Gene Regulation by the AGL15 Transcription Factor Reveals Hormone Interactions in Somatic Embryogenesis[1[OPEN]]

Qiaolin Zheng, Yumei Zheng, Huihua Ji, Whitney Burnie, and Sharyn E. Perry*

Department of Plant and Soil Sciences, University of Kentucky, Lexington, Kentucky 40546-0312

ORCID ID: 0000-0002-3011-8240 (S.E.P.).

The MADS box transcription factor Arabidopsis (*Arabidopsis thaliana*) *AGAMOUS-LIKE15* (*AGL15*) and a putative ortholog from soybean (*Glycine max*), *GmAGL15*, are able to promote somatic embryogenesis (SE) in these plants when ectopically expressed. SE is an important means of plant regeneration, but many plants, or even particular cultivars, are recalcitrant for this process. Understanding how (Gm)AGL15 promotes SE by identifying and characterizing direct and indirect downstream regulated genes can provide means to improve regeneration by SE for crop improvement and to perform molecular tests of genes. Conserved transcription factors and the genes they regulate in common between species may provide the most promising avenue to identify targets for SE improvement. We show that (Gm)AGL15 negatively regulates auxin signaling in both Arabidopsis and soybean at many levels of the pathway, including the repression of *AUXIN RESPONSE FACTOR6* (*ARF6*) and *ARF8* and *TRANSPORT INHIBITOR RESPONSE1* as well as the indirect control of components via direct expression of a microRNA-encoding gene. We demonstrate interaction between auxin and gibberellic acid in the promotion of SE and document an inverse correlation between bioactive gibberellic acid and SE in soybean, a difficult crop to transform. Finally, we relate hormone accumulation to transcript accumulation of important soybean embryo regulatory factors such as ABSCISIC ACID INSENSITIVE3 and FUSCA3 and provide a working model of hormone and transcription factor interaction in the control of SE.

AGAMOUS-LIKE15 (*AGL15*; Arabidopsis Genome Initiative identifier *At5g13790*) encodes a MADS domain transcription factor that is expressed at its highest level in zygotic embryos, although it is not unique to embryo development and has roles after the completion of germination (Adamczyk et al., 2007). When ectopically expressed via a *35S* promoter, *AGL15* can enhance somatic embryogenesis (SE) in two different systems in Arabidopsis (*Arabidopsis thaliana*) and can lead to long-term maintenance (over 19 years to date) of development in embryo mode (Harding et al., 2003; Thakare et al., 2008). Loss-of-function alleles of *agl15*, especially when present with a loss-of-function allele in *agl18*, the closest family member to AGL15, show significantly reduced SE (Thakare et al., 2008). Like *AGL15*, *AGL18* can promote SE when overexpressed in Arabidopsis (Adamczyk et al., 2007). Genes encoding putative orthologs of AGL15 and AGL18 were isolated from soybean (*Glycine max*; referred to as GmAGL15 [*Glyma12g17721* and *Glyma11g16105*] and GmAGL18 [*Glyma02g33040*]) and were able to enhance SE in this species when expressed via a *35S* promoter (Zheng and Perry, 2014). Recently, three AGL15 orthologs from cotton (*Gossypium hirsutum*) were shown to be preferentially expressed during SE, especially the induction phase in response to the synthetic auxin 2,4-D, and when overexpressed, all three orthologs led to more rapid production of embryogenic callus and better quality of callus (Yang et al., 2014). In addition, expression of an AGL15-like gene was correlated with early embryogenic culture initiation in maize (*Zea mays*; Salvo et al., 2014).

Somatic embryo development provides an accessible system that has been used as a model for zygotic embryogenesis (Vogel, 2005; Rose and Nolan, 2006), but how this process occurs is not well understood, even though this phenomenon of totipotency has been known since 1958 (Steward et al., 1958). Understanding SE is important because it is one means of regeneration of plants to meet agricultural challenges. In soybean, the transformability of different varieties is related directly to competence for SE (Ko et al., 2004; Kita et al., 2007; Klink et al., 2008), suggesting that improvement of regeneration will aid in transformation competence. In addition, testing the functions of genes generally includes studying results of increased/ectopic expression as well as loss of function. Both of these approaches

[1] This work was supported by the United Soybean Board (no. 0282/1282/2822), the National Science Foundation (grant no. IOS–0922845), and the University of Kentucky Microarray Pilot Grant Program. This is publication no. 16-06-027 of the Kentucky Agricultural Experiment Station.

* Address correspondence to sperr2@uky.edu.

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 (www.plantphysiol.org) is: Sharyn E. Perry (sperr2@uky.edu).

S.E.P. directed the project, supervised the research, and contributed some of the data; Q.Z. designed and performed the majority of the experiments and analyzed the data; Y.Z. performed some of the Arabidopsis work; W.B. contributed to the analysis of the *tir1* mutants; H.J. performed the auxin measurements; Q.Z. and S.E.P. contributed to writing the article with comments from Y.Z. and H.J.

[OPEN] Articles can be viewed without a subscription.
www.plantphysiol.org/cgi/doi/10.1104/pp.16.00564

 © 2016 American Society of Plant Biologists. All Rights Reserved.

include technologies that involve the transformation and regeneration of plants. However, little is known about what makes cells competent to respond to induction for SE, a trait that varies depending on plant, tissue, age, or even particular cultivar of a species. To better understand how (Gm)AGL15 promotes SE, we assessed transcriptomes in response to (Gm)AGL15 accumulation in Arabidopsis and soybean (Zheng et al., 2009; Zheng and Perry, 2014). Comparison of the results in these two species led to the discovery that ethylene has a role in SE (Zheng et al., 2013) and that key embryo transcription factors are controlled directly by (Gm) AGL15 in both species (Zheng and Perry, 2014). Here, we report on (Gm)AGL15 regulation of auxin signaling and integration with GA metabolism.

RESULTS

TRANSPORT INHIBITOR RESPONSE1 Is a Direct Target of AGL15 Repression

The expression of *AGL15* via a *35S* promoter ($35S_{pro}$) enhances SE in both Arabidopsis and soybean (Harding et al., 2003; Thakare et al., 2008; Zheng and Perry, 2014). In one Arabidopsis SE system, seeds are allowed to complete germination in liquid medium containing the synthetic auxin 2,4-D (Mordhorst et al., 1998). By 21 d after culture (dac), a fraction of the callused seedlings will have SE development from the region of the shoot apical meristem (SAM SE), and a positive correlation between SAM SE and AGL15 accumulation has been reported (Harding et al., 2003; Thakare et al., 2008). To understand how (Gm) AGL15 promotes SE, transcriptome analysis in response to $35S_{pro}$:*(Gm)AGL15* was performed in both species (Zheng et al., 2009; Zheng and Perry, 2014). Because of a central role for auxin, generally added as 2,4-D, in inducing SE, genes responsive to (Gm)AGL15 and involved in auxin biosynthesis or response were of particular interest. In both the Arabidopsis microarray experiment to assess gene regulation in response to AGL15 (Zheng et al., 2009) and the similar soybean experiment (Zheng and Perry, 2014; discussed below), a gene encoding TRANSPORT INHIBITOR RESPONSE1 (TIR1; *At3g62980*), an auxin receptor (Ruegger et al., 1998), was found to be repressed in response to (Gm)AGL15. For the Arabidopsis microarray experiment, 10-dac SAM SE tissue was used for RNA extraction, and this was before any obvious SE development. The ratio of *TIR1* transcript was 2.02 (significant at $P < 0.01$) for the *agl15 agl18* tissue compared with the Columbia (Col) wild type, with a slight (0.81) but not significant reduction for $35S_{pro}$:*AGL15* compared with the wild type (Zheng et al., 2009).

The results of the Arabidopsis chromatin immunoprecipitation (ChIP)-chip experiments to globally map AGL15 association with the genome indicated that regions that may correspond to regulatory regions of *TIR1* appeared to be bound by AGL15 (Supplemental Fig. S1). This gene was not identified as a direct target by Zheng et al. (2009) because the peaks that met the cutoffs used were assigned to *At3g62988*, which was closer. Primers specific for a weaker upstream peak that did not meet the cutoff used in the ChIP-chip analysis (about 260 bp 5′ of the ATG) but that includes several noncanonical CArG motifs were designed to verify the in vivo association with AGL15 by ChIP-quantitative PCR (qPCR) enrichment tests (Supplemental Table S1). As shown in Figure 1A, the fold change (comparison of suspected target in the immune precipitation to isolate AGL15-DNA complexes with the preimmune control) for association with this region of *TIR1* was 43.2 compared with 2.7 for the nonbound *TUBULIN ALPHA-3* (*TUA3*) fragment (or 17.9 if normalized to *TUA3*; both show significantly higher association of AGL15 with *TIR1* at $P < 0.05$). Another way to analyze the data involves comparing the amount of suspected target (*TIR1*) to nonbound fragment (*TUA3*) within the same immune precipitation. This ratio was 3 for the immune precipitation compared with 0.2 for the preimmune control (Fig. 1B). To allow the recovery of AGL15-DNA complexes independent of the AGL15 antiserum, we used a form of AGL15 with a C-terminal tandem affinity purification (TAP) tag (Puig et al., 2001). When immune precipitation was performed via the TAP tag of AGL15-TAP and using IgG-Sepharose to bind to the

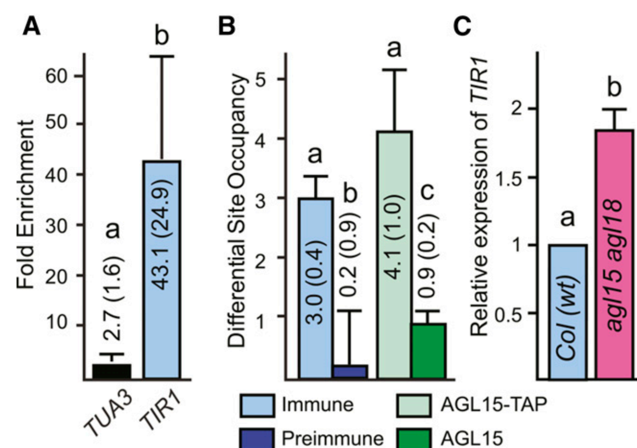

Figure 1. The gene encoding the auxin receptor TIR1 is a directly repressed target of AtAGL15. A, Fold enrichment from qPCR on three independent ChIP experiments. The level of product in the immune precipitation is compared with the control (preimmune) for a nonbound region (*TUA3*) and for the AGL15-bound target *TIR1*. B, Calculations comparing within the same precipitation the amount of target DNA fragment (*TIR1*) with the nonbound region (*TUA3*). Preimmune is shown as a control. AGL15-TAP tissue and control precipitation using AGL15 tissue provided a method independent of AGL15 antiserum to assess the AGL15-DNA association. In this experiment, IgG-Sepharose was used to isolate complexes via the protein A domain within the TAP tag added to the C-terminal end of AGL15. C, qRT-PCR to assess transcript abundance in the Col wild type (wt) and *agl15 agl18* double loss-of-function mutant developing seeds at 9 to 10 d after flowering (daf). Means and SE for at least three independent experiments are shown. Different letters within each part indicate significance at $P < 0.05$ as determined using Student's *t* test.

protein A domain of the TAP tag, the difference in occupancy values for the recovery of *TIR1* DNA fragments compared with the nonbound fragment *TUA3* was higher than 4, indicating a specific association, compared to 0.9 for ChIP using nontagged control tissue.

