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Research Article

The MADS-domain factor AGAMOUS-Like18 promotes somatic embryogenesis

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P.P. led the investigation of AGL18, SE development, and experiments to determine direct and indirect gene regulation by AGL18. P.P., S.J., and R.T. performed the experiments to determine AGL15 regulated targets genome-wide. P.P. and S.J. investigated the phosphorylation of AGL18. R.D. generated the AGL18 tagged constructs used in this study. M.C. provided training and advice on CLC Genomics workbench and analysis. S.E.P. assisted with and oversaw all aspects of the work.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is Sharyn E. Perry (sperr2@uky.edu).

Abstract

AGAMOUS-Like 18 (AGL18) is a MADS domain transcription factor (TF) that is structurally related to AGL15. Here we show that, like *AGL15*, *AGL18* can promote somatic embryogenesis (SE) when ectopically expressed in Arabidopsis (*Arabidopsis thaliana*). Based on loss-of-function mutants, AGL15 and AGL18 have redundant functions in developmental processes such as SE. To understand the nature of this redundancy, we undertook a number of studies to look at the interaction between these factors. We studied the genome-wide direct targets of AGL18 to characterize its roles at the molecular level using chromatin immunoprecipitation (ChIP)-SEQ combined with RNA-SEQ. The results demonstrated that AGL18 binds to thousands of sites in the genome. Comparison of ChIP-SEQ data for AGL15 and AGL18 revealed substantial numbers of genes bound by both AGL15 and AGL18, but there were also differences. Gene ontology analysis revealed that target genes were enriched for seed, embryo, and reproductive development as well as hormone and stress responses. The results also demonstrated that AGL15 and AGL18 interact in a complex regulatory loop, where AGL15 inhibited transcript accumulation of *AGL18*, while AGL18 increased *AGL15* transcript accumulation. Co-immunoprecipitation revealed an interaction between AGL18 and AGL15 in somatic embryo tissue. The binding and expression analyses revealed a complex crosstalk and interactions among embryo TFs and their target genes. In addition, our study also revealed that phosphorylation of AGL18 and AGL15 was crucial for the promotion of SE.

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Introduction

Because humans are dependent on seeds with at least 70% of our caloric input directly from seeds (Sreenivasulu and Wobus, 2013), understanding the developmental processes within the seed is of the utmost importance. Although Arabidopsis (*Arabidopsis thaliana*) is a potent molecular genetic model, studies on embryogenesis are still challenging mostly due to the small size of Arabidopsis embryos and the fact that they are embedded within layers of maternal tissues. In addition, screens for embryo lethal mutants tend to identify nonredundant gene products essential for life rather than for embryogenesis per se, limiting genetic approaches to studying this phase of the plant life cycle (Meinke, 2020). Somatic embryogenesis (SE) has been used as a model to understand developmental processes within the seed (Vogel, 2005; Rose and Nolan, 2006; Leljak-Levanić et al., 2015), albeit with mixed relevance at a molecular level (reviewed in Tian et al., 2020a). Regardless of relevance to zygotic processes, SE is an important tool of regeneration, though poorly understood, and therefore further investigation is important to understand and potentially improve SE for both applied and basic science to meet today's agricultural challenges.

An important approach to understanding gene function is to overexpress the gene of interest or remove the gene function (as in mutants, or via newer technologies such as Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein (CRISPR–Cas) systems). Overexpression and newer technologies require the ability to transform the plant. Transforming a cell either by biolistics or *Agrobacterium tumefaciens* then requires the ability to regenerate a plant from the transformed cell, and SE is one mechanism to do this. Some plants regenerate via SE or organogenesis, but many are recalcitrant to either process. Even particular cultivars (cvs) of plants can regenerate well or poorly, or not at all. One specific example is in soybean (*Glycine max*) where cv Jack is relatively efficient at SE (but still difficult), but other cvs (e.g. Stonewall) do not produce SE well or at all. For soybean, in general, early maturity group (MG) cvs are more efficient at SE, while late MG cvs do not produce SE as well, limiting the ability to genetically transform these cvs for enhanced agricultural performance for different climate zones (Meurer et al., 2001).

SE is also an important means to propagate some long-living species and to remove viral contamination. As specific examples, SE in coffee (*Coffea arabica*), cacao (*Theobroma cacao*), and poplar (*Populus spp.*) allows rapid propagation of desirable genotypes, but in some cases, improvement of SE efficiency is needed to allow this process to be more cost effective (Florez et al., 2015; Guan et al., 2016; Campos et al., 2017).

Despite difficulties identifying genes involved in embryo development by genetic approaches, a number of embryo-preferentially expressed genes encoding transcription factors (TFs) have been identified and these include the so-called LAFL factors. The LAFLs include a HEME-ACTIVATED PROTEIN3 subunit of CCAAT binding factors LEAFY COTYLEDON1 (LEC1) and three B3 domain factors; ABSCISIC ACID (ABA) INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON2 (LEC2), that are the key regulators of embryo development (Boulard et al., 2017), hence LAFL. These proteins are necessary and sufficient for embryo development. The genes encoding the LAFLs are expressed primarily during embryo development, although roles after completion of germination have also been reported (reviewed in Tian et al., 2020a). When ectopically expressed, *LAFLs* are sufficient to initiate embryogenic programs in somatic cells including promoting SE. Loss-of-function mutants in these genes have defects in later stages of embryogenesis, specifically maturation and desiccation processes, and in some cases, morphogenesis.

In addition to the key LAFL factors, a number of other genes when ectopically expressed, as well as some loss-of-function mutants, result in ectopic embryo development (reviewed in Tian et al., 2020a). Often the genes that promote SE when ectopically expressed do not have obvious zygotic phenotypes in loss-of-function mutants, suggesting the presence of redundant factors (Pickett and Meeks-Wagner, 1995; Meinke et al., 2003). Most of the genes that produce ectopic SE development when mutated (loss-of-function) are involved in epigenetic control of gene expression that normally would facilitate the developmental phase transition from embryo to postgerminative development. In these situations, often key TFs controlling embryogenesis are ectopically expressed.

Prior work in the lab characterized gene regulation by the MADS-factor AGAMOUS-Like 15 (AGL15; Zheng et al., 2009). AGL15 primarily accumulates during the early stages of embryo development (Heck et al., 1995; Perry et al., 1996) and ectopic expression promotes SE in several species (Harding et al., 2003; Zheng and Perry, 2014; Yang et al., 2014a, 2014b). Prior work determined targets of AGL15 regulation (Zheng et al., 2009). Interestingly, AGL15 can both directly express and directly repress target genes. AGL15 was found to directly express *LEC2*, *FUS3*, and *ABI3* (Zheng et al., 2009). Conversely, LEC2 and FUS3 directly upregulate *AGL15* (Braybrook et al., 2006; Wang and Perry, 2013).

Like many other MADS domain proteins, AGL15 forms dimers to bind DNA (Hill et al., 2008). *AGL18* encodes a MADS domain protein that is the closest putative paralog to AGL15, and shows overlapping expression patterns with *AGL15* in Arabidopsis (Lehti-Shiu et al., 2005; Adamczyk et al., 2007). This includes co-expression in the embryo, the shoot meristem, somatic embryo cultures, as well as other tissues (Supplemental Table S1). AGL18 shows redundancy with AGL15 in controlling developmental programs (Adamczyk et al., 2007; Thakare et al., 2008). Here we show that similar to *AGL15*, ectopic expression of *AGL18* promotes SE. However, the mechanism of function of AGL18 in embryogenesis and the relation, if any, with AGL15 is still unexplored. To understand how a TF promotes a developmental process, it is necessary to understand the genes regulated. This type of analysis for AGL15 has allowed us to develop means to improve SE through culture manipulation

(Zheng et al., 2013a, 2013b; Zheng et al., 2016). Here we show an in planta interaction between AGL15 and AGL18. We report on high-throughput methods to map genome-wide in vivo binding sites for AGL18 and AGL15, and to determine the response of genes to AGL18 accumulation. These approaches allow the discrimination of putative direct target genes from indirect targets and allow us to compare shared and unique sites for these factors. Interestingly, our findings suggest a complex regulatory loop between the two MADS-domain proteins, with different positive and negative feedback loops. In addition, our studies also revealed that phosphorylation of AGL18 and AGL15 is necessary for the promotion of SE in Arabidopsis.

