AGAMOUS-Like15 Promotes Somatic Embryogenesis in Arabidopsis and Soybean in Part by the Control of Ethylene Biosynthesis and Response[1][C][W][OA]

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Many of the regulatory processes occurring during plant embryogenesis are still unknown. Relatively few cells are involved, and they are embedded within maternal tissues, making this developmental phase difficult to study. Somatic embryogenesis is a more accessible system, and many important regulatory genes appear to function similar to zygotic development, making somatic embryogenesis a valuable model for the study of zygotic processes. To better understand the role of the Arabidopsis (*Arabidopsis thaliana*) MADS factor AGAMOUS-Like15 (AGL15) in the promotion of somatic embryogenesis, direct target genes were identified by chromatin immunoprecipitation-tiling arrays and expression arrays. One potential directly up-regulated target was *At5g61590*, which encodes a member of the ethylene response factor subfamily B-3 of APETALA2/ETHYLENE RESPONSE FACTOR transcription factors and is related to *Medicago truncatula SOMATIC EMBRYO-RELATED FACTOR1* (*MtSERF1*), which has been shown to be required for somatic embryogenesis in *M. truncatula*. Here, we report confirmation that *At5g61590* is a directly expressed target of AGL15 and that *At5g61590* is essential for AGL15's promotion of somatic embryogenesis. Because At5g61590 is a member of the ETHYLENE RESPONSE FACTOR family, effects of ethylene on somatic embryogenesis were investigated. Precursors to ethylene stimulate somatic embryogenesis, whereas inhibitors of ethylene synthesis or perception reduce somatic embryogenesis. To extend findings to a crop plant, we investigated the effects of ethylene on somatic embryogenesis in soybean (*Glycine max*). Furthermore, we found that a potential ortholog of AGL15 in soybean (GmAGL15) up-regulates ethylene biosynthesis and response, including direct regulation of soybean orthologs of *At5g61590/MtSERF1* named here *GmSERF1* and *GmSERF2*, in concordance with the *M. truncatula* nomenclature.

The inaccessibility of higher plant embryos during early development has made it difficult to study this stage of the life cycle. However, somatic embryogenesis (SE) offers an accessible alternative in which to study aspects of zygotic embryo development. SE is also important for the rapid multiplication and conservation of given genotypes and for the regeneration of transgenic plants (Ikeda and Kamada, 2005). While hormone and stress are common treatments that can stimulate the initiation of SE (Karami and Saidi, 2010), little is understood about how these triggers lead to the formation of somatic embryos, including the mechanisms by which the regulatory networks of transcription factors communicate to coordinate SE.

Potential or competence for SE varies with species, tissue, age of tissue, and even among closely related genotypes. It is thought that stress and hormones act first to promote the dedifferentiation of cells within the explants and then to allow competent cells to reprogram to follow an embryogenic pathway. The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is very commonly a component of medium for somatic embryo induction. In some systems, exogenous auxin must then be removed for embryo development to progress. The process of excising the explants, causing wounding, and placing into culture, often with high (e.g. nonphysiological for a natural auxin) concentrations of 2,4-D, all promote stress responses (for review, see Fehér et al., 2002; Karami and Saidi, 2010; Zavattieri et al., 2010). Several studies have been performed comparing embryogenic with nonembryogenic genotypes. In alfalfa (*Medicago sativa*), this type of study suggested that embryogenic genotypes may be hypersensitive to auxin (for review, see Fehér, 2005). Stress can enhance the response to low levels of 2,4-D that would normally not induce SE but with the addition of stress will promote SE (Pasternak et al., 2002). Along with auxin, ethylene and abscisic acid (ABA) are considered stress hormones that may be involved in cells acquiring competence for SE (Karami and Saidi, 2010).

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A number of transcription factors have been found to promote SE when ectopically expressed, and some ties to hormones have been made. For instance, in Arabidopsis (*Arabidopsis thaliana*), *LEAFY COTYLEDON1* (*LEC1*) and *LEC2*, which cause proliferation of SE on "seedlings" when ectopically expressed, have been found to induce the expression of genes involved in auxin synthesis (Stone et al., 2008; Junker et al., 2012). *LEC1* is also responsive to auxin (Ledwon and Gaj, 2011). *WUSCHEL* (*WUS*) is auxin responsive and in the presence of auxin promotes SE (Zuo et al., 2002; Gallois et al., 2004; Su et al., 2009). *AGAMOUS-Like15* (*AGL15*) and *FUSCA3* (*FUS3*) do not cause SE to form on seedlings when ectopically expressed but do lead to the proliferation of embryogenic tissue (*35Spro*:*AGL15*) or embryo features in seedlings (*ML1pro*:*FUS3*). Both of these genes have been found to be up-regulated in response to auxin (Gazzarrini et al., 2004; Zhu and Perry, 2005). These gene products, as well as LEC2, have been found to impact GA and/or ABA (Gazzarrini et al., 2004; Wang et al., 2004; Braybrook et al., 2006). The GA-ABA ratio has been reported to determine embryo or postembryo developmental mode (Gazzarrini et al., 2004). Less is known about other hormones, but in *Medicago truncatula*, *SOMATIC EMBRYO-RELATED FACTOR1* (*MtSERF1*) was found to be essential for SE (Mantiri et al., 2008b). *MtSERF1* encodes one of the ETHYLENE RESPONSE FACTOR (ERF) subfamily B-3 members of the APETALA2 (AP2)/ERF transcription factor family. Transcript accumulation from *MtSERF1* is ethylene responsive (Mantiri et al., 2008b) and also depends on auxin and cytokinin, hormones that induce SE in *M. truncatula* (Mantiri et al., 2008a).