The expression of *TIR1* was assessed in developing seeds. *TIR1* transcript accumulation in 9- to 10-d developing seeds of the *agl15 agl18* double mutant was increased significantly compared with the Col wild type (Fig. 1C).

Repression of *TIR1* Is Relevant for SE and May Be by Impact on GA Accumulation

AGL15/18 protein and *TIR1* transcript accumulation were inversely correlated, and ChIP data support *TIR1* as a direct AGL15 repressed gene. Does this repression impact SE? To test this, we used the system described by Mordhorst et al. (1998) described briefly above. Typically, 15% to 30% of the Col wild type has SAM SE development, whereas $35S_{pro}$:*AGL15* has twice this amount, while *agl15 agl18* shows a reduction to about one-half that found in the Col wild type (Harding et al., 2003; Thakare et al., 2008). Subsequently, we found that targets of AGL15 contribute to this SAM SE (Wang et al., 2004; Zheng et al., 2009).

To test whether the loss of *TIR1* expression may contribute to enhanced SAM SE, three independent loss-of-function alleles were obtained from the Arabidopsis Biological Resource Center and tested compared with the Col wild type in the SAM SE system. Two alleles, *tir1-10* (SALK_151603C) and *tir1-11* (SALK_090445C; up to *tir1-9* is described in Ruegger et al., 1998), are insertional mutants (Alonso et al., 2003) into the 5′ untranslated region or an exon, respectively, that showed reduced levels of transcript, while *tir1-1* (CS3798) is an ethyl methanesulfonate-generated mutant that causes a Gly-to-Asp substitution at position 147 and had no obvious reduction of transcript (Ruegger et al., 1998; Supplemental Fig. S2A). As shown in Figure 2A, all three alleles showed significant increases in the percentage of seedlings with SAM SE compared with the wild type.

Because auxin and GA impact upon each other and prior work demonstrated that a directly expressed target of AGL15 encodes a GA 2-oxidase (AtGA2ox6 encoded by *At1g02400*; Wang et al., 2004), we investigated the interaction between these hormones in the control of SAM SE. Prior work used a knockdown allele of *ga2ox6* in the Wassilewskija (Ws) ecotype and demonstrated a decrease in SAM SE relative to the Ws wild type (Wang et al., 2004). However, Ws is poorly embryogenic in this system compared with Col. We obtained a loss-of-function allele in Col that is also a knockdown allele rather than a knockout allele (SALK_059724; the insertion is within an intron; Supplemental Fig. S2B). As shown in Figure 2B, this *ga2ox6* allele showed a significant reduction of SAM SE.

Because auxin signaling impinges on GA accumulation (Frigerio et al., 2006; Weiss and Ori, 2007), we tested whether the increased SAM SE observed for *tir1*

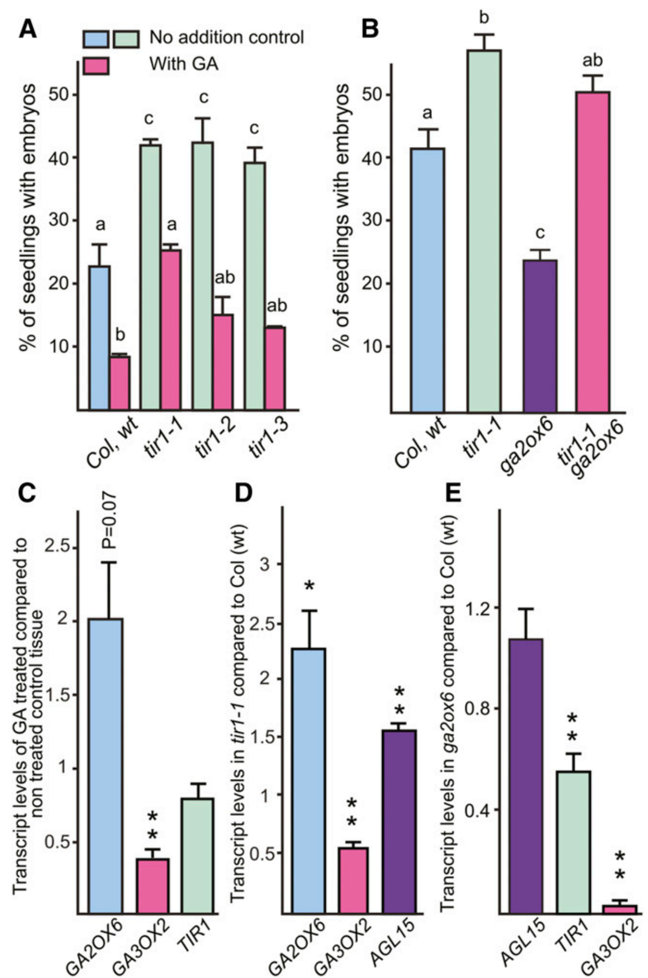

Figure 2. Repression of *TIR1* is relevant for SE, and this may be by the control of biologically active GA. A, Three different loss-of-function alleles of *tir1* show significantly increased SAM SE compared with the Col wild type (wt). With the addition of 5 μM GA_3, the frequency of SAM SE is decreased significantly. Different letters indicate significant differences at $P < 0.05$. B, A loss-of-function allele in a GA catabolic enzyme, *GA2ox6*, decreases SAM SE compared with the wild type as well as in the *tir1* background. Different letters indicate significant differences at $P < 0.01$. C to E, SAM SE tissue at 10 dac was used to examine transcript accumulation from a GA biosynthetic enzyme (*GA3ox2*), *GA2ox6*, *TIR1*, and *AGL15* in response to GA_3 treatment (C) or in response to loss of *tir1* (D) or loss of *ga2ox6* (E). Means and SE from three biological replicates are shown. Asterisks indicate significant differences from the control at $P < 0.05$ (*) and $P < 0.01$ (**) as determined using Student's *t* test.

loss-of-function alleles may be via GA. SAM SE cultures supplemented with 5 μM GA_3, a biologically active GA, led to a significant decrease in the frequency of SAM SE compared with nonsupplemented medium for both the Col wild type and all three *tir1* alleles (Fig. 2A). We examined transcript accumulation in response to GA treatment and found that a gene encoding a GA catabolic enzyme (*GA2ox6*) showed an increase in transcript accumulation with GA_3 treatment compared with the control, and a gene encoding a GA biosynthetic enzyme

(*GA3ox2*) showed a significant decrease, as expected by feedback control (Hedden and Phillips, 2000; Olszewski et al., 2002), demonstrating that GA treatment was effective. Although the fold change was not significant for *GA2ox6* when all biological replicates were considered together, all individual experiments showed significant increases compared with nontreated samples when analyzing the technical replicates. While somewhat decreased, *TIR1* did not show a significant change (Fig. 2C). However, both the catabolic and biosynthetic enzymes showed significant responses to loss of *tir1*: in *tir1-1* compared with Col wild-type 10-dac SAM SE tissue, the genes responded in a manner that would indicate a decrease of biologically active GA in response to loss of TIR1 (Fig. 2D). This is consistent with auxin signaling leading to bioactive GA accumulation (for review, see Weiss and Ori, 2007). Furthermore, AGL15 showed a slight but significant increase in the *tir1-1* background. We also examined the transcript accumulation of select genes in the *ga2ox6* knockdown SAM SE tissue and found a significant decrease in *TIR1* transcript in this situation where endogenous GA accumulation is perturbed (Fig. 2E). Consistent with the exogenous GA treatment, increased GA led to a reduction of *TIR1* transcript, although with the exogenous GA the reduction in TIR1 transcript was not significant (Fig. 2, C and E). As expected based on feedback regulation, expression of the biosynthetic enzyme as measured by transcript abundance was decreased in a situation with increased GA (Fig. 2, C and E). Finally, we generated a double mutant between *tir1-1* and *ga2ox6* and, as shown in Figure 2B, the double mutant produced SAM SE at a frequency intermediate, and not significantly different at $P < 0.01$, to the Col wild type or *tir1-1*. In summary, these results indicate an interaction between auxin signaling and GA with feedback on AGL15 and developmental consequences affecting SAM SE.

TIR1 and a *GA2ox* Also Are Direct Targets of GmAGL15

We performed a microarray experiment using the Affymetrix Soybean Array and comparing explants suitable for inducing SE on D40 induction medium (so-called due to the concentration of 2,4-D) from the soybean cv Jack compared with tissue expressing a $35S_{pro}$:*GmAGL15* transgene in cv Jack. This *GmAGL15* overexpression transgene led to a significant increase in SE on D40 medium and enhanced proliferation upon subculturing (Zheng and Perry, 2014). For the uninduced explant tissue, cotyledons of the stage used for SE (from 4- to 5-mm embryos) were collected. We also compared tissue induced for 3 dac on D40 medium.

At least two putative orthologs of *TIR1* (*Glyma07g30910* and *Glyma08g06390*) were repressed significantly in response to GmAGL15 at 0 dac. By 3 dac on D40 medium, *Glyma07g30910* showed a slight but significant increase for two of the three probe sets in $35S_{pro}$:*GmAGL15* compared with cv Jack. A third putative ortholog (*Glyma19g39420*) that was present on the microarray showed no significant change. One putative ortholog of GA2ox6, *Glyma02g01330*, showed significantly increased transcript in $35S_{pro}$:*GmAGL15* compared with cv Jack at 0 dac (1.9-fold and significant at $P < 0.01$) followed by a significant decrease at 3 dac (0.4-fold and significant at $P < 0.01$). Other putative orthologs were not present or signal was absent on the array. Supplemental Table S2 shows the identity and similarity between the soybean genes and Arabidopsis genes used in this study.

qRT-PCR was used to confirm the microarray results using independently generated tissue for *Glyma07g30910* (referred to as *GmTIR1*) and *Glyma02g01330* (referred to as *GmGA2ox6*). As shown in Figure 3A, the qRT-PCR results agree well with what the microarrays revealed. *GmTIR1* appeared to be initially repressed by $35S_{pro}$:*GmAGL15*, whereas *GmGA2ox6* was expressed at 0 dac. By 3 dac, the pattern reversed and was significant for both genes. The reversal for *GmTIR1* was due at least in part to differences in the response to culture on 2,4-D. As shown in Figure 3B, when transcript was compared between culture for 3 d on D40 medium or on the same medium but lacking 2,4-D (D0), *GmTIR1* showed a

