One possibility is that the mutation changes the binding preferences of ASIL2, leading to binding and interfering with ASIL1 and possibly other trihelix proteins. This hypothesis, however, will remain speculative until protein–protein interaction experiments are conducted.

3.7 | Possible roles of the *ASIL* genes during vegetative development

Even though, contrary to our expectations, the *ASIL* genes do not repress the embryonic maturation program during vegetative development, they may have other functions during this phase. Our analyses of the transcriptome of mutant seedlings allow us to hypothesize what some of those roles may be and whether there is any redundancy between these closely related genes. The best approach is to analyze the similarities and differences between the transcriptomic phenotypes and biological processes affected in the putative null alleles, *asil1-1* and *asil2-2*.

Overall, mutations in *asil1-1* have a much bigger effect on the transcriptome (3,692 DEGs) than mutations in *asil2-2* (1,381 DEGs) (Table 1). We did not observe a big overlap between the lists of DEGs in *asil1-1* and those in *asil2-2* (Figure 5d), suggesting that *ASIL1* regulates a different set of genes than *ASIL2*. It is important to keep in mind that this regulation could be direct or indirect, and we do not have chromatin immunoprecipitation data to determine which of the genes are direct targets of these factors. The list of gene ontology (GO) terms enriched for each of these mutants, generated using the agriGO analysis toolkit (Du et al., 2010), is also largely nonoverlapping (except for response to auxin) (Table 4). Broadly speaking, *ASIL1* appears to be more involved in the regulation of development, whereas *ASIL2* may have more to do with response to hormones and biotic and some abiotic stresses (Table 4). This is consistent with previous work that showed that *ASIL2* is involved in the response to iron deficiency (Koryachko et al., 2015). When looking at the *asil1-1 asil2-2* double mutant, there are 1,283 DEGs that are unique to this genotype (counting only genes misregulated when compared with both Col and L*er*) (Figure 5d). These genes are candidates to be regulated redundantly by both genes. The GO terms enriched for this subset of genes are largely related to responses to pathogens (Table 4).

Another way to make educated guesses about the role of a gene is to see whether its expression changes in response to an experimental condition. A significant change may indicate a function in the response to the treatment. However, a lack of alteration in expression is not necessarily informative, because the already translated protein may be sufficient for the action, without the need for a transcriptional response. One approach to assess variation in transcript levels in many conditions is through the Arabidopsis eFP Browser (Winter et al., 2007), which collects a number of transcriptomic experiments that addressed responses to abiotic and biotic stresses and chemical and hormonal treatments. When looking at this resource, it is clear that the transcript levels of *ASIL1* are remarkably stable across most conditions tested (osmotic, salt, drought, ultraviolet B (UVB), and wounding stresses; gamma irradiation; infection by several pathogens; applications of most plant hormones). It does respond to temperature (being higher after a heat shock and lower in the cold) and oxidative stress (Kilian et al., 2007). When looking at specific root tissues, salt exposure leads to transcript increases in the epidermis but decreases in the cortex. There is also an increase in response to iron deficiency (Dinneny et al., 2008). Interestingly, none of these are represented in the list of GO-enriched terms for *asil1-1* seedlings (Table 4). The mRNA levels of *ASIL2* are also fairly unresponsive. However, there is a general increase in amounts in response to various abiotic stresses (osmotic, salt, drought, genotoxic, oxidative) (Kilian et al., 2007). "Response to salt stress" is among the enriched terms for *asil2-2* in Table 4. In the root, similarly to *ASIL1*, there is an increase in the