AGL15 promotes SE in Arabidopsis, as does the ortholog in soybean (*Glycine max* [referred to as GmAGL15]; Harding et al., 2003; Thakare et al., 2008; Q. Zheng and S.E. Perry, unpublished data). To understand how AGL15 promotes SE, direct and indirect regulated genes were identified (Wang et al., 2004; Zhu and Perry, 2005; Zheng et al., 2009). *At5g61590*, a potential ortholog of *MtSERF1*, was found to be a direct up-regulated target of AGL15 in Arabidopsis based on the chromatin immunoprecipitation (ChIP)-tiling array and expression array data. A number of genes related to ethylene biosynthesis and response were identified as regulated by GmAGL15, including potential *MtSERF1* orthologs (referred to as *GmSERF1* and *GmSERF2*). Here, results are presented to identify the connection between (*Gm*)*AGL15* and *At5g61590*/*GmSERF1*/*GmSERF2* and to better understand the function of *At5g61590* and hormone interaction in SE in Arabidopsis and soybean.

RESULTS

At5g61590 Is a Directly Up-Regulated Target of AGL15

To better understand how AGL15 promotes SE, we performed ChIP-tiling array experiments to map in vivo binding sites for AGL15. We also performed expression microarray analysis to determine transcriptome changes in response to increased (*35Spro*:*AGL15*) and decreased (*agl15 agl18*; AGL18 is a redundant factor to AGL15) AGL15 accumulation in a shoot apical meristem somatic embryo (SAM SE) system (Zheng et al., 2009). The SAM SE system involves allowing mature seed to complete germination in culture medium that contains the synthetic auxin 2,4-D as described by Mordhorst et al. (1998). By 3 weeks after the start of culture, seedlings have callused cotyledons and hypocotyls, and a fraction will have green somatic embryo tissue at the shoot apical meristem region. Prior work found that the percentage of seedlings with SAM SE development was positively correlated with AGL15 accumulation (Harding et al., 2003; Thakare et al., 2008). For the expression arrays, an early stage of SAM SE (10 d in culture) was used, before there was obvious SE development (Zheng et al., 2009).

One gene of interest for which regulatory regions were directly bound by AGL15 was *At5g61590*, which encodes a member of the AP2/ERF family of transcription factors. The gene product is one of two in Arabidopsis that is most closely conserved over the majority of the protein (amino acid residues 1–182 of 201 total) to MtSERF1. MtSERF1 was found to have a role in *M. truncatula* SE (Mantiri et al., 2008b), making potential regulation of an Arabidopsis ortholog (*At5g61590*) by AGL15 especially interesting. The region of *At5g61590* identified by CisGenome (Ji et al., 2008) as bound by AGL15 on all three replicates of the ChIP-chip experiment was 362 bp in length and included the 5′ untranslated region (UTR) as well as part of the promoter region including a CArG motif of sequence CTTTATATAG, a C-8-G type binding site that was found to be preferentially bound by AGL15 in vitro (Tang and Perry, 2003). The expression microarrays indicated that *At5g61590* might be up-regulated in response to AGL15 accumulation (the change of transcript in *35S*:*AGL15* compared with wild-type tissue was 2.1-fold [$P = 0.03$]; no significant change was observed for the comparison of the *agl15 agl18* double mutant with the wild type; Gene Expression Omnibus data set GSE17742; Zheng et al., 2009).