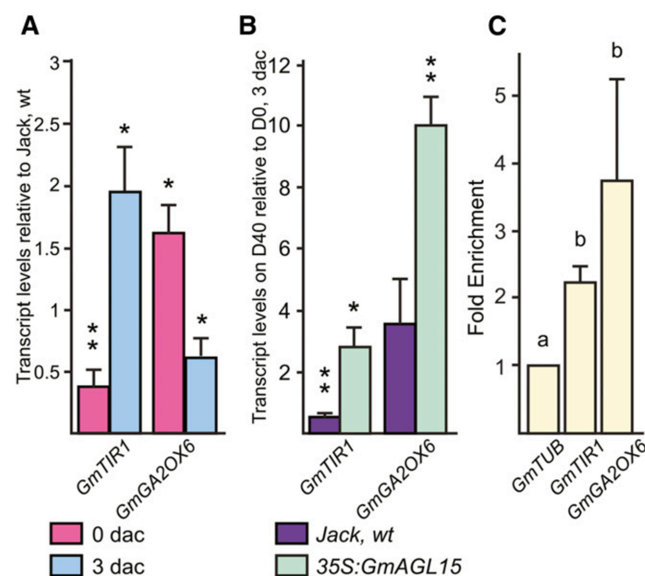

Figure 3. *GmTIR1* and *GmGA2ox6* are directly responsive targets of GmAGL15. A, Transcript accumulation from soybean orthologs of *TIR1* and *GA2ox6* in $35S_{pro}$:*GmAGL15* compared with the cv Jack wild type (wt) for explants (0 dac) and after 3 d on D40 medium. B, Transcript accumulation from *GmTIR1* and *GmGA2ox6* in response to 2,4-D. The data compare transcripts after 3 d on D40 medium with 3 d on the same medium but lacking 2,4-D. Asterisks indicate significant differences at $P < 0.01$ (**) and $P < 0.05$ (*). C, Fold enrichment calculations from qPCR on at least three independent ChIP populations to determine the amounts of suspected target in the immune precipitation compared with the preimmune control precipitation. *GmTUB* is the nonbound control fragment, and the results are normalized to this control. Data shown are means and SE for three to five biological replicates. Different letters within each part indicate significance.

reduction in cv Jack in response to 2,4-D but showed an up-regulation in the *35S*$_{pro}$:*GmAGL15* background in response to 2,4-D. *GmGA2ox6* responded positively to 2,4-D in both cv Jack and *35S*$_{pro}$:*GmAGL15*, so the response to 2,4-D alone cannot explain the switch from increased to decreased transcript in *35S*$_{pro}$:*GmAGL15* compared with cv Jack between 0 and 3 dac. If GmTIR1 leads to the repression of GA catabolism, as reported in other tissues (Weiss and Ori, 2007) and confirmed in Arabidopsis SAM SE (Fig. 2D), one might expect a reduction of *GmGA2ox6* correlating with increased *GmTIR1* in *35S*$_{pro}$:*GmAGL15* at 3 dac, as was observed for the comparison of *35S*$_{pro}$:*GmAGL15* and the control (Fig. 3A). But when comparing 3 dac on D40 with 3 dac on D0 (Fig. 3B), the expected pattern was observed for the cv Jack wild type (i.e. decreased *GmTIR1* and increased *GmGA2ox6*), but why do transcripts from both genes increase in the *35S*$_{pro}$:*GmAGL15* background? In the Arabidopsis eFP browser at the Bio-Analytic Resource for Plant Biology (Winter et al., 2007), *GA2ox6* shows a large increase in transcript in response to wounding. Because the data in Figure 3A compare explants (wounded) on D40 for 3 dac with explants (0 dac) flash frozen, minimizing the wound response, whereas the data in Figure 3B compare explants at 3 dac with and without 2,4-D, the wounding responses also may be reflected in the data in Figure 3B.

Is the regulation of *GmGA2ox6* and *GmTIR1* by GmAGL15 direct, as found for Arabidopsis (Wang et al., 2004; Fig. 1)? In order to investigate whether these genes are bound by GmAGL15 in soybean, three independent biological replicates of the ChIP experiment were performed using anti-AGL15-specific antiserum (raised against *Brassica napus* AGL15; Perry et al., 1996) or preimmune serum as a control. ChIP-qPCR was used to quantitate the association of DNA fragments with AGL15 in the immune precipitation compared with controls. Specific primers (Supplemental Table S1), which can amplify the DNA fragments in regulatory regions of the soybean genes that contain potential binding sites for MADS domain proteins (CArG motifs), were designed to verify the in vivo association with GmAGL15. As shown in Figure 3C, when one compares the amount of the suspected target in the immune precipitation compared with the preimmune precipitation, both *GmTIR1* and *GmGA2ox6* regulatory regions were associated directly with GmAGL15. These results indicate that these genes are regulated directly by GmAGL15 in soybean as they are by AGL15 in Arabidopsis.

Manipulation of GA Affects Soybean Somatic Embryogenesis

Both endogenous and exogenous GA decrease Arabidopsis SAM SE (Wang et al., 2004; Fig. 2, A and B). To investigate whether GA has any effect on SE from cotyledon explants in soybean, explants from young embryos from a *35S*$_{pro}$:*GmAGL15* transgenic line (8981) and wild-type cv Jack were cultured on D40 supplemented with or without 10 μM GA_3. To quantitate, somatic embryo induction was scored as described by Meurer et al. (2001). Approximately 21 to 25 explants were placed per culture plate. Individual explants were scored as 0 if no embryos were produced, 1 if one to five embryos were present, 2 if six to 15 embryos were present, and 3 if more than 15 embryos were present. The score for each plate was calculated by summing the score for each explant on the plate and then dividing by the total number of explants. The average was calculated among all plates of a given genotype and treatment. The score of the embryos from cv Jack was less than 0.5 on D40 medium containing GA_3 at all time points but was greater than 1 on D40 medium lacking GA_3. This difference is significant at $P < 0.001$ (Fig. 4A). Similarly, the score of embryos from *35S*$_{pro}$:*GmAGL15* (line 8981) on D40 medium with GA_3 was significantly less than that on D40 medium (Fig. 4B). Furthermore, the effect of paclobutrazol, an inhibitor of GA biosynthesis, on SE was investigated. Addition of 150 nM paclobutrazol to the D40 medium significantly increased the SE score for nontransgenic cv Jack at 30 dac, with a score of 2.27 for explants with paclobutrazol compared with 1.94 for unsupplemented D40 medium (significant at $P < 0.01$; Fig. 4C). The numbers of embryos on explants with or without paclobutrazol increased at 45 dac, with slightly, but not significantly, higher numbers with paclobutrazol. Therefore, as found in Arabidopsis, the production of SE is negatively correlated with biologically active GA in soybean.

Consistent with the effect of GA or paclobutrazol on soybean SE, *GmAGL15/18*, *ABSCISIC ACID INSENSITIVE3* (*ABI3*), and *FUSCA3* (*FUS3*) were down- or up-regulated by treatment with GA_3 or paclobutrazol at 7 dac on D40 medium (Fig. 4D). Specifically, GA treatment significantly decreased the transcripts of *GmAGL18*, *GmABI3*, and *GmFUS3*. Conversely, *GmAGL18*, which can promote SE (Zheng and Perry, 2014), was significantly up-regulated by paclobutrazol treatment (Fig. 4D). As expected, *GA2ox6* was negatively regulated by paclobutrazol treatment, since paclobutrazol is an inhibitor of GA biosynthesis and GA metabolism shows much feedback regulation (Hedden and Phillips, 2000). *GmTIR1* also showed significantly reduced transcript abundance in response to paclobutrazol, consistent with a role in the repression of this gene being involved in SE (Fig. 4D). While transcript abundance from some genes did not show significant changes in response to GA or paclobutrazol when the results from three to four biological replicates were considered together, in the individual experiments, deriving significance from the technical replicates, transcripts from *GmAGL15*, *GmABI3*, and *GmFUS3* generally were increased significantly in response to paclobutrazol compared with untreated controls. Similarly, the transcript from *GmAGL15* was reduced in response to GA_3 when the change within a biological replicate was significant.

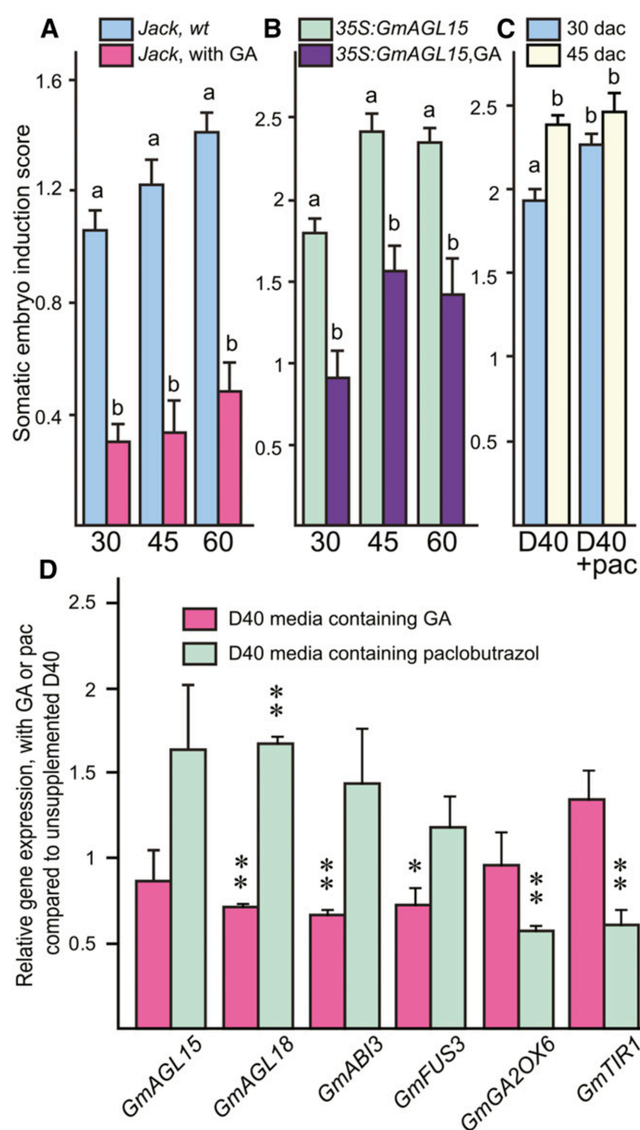

Figure 4. Effects of GA on SE in soybean. A to C, Somatic embryo production in immature cotyledon explants of the cv Jack wild type (wt; A) and $35S_{pro}$:*GmAGL15* (line 8981; B) with and without 10 μM GA_3 or the cv Jack wild type with and without 150 nM paclobutrazol (pac; C) in D40 medium. Means and SE for at least five plates of explants per genotype/time point are shown. Different letters indicate significant differences at $P < 0.0001$ (A) and $P < 0.01$ (B and C). D, Gene expression in response to bioactive GA accumulation in soybean. Immature cotyledon explants (cv Jack wild type) were placed onto D40 medium with and without GA_3 or paclobutrazol supplement. Tissue was collected at 7 dac, and qRT-PCR was performed for soybean genes of interest. Comparisons were with unsupplemented D40 medium. Means and SE are shown for three to four biological replicates. Asterisks indicate significant differences at $P < 0.05$ (*) and $P < 0.01$ (**).