Results

AGL18 promotes somatic embryogenesis

Prior work demonstrated that a *35S:AGL15* transgene enhanced production of somatic embryo tissue from shoot apical meristems (SAMs) of seedlings in liquid culture (Harding et al., 2003; Thakare et al., 2008). In short, when seeds are allowed to complete germination in liquid media containing the synthetic auxin 2,4-D, the shoot apical region will, at some frequency, produce somatic embryos by 3 weeks in culture (21-d-old culture [dac]: days in culture, Mordhorst et al., 1998). Because *AGL18* (*At3g57390*) shows redundancy with *AGL15* in controlling developmental programs (Adamczyk et al., 2007; Thakare et al., 2008), and is expressed in overlapping developmental contexts (Lehti-Shiu et al., 2005), we looked at the effect of AGL18 accumulation on SAM SE.

As shown in Figure 1, an average of 19.8% of Columbia (Col) wild-type (WT) seedlings had somatic embryo tissue at the apex. Also as expected, the *35S:AGL15* transgene significantly increased the number of seedlings with SAM somatic embryos (64.4%). An *agl15/agl18* double mutant showed significant reduction compared to the WT (13%), although neither single mutant showed a significant effect in these experiments. Moreover, *35S:AGL18* was able to promote SAM SE with 40.8% ($P < 0.01$) of seedlings showing somatic embryo development (Figure 1). Therefore, AGL18 accumulation, like AGL15, is positively correlated with somatic embryo development.

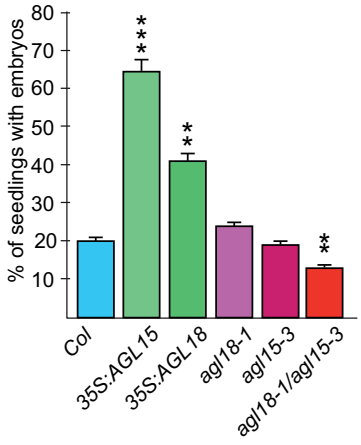


Figure 1 AGL18 promotes SE. Different lines of seeds were grown in 2,4-D medium and the embryos were counted after 21d. Means and standard error of the mean derived from three independent experiments are shown. ***$P < 0.001$, **$P < 0.01$ as determined by Student's *t* test.

Genome-wide mapping of regions bound by AGL18

We hypothesized that AGL18 with AGL15 may play a key role in the regulation of Arabidopsis embryogenesis. To test our hypothesis, we mapped genome-wide in vivo binding sites of AGL18 using chromatin immunoprecipitation (ChIP), followed by deep sequencing (ChIP-SEQ), which was then combined with RNA-SEQ data to distinguish the direct or indirect and responsive targets (see below).

Prior work (ChIP–chip) to map AGL15 binding sites used culture tissue that expresses embryo programs (referred to as embryonic culture tissue [ECT]; Wang et al., 2002; Harding et al., 2003). ECT provides an abundant and robust tissue for characterization of genome-wide binding sites by ChIP, and has been used not only for AGL15 (Zheng et al., 2009), but also for the key embryo B3 domain TFs FUS3 and ABI3 (Wang and Perry, 2013; Tian et al., 2020b). A *35S:AGL15* transgene allows the establishment of ECT, but despite the *35S* promoter, the ECT does not over-accumulate AGL15 protein compared to zygotic embryos (Wang et al., 2002). We engineered ECT to map binding sites for AGL18 (described in the "Materials and methods") by introducing a *35S:AGL18-10x-c-Myc* transgene to allow the use of a commercial antibody for ChIP (Cell Signaling Technology, Inc. Danvers, MA, USA). Although a *35S* promoter was used, the resulting ECT did not show any significant increase in *AGL18* transcript accumulation compared to the tissue without the *35S:AGL18-10x-c-Myc* transgene and had less transcript accumulation than found in 7–8 d after flowering (daf) developing Col seeds (Supplemental Figure S1, example of tissue is shown in Supplemental Figure S2). ChIP was performed as described in the previous report (Wang and Perry, 2013), using anti-c-Myc antibody to immunoprecipitate AGL18-10x-c-Myc–DNA complexes, and the DNA was recovered.

Three biological replicates of the ChIP-SEQ experiment were analyzed using the CLC genomic workbench-12 (ChIP-SEQ Analysis). AGL18 binds to thousands of regions in the Arabidopsis genome. Although there are large differences in the number of sites in each biological replicate, the sequencing data were of good quality. We used a majority rule to further narrow the number of sites considered. The majority rule in our case means at least two of the three replicates have the peak and are further considered as potential targets. Unlike the strategy that a peak must be identified in all replicates, this majority rule could avoid some important peak positions that failed to be detected due to low reads or high noise background (Yang et al., 2014a, 2014b). In our study, we found 3,446 potential peaks that were common among the three AGL18 ChIP-SEQ datasets. A comprehensive list is given

in Supplemental Dataset S1. Location relative to the nearest gene(s) is shown in Supplemental Table S2.

To allow comparison on the same platform, we also performed ChIP-SEQ for AGL15 using ECT (Supplemental Dataset S2; Wang et al., 2002; Harding et al., 2003). The majority (87%) of genes associated with AGL15 binding sites identified previously using a ChIP–chip approach (Zheng et al., 2009; Supplemental Dataset S2) were also identified as potential direct targets in ChIP-SEQ. A larger number of sites were identified in ChIP-SEQ, but the ChIP–chip data considered all three biological replicates, whereas the majority rule applied to the ChIP-SEQ data, and cutoffs for the ChIP–chip peak size were larger.

When the AGL18 and AGL15 ChIP-SEQ datasets were compared, of the 3,446 AGL18-bound genes, more than one-third (1,254) were also associated with AGL15. When the list of genes with potential regulatory regions associated with both AGL15 and AGL18 were analyzed using the Gene ontology (GO) term enrichment tool by Protein Analysis Through Evolutionary Relationships (PANTHER; Mi et al., 2019), many categories were overrepresented, including "Post-embryonic development" (GO:0009791; 1.60-fold enrichment (FE); false discovery rate (FDR) 1.40E-03) and "Regulation in gene expression" (GO:0010468; 1.64 FE; FDR 9.07E-07). Other selected categories within "biological processes" are shown in Figure 2. These categories include reproductive processes, hormone, and stress-related processes. Overrepresented GO categories from "biological processes" for genes with binding sites for only AGL18 or AGL15 also are shown in Figure 2. In summary, the genes bound by AGL18 but not AGL15 were enriched for "seed development" (GO: 0048316; 4.62 FE, FDR 4.24E-02), "plant ovule morphogenesis" (GO: 0048482; 6.93 FE, FDR 1.89E-01), and "regulation of abscisic acid biosynthetic process" (GO: 0010115; 2.13 FE, FDR 2.68E-01). Interestingly, overrepresentation of genes in GO categories "response to gibberellin" (GO: 0009739; 1.90 FE, FDR 1.25E-03), "ethylene-activated signaling pathway" (GO:0009873; 2.12 FE, FDR 1.06E-03), and "response to auxin" (GO:0009733; 1.62 FE, FDR 2.80E-04) were present for AGL15-bound genes (Figure 2).

To determine if any potential cis-motifs were overrepresented in the AGL18 and AGL15-bound regions, sequences corresponding to binding regions were obtained using CisGenome (Ji et al., 2008; Jiang et al., 2010) and analyzed with MEME-Suite 5.3.3 and default settings (Bailey et al., 2009). As shown in Figure 3, both of these MADS TFs showed an overrepresentation of CArG motifs (binding sites for MADS domain proteins with a canonical sequence of CC (A/T)6GG; Shore and Sharrocks, 1995). Variant CArGs with sequences C(A/T)7GG and C(A/T)8G were also identified for AGL18 and AGL15, respectively. Binding site selection studies had previously shown that AGL15 preferentially binds these forms of CArG motifs with longer A/T-rich cores (Tang and Perry, 2003). We then compared the location of the site between the AGL18 and AGL15 data to see if they may potentially overlap. Of the 84% of AGL18 sites with which AGL15 also associates, the majority show binding within 200 bp by both TFs (95%; 835 sites for at least one site for those genes with multiple sites).

Gene expression changes in response to AGL18 accumulation

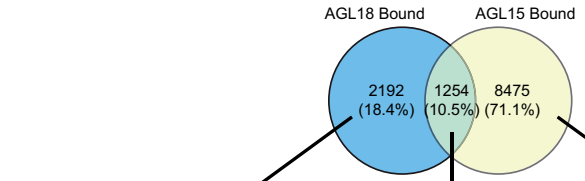
Prior studies on transcriptional regulation indicate that binding may occur without consequences for gene expression, at least under the specific conditions evaluated (Wyrick and Young, 2002; Lee et al., 2007; Oh et al., 2009). Therefore, it is important to determine the genes that respond to changes in AGL18 accumulation. RNA-SEQ was used to assess the transcriptome in response to AGL18 accumulation in Col WT, the *35S:AGL18* line, and the *agl15/18* double mutant. The double mutant *agl15/18* was used because it consistently showed a reduction in SE (Thakare et al., 2008). For this purpose, we used two different tissue systems (1) the SAM SE system (Mordhorst et al., 1998), 10 dac, before any obvious embryo development was apparent and (2) developing seeds collected from 7 to 8 daf siliques. Three biological replicates were performed for each genotype and the percentage of total mapped with genes for each replicate was determined.