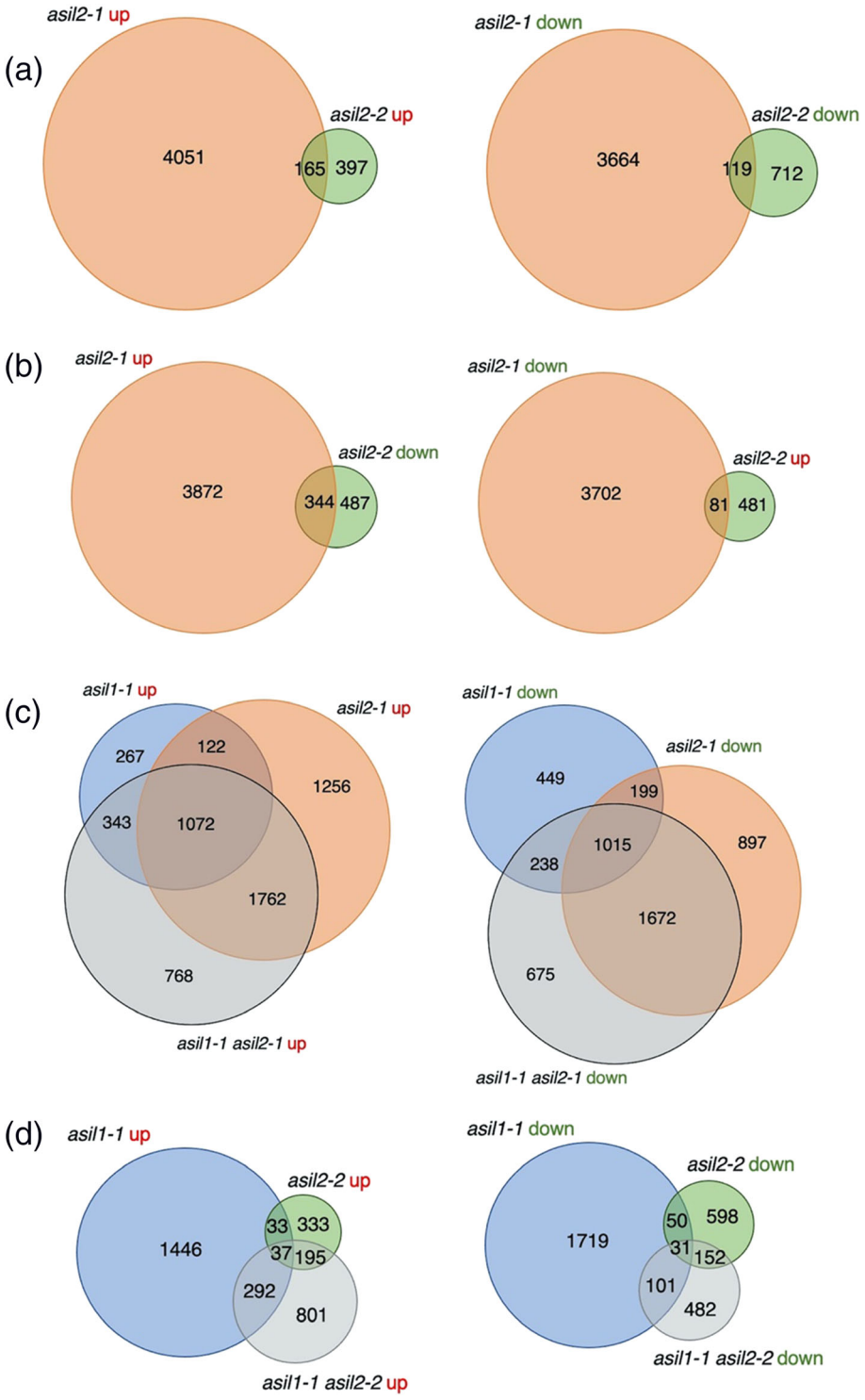

FIGURE 5 Comparisons and overlaps of differentially expressed genes (DEGs) in 14 days after germination (DAG) seedlings. Comparisons of sets of DEGs derived from the RNAseq experiment, overlaps of these sets between single and double *asil* mutants. (a,b) *asil2* alleles: *asil2-1* versus *asil2-2*, (a) up versus up and down versus down, (b) up versus down and vice versa, (c) *asil1-1* versus *asil2-1* versus *asil1-1 asil2-1*. (d) *asil1-1* versus *asil2-2* versus *asil1-1 asil2-2*. Overlap lists generated with BioVenn

epidermis (but not the cortex) in response to salt, and in contrast to *ASIL1*, there is a decrease in response to iron deficiency (Dinneny et al., 2008).

The conclusion from these analyses is that, contrary to our initial predictions of redundancy, during early vegetative development, *ASIL1* and *ASIL2* mostly regulate and respond to different processes. They may, however, be involved redundantly in the response to biotic stresses, like infections by fungi and bacteria.