Other Genes Encoding Products Involved in Auxin Response Are Regulated by Gm(AGL15)

(Gm)TIR1 was repressed by (Gm)AGL15 in Arabidopsis and soybean. Prior work demonstrated that the AGL15 directly expressed target *INDOLE-3-ACETIC ACID INDUCIBLE30* (*IAA30*) has a role in the promotion of SE. *IAA30* encodes an AUX/IAA protein that binds to auxin response factors (ARFs) and represses the activity of the ARF until auxin-mediated signaling leads to ubiquitination and degradation of the AUX/IAA. IAA30 lacks the domain that leads to degradation and, consequently, is more stable (Sato and Yamamoto, 2008). We examined the expression array data to identify genes that respond to (Gm)AGL15 similarly in Arabidopsis and soybean. We found that *At1g30330*, which encodes ARF6, had a small (1.42) but significant increase in transcript in the *agl15 agl18* double mutant that has reduced SAM SE compared with the Col wild type. The $35S_{pro}$:*AGL15* tissue showed reduction in transcript from this gene compared with the wild type, but this was not significant in the microarray experiment (0.87). The most closely related ARF, *ARF8* (*At5g37020*; 74% identical and 81% similar to ARF6 at the protein level), which has redundant functions in flowering (Nagpal et al., 2005), showed consistent but not significant changes in transcript accumulation (1.17 for *agl15 agl18* and 0.68 for $35S_{pro}$:*AGL15* compared with the wild type). Putative orthologs of these genes showed significant changes in transcript accumulation in $35S_{pro}$:*GmAGL15* compared with cv Jack in soybean at 0 dac. These include *Glyma11g31940* (putative ortholog of *At5g37020*) and *Glyma15g09750* (*At1g30330*), which both showed significant fold change at 0 dac for $35S_{pro}$:*GmAGL15* compared with cv Jack of 0.6. By 3 dac, while there was still a modest (0.8) reduction, this was no longer significant. A number of other genes with the best Arabidopsis match being ARF6 or ARF8 also showed reductions in transcript, significant for some genes but not for others.

Also intriguing was the fact that *ARF6* appeared to be a direct target of AGL15 based on the Arabidopsis ChIP-chip, whereas *ARF8* did not appear bound (Zheng et al., 2009; Supplemental Fig. S3, A and B, respectively). Finally, a gene encoding a microRNA that posttranscriptionally regulates *ARF6* and *ARF8* (Wu et al., 2006) also was bound directly by AGL15 (Supplemental Fig. S3C). This microRNA is not on the expression array, so no data on whether the gene was responsive to AGL15 or not was available. Therefore, we further tested the regulation of these genes in Arabidopsis and soybean and examined their impact on SE.

In 10-dac SAM SE, there was a significant decrease in *ARF6* transcript in $35S_{pro}$:*AGL15* compared with the wild type, while the *agl15-3* mutant showed a significant increase (Fig. 5A). The trends were the same for *ARF8* transcript, but the changes were not significant when all data were considered together. Transcript from the *microRNA167A* gene was increased in $35S_{pro}$:*AGL15* compared with the Col wild type (Fig. 5A). There was no significant change in the *agl15-3* mutant compared with the Col wild type, but based on high quantitation cycle values, this gene may not be expressed in these genotypes and only shows ectopic expression in $35S_{pro}$:*AGL15*. In the context of developing seeds (9–10 daf), transcript from the *microRNA167A*

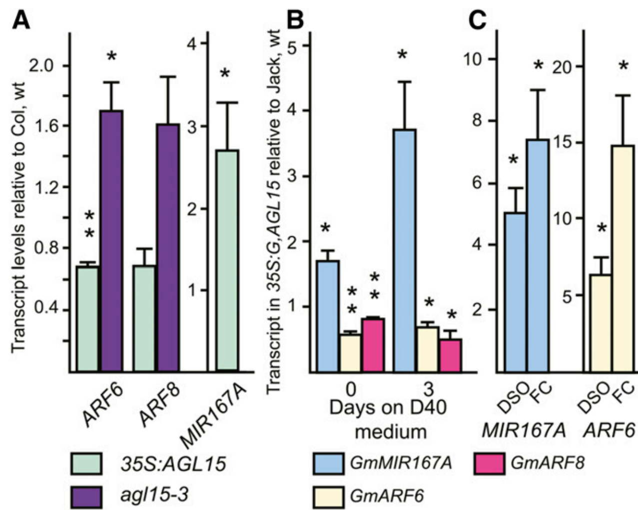

Figure 5. Regulation of a microRNA167A-encoding gene and its targets *ARF6* and *ARF8* by (Gm)AGL15. A, Transcript accumulation in response to increased AGL15 ($35S_{pro}$:*AGL15*) or decreased AGL15 (*agl15-3*) compared with the Col wild type (wt) in SAM SE tissue. B, Transcript accumulation of putative soybean orthologs of the genes of interest in response to $35S_{pro}$:*GmAGL15* in cotyledon explants (0 d in culture) and after 3 d on D40 induction medium. C, AGL15 associates with the *miRNA167A* and *ARF6* genes directly as measured by ChIP-qPCR. FC indicates fold change and compares the amount of the target in the immune ChIP with the preimmune control. DSO indicates the different site occupancy of AGL15 at the presumed target relative to a nonbound DNA fragment (*TUA3*) within the immune precipitation. Results shown are means and SE for at least three independent biological replicates. Asterisks indicate significant differences at $P < 0.01$ (**) and $P < 0.05$ (*).

gene was increased significantly in $35S_{pro}$:*AGL15* compared with the Col wild type by 3.2-fold and was decreased significantly in the *agl15-3* mutant compared with the Col wild type by 2.5-fold, but there were no significant changes in *ARF6* or *ARF8* transcript. Soybean explants (0 dac) and explants placed in culture showed consistent and significant changes, with *pri-miRNA167A* showing increased transcript in $35S_{pro}$:*GmAGL15* and *GmARF6* and *GmARF8* showing significant decreases (Fig. 5B).

We performed ChIP-qPCR experiments on three biological replicates to confirm that the *miRNA167A*-encoding gene and *ARF6* are direct targets of AGL15 in Arabidopsis. As shown in Figure 5C, both are indeed bound directly by AGL15 when targeting in immune compared with preimmune precipitation, or when targeting compared with nonbound control in the immune precipitation, was calculated.

Repression of *ARF6* Is Relevant for SE

To test whether decreased ARF6 and/or ARF8 is relevant for the promotion of SE, we obtained *arf6* and *arf8* seeds as well as seeds homozygous for *arf6* and segregating for *arf8* (a double homozygous knockout is sterile; Nagpal et al., 2005) from the Arabidopsis Biological Resource Center and as a gift from Dr. Jason Reed. As shown in Figure 6, *arf6-1* and seeds obtained from *arf6-1* homozygous/*arf8-2*,*ARF8* plants showed significant increases in SAM SE production compared with Col, but *arf8-2* did not have a significant increase in SAM SE. The results of second alleles (*arf6-2* and *arf8-3*) are shown in Supplemental Figure S4. We added GA_3 to see if, as for *tir1*, GA may be downstream of ARF6/8 and found that GA_3 reduced SAM SE to the same level as the Col wild type with GA_3 (Fig. 6).

We assessed *ARF6*, *ARF8*, and *pri-miRNA167A* in *tir1-1* tissue compared with the wild type. For both 10-dac SAM SE and developing seeds collected 9 to 10 daf, there were significant increases in *pri-miRNA167A* and significant decreases in *ARF6* and *ARF8* transcripts in *tir1* loss-of-function alleles compared with the wild type (Fig. 7). These results indicate positive feedback of TIR1 upon auxin signaling and are consistent with the reduction in this pathway promoting SE, possibly through GA accumulation.

Overexpression of GmAGL15 Does Not Lead to Increased IAA

Prior analysis in a soybean microarray study where $35S_{pro}$:*GmAGL15* and the cv Jack wild type were compared at 0 dac (isolated explants) and then after 3 d on D40 medium that contains 40 mg L^{-1} 2,4-D revealed extensive overlap between genes expressed in response to increased GmAGL15 compared with cv Jack at 0 dac and genes up-regulated in cv Jack in response to culture on the synthetic auxin 2,4-D. Similarly, there was much overlap between genes with reduced transcript in these

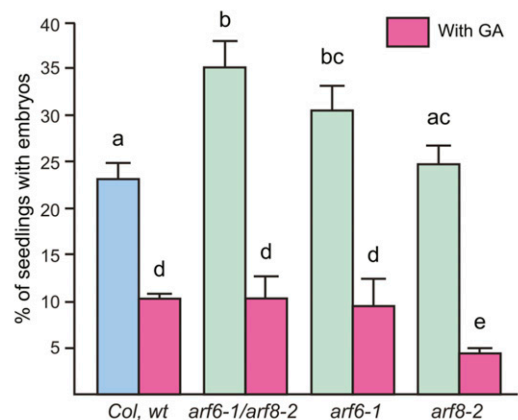

Figure 6. The repression of *ARF6* and *ARF8* is relevant for SE and may be by the control of biologically active GA. Seeds from homozygous *arf6-1* or from a double mutant homozygous for *arf6-1* and segregating for *arf8-2* show increased SAM SE compared with the Col wild type (wt). The addition of GA_3 reduces the frequency of SAM SE. Results shown are means and SE for three biological replicates of the experiment. Different letters indicate significant differences at $P < 0.05$ as determined by Student's *t* test.

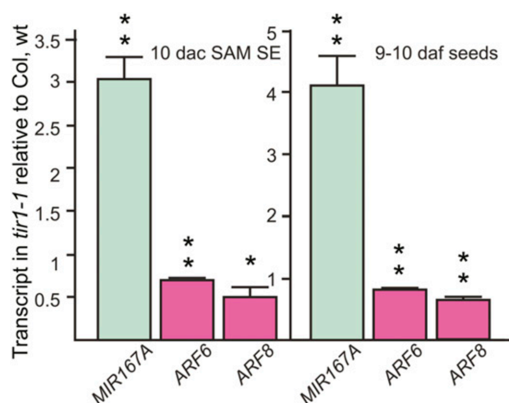

Figure 7. Transcript accumulation from *MIR167A*, *ARF6*, and *ARF8* is perturbed in *tir1-1*. The transcript accumulation in *tir1-1* developing seeds and SAM SE was compared with that in Col wild-type (wt) tissue. Results shown are means and SE for three biological replicates. Asterisks indicate significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) as determined using Student's *t* test.

two data sets (Zheng and Perry, 2014). How 2,4-D induces SE is not well understood. It may act as an auxin, induce endogenous auxin production, or act as a stressing agent. A number of genes demonstrated to be responsive to IAA in Arabidopsis (Nemhauser et al., 2006) have putative orthologs in soybean that are up-regulated in $35S_{pro}$:*GmAGL15* compared with cv Jack as well as in response to culture on D40 medium, including *IAA11*, *IAA13*, *ACS6*, and *GH3-1*. Thus, a possible explanation for enhanced SE by $35S_{pro}$:*GmAGL15* may be increased auxin within this tissue.

Unwounded 4- to 5-mm embryos were isolated from cv Jack and from $35S_{pro}$:*GmAGL15* (line 8981 in cv Jack). Extraction for ultra-performance liquid chromatography-tandem mass spectrometry was performed as described by Fu et al. (2012) with some modifications. As shown in Figure 8, contrary to expectations, there was a significant decrease in IAA content in the $35S_{pro}$:*GmAGL15* tissue compared with cv Jack. We also analyzed a transgenic $35S_{pro}$:*GmAGL15* in a different soybean cultivar, Williams 82 (line 1041), and compared it with nontransgenic cv Williams 82. The results were similar to those in cv Jack. The GmAGL15-overaccumulating tissue contained significantly less IAA (Fig. 8).