Approximately 4,000 potential genes were reported as having significant changes ($P < 0.01$) between the three populations of seeds (7–8 daf) when sorted for those showing at least a two-fold difference between Col WT and the *35S:AGL18* line. The list of 3,106 potential expressed genes (i.e. *35S:AGL18*/Col two–fold or greater) and 789 potential repressed genes (i.e. *35S:AGL18*/Col $\leqslant$0.5-fold) is given in Supplemental Dataset S3. About 350 additional genes were significantly changed with a less stringent fold-cutoff ($P < 0.05$, fold change at least 1.5-fold cutoffs) in three seed (7–8 daf) populations of Col WT and the *agl15/18* double mutant. An additional 106 genes that were potentially expressed (i.e. *agl15agl18*/Col $\leqslant$0.66–fold) and 242 genes that were potentially repressed (i.e. *agl15agl18*/Col $\geqslant$1.5–fold) are given in Supplemental Dataset S3.

SE is a different context than seed development, so we also examined transcript accumulation in response to AGL18 in 10-d-old SAM SE tissues of the *35S:AGL18* line compared to those of Col WT and found a total of 459 genes to be significantly different between these two groups. Two-hundred and sixty-two genes showed increased transcripts, and 197 genes showed decreased transcript levels ($P < 0.05$ and at least 1.5-fold cutoffs; Supplemental Dataset S4). When comparing 10-d-old SAM SE tissue of the *agl15/18* mutant to Col WT using the same cutoffs, there were approximately 3,740 genes with altered transcript accumulations. The result showed a total of 1,629 genes that are upregulated in response to the loss of AGL18/15 (i.e. repressed targets), and 2,111 genes are downregulated (i.e. expressed targets; Supplemental Dataset S4).

Identification of putative direct and indirect targets of AGL18

Combining the ChIP-SEQ and RNA-SEQ results allows discrimination of genes that are directly bound by AGL18 as



GO categories for genes with unique binding sites for AGL18 enriched for:	Fold enrich-ment	P value
Seed development GO:0048316	4.62	4.24E-02
Seed morphogenesis GO:0048317	2.77	1.91E-01
Plant ovule morphogenesis GO:0048482	6.93	1.89E-01
Regulation of embryonic development GO:0045995	2.77	1.17E-01
Meristem development GO:0048507	4.07	1.38E-02
Positive regulation of unsaturated fatty acid biosynthetic process GO:2001280	6.93	1.89E-01
Regulation of abscisic acid biosynthetic process GO:0010115	2.13	2.68E-01

GO categories for genes with unique bindingh sites for AGL15 enriched for:	Fold enrich-ment	FDR
Regulation of post-embryonic development GO:0048580	1.47	3.76E-03
Flower development GO:0009908	1.48	2.53E-03
Post-embryonic plant morphogenesis GO:0090698	1.59	2.21E-02
Response to gibberellin GO:0009739	1.90	1.25E-03
Ethylene-activated signaling pathway GO:0009873	2.12	1.06E-03
Response to carbohydrate GO:0009743	1.70	1.48E-02
Response to salt stress GO:0009651	1.54	4.63E-05
Response to auxin GO:0009733	1.62	2.80E-04

GO categories for genes with regulatory regions bound by AGL18 and AGL15 enriched for:	Fold enrich-ment	FDR
Post-embryonic development GO:0009791	1.60	1.40E-03
Flower development GO:0009908	2.30	1.61E-03
Regulation of post-embryonic development GO:0048580	1.97	4.53E-02
Reproductive shoot system development GO:0090567	2.33	9.23E-04
Response to hormone GO:0009725	1.72	1.34E-04
Response to lipid GO:0033993	1.91	1.76E-03
Response to water deprivation GO:0009414	2.04	4.03E-02
Regulation in gene expression GO:0010468	1.64	9.07E-07

Figure 2 GO enrichment analysis for genes with regulatory regions associated with AGL15, AGL18, or both MADS-domain proteins. PANTHER classification system was used to find the significantly overrepresented categories of genes. FE compares the dataset to the whole Arabidopsis genome (release February 22, 2020). The number of genes bound by either AGL15, AGL18, or both is shown as well as the percentage of fragments of the total (AGL15 and AGL18 targets) bound by one or both.

well as those that are responsive, from genes that change in transcript amounts, but are potentially indirect targets. As shown in Supplemental Figure S3, in Arabidopsis seeds (7–8 daf) 314 genes of the total 3,211 expressed targets were also associated directly with AGL18 (9.8%). On the other hand, 130 genes of the 1,030 (12.6%) repressed targets appeared to be directly regulated by AGL18. Similarly, 238 genes of the 2,373 (10.0%) expressed targets and 197 genes of the 1,826 (10.8%) repressed targets were also directly bound with AGL18 in Arabidopsis SAM SE (Supplemental Figure S3). Thus, overall, ∼10% of genes responsive to AGL18 also showed a direct association with AGL18.

When the lists of direct and responsive targets were examined for overrepresentation of GO terms, directly expressed genes in seeds were overrepresented in the regulation of short-day photoperiodism, flowering (GO: 0048587), and directly repressed genes were overrepresented in the transporter activity category (ABA and auxin transport; Figure 4). In SAM SE, the GO terms hormone catabolic process and gibberellin biosynthesis process were overrepresented in directly expressed genes. Directly repressed genes were overrepresented in developmental process and stress response (Figure 4).

Functional verification of selected AGL18–DNA interactions

A number of genes involved in embryogenesis were bound by AGL18 in the ChIP-SEQ experiment, such as genes encoding the MADS domain TF *AGL15*; *At5g13790* (Heck et al., 1995), *AGL16*; *At3g57230* (Alvarez-Buylla et al., 2000),

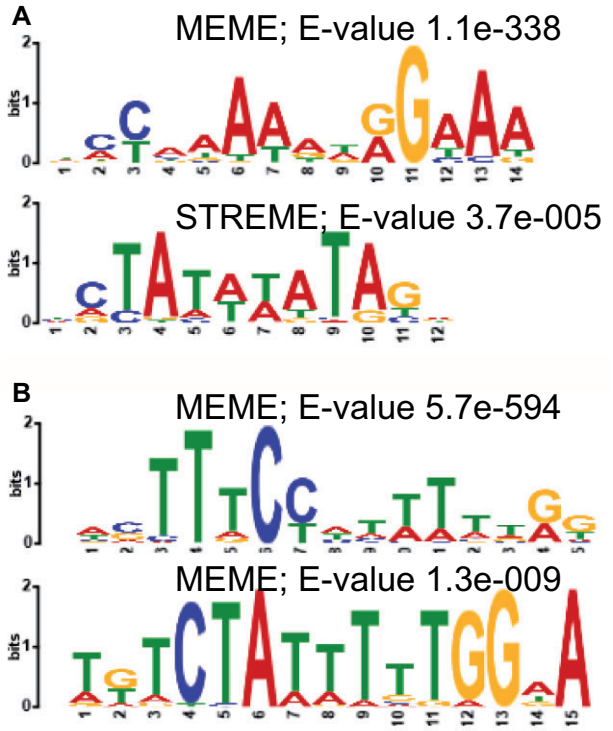


Figure 3 Cis-motifs overrepresented in genomic regions associated with AGL15 or AGL18. A, Overrepresented cis-motifs in regions bound by AGL15. B, Overrepresented cis-motifs in regions bound by AGL18.

seed storage protein *Cruciferin2*; *At1g03880* (Pang et al., 1988), and a gene encoding a gibberellic acid (GA) biosynthesis gene, *GIBBERELLIN 3-OXIDASE2* (*GA3OX2*); *At1g80340* (Curaba et al., 2004). To verify, the sequences corresponding to these genes were occupied in vivo by AGL18, ChIP-qPCR enrichment tests were performed. In this approach, after enrichment of in vivo associated sites by ChIP, the DNA fragment of interest should be present in greater abundance in the *35S:AGL18-10x-c-Myc* immunoprecipitation than the negative control, which lacked the c-Myc antibody. Then quantitative PCR (qPCR) was used to assess the amount of DNA fragment recovered by ChIP using three biological replicates of the ChIP experiments, which were independent of those used for ChIP-SEQ. The term "fold enrichment" is the amount of DNA recovered from the AGL18-10x-c-Myc tissue compared with the negative (no primary antibody) control and normalized to a fragment that had been characterized for AGL15 as nonbound (subsequently verified as nonbound by AGL18 from the ChIP-SEQ data). The FE values of all genes tested were significantly higher when ChIP was performed using c-Myc antibody and AGL18-10x-c-Myc tissue than the no antibody control, suggesting all of the targets tested were associated with AGL18 (Figure 5A).