3.8 | GSL levels are not altered in *asil* seedlings

After looking at the GO-term enrichment in the lists of DEGs (Table 4), we decided to analyze GSL content in vegetative plants, to test whether there were differences that are worth more in-depth study in future work. We concentrated our studies on the putative null alleles (*asil1-1*, *asil2-2*) and avoided *asil2-1* and its combinations, because of its peculiar nature.

TABLE 4 Biological process GO term enrichment for DEGs for the different sets of *asil* mutants

Terms for upregulated DEGs common to *asil1-1* and Gao et al. (2009)	Terms for downregulated DEGs common to *asil1-1* and Gao et al. (2009)
-Response to auxin stimulus (5e-06) -Glucosinolate biosynthetic processes (5e-05) -Response to light stimulus (0.0003)	None
Terms for upregulated DEGs unique to *asil1-1* and not in Gao et al. (2009)	Terms for downregulated DEGs unique to *asil1-1* and not in Gao et al. (2009)
-Seed development (5e-19) -Chloroplast organization (3e-16) -tRNA aminoacylation (2e-08) -rRNA processing (1.6e-07) -RNA splicing (9e-07) -Embryo sac development (1e-05)	-Cell wall organization (4e-09) -Root development (4e-08) -Glucosinolate metabolic process (4e-06) -Response to hypoxia (2e-05) -Root cell hair differentiation (4e-05) -Photosynthesis, light harvesting (7e-05) -Response to auxin stimulus (1e-04)
Terms for upregulated DEGs unique to Gao et al. (2009) and not in *asil1-1*	Terms for downregulated DEGs unique to Gao et al. (2009) and not in *asil1-1*
-Response to ABA (1.42e-12) -Defense response to fungus (3e-08) -Defense response to bacteria (2e-06) -Response to hypoxia (7e-06) -Response to salt stress (3e-05) -Plant type hypersensitive response (0.0002) -Defense response by callose deposition (0.0009) -Ethylene signaling pathway (0.006)	-Response to jasmonic acid (2e-06) -Response to ABA (4e-05) -Response to ethylene (0.0001) -Regulation of transcription (0.0002) -Chloroplast organization (0.0003) -Response to salicylic acid (0.001) -Response to GA (0.005)
Terms for upregulated DEGs in *asil2-2*	Terms for downregulated DEGs in *asil2-2*
-Protein aminoacid phosphorylation (3e-07) -Post-embryonic development (9e-05) -Innate immune response (0.0016) -Defense response to bacterium (0.004)	-Response to chitin (8e-36) -Regulation of transcription (3e-14) -Response to ABA stimulus (9e-11) -Response to ethylene stimulus (5e-09) -Cold acclimation (2e-08) -Response to salt stress (4e-07) -Response to auxin stimulus (8e-05) -Response to GA stimulus (4e-05) -Response to R/FR light (0.0002) -Defense response to bacterium (0.005)
Terms for upregulated DEGs in *asil1-1 asil2-2* (unique to double mutant)	Terms for downregulated DEGs in *asil1-1 asil2-2* (unique to double mutant)
-Defense response to bacterium (3e-19) -Protein phosphorylation (4e-12) -Defense response to fungus (1e-09) -Response to hypoxia (4e-07) -Callose deposition in cell wall during defense response (1e-06) -Response to salicylic acid (3e-05) -Induced systemic resistance (7e-07) -Response to ABA stimulus (0.0001) -Activation of innate immune response (0.001) -Positive regulation by symbiont of host immune response (0.002)	-Response to light intensity (0.002)

Note: Only terms with $p < 0.01$ were considered (p value in parenthesis). Mostly, terms related to development, hormones, and responses to stress are included. The GO enrichment was determined with the agriGO algorithm. For *asil1-1 asil2-2*, only DEGs present in comparisons with both Col and L*er* were used.

Abbreviations: DEGs, differentially expressed genes; GO, gene ontology.