We used the Arabidopsis SAM SE system to test whether $35S_{pro}$:*AGL15* would respond differently to different amounts of 2,4-D than does the Col wild type. The level of 2,4-D in this system is usually 4.5 μM (1× amount), and $35S_{pro}$:*AGL15* typically has about twice the number of seedlings with SAM SE development than does the wild type (Fig. 9; 33.5% compared with 17.4%, or 1.93-fold). We tested twice the normal amount and found that both $35S_{pro}$:*AGL15* and the Col wild type showed decreased percentages of SAM SE, but while the decrease was to 53% for the Col wild type, it was only to 73% for $35S_{pro}$:*AGL15*. At lower than normal 2,4-D (2.2 μM, or 0.5×), $35S_{pro}$:*AGL15* showed a significant increase in SAM SE, and it was even a little higher at 0.25× 2,4-D (Fig. 9). However, the Col wild type did not change significantly over these 2,4-D concentrations (Fig. 9). Not only was the percentage of $35S_{pro}$:*AGL15* seedlings with SAM SE development higher at lower 2,4-D amounts, but the typical extent of development was increased at 0.25× and 0.5× 2,4-D as well (Supplemental Fig. S5).

DISCUSSION

(Gm)AGL15 Regulates Auxin Signaling at Several Levels

Prior work in Arabidopsis indicated that overexpression of AGL15 may limit auxin responses by direct up-regulation of the AUX/IAA transcriptional repressor, *IAA30*, and this is relevant for SE (Zheng et al., 2009). *AUX/IAAs* encode proteins that bind to ARFs that, in turn, are associated with DNA via auxin response elements on auxin-responsive genes (Piya et al., 2014). The IAA keeps ARF from regulating gene expression until auxin perception leads to degradation of the IAA.

Here, we report on results indicating that *(Gm)AGL15* may limit the auxin response at several other levels of the signaling pathway. Both AGL15 and GmAGL15 directly repress *(Gm)TIR1*, which encodes an auxin receptor and regulates the degradation of AUX/IAA transcriptional repressors in response to auxin (for review, see Hayashi, 2012). Thus, repression of *TIR1* also would limit the auxin response. *(Gm)ARF6* and *(Gm) ARF8* are repressed by (Gm)AGL15, and this is direct for *(Gm)ARF6*. In addition, transcripts from these genes are targets for *(Gm)miR167A*, a microRNA that is expressed directly in response to (Gm)AGL15. Because ARF6 and ARF8 are thought to function as transcriptional activators (Piya et al., 2014), the direct and indirect regulation by (Gm)AGL15 would be predicted to further repress the auxin response.

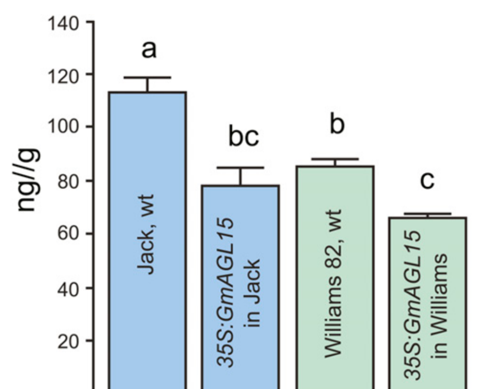

Figure 8. $35S_{pro}$:*GmAGL15* does not increase endogenous IAA content. Data shown are means and SE for at least three biological replicates. Different letters indicate significant differences at $P < 0.05$. wt, Wild type.

Because the ARFs and TIR1 are members of multigene families, it is possible that other members are regulated in response to (Gm)AGL15 in a manner that promotes the auxin response. Auxin signaling involves not only TIR1 but also the related auxin signaling F-box proteins (AFBs; for review, see Weiss and Ori, 2007). In both soybean at 0 dac (when orthologs are present on the array) and Arabidopsis, for genes with a significant change, (Gm)AGL15 appears to decrease *AFB* transcript accumulation. The one exception is *AFB3*, which shows significantly increased transcript for the comparison of both *agl15 agl18* and $35S_{pro}$:*AGL15* with the Col wild type. Besides ARF6 and ARF8, three other ARFs have Gln-rich regions and are thought to function as transcriptional activators (Tiwari et al., 2003; Piya et al., 2014). Two of these (*ARF5* and *ARF19*) show significant reduction in transcript in response to the loss of *agl15/18*, indicating that they may be expressed in response to AGL15 (Zheng et al., 2009). However, this is after incubation in 2,4-D for 10 d. The microarray experiment described by Zheng and Perry (2014) indicates that soybean orthologs of these genes are repressed by $35S_{pro}$:*GmAGL15* at 0 dac and, upon incubation on 2,4-D, are up-regulated, so that there is no difference by 3 dac. Even though *ARF5* and *ARF19* are expressed in response to AGL15/18 in the Arabidopsis SAM SE system (10 dac 2,4-D), *IAA12* and *IAA30* gene products that interact with ARF5 and are coexpressed in embryos or the shoot apical meristem (Piya et al., 2014) also appear to be expressed in response to AGL15 (Zheng et al., 2009). Thus, ARF5 activity may not increase in response to AGL15. Likewise, IAAs that interact with *ARF19* also appear to be expressed in response to AGL15 accumulation. ARF6 and ARF8 are repressed by (Gm)AGL15, but their activity also would be reduced due to AGL15's up-regulation of *IAA12* and *IAA30* (Zheng et al., 2009). Thus, overall, (Gm)AGL15 appears to reduce auxin signaling.

The soybean experiment allowed us to examine not only gene responses to increased GmAGL15 in explants but also allowed a short time course after the placement of explants on SE induction medium. Interestingly, *GmTIR1* transcript was actually increased at 3 dac in $35S_{pro}$:*GmAGL15* compared with the wild type. This is due at least in part to a down-regulation of *GmTIR1* at 3 dac compared with 0 dac in cv Jack. The repression of *TIR1* may be relevant for SE. As shown in Arabidopsis, loss of function of *TIR1* led to increased SAM SE (Fig. 2A). While we observed increased SAM SE, in other SE systems, *tir1* showed abnormal or impaired SE (Su et al., 2009; Wójcik and Gaj, 2016). The difference may be due to the tissue and potential redundancy with TIR1. Here, we used the SAM SE system of Mordhorst et al. (1998). Based on the Arabidopsis eFP browser, not only *TIR1* but also all of the *AFBs* show transcript accumulation in the vegetative shoot apex (Winter et al., 2007). The other studies used developing zygotic embryos as explants and induced primary somatic embryos on solid medium with 4.5 to 5 μM 2,4-D. The eFP browser indicates that *TIR1*, *AFB3*, and *AFB5* show transcript accumulation in

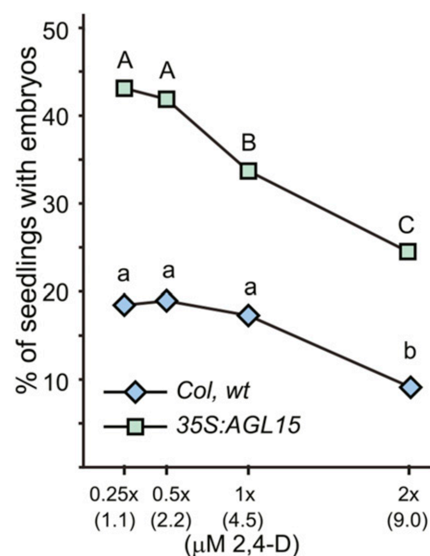

Figure 9. $35S_{pro}$:*AGL15* shows different 2,4-D sensitivity from the Col wild type (wt) in SAM SE culture. SAM SE cultures with normal (1×, 4.5 μM) and lower and higher 2,4-D concentrations were used to test the 2,4-D sensitivity of Arabidopsis *AGL15*-overexpressing tissue compared with the wild type. Means of three to four biological replicates are shown. Different letters within a genotype indicate significance at $P < 0.05$ as determined using Student's *t* test.

green maturing embryos (the stage used for explants) but at lower levels than in the shoot apex. *AFB2* and *AFB1* have very low or no transcript in green maturing embryos. It is interesting that Wójcik and Gaj (2016) found that *TIR1* transcript was reduced initially early in the induction of SE (5 dac) followed by a later increase.

As found for *tir1*, loss of ARF6 and ARF8 was relevant for SE, with a significant increase in SAM SE in the *arf6* mutant in the *arf8/ARF8* background (Fig. 6; Supplemental Fig. S4). In contrast to our findings, *arf6 arf8* has been found to be defective in SE in some systems (Su and Zhang, 2014; Su et al., 2016), emphasizing the importance of tissue and developmental context. Like *TIR1*, genes encoding ARFs that are thought to be transcriptional activators generally show increased signal in the shoot apex in microarray experiments compared with green maturing embryos, perhaps reflecting an optimal amount of auxin signaling needed to promote SE.

In summary, (Gm)AGL15 appears to regulate auxin signaling at several points in the pathway, including direct repression of *(Gm)TIR1* and *(Gm)ARF6* and indirect repression of *(Gm)ARF8*. The expression of *miR167A* and *AUX/IAAs*, which act to block activating ARF activity, also would impact on signaling. Some of these interactions are diagrammed in Figure 10.

How Does Repression of the Auxin Response Lead to Increased SE?

A common feature of SE systems is the induction of SE with auxin, usually using the synthetic auxin 2,4-D

(Karami and Saidi, 2010). It is unclear whether 2,4-D acts as an auxin, to induce endogenous auxin metabolism or localization, and/or as a stressing agent. Given the necessity of 2,4-D in many SE systems, if the main function of 2,4-D in SE is to act as an auxin or to induce endogenous auxin, the finding that increased GmAGL15 represses auxin responses is surprising.

It is interesting that while auxin is important for SE, and there are reports that embryogenic genotypes are more sensitive to auxin than nonembryogenic genotypes (Fehér, 2005), it may actually be the cells that are somewhat resistant to auxin that are competent to form embryos (Emons, 1994). The concentration of 2,4-D is important for the dedifferentiation and redifferentiation process. Too high levels of 2,4-D can block the embryogenesis of cells that already have acquired embryogenic potential (Iantcheva et al., 2005). There may be a complex interaction between cells that form the embryo and nonembryogenic cells that support the competent cells. There are reports of situations where some cell types provide an essential role as nurse cells for the cells that actually form the embryo (McCabe et al., 1997; van Hengel et al., 1998; Passarinho et al., 2001). Potentially, different cells within the tissue have different responses to 2,4-D.