Quantitative PCR enrichment tests were used to test whether *At5g61590* regulatory regions were directly bound by AGL15 and to quantitate this interaction. The *TUBULIN3* (*TUA3*) coding sequence, which is not a direct target of AGL15, was used as a nonbound control. As shown in Figure 1A, the change between the immunoprecipitation and preimmune control was 30.2-fold, indicating more of the *At5g61590* target in the immunoprecipitation than in the preimmune precipitation. As expected, the amount of amplicon generated from the control (*TUA3*) was approximately equal in the immunoprecipitation compared with the preimmune precipitation (1.3 ± 0.5; Fig. 1A). The fold enrichment for *At5g61590* was significantly higher than that for *TUA3* at $P < 0.05$. Differential site occupancy (DSO) compares the occupancy of AGL15 at the

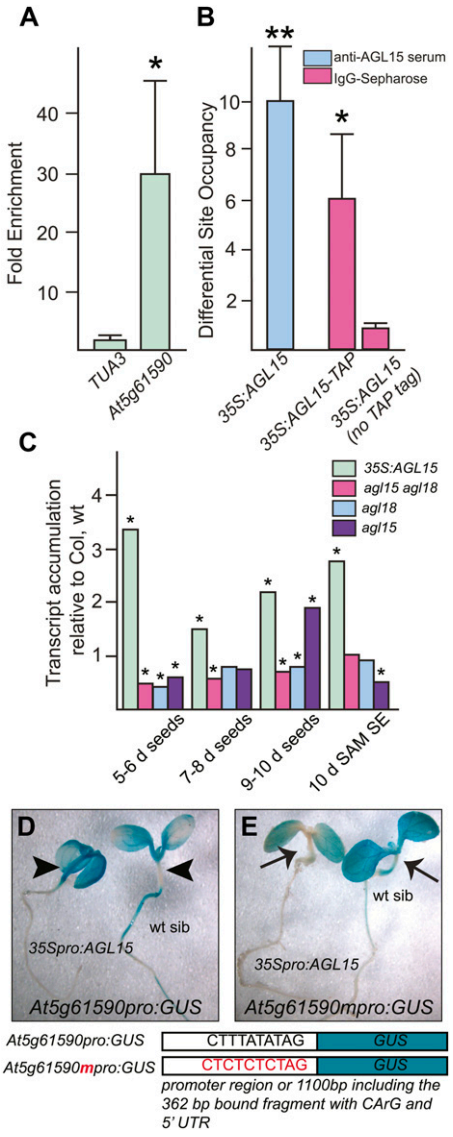

Figure 1. Verification of the direct association of AGL15 with regulatory regions of *At5g61590* and confirmation of the response of *At5g61590* transcript accumulation to AGL15 accumulation. A, Means and SE of fold enrichment calculated from qPCR on three independent ChIP experiments. B, DSO was calculated from qPCR on three to four independent ChIP experiments. The left bar compares the amount of the regulatory region of *At5g61590* recovered by ChIP using AGL15 antiserum to a nonbound control (*TUA3*) in the same immunoprecipitation. The right two bars represent the DSO calculated by performing ChIP with IgG-Sepharose to precipitate AGL15-TAP-DNA complexes via the protein A domains in the TAP tag. The control is nontagged *AGL15*-expressing tissue. Mean and SE values are shown. C, Transcript accumulation of *At5g61590* in gain- and loss-of-function *AGL15/AGL18* tissue compared with the wild type (set to 1) as evaluated by qRT-PCR. A representative experiment of three independent biological replicates is shown. For A to C, asterisks indicate significance as follows: $*P < 0.05$ and $**P < 0.01$ as determined using Student's *t* test. D and E, The promoter (approximately 1,100 bp) that includes the 362-bp DNA fragment identified as bound by AGL15 was fused to the reporter gene *GUS*. The bound fragment included a C-8-G AGL15-binding site. This site was mutated (*At5g61590mpro:GUS*). Plants with the reporter transgenes were crossed to hemizygous *35Spro:AGL15*, and the overexpressor and wild-type siblings were stained for GUS activity and separated visually. GUS activity was increased from *At5g61590pro:GUS* in response to ectopic AGL15 compared with the wild type (compare regions marked with arrowheads for the hypocotyls in D). The mutated binding site no longer showed this response (compare regions marked with arrows in E to note that *35Spro:AGL15* does not cause increased GUS activity compared with the wild type in this tissue). [See online article for color version of this figure.]

target with the nonbound control by assessing the amplification of the *At5g61590* target fragment and that of *TUA3* in the same immunoprecipitation. The average DSO from three independent experiments was 10.1, indicating that AGL15 preferentially binds to *At5g61950* compared with *TUA3*, and the difference was significant ($P < 0.01$) based on Student's *t* test (Fig. 1B). In order to confirm the specificity of the immunoprecipitation, a method that is independent of the AGL15 antiserum was used to isolate AGL15-DNA fragment complexes. A form of AGL15 with a C-terminal tandem affinity purification (TAP) tag and IgG-Sepharose that binds to the protein A domain within the TAP tag was used (Puig et al., 2001). The control in this case was tissue accumulating untagged AGL15. As shown in Figure 1B, the DSO of *At5g61590* compared with *TUA3* for the TAP-tagged tissue averaged 6.1 compared with 0.8 for the sample with tissue from *35Spro:AGL15* plants, indicating that the AGL15-TAP-*At5g61590* DNA fragment was immunoprecipitated to a greater extent with the IgG-Sepharose (significant at $P < 0.05$).