Verification of the response of target genes to loss- and gain-of-function of AGL18

To confirm whether the key regulators of embryogenesis listed in Supplemental Dataset S5 were responsive to the accumulation of AGL18, reverse transcription-qPCR (RT-qPCR) was performed. We studied transcript accumulation in staged 7- to 8-daf 35S:*AGL18* and *agl15agl18* seeds, and 10-d-old 35S:*AGL18* and *agl15agl18* SAM SE culture tissues with Col WT seeds and SAM SE tissues, respectively. The genes, including *AGL15*, *AGL16*, and *Cru2* showed significantly increased levels of transcript in the 35S:*AGL18* line and reduced levels of transcript in the *agl15agl18* double mutant compared to the WT in both seed and SAM SE tissues (Figure 5, B and C), with the exception of *GA3OX2* in SAM SE, for which the transcript abundance showed a significant reduction in the *35S:AGL18* line, but a nonsignificant difference in the *agl15/18* double mutant (Figure 5C).

AGL18 targets are relevant for SAM SE

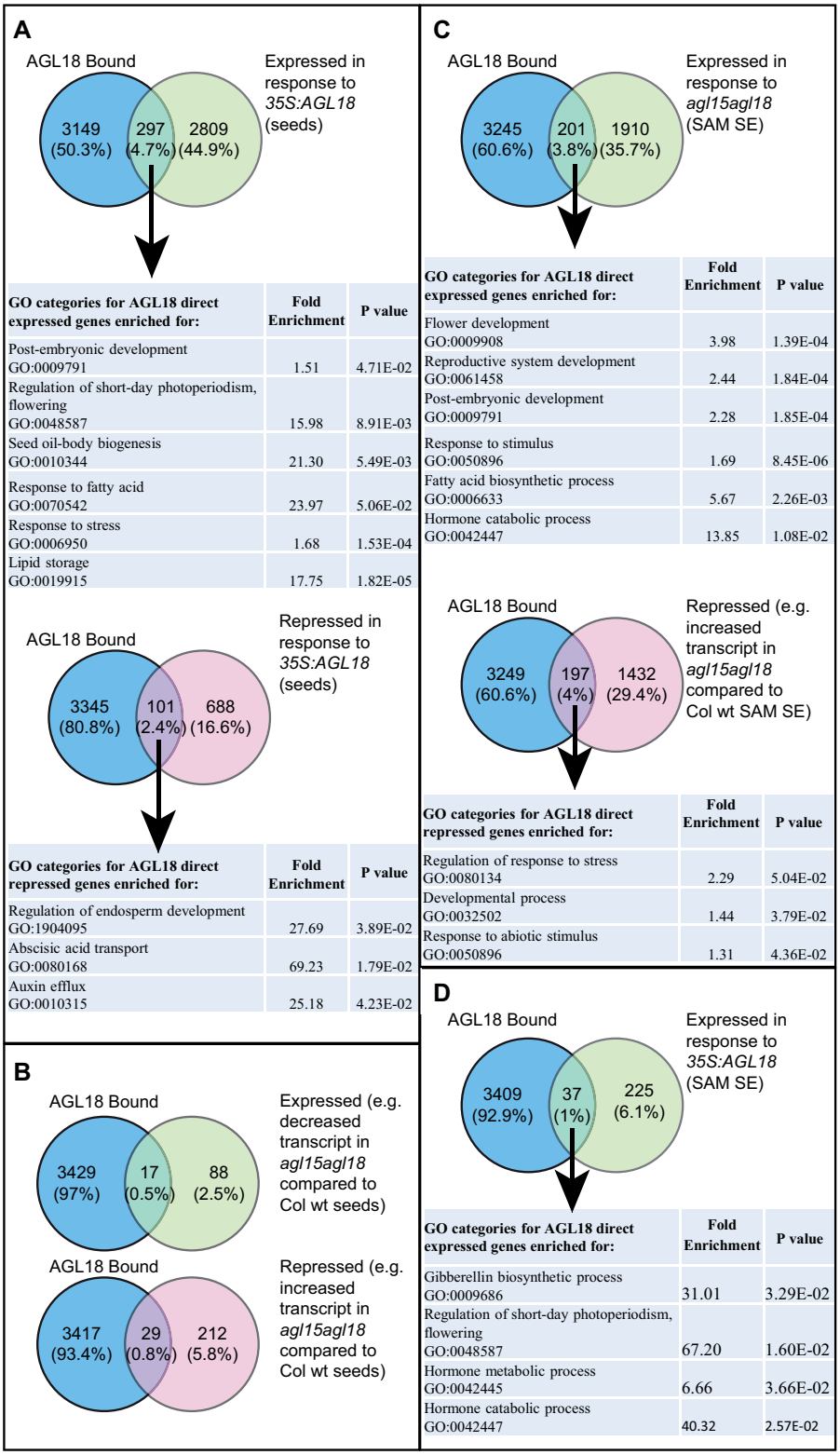
Prior studies showed that AGL15 directly represses a GA biosynthetic gene, *GA3ox2* (Zheng et al., 2009). Notably, Arabidopsis *GA3ox2* is also repressed in response to LEC2 and FUS3, showing lower GA/ABA ratios and embryo identity of organs (Curaba et al., 2004; Gazzarrini et al., 2004; Lumba and McCourt, 2005). Here, we found that regulatory regions of the GA biosynthetic enzyme *GA3ox2* (*At1G80340*) were directly bound by AGL18 (Figure 5A), and the *35S:AGL18* line showed a significant decrease in transcript (0.59-fold, $P < 0.01$) in SAM SE and confirmed by RT-qPCR (Figure 5C). We tested whether *GA3OX2* was potentially involved in the somatic embryo development from the shoot apical region of seedlings (SAM SE) in liquid media containing 2,4-D. A confirmed homozygous knockout line, *ga3ox2*, was obtained from the ABRC (SALK_20777). Our results demonstrated that the *ga3ox2* mutant produced significantly higher SAM SE than Col WT (~50% compared to 27% for the Col control; Figure 5D). Therefore, AGL18 (and AGL15) promotes SE in part by directly repressing the expression of *GA3OX2*.

Meanwhile, *AGL16* was directly expressed by AGL18. A loss-of-function mutant in *AGL16* (SALK_104701C) led to a significant reduction in SAM SE as shown in Figure 5D. These results support the importance of AGL18 upregulating this gene for SAM SE.

Direct regulatory interactions among MADS-domain transcription factors

Notably, MADS domain proteins do not function as monomers, but rather they form multimeric protein complexes that include other MADS domain proteins (Riechmann et al., 1996; Egea-Cortines et al., 1999; de Folter et al., 2005; Immink et al., 2010). While prior work indicated interactions between AGL15 and AGL18 (Serivichyaswat et al., 2015), co-immunoprecipitation (co-IP) data from plant tissue was still lacking. To test whether AGL15 and AGL18 can interact in planta, we performed co-IP using the ECTs expressing both *35S:AGL15* and *35S:AGL18*. As shown in Figure 6, when IP is performed with anti-AGL15 antibody, AGL18-10x-c-Myc can be detected, but not in the controls.

Previous *35S:AGL15* ChIP–chip (Zheng et al., 2009) and current *35S:AGL15* ChIP-SEQ data (Supplemental Dataset



GO categories for AGL18 direct expressed genes enriched for:	Fold Enrichment	P value
Post-embryonic development GO:0009791	1.51	4.71E-02
Regulation of short-day photoperiodism, flowering GO:0048587	15.98	8.91E-03
Seed oil-body biogenesis GO:0010344	21.30	5.49E-03
Response to fatty acid GO:0070542	23.97	5.06E-02
Response to stress GO:0006950	1.68	1.53E-04
Lipid storage GO:0019915	17.75	1.82E-05

GO categories for AGL18 direct repressed genes enriched for:	Fold Enrichment	P value
Regulation of endosperm development GO:1904095	27.69	3.89E-02
Abscisic acid transport GO:0080168	69.23	1.79E-02
Auxin efflux GO:0010315	25.18	4.23E-02

GO categories for AGL18 direct expressed genes enriched for:	Fold Enrichment	P value
Flower development GO:0009908	3.98	1.39E-04
Reproductive system development GO:0061458	2.44	1.84E-04
Post-embryonic development GO:0009791	2.28	1.85E-04
Response to stimulus GO:0050896	1.69	8.45E-06
Fatty acid biosynthetic process GO:0006633	5.67	2.26E-03
Hormone catabolic process GO:0042447	13.85	1.08E-02