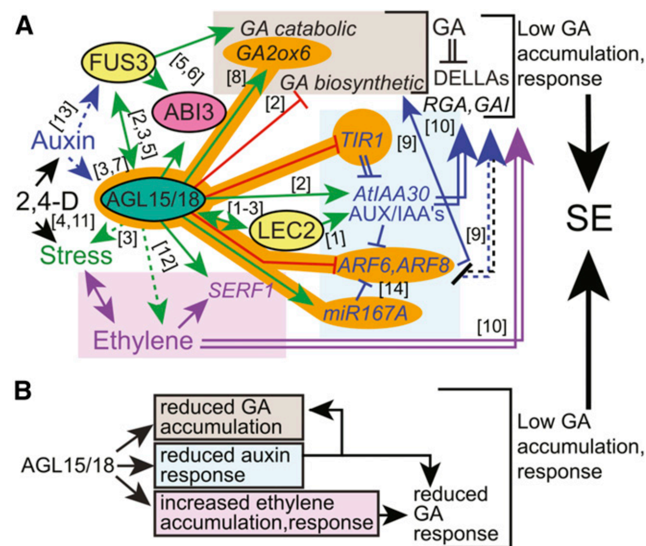

Figure 10. Model diagramming some of the interactions between key embryo transcription factors and hormones. A, The orange highlighted region indicates new contributions from this article. Many of the other interactions have been described in other contexts but are verified for a role in SE in this study. Numbers in brackets indicate references for these previously described interactions: [1], Braybrook et al. (2006); [2], Zheng et al. (2009); [3], Zheng and Perry (2014); [4], Karami and Saidi (2010); [5], Wang and Perry (2013); [6], Yamamoto et al. (2010); [7], Zhu and Perry (2005); [8], Wang et al. (2004); [9], Oh et al. 2014; [10], Weiss and Ori (2007); [11], Fehér (2005); [12], Zheng et al. (2013); [13] Gazzarrini et al. (2004); [14], Wu et al. (2006). Direct targets of LEC2, FUS3, and AGL15 are shown by solid lines with arrowheads for expressed and bars for repressed. Dotted lines indicate interactions that may be direct or indirect. The auxin-related pathway is in blue, the ethylene pathway is in purple, and the GA pathway is in black. The double solid lines indicate effects on protein stability. The dashed line represents an interaction whereby DELLA proteins interact with activating ARFs and may prevent binding to DNA, while *GAI* may be a direct target of ARF6. B, A simplified version of A focusing on outputs of AGL15/18 on hormones and interactions between hormones. For the sake of clarity, not all interactions are shown.

The modulation of tissue sensitivity to auxin (2,4-D) has been proposed to be important for the early stages of SE. Wójcik and Gaj (2016) reported that miR393, which targets *TIR1/AFB* transcripts, is initially increased during SE induction (5 dac), followed by a decrease (10 dac) in this microRNA. This modulation may be important; both the loss and gain of function of miR393a/b showed impaired SE efficiency and productivity (Wójcik and Gaj, 2016). *TIR1* transcript showed an opposite pattern at 5 and 10 dac compared with *miR393* (Wójcik and Gaj, 2016). We observed a similar pattern in soybean, with reduced *GmTIR1* associated with the earliest stages of SE.

Although 2,4-D may act as an auxin, the evidence is good that 2,4-D also acts as a stressing agent (for review, see Karami and Saidi, 2010; Fehér, 2015), and SE has been proposed to be an extreme response to stress (for review, see Fehér, 2015). Overexpression of *GmAGL15* in explants, as well as the induction of SE in the cv Jack wild type, both up-regulate a common set of genes that are significantly overrepresented for response to stress (Zheng and Perry, 2014). Additionally, soybean orthologs of Arabidopsis genes encoding transcription factors associated with dedifferentiation show increased transcript in $35S_{pro}$:*GmAGL15* explants compared with the cv Jack wild type and in response to the induction of SE in the cv Jack wild type for 3 dac (Perry et al., 2016). Thus, $35S_{pro}$:*(Gm)AGL15* may prime tissue to be more competent to 2,4-D by the expression of stress- and dedifferentiation-associated genes, yet it may allow an appropriate level of auxin signaling. Repression of the auxin response contributes to a reduction of biologically active GA that would promote SE.

GA and Auxin Interactions in the Control of SE

AGL15 directly regulates a gene involved in GA catabolism (*GA2ox6*), and a decrease in biologically active GA is observed in $35S_{pro}$:*AGL15* compared with the control (Wang et al., 2004). AGL15 also represses a GA biosynthetic gene, *GA3ox2* (*At1g80340*; Zheng et al., 2009). GmAGL15 directly expressed an ortholog of *GA2ox6* in soybean (Fig. 3; 0 dac). As found in Arabidopsis (Wang et al., 2004), the accumulation of biologically active GA was inversely correlated with SE, with the addition of GA_3 to the medium reducing SE on soybean explants in both wild-type and $35S_{pro}$:*GmAGL15* tissue (Fig. 4). In $35S_{pro}$:*GmAGL15*, the decrease was to about the level of production of SE in cv Jack without GA_3 supplement. The experiments illustrated in Figure 4, A and B, were performed in an overlapping time frame, allowing some comparison of

results. The addition of paclobutrazol, an inhibitor of GA biosynthesis, to the D40 medium was performed separately from the GA_3 experiments, and the production of SE in cv Jack was higher in this set of experiments than in the GA experiments, yet paclobutrazol led to an increase in SE at 30 dac (Fig. 4C). The addition of GA_3 to the Arabidopsis *tir1*, *arf6*, and *arf8* loss-of-function mutants also decreased SAM SE (Figs. 2A and 6), indicating that GA accumulation may be downstream of TIR1 signaling. Part of this is through *GA2ox6*; when the knockdown of *GA2ox6* is combined with *tir1-1*, it reduced the frequency of SAM SE to between the Col wild type and *tir1-1* (Fig. 2B). While biologically active GA accumulation did not have a consistent effect on *TIR1* transcript accumulation, the *tir1* mutant showed a significant increase in a GA catabolic enzyme and a decrease in a GA biosynthetic enzyme in 10-dac SAM SE cultures (Fig. 2D) that would be consistent with decreased biologically active GA and increased SAM SE (Fig. 2A) and with findings in other species and tissue contexts (for review, see Weiss and Ori, 2007). One gene directly associated with ARF6 and up-regulated in response to auxin is *GA20ox1* (*At4g25420*), which encodes a GA biosynthetic enzyme (Oh et al., 2014). Therefore, (Gm)AGL15 controls GA accumulation not only by directly regulated genes involved in this process (e.g. *GA2ox6*) but also by control of the auxin response.

Auxin also leads to the destabilization of DELLAs in response to GA, thereby promoting the GA response (Weiss and Ori, 2007). GA also is involved in auxin responses. The DELLA regulatory factor REPRESSOR OF GA1-3 1 (RGA) prevents the binding of ARF6 to DNA (Oh et al., 2014). Therefore, upon the degradation of DELLAs in response to GA, ARF6 would be able to bind target genes and, since it acts at least mainly as an activator, cause auxin-responsive gene regulation. Interestingly, a putative direct target of ARF6 that is activated by auxin treatment is the gene encoding another DELLA protein, *GIBBERELLIC ACID INSENSITIVE* (*GAI*), while ARF6 binds to regulatory regions of *RGA*, but a response was not demonstrated (Oh et al., 2014). Both *(Gm)RGA* and *(Gm)GAI* transcript accumulation show responses to $35S_{pro}$:*GmAGL15*, with apparent repression at 0 dac but significantly increased transcript at 3 dac on 2,4-D (Zheng et al., 2013). Thus, in a time frame in soybean from 0 to 3 dac on 2,4-D medium, when *GmTIR1* is going from repressed to expressed in $35S_{pro}$:*GmAGL15* compared with cv Jack and *GmGA2ox6* is showing the opposite pattern (Fig. 3A), the DELLA-encoding genes *GmRGA* and *GmGAI* may show increased transcript in the overexpressors based on microarray results (Zheng et al., 2013). *GmARF6* and *GmARF8* transcript accumulation both are decreased at 0 and 3 dac in $35S_{pro}$:*GmAGL15* compared with the wild type (Fig. 5), and the increased DELLAs at 3 d may reduce their activity further, thereby ameliorating the potential increased auxin response from the up-regulation of *TIR1*. This highlights the complex interaction between hormones that would also include feedback mechanisms.

Decreased biologically active GA accumulation or response has been correlated with embryo development. The ratio of GA to abscisic acid (ABA) determines embryonic or adult leaf identity (Gazzarrini et al., 2004). The *pkl* mutant that is defective in a CHD3 chromatin-remodeling factor shows increased accumulation of bioactive GA but has a phenotype indicating reduced response to GA. One phenotype of *pkl* is an enlarged green root that, when excised, forms SE, and the penetrance of this phenotype is increased by repressing bioactive GA synthesis (Ogas et al., 1997, 1999; Henderson et al., 2004).

Ethylene Interactions with GA and Auxin

Previous work demonstrated that (Gm)AGL15 impacts ethylene biosynthesis and perception, and this is relevant to SE (Zheng et al., 2013). Even if 2,4-D acts only as a stressing agent, an increase in ethylene would be expected (Raghavan, 2006; Karami et al., 2009). Ethylene and auxin impact each other's accumulation and interact in a complex cooperative manner (Stepanova et al., 2008; Vandenbussche et al., 2012). Ethylene and GA interactions may be cooperative or antagonistic depending on the context (Weiss and Ori, 2007). In the context of SE, the interaction appears antagonistic. One mechanism for this antagonism is by the stabilization of DELLAs that repress GA signaling as well as the auxin response. However, ethylene and auxin also impact each other's biosynthesis (Stepanova et al., 2008). Arabidopsis (*At5g61590*) and soybean (*Glyma20g16920* and *Glyma10g24360*) orthologs of *Medicago truncatula SOMATIC EMBRYO-RELATED FACTOR1* were found to be directly up-regulated targets of (Gm)AGL15, and this expression was found to be relevant to SAM SE (Zheng et al., 2013) as it was to *M. truncatula* SE (Mantiri et al., 2008). In both Arabidopsis and soybean, these genes respond not only to ethylene but also to GA, with significantly reduced transcript in response to exogenous GA (Zheng et al., 2013).

Hormone and Transcription Factor Interactions

(Gm)AGL15 and (Gm)AGL18 promote SE in Arabidopsis and soybean, at least in part by directly regulating *(Gm)FUS3* and *(Gm)ABI3* and, in Arabidopsis, *LEAFY COTYLEDON2* (*LEC2* [not tested in soybean]; Harding et al., 2003; Adamczyk et al., 2007; Thakare et al., 2008; Zheng et al., 2009; Zheng and Perry, 2014). These transcription factors are key regulators of embryogenesis in diverse species (Gazzarrini et al., 2004; Moreno-Risueno et al., 2008; Stone et al., 2008; Kim et al., 2013; Zhang et al., 2014) and interact in a complex manner. *AGL15* may be a directly expressed target of LEC2 (Braybrook et al., 2006), and in turn it may

directly up-regulate *LEC2* as well as putative LEC2 indirect targets *FUS3* and *ABI3* (Zheng et al., 2009; Zheng and Perry, 2014). *AGL15* was identified as a directly expressed target of FUS3, as was *ABI3* and other important embryo transcription factors (Wang and Perry, 2013). The situation becomes even more complex when downstream targets of these factors are considered.

Jia et al. (2013) provide an overview of hormone pathway genes regulated by LEC1, LEC2, and FUS3. We focus here on those likely or shown to be directly responsive targets. While some biosynthetic genes respond to these regulators (e.g. *YUCCA4*, which may be a directly expressed gene of LEC2 and FUS3), *IAA CARBOXYLMETHYLTRANSFERASE1*, which encodes a protein involved in auxin catabolism, is a directly up-regulated target of FUS3. LEC2 and FUS3 both also impact auxin signaling, with LEC2 potentially directly up-regulating *IAA17*, *IAA30*, and *IAA31* (Braybrook et al., 2006) and FUS3 directly up-regulating *IAA12*, *IAA17*, and *IAA30* (Yamamoto et al., 2010; Wang and Perry, 2013). While FUS3 regulates a number of genes involved in GA biosynthesis, these appear to be indirect, with the one *GA 3-oxidase* identified as direct by Wang and Perry (2013) not showing a response according to Yamamoto et al. (2010). Another *GA 3-oxidase* (*GA3ox2*; *At1g80340*) was found to be repressed by FUS3 and LEC2 and proposed to be direct for FUS3 based on gel mobility shift assays (Curaba et al., 2004). The two genes involved in GA catabolism highlighted by Jia et al. (2013) are both directly responsive targets of FUS3 that would reduce biologically active GA in midstage seeds. While a number of genes involved in ethylene biosynthesis and signaling were identified as FUS3 targets by Jia et al. (2013) that would be repressed by FUS3, these results were from a study where FUS3 was ectopically expressed and posttranslationally controlled, and none of the identified genes showed significant changes in seeds. Also, FUS3 appears to be a transcriptional activator, so it is likely that the repressive effects are indirect.