GSLs are nitrogen- and sulfur-containing secondary metabolites that are present almost exclusively in the order Capparales, which includes the crucifers, like *Arabidopsis*. GSLs are used by the plants as defense against herbivores and other pests. They also have commercial importance, because they are responsible for the flavors of the crops of this family (such as broccoli or cabbage) (Halkier & Gershenzon, 2006; Kliebenstein et al., 2005). Terms related to GSL metabolism are enriched in *asil1-1* mutants (Table 4). Many of the GSL metabolic genes are also affected in *asil2-2* or *asil1-1 asil2-2*, even though they do not make the cutoff for enrichment. Broadly speaking, biosynthetic genes tend to be downregulated in *asil1-1* and upregulated in *asil2-2* or *asil1-1 asil2-2*, whereas genes encoding hydrolytic enzymes are downregulated in all mutants (Table S2). We therefore explored the possibility that loss of *ASIL1*

and/or *ASIL2* functions might result in changes in GSL content in seedlings.

We measured GSL content in 3-week-old *asil1-1* and *asil2-2* plants and compared them with their corresponding wild type backgrounds (Col and L*er*, respectively). Because Col and L*er* synthesize different repertoires of GSLs (Table 5) (Kliebenstein et al., 2001), we did not take measurements in *asil1-1 asil2-2*, which is in a mixed background, and thus very difficult to interpret. Two independent experiments were carried out, with the same qualitative outcome: there were almost no differences between the mutants and the wild types in GSL content. The only exception was slightly higher levels of 4MOI3M in *asil2-2* in one of the experiments (Table 5 shows the numbers for that experiment). In conclusion, single *ASIL* genes do not appear to affect the amounts of GSLs.

4 | DISCUSSION

The tight control of developmental transitions is of crucial importance in the life history of any organism. Failure to do so may lead to significant abnormalities or death. In this study, we were interested in understanding the regulation of the embryonic maturation program. This shift in cell programing needs to be turned on at the right time for the production of viable and healthy seeds. It also needs to be repressed after germination, to prevent growth arrest. While a number of nonsequence-specific DNA-modifying and remodeling factors and a couple of transcription factors are known to be involved in postgerminative repression, those acting during embryogenesis are mostly unknown. It is also unclear whether the mechanisms regulating the maturation program at these two stages are the same.

In the present study, we set out to test the hypothesis that the *ASIL* genes redundantly controlled embryonic maturation both during early embryogenesis and after germination. Our analyses of embryos and seedlings failed to detect any effect whatsoever of mutating the *ASIL* genes on the expression of the embryonic maturation program at either stage of development.

Our previous review of the published literature had indicated a possible role for trihelix transcription factors in embryo maturation (Barr et al., 2012). At that time, Gao et al. (2011) had reported that during embryogenesis *asil1-1* embryos displayed what could interpreted as premature expression of *LEC2*, *FUS3*, *ABI3*, *OLEO 2*, *At2S3*, and *CRC*. We had also found that *ASIL1* and *ASIL2* were underexpressed at the torpedo stage of *dcl1-15* embryos, which mature very early, that chlorophyll accumulated early in *asil1-1* and *asil1-1 asil2-1* embryos, and that *At2S3* appeared to be expressed earlier in *asil1-1 asil2-1* embryos (Willmann et al., 2011). The experiments presented here contradict those findings. Our RNAseq experiment showed no differences at the late heart stage in the mutants, which is not what we would have expected if the *ASIL* genes were acting as repressors during early embryogenesis. The RT-qPCR follow-up, although noisier, broadly supported the transcriptomic analyses. In addition to the mRNA expression analyses, our reexamination of chlorophyll and *At2S3p:GFP* accumulation, using better techniques and/or larger sample sizes, refuted our own previously published data. Finally, the fact that overexpressing the ASIL proteins in the embryos had essentially no effect on the genes they were hypothesized to regulate provides more evidence that the *ASIL* genes are not controlling the maturation program in this phase of the life cycle.