GO categories for AGL18 direct repressed genes enriched for:	Fold Enrichment	P value
Regulation of response to stress GO:0080134	2.29	5.04E-02
Developmental process GO:0032502	1.44	3.79E-02
Response to abiotic stimulus GO:0050896	1.31	4.36E-02

GO categories for AGL18 direct expressed genes enriched for:	Fold Enrichment	P value
Gibberellin biosynthetic process GO:0009686	31.01	3.29E-02
Regulation of short-day photoperiodism, flowering GO:0048587	67.20	1.60E-02
Hormone metabolic process GO:0042445	6.66	3.66E-02
Hormone catabolic process GO:0042447	40.32	2.57E-02

Figure 4 Overrepresented GO categories for genes associated with AGL18 by ChIP-SEQ and responsive to AGL18 accumulation in 10 dac SAM SE or 7–8 daf developing seeds. PANTHER classification system was used to find the significantly ($P < 0.05$) overrepresented categories of genes. Here FE compared the direct responsive populations to the Arabidopsis genome. Release February 22, 2020). The number of genes bound by AGL18 and/or responsive or both are shown as well as the percentage of the total of bound and/or responsive. A, Direct expressed and repressed in response to a *35S:AGL18* transgene in developing seeds. B, Overlap of directly associated and responsive to loss of function *agl15 agl18* in developing seeds. No GO categories were overrepresented. C, Direct expressed and repressed in response to loss of function *agl15 agl18* in SAM SE. D, Direct expressed in response to a *35S:AGL18* transgene in SAM SE.

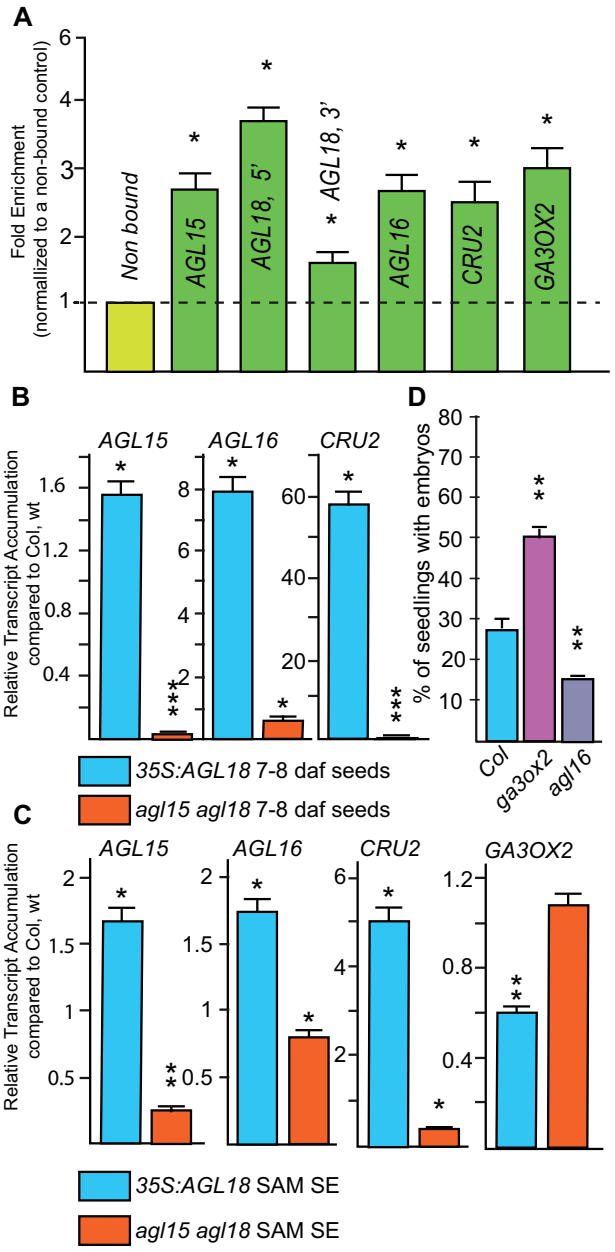


Figure 5 Verification of association of AGL18 with select target genes and response to AGL18 accumulation. A, In vivo association of AGL18 with select putative targets. FE calculations from qPCR on three independent ChIP experiments using c-Myc antibody and AGL18-10x-c-Myc tissue. Data are normalized to a nonbound region of the genome. $^{**}P < 0.01$, $^{*}P < 0.05$ as determined by Student's *t* test. B and C, Response of selected putative targets to AGL18. qRT-PCR was used to assess transcript abundance in gain- and loss-of-function developing seed (7–8 d old) (B) or SAM SE tissue (C) and compared with Col WT in three biological replicates. ***significant at $P < 0.001$; **significant at $P < 0.01$; *significant at $P < 0.05$, as determined using a Student's *t* test. D, AGL18 direct responsive genes impact on SAM SE. Loss-of-function alleles of *ga2ox3* and *agl16* were assessed for SAM SE production. Different lines of seeds are grown in 2,4-D medium and the embryos were counted after 21 d. Means derived from three independent experiments are shown. $^{***}P < 0.001$, $^{**}P < 0.01$ and as determined by Student's *t* test. Error bars in (A) through (D) indicate SE and three biological replicates were used for each sample.

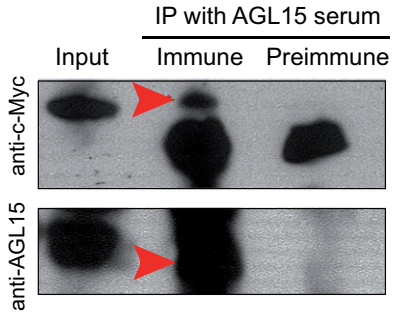


Figure 6 AGL18 interacts with AGL15 in vivo. Precipitation was performed using AGL15 immune serum or preimmune as a control, with protein-A Sepharose beads. Eluted fractions were analyzed by protein gel blot. Detection was first using c-Myc antibody with goat-anti-rabbit-HRP. The arrowhead indicates the co-immunoprecipitated AGL18-10x-c-Myc (top). The lower band is the heavy chain of IgG from the immunoprecipitation. The blot was then probed with anti-AGL15 immune serum to confirm precipitation of AGL15 (bottom).

S2) both showed that AGL15 binds to potential regulatory regions for *AGL18*. Interestingly, *35S:AGL18* ChIP-SEQ data showed that AGL18 binds to potential regulatory regions of *AGL15* (Supplemental Dataset S1). Also, as shown in Figure 5A, AGL18 bound to its own promoter, both at the 5′- and 3′-ends. Because these proteins also interact (Figure 6) and prior work indicated that AGL15 represses its own expression (Zhu and Perry, 2005), we set out to test the regulatory interactions between these genes. To do this, we generated transgenic plants containing *35S:AGL15* or *35S:AGL18* or *35S:AGL15*::*35S:AGL18*. We also looked at the loss-of-function *agl18* and/or *agl15* mutants to further test the relation between AGL15 and AGL18. RNA was isolated from these transgenic, mutant, and Col WT Arabidopsis seedlings grown for 10 d. As shown in Figure 7, the transcripts of *AGL18* in the *35S:AGL15* line were significantly downregulated (−1.5-fold), while significantly upregulated in the *agl15* mutant (10.5-fold) compared with the Col WT seedlings. On the contrary, *AGL15* transcripts in the *35S:AGL18* line were significantly upregulated (1.55-fold) and significantly downregulated (approximately four-fold) in the *agl18* mutant compared with the Col WT seedlings. In the *35S:AGL15*::*35S:AGL18* line, both *AGL15* and *AGL18* were significantly upregulated compared to WT seedlings. Thus, it appears that AGL15 directly represses *AGL18*, the gene product of which can in turn directly activate *AGL15* in a feedback loop (Figure 7).