Hormones, in turn, impact the transcript accumulation of the key embryo transcription factors. Gazzarrini et al. (2004) demonstrated that *FUS3* responds to auxin, positively regulates ABA synthesis, and negatively regulates GA synthesis. These hormones feed back to stabilize (ABA) or destabilize (GA) FUS3. We show here that GA significantly decreased transcript accumulation from the soybean orthologs of *AGL18*, *ABI3*, and *FUS3* (Fig. 4). These genes, along with *GmAGL15*, also respond to ethylene with significantly reduced transcript in response to the ethylene biosynthesis inhibitor aminoethoxyvinylglycine, which inhibits 1-aminocyclopropane-1-carboxylic acid synthase. This decrease can be rescued by the addition of 1-aminocyclopropane-1-carboxylic acid (Zheng et al., 2013). These and other interactions discussed above are diagrammed in Figure 10.

CONCLUSION

AGL15 directly and indirectly controls components of the auxin signaling pathway in both Arabidopsis and soybean in a manner that would limit auxin signaling. This pathway interacts with GA metabolism to influence somatic embryo development. In turn, hormones impact the transcription factors involved in embryogenesis.

MATERIALS AND METHODS

Plant Material and SE Systems

For soybean (*Glycine max*), the $35S_{pro}$:*GmAGL15* transgenic line 8981 was described by Thakare et al. (2008). Subsequent transformations of a related construct that included a 10× c-myc tag were performed using cv Jack and cv Williams 82 (obtained from the U.S. Department of Agriculture Soybean Germplasm Collection [http://www.ars-grin.gov/npgs/]; Zheng et al., 2013). Transformation of soybean was carried out as described (Thakare et al., 2008) with minor modifications. Briefly, green somatic embryo tissue induced on D40 medium from the immature cotyledon explants of cv Jack and proliferated for 1 to 2 months on D20 medium was placed at the center of a plate containing D20 medium. Gold microcarriers (9 mg of 0.6 μm; Bio-Rad) were washed with 100% ethanol and sterilized water. DNA (12.5 μL at a concentration of 80 ng μL^{-1}), 220 μL of sterile water, 250 μL of 2.5 M $CaCl_2$, and 100 μL of 0.1 M spermidine were added in that order to the gold microcarriers while gently mixing. After mixing and washing, the carriers were pelleted by centrifugation, resuspended in 36 μL of ethanol, and incubated on ice for 1 h. The mixtures were resuspended by pipetting, and 10 μL was used per macrocarrier (Bio-Rad) for bombardment using a DuPont biolistic particle delivery system (model PSD-1000; Bio-Rad) and 1,100-p.s.i. rupture discs (Inbio Gold). The recovery of plants was as described (Thakare et al., 2008).

Transgenic and wild-type control soybeans were grown in a greenhouse in the same conditions as described (Zheng et al., 2013). The cotyledon explant culture and scoring strategy were as described (Meurer et al., 2001; Zheng and Perry, 2014) with supplements as described in the text. Cultures were scored at 30, 45, and 60 dac, and tissue was frozen at appropriate time points for RNA extraction. Time points for transcript analysis allowed us to compare explants (0 dac) with a short time course on the SE induction medium (3 and 7 dac) with the response to $35S_{pro}$:*GmAGL15* at each time point. All time points were prior to any visible embryo development. Embryos first become apparent by 21 dac for nontransgenic tissue and 17 dac for $35S_{pro}$:*GmAGL15*.

Arabidopsis (*Arabidopsis thaliana*) wild-type, insertional loss-of-function alleles (*tir1*, *ga2ox6*, *arf6*, *arf8*, *arf6 arf8*/+, and the *agl15 agl18* double mutant), and $35S_{pro}$:*AGL15* plants (all Col ecotype) were sown on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 10 g L^{-1} Suc, 0.5 g L^{-1} MES, and 7 g L^{-1} agar, pH 5.8, with 50 $\mu g\ mL^{-1}$ kanamycin for $35S_{pro}$:*AGL15* seed, chilled for 2 to 3 dac at 4°C, and transferred to a growth room with a 16-h-light/8-h-dark cycle at 23°C to 24°C. After 7 to 10 d, seedlings were transferred to potting mix (Promix BX; Premier Brands) and grown under a 16-h-light (20°C)/8-h-dark (18°C) cycle in a growth chamber. To stage seed, flowers were tagged on the day they opened and seeds were collected and flash frozen in liquid nitrogen for RNA at 9 to 10 daf. Seeds for SAM SE were allowed to develop to dry seed, and SAM SE was performed as described (Mordhorst et al., 1998). For qRT-PCR, tissue was collected at 10 d after the start of culture and flash frozen. To score for embryo production, tissue was examined at 21 d after the initiation of culture.

For all experiments, biological replicates (independently generated) refers to tissue collected as sets grown together but replicates were generated at different times.

ChIP, ChIP-qPCR, and qRT-PCR

The details of ChIP and qPCR to measure the amounts of targets relative to controls for Arabidopsis and soybean were as described by Zheng et al. (2009) and Zheng and Perry (2014), respectively. RNA extraction and reverse transcription-PCR also were described previously (Zheng et al., 2009; Zheng and Perry, 2014). The specific oligonucleotides used for qPCR and qRT-PCR are listed in Supplemental Table S1. Data analysis was performed using the REST

software for qRT-PCR (Pfaffl et al., 2002) or as described (Zheng et al., 2009) for ChIP-qPCR.

IAA Measurements

For IAA production determination in soybean (transgenic lines 8981 and 1041 and wild-type cv Jack and cv Williams 82), fresh 4- to 5-mm immature embryos were isolated from developing seeds, taking care to avoid any injury, frozen in liquid nitrogen, and stored at −80°C until used. IAA extraction and measurement were performed as described by Fu et al. (2012) with some modifications. Frozen immature embryos were ground to powder in liquid nitrogen with a mortar and pestle, and 0.4 g of ground tissue was extracted with 3 mL of methanol for 1 h with shaking. IAA-d2 was used as an internal standard. The suspension was dried under nitrogen and reconstituted with 1 mL of 5% acetic acid in water. Extract solution was cleaned up on an SPE C18 column. Eluent was dried with nitrogen again and reconstituted with methanol. The methanol extract was filtered through a 0.22-μm PTFE filter, and 5 μL of eluent was injected into the Waters ACQUITY UPLC-XEVO TQD system with an ACQUITY UPLC BEH C18 column (2.1 × 50 mm with 1.7-μm particle size). Separation was achieved using a gradient mobile phase consisting of 0.05% acetic acid in water (mobile phase A) and 0.05% acetic acid in acetonitrile (mobile phase B). The gradient program starts with initial conditions of 95% mobile phase A and 5% mobile phase B, which were held for 1 min. Mobile phase B was increased at a linear rate to 50% in 0.5 min and then increased further to 90% in 1.5 min and held for 1 min. Finally, mobile phase B was rapidly returned to 5% and maintained for 2.5 min. The mobile phase flow rate was set at 0.35 mL min^{-1}. The mass spectrometer was operated in electrospray capillary voltage at 3.9 kV in the positive ion mode; ion source temperature and desolvation temperature were set at 150°C and 300°C, respectively; desolvation gas and cone gas flow were set at 600 and 30 L h^{-1}, respectively. Quantitative analysis was performed by MRM mode with 176 > 130 quantification transition for IAA and 178 > 132 for IAA-d2. The qualification transition of IAA was 176 > 103.

Accession Numbers

Accession numbers are listed in Supplemental Table S2.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. CisGenome screenshot of AGL15 binding to *TIR1*.

Supplemental Figure S2. Confirmation of knockdown alleles of *tir1* and *ga2ox6*.

Supplemental Figure S3. CisGenome screenshots for AGL15 binding to *ARF6*, *ARF8*, and *miRNA167A*.

Supplemental Figure S4. SAM SE data for *arf6-2* and *arf 8-3* and seeds from *arf6-2* homozygous and segregating for *arf8-3*.

Supplemental Figure S5. SAM SE development at different concentrations of 2,4-D.

Supplemental Table S1. Oligonucleotide primers used in this study.

Supplemental Table S2. Accession numbers for putative orthologs in Arabidopsis and soybean used in this study.

ACKNOWLEDGMENTS

We thank Jeanne Hartman, Olivia Jones, and Rachel Mueller for assistance with cultures and Donna Wall and the UK Microarray Facility for probe generation, array hybridization, and data collection; Dr. Nakagawa (Shimane University) for the gift of the GWB vectors, Dr. Fernandez (University of Wisconsin-Madison) for the *ga2ox6* seed, Dr. Jason Reed (University of North Carolina at Chapel Hill) for *arf6-2*, *arf8-3*, and *arf6-2 arf8-3*/+ seed, and the Arabidopsis Biological Resource Center for other Arabidopsis mutants; and two anonymous reviewers for their constructive comments to strengthen the article.

Received April 11, 2016; accepted October 25, 2016; published October 28, 2016.

LITERATURE CITED

Adamczyk BJ, Lehti-Shiu MD, Fernandez DE (2007) The MADS domain factors AGL15 and AGL18 act redundantly as repressors of the floral transition in Arabidopsis. Plant J **50:** 1007–1019

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:** 653–657

Braybrook SA, Stone SL, Park S, Bui AQ, Le BH, Fischer RL, Goldberg RB, Harada JJ (2006) Genes directly regulated by LEAFY COTYLEDON2 provide insight into the control of embryo maturation and somatic embryogenesis. Proc Natl Acad Sci USA **103:** 3468–3473

Curaba J, Moritz T, Blervaque R, Parcy F, Raz V, Herzog M, Vachon G (2004) *AtGA3ox2*, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by LEAFY COTYLEDON2 and FUSCA3 in Arabidopsis. Plant Physiol **136:** 3660–3669

Emons AMC (1994) Somatic embryogenesis: cell biological aspects. Acta Bot Neerl **43:** 1–14

Fehér A (2005) Why somatic plant cells start to form embryos? *In* A Mujib, J Samaj, eds, Plant Cell Monographs: Somatic Embryogenesis. Springer-Verlag, Berlin, pp 85–101

Fehér A (2015) Somatic embryogenesis: stress-induced remodeling of plant cell fate. Biochim Biophys Acta **1849:** 385–402

Frigerio M, Alabadí D, Pérez-Gómez J, García-Cárcel L, Phillips AL, Hedden P, Blázquez MA (2006) Transcriptional regulation of gibberellin metabolism genes by auxin signaling in Arabidopsis. Plant Physiol **142:** 553–563

Fu J, Chu J, Sun X, Wang J, Yan C (2012) Simple, rapid, and simultaneous assay of multiple carboxyl containing phytohormones in wounded tomatoes by UPLC-MS/MS using single SPE purification and isotope dilution. Anal Sci **28:** 1081–1087