The hypothesis of the postgerminative repression of embryonic traits by the ASIL genes was based primarily on the work of Gao et al. (2009). They had indicated that *asil1-1* seedlings ectopically expressed the *LAFL* and some maturation product genes (*OLEO1* and *2*, *At2S3*, *CRA1*, *CRC*). These plants also showed increased TAGs and 2S albumin accumulation but only after ABA treatment. In support of this repressive role, they found that ASIL1 protein could bind to the promoter of *At2S3* in a yeast one-hybrid system and in vitro (Gao et al., 2009). Our intention was to replicate their findings for *asil1-1* and to expand on them by analyzing *asil2* seedlings. However, our transcriptomic, RT-qPCR, and lipid staining data do not agree with

TABLE 5 Glucosinolate content in *asil* mutants

Glucosinolate	Col	*asil1-1*	L*er*	*asil2-2*
3-OH	np	np	10.3 ± 5.1	10.9 ± 7.2
3MSO	3.7 ± 1.5	3.1 ± 1.5	np	np
4MSO	24.8 ± 10.8	20.6 ± 11.5	np	np
5MSO	1.2 ± 0.5	1.0 ± 0.5	np	np
7MSO	0.8 ± 0.4	0.7 ± 0.3	np	np
I3M	10.1 ± 3.1	8.5 ± 4.6	7.0 ± 4.8	5.5 ± 2.5
4MOI3M	0.8 ± 0.2	0.8 ± 0.3	0.4 ± 0.2	0.6 ± 0.2*
NMOI3M	0.4 ± 0.4	0.3 ± 0.3	0.4 ± 0.2	0.3 ± 0.1

Note: The table indicates the average concentration (as nmol/cm^2) ± SD. np: not present in that background. $n = 8$ per genotype.
Nomenclature:
Aliphatic GSLs: 3-OH (3-Hydroxypropyl glucosinolate); 3MSO (3-Methylsulfinylpropyl glucosinolate); 4MSO (4-Methylsulfinylbutyl glucosinolate); 5MSO (5-Methylsulfinylpentyl glucosinolate); 7MSO (7-Methylsulfinylheptyl glucosinolate).
Indolic GLSs: I3M (Indol-3-ylmethyl glucosinolate); 4MOI3M (4-Methoxyindol-3-ylmethylglucosinolate); NMOI3M (1-Methoxyindol-3-ylmethylglucosinolate).
*$p < .5$ (ANOVA test).

theirs and indicate that neither of the *ASIL* genes play a role in the control of maturation during the vegetative phase.

In order to better understand the similarities and differences between the sets of DEGs between our experiment and theirs, we looked at which DEGs overlap and which were unique to each dataset. We then searched for GO terms for biological process enriched in each of these lists. The only significant difference between the two experimental setups was a 2-day-long treatment with ABA in their case. Only a very small percentage of DEGs was common to both experiments, although the overlaps between DEG lists were significant for both the upregulated and downregulated gene sets (down: $p = 2.7 \times 10^{-44}$, up: $p = 2 \times 10^{-08}$; Fisher's exact test) (Figure 6). The list of common genes showed very few biological processes for which there was GO enrichment (Table 4). Consistent with the experimental designs, "response to ABA" prominently figured among the GO-enriched terms present only in (Gao et al., 2009) experiment (74/1,026 genes), other terms being responses to hormones and abiotic and biotic stresses. Surprisingly, GO terms related to development (including seed/embryo development or maturation) were not present. Among the GO terms enriched for those DEGs present in our experiment only, one term that stands out is "seed development." Interestingly, this list of 118 genes does not include any maturation or patterning genes, but mostly genes involved in basic cellular processes, which fall under the category of *EMBRYO LETHAL* (*EMB*) genes (Meinke, 2020). Thus, our analyses of DEG overlaps and GO-enrichment points to different outcomes for very similar experiments using the same alleles.

This begs the question of why we obtained different results for *asil1-1* (they did not analyze *asil2*). The different transcriptomic technologies used (microarray vs. RNAseq) are unlikely to be the culprit (Giorgi et al., 2013). Gao et al. (2009) reported the fold-level upregulation of genes from their microarray data, not absolute levels of expression, and did not deposit the data in a public repository. This makes it hard to evaluate their results critically. Their RT-qPCR analyses are curious. In their hands, *ASIL1* is expressed in seedlings at higher levels than the gene they used to normalize, *ACTIN2* (*ACT2*, *At3g18780*). In our seedlings, *ACT2* mRNA levels are 10 times higher than those of *ASIL1* (Dataset S3). A similar phenomenon is observed in their 4–6 DAP embryos (Gao et al., 2011), with *LAFL* genes expressed 50–100 times higher than *ACT2* (but only 0.5–1 times in our embryos, see Dataset S1). Neither can we explain their observed increases of 2S albumin using Western blots or TAGs using thin layer chromatography, which is not what we would predict (or what we observed, in the case of TAGs; Figure 4e–l). In sum, we do not have a good hypothesis to explain the contradictory results. At this point, all we can do is report the data we obtained and point out the discrepancies.