Phosphorylation is potentially involved in regulation of embryogenesis

It has been previously shown that AGL15 is phosphorylated by MITOGEN-ACTIVATED PROTEIN KINASE3/6 (MAPK3/6) on serine residues 231 and 257 in Arabidopsis in floral receptacles and this is relevant for AGL15 function (Patharkar and Walker, 2015; Patharkar et al., 2016). MAP kinases preferentially phosphorylate serines or threonines

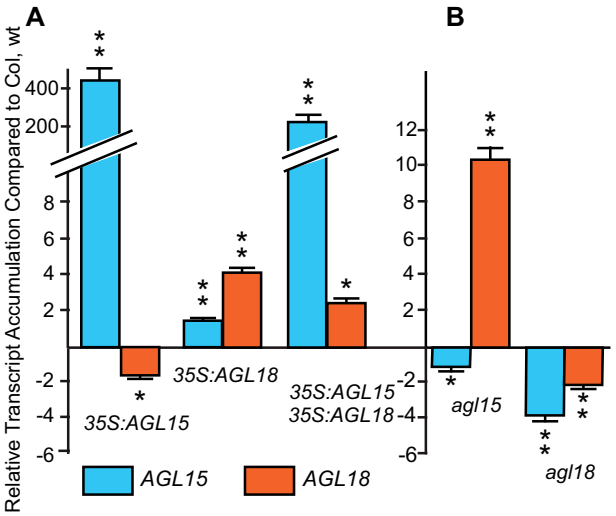


Figure 7 The relation between AGL15 and AGL18. Relative expression of *AGL15* and *AGL18* compared to Col WT in 10 d Arabidopsis seedlings as measured by quantitative real-time PCR. A, Overexpression and (B) mutant lines of *AGL15* and *AGL18*. $^{**}P < 0.01$; $^{*}P < 0.05$ as determined by Student's *t* test. At least three biological replicates were used for each sample and error bars indicate SE.

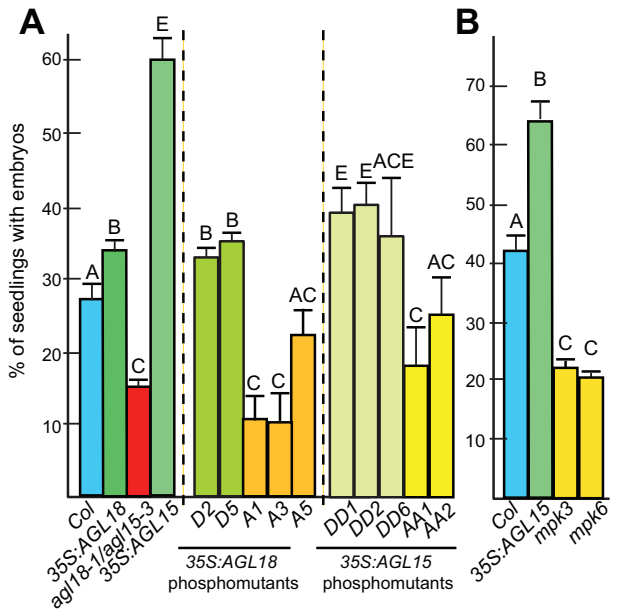


Figure 8 Phosphorylation of AGL18 is important for the promotion of SE. Different lines of seeds were grown in 2,4-D medium and the embryos were counted after 21 d. Means and standard error of the mean derived from at least three biological replicates are shown. Different letters indicate significant difference at $P < 0.05$ as determined by Student's *t* test. A, Phospho-null and phospho-mimic lines of *AGL18* and *AGL15*; the dotted lines are meant to indicate that the AGL18 and AGL15 phosphomutants were compared to the controls of Col, *35S:AGL18*, *35S:AGL15*, and *agl18 agl15* for statistical analysis, but not to each other. B, loss-of-function alleles of *mpk3* and *mpk6*.

followed by a proline (S/T-P; Sheridan et al., 2008; Paul et al., 2017). AGL18 contains one potential MAPK phosphorylation site at Ser 198 (NetPhos version 2.0 algorithm, Blom et al., 1999; Patharkar and Walker, 2015. Whether phosphorylation of AGL18 is important for its function is unknown. To study whether this posttranslational modification is needed for AGL18 to promote SE, we substituted the potential phosphorylation site (S) to alanine (A), which serves as a phospho-null site (Paul et al., 2017) or to aspartic acid (D), a constitutively active phosphorylation mimic, also known as a phospho-mimic (Chan et al., 2017). A least three independent homozygous lines of each mutation were selected for SAM SE experiments. Two of the three transgenic lines of *35S:AGL18*$^{S>D}$ in the Col background showed significantly increased SAM SE (~34%) compared to Col WT (23%) as shown in Figure 8A. This was comparable to *35S:AGL18* with the native gene sequence. However, the *35S:AGL18*$^{S>A}$ plants showed significant decreases in SAM SE production compared to Col WT for two of the three lines tested (Figure 8A). Production of SAM SE in these lines was comparable to that found for the *agl15 agl18* double mutant.

Phosphorylation of AGL15 also impacted SAM SE (Figure 8A). Two phosphorylation sites were identified: Ser 231 and Ser at 257 and were changed to alanine (AA lines) or aspartic acid (DD lines; Patharkar et al., 2016). These constructs were kindly provided by Prof. John Walker whose lab demonstrated the necessity of phosphorylation of AGL15 for processes in abscission (University of Missouri). We generated transgenic lines in Col WT and tested them for SAM SE. As shown in Figure 8, the DD lines showed significant increase in SAM SE compared to Col WT, whereas at least one of the AA lines tested showed decrease in SAM SE and was comparable to the *agl15 agl18* double mutant .

Previous studies established that AGL15 is phosphorylated by the MAPK cascade consisting of MPKK4/5 and MPK3/6 (Patharkar and Walker, 2015). To determine whether this MAPK cascade has a potential role in somatic embryo development, confirmed homozygous knockout lines of *mpk3* (SALK_209371) and *mpk6* (SALK_062471.45.25.x) were obtained from the ABRC. Seeds from homozygous insertional lines were allowed to complete germination in the SAM SE system. Our results showed that *mpk3* and *mpk6* loss-of-function mutants produce significantly reduced SAM SE compared to Col WT (Figure 8B). Therefore, phosphorylation may be necessary to promote SE in Arabidopsis.

Discussion

Numbers and types of genes regulated by AGL18

AGL18 is the closest homolog to AGL15 in soybean and Arabidopsis, and overexpression of this gene promotes SE in both soybean (Zheng and Perry, 2014) and, as reported here, in Arabidopsis. These two genes also show some redundancy in the control of flowering time (Adamczyk et al., 2007) and promotion of SE (Figure 1). To understand the nature of the redundancy at a molecular level, we assessed transcript accumulation and genome-wide binding by this TF. We also extended prior ChIP–chip work on AGL15 by performing ChIP-SEQ for this factor to allow us (1) to

compare binding for AGL18 and AGL15 using the same platform and (2) To compare ChIP–chip and ChIP-SEQ for AGL15. Few, if any, prior studies looked at targets of a heterodimer genome-wide in plants. Here we identified more than 3,000 binding sites for AGL18 and approximately 9,000 AGL15-associated binding sites using ChIP-SEQ. Based on previous work with other DNA binding factors (Lee et al., 2007; Kaufmann et al., 2009; Oh et al., 2009; Zheng et al., 2009), the large number of binding sites for AGL15 and AGL18 is common. For AGL15, the majority of sites identified by ChIP–chip (Zheng et al., 2009) were also identified by ChIP-SEQ. More than one-third of the sites bound by AGL15 were also identified as sites associated with AGL18, and typically the sites were within 200 bp, supportive of binding as a heterodimer. Serivichyaswat et al. (2015) demonstrated an in vitro interaction between AGL15 and AGL18 by yeast two-hybrid and pull-down and confirmed the potential for an in vivo interaction via bimolecular fluorescence complementation (BiFC) (Serivichyaswat et al., 2015). Here we demonstrated an in planta interaction by co-IP (Figure 6). If these MADS TFs form heterodimers, why the discrepancy in the number of sites bound by both AGL15 and AGL18? First, data to date suggest that AGL18 may not directly interact with DNA. Serivichyaswat et al. (2015) was not able to demonstrate an interaction with CArG motifs for AGL18 by gel shift assays, and suggested that AGL18 may interact with other factors that can bind to DNA in a sequence-specific manner, to regulate gene expression. Because the fixation step with formaldehyde can stabilize protein–protein, as well as protein–DNA interactions, ChIP can identify DNA fragments with which TFs associate indirectly via protein–protein interaction, but this may be expected to be less efficient. AGL15 can form homodimers to bind DNA, although thus far no evidence has been provided that AGL18 can do the same (Serivichyaswat et al., 2015). Importantly, Serivichyaswat et al. (2015) also propose that other factors may be involved in the complexes that include AGL15 and/or AGL18 to control gene expression, and, if AGL18 does not bind DNA directly, it would be interesting to determine how AGL18 associates with gene regions not bound by AGL15. When regions associated with just AGL18 were assessed using MEME-Suite, sequences related to CArG motifs were identified, suggesting that either AGL18 can associate directly with DNA in planta, or associate via interaction with another MADS-domain protein. Finally, *AGL15* transcript accumulates to higher amounts than *AGL18* transcript in embryo tissue and this may reflect protein abundance and resulting stoichiometry of complexes and occupancy of sites.