Gazzarrini S, Tsuchiya Y, Lumba S, Okamoto M, McCourt P (2004) The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. Dev Cell **7:** 373–385

Harding EW, Tang W, Nichols KW, Fernandez DE, Perry SE (2003) Expression and maintenance of embryogenic potential is enhanced through constitutive expression of *AGAMOUS-Like 15*. Plant Physiol **133:** 653–663

Hayashi K (2012) The interaction and integration of auxin signaling components. Plant Cell Physiol **53:** 965–975

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci **5:** 523–530

Henderson JT, Li HC, Rider SD, Mordhorst AP, Romero-Severson J, Cheng JC, Robey J, Sung ZR, de Vries SC, Ogas J (2004) PICKLE acts throughout the plant to repress expression of embryonic traits and may play a role in gibberellin-dependent responses. Plant Physiol **134:** 995–1005

Iantcheva A, Vlahova M, Atanassov A (2005). Somatic embryogenesis in genera *Medicago*: an overview. *In* A Mujib, J Samaj, eds, Plant Cell Monographs: Somatic Embryogenesis. Springer-Verlag, Berlin, pp 285–304

Jia H, McCarty DR, Suzuki M (2013) Distinct roles of LAFL network genes in promoting the embryonic seedling fate in the absence of VAL repression. Plant Physiol **163:** 1293–1305

Karami O, Aghavaisi B, Mahmoudi Pour A (2009) Molecular aspects of somatic-to-embryogenic transition in plants. J Chem Biol **2:** 177–190

Karami O, Saidi A (2010) The molecular basis for stress-induced acquisition of somatic embryogenesis. Mol Biol Rep **37:** 2493–2507

Kim HU, Jung SJ, Lee KR, Kim EH, Lee SM, Roh KH, Kim JB (2013) Ectopic overexpression of castor bean *LEAFY COTYLEDON2* (*LEC2*) in Arabidopsis triggers the expression of genes that encode regulators of seed maturation and oil body proteins in vegetative tissues. FEBS Open Bio **4:** 25–32

Kita Y, Nishizawa K, Takahashi M, Kitayama M, Ishimoto M (2007) Genetic improvement of the somatic embryogenesis and regeneration in soybean and transformation of the improved breeding lines. Plant Cell Rep **26:** 439–447

Klink VP, MacDonald MH, Martins VE, Park SC, Kim KH, Baek SH, Matthews BF (2008) MiniMax, a new diminutive *Glycine max* genotype with a rapid life cycle, embryogenic potential and transformation capabilities. Plant Cell Tissue Organ Cult **92:** 183–195

Ko TS, Nelson RL, Korban SS (2004) Screening multiple soybean cultivars (MG 00 to MG VIII) for somatic embryogenesis following Agrobacterium-mediated transformation of immature cotyledons. Crop Sci **44:** 1825–1831

Mantiri FR, Kurdyukov S, Lohar DP, Sharopova N, Saeed NA, Wang XD, Vandenbosch KA, Rose RJ (2008) The transcription factor MtSERF1 of the ERF subfamily identified by transcriptional profiling is required for somatic embryogenesis induced by auxin plus cytokinin in *Medicago truncatula*. Plant Physiol **146:** 1622–1636

McCabe PF, Valentine TA, Forsberg LS, Pennell RI (1997) Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. Plant Cell **9:** 2225–2241

Meurer CA, Dinkins RD, Redmond CT, McAllister KP, Tucker DT, Walker DR, Parrott WA, Trick HN, Essig JS, Frantz HM, et al (2001) Embryogenic response of multiple soybean *Glycine max* (L.) Merr. cultivars across three locations. In Vitro Cell Dev Biol Plant **37:** 62–67

Mordhorst AP, Voerman KJ, Hartog MV, Meijer EA, van Went J, Koornneef M, de Vries SC (1998) Somatic embryogenesis in *Arabidopsis thaliana* is facilitated by mutations in genes repressing meristematic cell divisions. Genetics **149:** 549–563

Moreno-Risueno MA, González N, Díaz I, Parcy F, Carbonero P, Vicente-Carbajosa J (2008) FUSCA3 from barley unveils a common transcriptional regulation of seed-specific genes between cereals and Arabidopsis. Plant J **53:** 882–894

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, et al (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development **132:** 4107–4118

Nemhauser JL, Hong F, Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell **126:** 467–475

Ogas J, Cheng JC, Sung ZR, Somerville C (1997) Cellular differentiation regulated by gibberellin in the *Arabidopsis thaliana pickle* mutant. Science **277:** 91–94

Ogas J, Kaufmann S, Henderson J, Somerville C (1999) PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in Arabidopsis. Proc Natl Acad Sci USA **96:** 13839–13844

Oh E, Zhu JY, Bai MY, Arenhart RA, Sun Y, Wang ZY (2014) Cell elongation is regulated through a central circuit of interacting transcription factors in the Arabidopsis hypocotyl. eLife **3:** e03031

Olszewski N, Sun TP, Gubler F (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell (Suppl) **14:** S61–S80

Passarinho PA, Van Hengel AJ, Fransz PF, de Vries SC (2001) Expression pattern of the *Arabidopsis thaliana AtEP3/AtchitIV* endochitinase gene. Planta **212:** 556–567

Perry SE, Nichols KW, Fernandez DE (1996) The MADS domain protein AGL15 localizes to the nucleus during early stages of seed development. Plant Cell **8:** 1977–1989

Perry SE, Zheng Q, Zheng Y (2016) Transcriptome analysis indicates that GmAGAMOUS-Like 15 may enhance somatic embryogenesis by promoting a dedifferentiated state. Plant Signal Behav **11:** e1197463

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res **30:** e36

Piya S, Shrestha SK, Binder B, Stewart CN Jr, Hewezi T (2014) Protein-protein interaction and gene co-expression maps of ARFs and Aux/IAAs in Arabidopsis. Front Plant Sci **5:** 744

Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E, Wilm M, Séraphin B (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods **24:** 218–229

Raghavan V (2006) Can carrot and *Arabidopsis* serve as model systems to study the molecular biology of somatic embryogenesis? Curr Sci **90:** 1336–1343

Rose RJ, Nolan KE (2006) Genetic regulation of somatic embryogenesis with particular reference to *Arabidopsis thaliana* and *Medicago truncatula*. In Vitro Cell Dev Biol Plant **42:** 473–481

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grr1p. Genes Dev **12:** 198–207

Salvo SAGD, Hirsch CN, Buell CR, Kaeppler SM, Kaeppler HF (2014) Whole transcriptome profiling of maize during early somatic embryogenesis reveals altered expression of stress factors and embryogenesis-related genes. PLoS ONE **9:** e111407

Sato A, Yamamoto KT (2008) Overexpression of the non-canonical Aux/IAA genes causes auxin-related aberrant phenotypes in Arabidopsis. Physiol Plant **133:** 397–405

Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, Dolezal K, Schlereth A, Jürgens G, Alonso JM (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell **133:** 177–191

Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Am J Bot **45:** 705–708

Stone SL, Braybrook SA, Paula SL, Kwong LW, Meuser J, Pelletier J, Hsieh TF, Fischer RL, Goldberg RB, Harada JJ (2008) Arabidopsis LEAFY COTYLEDON2 induces maturation traits and auxin activity: implications for somatic embryogenesis. Proc Natl Acad Sci USA **105:** 3151–3156

Su YH, Liu YB, Zhou C, Li XM, Zhang XS (2016) The microRNA167 controls somatic embryogenesis in Arabidopsis through regulating its target genes ARF6 and ARF8. Plant Cell Tissue Organ Cult **124:** 405–417

Su YH, Zhang XS (2014) The hormonal control of regeneration in plants. Curr Top Dev Biol **108:** 35–69

Su YH, Zhao XY, Liu YB, Zhang CL, O'Neill SD, Zhang XS (2009) Auxin-induced *WUS* expression is essential for embryonic stem cell renewal during somatic embryogenesis in Arabidopsis. Plant J **59:** 448–460

Thakare D, Tang W, Hill K, Perry SE (2008) The MADS-domain transcriptional regulator AGAMOUS-LIKE15 promotes somatic embryo development in Arabidopsis and soybean. Plant Physiol **146:** 1663–1672

Tiwari SB, Hagen G, Guilfoyle T (2003) The roles of auxin response factor domains in auxin-responsive transcription. Plant Cell **15:** 533–543

Vandenbussche F, Vaseva I, Vissenberg K, Van Der Straeten D (2012) Ethylene in vegetative development: a tale with a riddle. New Phytol **194:** 895–909

van Hengel AJ, Guzzo F, van Kammen A, de Vries SC (1998) Expression pattern of the carrot EP3 endochitinase genes in suspension cultures and in developing seeds. Plant Physiol **117:** 43–53

Vogel G (2005) How does a single somatic cell become a whole plant? Science **309:** 86

Wang F, Perry SE (2013) Identification of direct targets of FUSCA3, a key regulator of Arabidopsis seed development. Plant Physiol **161:** 1251–1264

Wang H, Caruso LV, Downie AB, Perry SE (2004) The embryo MADS domain protein AGAMOUS-Like 15 directly regulates expression of a gene encoding an enzyme involved in gibberellin metabolism. Plant Cell **16:** 1206–1219

Weiss D, Ori N (2007) Mechanisms of cross talk between gibberellin and other hormones. Plant Physiol **144:** 1240–1246

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE **2:** e718

Wójcik AM, Gaj MD (2016) miR393 contributes to the embryogenic transition induced in vitro in Arabidopsis via the modification of the tissue sensitivity to auxin treatment. Planta **244:** 231–243

Wu MF, Tian Q, Reed JW (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. Development **133:** 4211–4218

Yamamoto A, Kagaya Y, Usui H, Hobo T, Takeda S, Hattori T (2010) Diverse roles and mechanisms of gene regulation by the Arabidopsis seed maturation master regulator FUS3 revealed by microarray analysis. Plant Cell Physiol **51:** 2031–2046

Yang Z, Li C, Wang Y, Zhang C, Wu Z, Zhang X, Liu C, Li F (2014) *GhAGL15s*, preferentially expressed during somatic embryogenesis, promote embryogenic callus formation in cotton (*Gossypium hirsutum* L.). Mol Genet Genomics **289:** 873–883

Zhang Y, Clemens A, Maximova SN, Guiltinan MJ (2014) The Theobroma cacao B3 domain transcription factor TcLEC2 plays a dual role in control of embryo development and maturation. BMC Plant Biol **14:** 106

Zheng Q, Perry SE (2014) Alterations in the transcriptome of soybean in response to enhanced somatic embryogenesis promoted by orthologs of Agamous-like15 and Agamous-like18. Plant Physiol **164:** 1365–1377

Zheng Q, Zheng Y, Perry SE (2013) AGAMOUS-Like15 promotes somatic embryogenesis in Arabidopsis and soybean in part by the control of ethylene biosynthesis and response. Plant Physiol **161:** 2113–2127

Zheng Y, Ren N, Wang H, Stromberg AJ, Perry SE (2009) Global identification of targets of the *Arabidopsis* MADS domain protein AGAMOUS-Like15. Plant Cell **21:** 2563–2577

Zhu C, Perry SE (2005) Control of expression and autoregulation of *AGL15*, a member of the MADS-box family. Plant J **41:** 583–594