If the *ASIL* genes do not regulate maturation, what other biological functions might they have? They do not seem to be playing a big part during embryogenesis, judging from the lack of patterning defects and the short list of DEGs in the mutants. However, it is possible that they are involved in pathways that we have not analyzed or that they have subtle effects that we could not detect. Both genes are expressed in the endosperm (Figure S1C–E), but there were no obvious defects in the endosperms of mutant seeds. There is also room for further redundancy, as at least one of the other genes in the same clade, *At3g24490*, is also highly expressed in embryos (Belmonte et al., 2013). Analyses of single mutants for this gene and multiple combinations with the *ASIL* genes could be used to test this idea.

During vegetative development, the list of genes whose expression is affected in the mutants is much more extensive, hinting at a more prominent role. The list of GO-enriched terms (Table 4) and the changes of the levels of *ASIL* transcripts in response to various experimental conditions, as discussed above, offer a resource to generate educated hypotheses. Generally speaking, both sets of data refer to responses to stimuli or stresses, abiotic and biotic, and hormones. This is consistent with what have been described for several members of the family (Kaplan-Levy et al., 2012). For instance, in the case of response to salt stress, a trihelix factor from *Brassica napus*, part of the same clade as the *ASIL* genes, conferred salt tolerance when overexpressed (Luo et al., 2017). Some trihelix factors in other clades have also been reported to be involved in salt tolerance in rice and soybean (Fang et al., 2010; Xie et al., 2009). For the one biological process we tested, overall GSL content, we found no obvious differences in the mutants (Table 5). It is possible that decreases in the activity of one part of the pathway are compensated by increases in another.

This work started with the goal of testing whether the *ASIL* genes, alone or redundantly, controlled the embryonic maturation program both embryonically and postembryonically. The sum of our experiments demonstrates that they do not. This was surprising, considering what was known previously, but all our data are consistent with this conclusion. Our findings suggest that, although the *ASIL* genes do not play a major role during embryogenesis, they do have functions during the vegetative phase. GO-enrichments and transcriptomic dataset analyses indicate their placement in pathways related to the response to biotic and abiotic stimuli. This work thus serves as a starting point, suggesting future directions of research to elucidate the contributions of the trihelix family of factors to the biology of the plant.

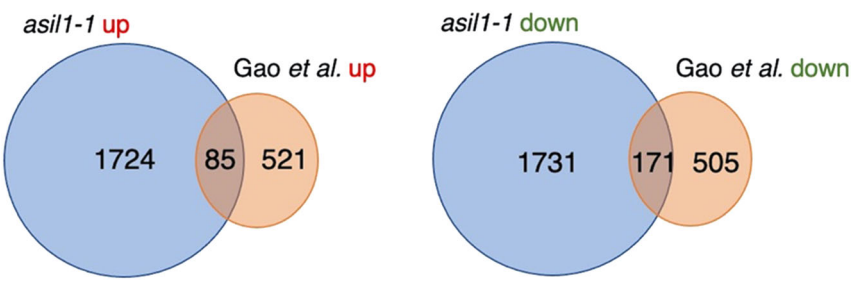

FIGURE 6 Overlaps of differentially expressed genes (DEGs) in our experiment versus Gao's. Comparison of our RNAseq data (*asil1-1*) versus microarray data from Gao et al. (2009)

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CONFLICT OF INTEREST

The authors have no conflicts of interests to declare.

AUTHOR CONTRIBUTIONS

JMP, JJH, DK, and PDJ designed the research. KAR, JMP, YW, MJF, JSB, TQD, BL, and PDJ performed the research. All the authors contributed to data analysis. PDJ conceived and supervised the project and wrote the article, with input from all authors.

CONFLICT OF INTEREST

The Authors did not report any conflict of interest.

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