Although a *35S:AGL18* transgenic line was used to map binding sites, there is not an overaccumulation of *AGL18* transcript compared to SE tissue or to developing seeds without this transgene. Why would there be less transcript than in developing seeds? This may reflect the fact that *AGL18* transcript accumulation occurs at the highest levels in the endosperm although it, like *AGL15*, is expressed in the zygotic embryo as well as SE and other tissues (Alvarez-Buylla et al., 2000; Lehti-Shiu et al., 2005; Adamczyk et al, 2007; Supplemental Table S1). Conversely, there is an increase in transcript accumulation for *AGL15* in *35S:AGL15* SE tissue compared to seeds and this may reflect the fact that *AGL15* mRNA within the seed is mostly in the developing embryo with little in the endosperm at the early stages of development. However, protein accumulation in developing zygotic embryos and ECT appears similar (Wang et al., 2002).

We next determined the targets that respond to AGL18 accumulation. By combining this information with the ChIP data, we determined direct compared to indirect responsive targets. As is typical in these studies, a minority of sites associated with AGL18 showed a significant response in target transcript accumulation compared to Col WT under the conditions tested and cutoffs used. Less than 10% of the AGL18 direct targets responded to AGL18 accumulation in developing seeds and mid-stage SAM SE tissue at the cutoffs we used. About 10% of the responsive genes show evidence of direct association. This is comparable with the fraction of AGL15-associated genes that showed response to AGL15 (Zheng et al., 2009) as well as in other studies (Lee et al., 2007; Oh et al., 2009; Yamamoto et al., 2010; Wang and Perry, 2013; Tian et al., 2020b). Therefore, this appears to be a general trend for TFs, or reflects the particular conditions under which we measure response. One possible reason for this discrepancy between binding and response is addressed in a review by Wyrick and Young (2002). Here they review the literature to show that binding without obvious regulation may be common and may represent conditions where particular cofactors or signals are required to activate a response. The observation that relatively few direct responsive genes were discovered using *agl15 agl18* developing seed compared to WT suggests that there may be higher levels of redundancy in this developmental context (Supplemental Dataset S5). Our finding that AGL16 is a direct target of AGL18 and is involved in promotion of SAM SE (Figure 5), and is structurally related to AGL15 and AGL18, including containing an EAR domain that is involved in repression of gene expression (Hill et al., 2008), provides a direction to look for increased redundancy of MADS-factor controlling embryo development.

Putative AGL18 direct targets and the MADS-domain TFs regulatory network

AGL15 and AGL18 appear to interact in a complex regulatory loop where AGL18 increases mRNA accumulation of *AGL15*, but AGL15 has a repressive effect upon the expression of *AGL18* (Figure 7). AGL15 also represses its own expression (Zhu and Perry, 2005). In addition, AGL15 can form DNA-binding homodimers as well as heterodimers with AGL18, but to date, there is no evidence for AGL18 forming homodimers or binding directly to DNA. Other genes encoding MADS domain proteins are also impacted upon by AGL15 and/or AGL18 accumulation. SEPALLATA3 (SEP3) is involved in many MADS-factor complexes including those

that incorporate AGL15 (Immink et al., 2009), and expression patterns of *SEP3* and *AGL15* overlap (Lehti-Shiu et al., 2005; Immink et al., 2009). AGL15 directly represses *SEP3*, and AGL18 may be involved in this regulation. Interaction of AGL15 with SEP3 has been confirmed by co-IP (Supplemental Figure S4). Conversely, SEP3 associates with regulatory regions of both *AGL15* and *AGL18* (Kaufmann et al., 2009). *AGL16*, which is directly induced by AGL18, has a role in the promotion of SAM SE as shown in Figure 5D. While it is intriguing to consider, additional experiments will need to be performed to look at the function of particular complexes (e.g. sequential ChIP) as well as higher levels of redundancy in embryo development. One possibility may be to check for the phenotype in *agl15/16/18* triple mutant lines.

AGL15 was found to directly regulate several key regulators of embryogenesis including FUS3, and ABI3 (Zheng et al., 2009). The ChIP-SEQ experiment reported here also shows the association of AGL15 with LEC1, and BABY BOOM (BBM), but not LEC2. AGL18 does not show association with any of these genes with the exception of LEC2 (AGL15 was shown to associate with LEC2 in ChIP-qPCR experiments, but binding to this site is weaker and in fact was not identified with cutoffs used for ChIP–chip; Zheng et al., 2009). LEC2 is sufficient to promote SE and may explain part of AGL15 and AGL18's ability to increase SAM SE. Several genes appear to be directly repressed by both AGL15 and AGL18 in SAM SE tissue. Excluding the MADS discussed above, these are overrepresented for "regulation of transcription" as assessed by DAVID Bioinformatics Resources 6.8 ($P = 0.04$; Huang et al., 2009).

Some other genes encoding TFs relevant for seed/SE development that appear directly responsive to AGL18 include *PLETHORA2* (*PLT2*) and *ABI4*. *PLT2* has been shown to induce SE when ectopically expressed (Horstman et al., 2017), and this is a gene that appears directly upregulated by AGL18 in SAM SE. ABI4 has roles in seed development (Soderman et al., 2000; Penfield et al., 2006). In addition, several factors involved in ethylene biosynthesis and response are encoded by directly expressed targets including *At1g05010* (*1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE44*; ethylene biosynthesis) and two ethylene response factors (*At1g19210* and *At3g16280*), that based on data in eBAR (Winter et al., 2007) are preferentially expressed in seeds. Ethylene has been found to promote SE in our system (Zheng et al., 2013a, 2013b). A gene encoding an NF-YC5 subunit of CCAAT binding factors that interacts with LEC1 (Calvenzani et al., 2012) and is expressed primarily in seeds based on eBAR (Winter et al., 2007) is an additional directly expressed AGL18 target.

Based on our findings, we propose a model (Figure 9) demonstrating the transcriptional regulation by AGL15 and AGL18. This model outlines the finding that phosphorylation is important for AGL18's ability to enhance SE, and we have verified binding and response, as well as demonstrated a role in SE for one expressed target (*AGL16*) and one repressed target (*GA3ox2*). It also highlights some in planta complexes involved in embryogenesis, and a feedback loop to potentially control AGL15 homodimer and AGL15–AGL18 heterodimer formation.

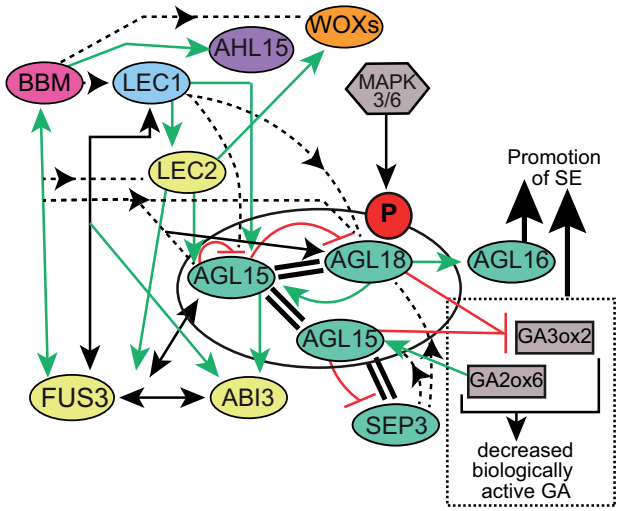


Figure 9 Working model showing interactions between major factors that influence SE. TF are in ovals; MADS in green, B3 domain in yellow, AP2 in pink, AT-Hook in purple, WUSCHEL-related homeobox in orange, and CCAAT in blue. Solid green arrows or red bars are meant to indicate direct up- or downregulation, respectively. Dotted lines indicate putative direct association with regulatory regions of the gene and arrowhead is meant to indicate what TF binds which gene. Double bars indicate protein–protein interactions. The circle around AGL15 and AGL18 indicates importance of phosphorylation for function. Not all interesting interactions of proteins are shown in the interest of clarity. Relevant references include Pelletier et al. (2017), Braybrook et al. (2006), Yamamoto et al. (2010), Wang and Perry (2013), Mönke et al. (2012), Tian et al. (2020a, 2020b), Zheng et al. (2009), Horstman et al. (2017), Karami et al. (2021), and Wang et al. (2020). AHL15 (also called AGF2; AT-HOOK PROTEIN OF GA FEEDBACK2); WOXs, WUSCHEL-RELATED HOMEOBOXes.

Hormonal regulation of embryogenesis

GA is one of the key hormones in plant development. The GA/ABA ratio may determine whether tissue develops as embryonic or postembryonic tissue (Curaba et al., 2004; Gazzarrini et al., 2004; Lumba and McCourt, 2005). Arabidopsis *GA2ox6*, a GA catabolic enzyme, was directly upregulated in response to AGL15 (Wang et al., 2004). Biologically active GA inversely corresponded with competence for somatic embryo development and seed dormancy (Wang et al., 2004). Similar to the previous report on AGL15 (Zheng et al., 2016), AGL18 directly represses transcript accumulation from the GA biosynthetic gene *GA3ox2*, with decreased transcript observed in the *35S:AGL18* line compared with the WT in SAM SE. However, AGL18 does not directly regulate *GA2ox6*. Finally, *ga3ox2* homozygous knockout (KO) lines showed a significantly higher number of embryo development compared to Col WT in SAM SE culture (Figure 5D).

Role of phosphorylation in embryogenesis

AGL15 (likely AGL18 also) is itself phosphorylated by MPK3 and MPK6 (Patharkar and Walker, 2015). Transgenic knockout lines of both *mpk3* and *mpk6* showed significantly reduced embryos development in SAM SE (Figure 8B). To further understand the role of AGL15/AGL18 phosphorylation by MAPK3/6, we transformed Col WT *35S:AGL18*$^{S>D}$ phospho-mimic and *35S:AGL18*$^{S>A}$ phospho-null constructs as well as similar constructs for *AGL15* (provided by Prof. J. Walker: Partharkar and Walker, 2015). Our SAM SE studies indicate that phosphorylation is necessary for embryo development with phospho-mimic lines promoting SAM SE, whereas phospho-null lines actually show significantly reduced SAM SE (Figure 8A). It would be practical and very interesting to compare transcriptomes of the phospho-mimic and -null mutant to Col WT in future studies and study the interdependence of the different forms in a variety of backgrounds.

Materials and methods

Plant material

Arabidopsis (*A. thaliana*) Col WT, insertional loss-of-function alleles (*agl15-3*, *agl18-1*, the *agl15agl18* double mutant, *agl16*, *ga3ox2*, *mpk3*, and *mpk6*) and *35S:AGL15*, *35S:AGL18*, *35S:AGL15::35S:AGL18* and phospho-mutant plants (all Col ecotype) were sown on MS (Classic Murashige and Skoog, 1962) supplemented with 10 g L^{-1} sucrose, 0.5 g L^{-1} MES, and 7 g L^{-1} agar, pH 5.8, with 50 mg/mL kanamycin for *35S:AGL15*, *35S:AGL18* and *35S:AGL15::35S:AGL18* seeds. These seeds were chilled at 4°C for 3 d and transferred to a growth room with a 16-h-light/8-h-dark cycle (long-day light conditions). Seedlings (10 d) were then transferred to a potting mix (ProMix BX; Premier Brands) and grown in a chamber with a 16-h-light (20°C)/8-h-dark (18°C) cycle.

To stage seed, flowers were tagged on the day of anthesis and seeds were collected and flash-frozen in liquid nitrogen for RNA extraction at 7–8 daf. SAM SE was performed as described in the previous report (Harding et al., 2003) and tissue collected for RT-PCR and RNA-SEQ at 10 d after the start of culture and flash frozen. Tissue was examined at 21 d after the start of culture to score for embryo production.

To generate ECT accumulating AGL18, *35S:AGL18-10x-c-Myc* transgenic plants were crossed with *35S:AGL15* plants, and the *35S:AGL18-10x-c-Myc:: 35S:AGL15* transgenic lines were recovered by demonstration of hygromycin resistance and characteristic phenotype of *AGL15* overexpressors (Wang et al., 2002). Developing embryos were cultured as previously described (Harding et al., 2003) to initiate ECT. Please see Supplemental Figure S2 for images of the tissue.

Transgene constructs

For the *35S:AGL18*-10x-c-Myc construct, about 2.5 kb of the genomic region was amplified and cloned into pENTR/D-TOPO vector (Invitrogen). The insert was transferred into destination vector pGWB19 (10x-c-Myc) under the CaMV35S promoter and *rbcS* terminator, obtained from Dr T. Nakagawa, Shimane University (Nakagawa et al., 2009) following the manufacturer's instructions for Gateway LR Clonase II Enzyme Mix (Invitrogen, Waltham, MA USA).

For *35S:AGL18*$^{S>A/D}$ phosphomutants, the *AGL18* gDNA was amplified and cloned into pENTR/D-TOPO vector (Invitrogen). Site-directed mutation was performed by using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's instructions. The inserts were transferred into destination vector pGWB19 (Nakagawa et al., 2009) as described above. The similar constructs for AGL15 were generated by Prof. J. Walker's lab and generously provided to our lab.

ChIP and co-IP

ECT with *35S:AGL18*-10x-c-Myc was fixed in MC buffer as described previously (Zheng and Perry, 2011). ChIP was performed as described previously (Tian et al., 2020b). The control was the same tissue, but ChIP was performed without a primary antibody. co-IP was done by the protocol described in (Fiil et al., 2008) using *35S:AGL18-10x-c-Myc::35S:AGL15* ECT tissue.

ChIP-SEQ and data analysis

To analyze the ChIP-SEQ data CLC genomic workbench 12 (Epigenomic Analysis–ChIP-SEQ Analysis) was used, following the workflow as described in the CLC Manual using default settings (https://resources.qiagenbioinformatics.com/tutorials/ChIPSEQ_peakshape.pdf). In short, we mapped the reads to the reference (Arabidopsis TAIR10) genome using default setting parameters, Match core = 1, Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3, Length fraction = 0.5, Similarity fraction = 0.8, and Ignore the Nonspecific match handling thresholds. The results of read mapping were then used as input to the TF ChIP-SEQ tool to detect significant peaks with Maximum *P*-value for peak calling to the value of 0.05 instead of the default value 0.1. Thus, we used a more stringent threshold. We verified the quality measures for control and samples that were acceptable for the quality threshold. Because we and others have found relevant TF binding sites not only at the 5′-region, but also at the 3′-region and within the gene (Zheng et al., 2009), peaks were associated with both nearby genes, using the Epigenomic analysis tool. The DNA sequences associated with peaks in the ChIP-SEQ data were extracted using the Utility tool. The number of significant peaks for the ChIP-SEQ datasets is given in Supplemental Table S3.

RNA isolation and RNA-SEQ

Developing seeds (7–8 daf) or SAM SE tissues were collected as described above. RNA was extracted using the QIAGEN RNeasy Plant Mini Kit and supplementing the RLC buffer with 1% final (w/v) high MW PEG. Two to three biological replicates were prepared and sent for library preparation and RNA-SEQ (Novogene, California). Data were analyzed by using CLC genomics workbench 12. The percentage of total mapped reads with genes for

each replicate was determined. The mapped reads, which serve as a measure of sequencing accuracy and lack of contaminating DNA, were in the range of 70%–90% (Conesa et al., 2016). To cast the broadest net, we used $P < 0.05$ and fold change at least 1.5-fold cutoffs. GO term analysis was performed using PANTHER (Mi et al., 2019).

ChIP-qPCR and RT-qPCR

Experiments to confirm binding by AGL18 (ChIP-qPCR) and response to AGL18 accumulation (RT-qPCR) were performed as described in Wang and Perry (2013). For verification of ChIP-SEQ targets experiments, ECT expressing *35S:AGL18-10x-c-Myc* was used with anti-c-Myc (Cell Signaling Technologies, Inc., Danvers, MA, USA). The specific primers used for these experiments are listed in Supplemental Table S4.

Statistical analysis

Information on the statistical analysis for the ChIP-SEQ and RNA-SEQ is provided in the relevant "Materials and methods". Statistical analysis for verification of binding and response of targets, as well as SAM SE, is described in Zheng et al. (2009) and Wang and Perry (2013), and the Figure Legends.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the Bioproject accession number PRJNA777254.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. *AGL15* and *AGL18* transcript accumulation in tissues used in this study.

Supplemental Figure S2. Images from the SE systems used in the study.

Supplemental Figure S3. Direct versus responsive AGL18 targets.

Supplemental Figure S4. AGL15 interacts with SEP3 in vivo.

Supplemental Table S1. Summary of expression data for *AGL18* and *AGL15*.

Supplemental Table S2. Location summary of binding sites for AGL18 and AGL15.

Supplemental Table S3. Number of significant peaks in the AGL18 ChIP-SEQ Datasets.

Supplemental Table S4. Oligonucleotides used in this study.

Supplemental Dataset S1. AGL18 bound potential targets::3,447 (2 out of 3 method).

Supplemental Dataset S2. AGL15 bound potential targets::9729 (2 out of 3 method).

Supplemental Dataset S3. Genes with transcript accumulation responsive to AGL18 accumulation in seeds.

Supplemental Dataset S4. Genes with transcript accumulation responsive to AGL18 accumulation in SAM SE.

Supplemental Dataset S5. Direct responsive targets of AGL18.

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