To confirm the response of *At5g61590* to AGL15 accumulation, quantitative reverse transcription (qRT)-PCR was performed on 10-d SAM SE tissue to verify the microarray results and on staged developing seed to extend the findings to zygotic embryo development. As shown in Figure 1C, *At5g61590* showed consistent increased transcript accumulation that was significant in *35Spro:AGL15* compared with the wild type in different-age developing seeds and in seedlings from the SAM SE system. The developing seeds also showed a consistent decrease in *At5g61590* transcript accumulation in the *agl15 agl18* double mutant. However, there was no significant change in the *agl15 agl18* SAM SE tissue compared with the wild type, in agreement with the microarray results.

To test whether the C-8-G cis-motif within the DNA fragment bound by AGL15 may be involved in the increased gene expression, as measured by transcript abundance in response to AGL15, two reporter constructs were made. The approximately 1,100-bp region upstream of the start codon of *At5g61590* and including the 5′ UTR and the region identified as bound by AGL15 was used as the regulatory region controlling *GUS* expression (*At5g61590pro:GUS*). Site-directed mutagenesis generated a second construct where the C-8-G CArG motif was changed to CTCTCTCTAG, which is not bound by MADS domain proteins

(*At5g61590mpro*:*GUS*). Lines were generated where there was a single transgenic insert bred to homozygosity. These lines were crossed to hemizygous *35Spro*:*AGL15* plants. The subsequent progeny were hemizygous for the reporter transgene and segregated 1:1 for the *35Spro*:*AGL15* or "wild-type sibling" lacking the *AGL15* transgene, and these seedlings could be visually separated by shorter hypocotyls and cotyledon epinasty in the overexpressors. As shown in Figure 1D, *At5g61590pro*:*GUS* responded to ectopic AGL15, as evidenced by increased GUS activity in the cotyledons and hypocotyls compared with the wild-type sibling. When the C-8-G binding site was mutated, this increased GUS activity was not observed (Fig. 1E).

Therefore, qRT-PCR and quantitative PCR (qPCR) results are consistent with the array data. *At5g61590* is a direct downstream target of AGL15, and its expression is increased (as measured by transcript abundance) in response to AGL15 in developing seeds and in SAM SE at least when AGL15 is ectopically expressed for this later context. A C-8-G motif appears to be at least in part responsible for the regulation by AGL15.

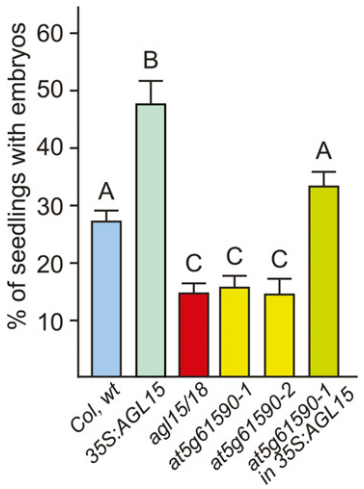

Figure 2. At5g61590 is involved in the promotion of SAM SE. Means and SE from at least three independent experiments with independently generated seed lots are shown. Different letters indicate significant differences in the production of SAM SE comparing the different genotypes at $P < 0.001$ as determined using Student's *t* test. wt, Wild type. [See online article for color version of this figure.]

At5g61590 and AGL15 in the Promotion of Somatic Embryogenesis in Arabidopsis

MtSERF1 was found to promote SE (Mantiri et al., 2008b), and *At5g61590* is one of the two closest orthologs in Arabidopsis that responded to AGL15 accumulation in a manner consistent with the promotion of SE. The other closest ortholog to MtSERF in Arabidopsis, *At5g07580*, did not respond to AGL15 accumulation. Does *At5g61590*, a direct target of AGL15, promote SE in Arabidopsis? The SAM SE system of Mordhorst et al. (1998) was used to test the effect of the accumulation of the *At5g61590* gene product on SE. As found previously and shown in Figure 2, *35Spro*:*AGL15* significantly increased the percentage of seedlings with SAM SE development, while *agl15 agl18* double loss of function led to a significant decrease (Harding et al., 2003; Thakare et al., 2008). Two separate loss-of-function alleles of *At5g61590* were obtained from the Arabidopsis Biological Resource Center and are referred to as *at5g61590-1* and *at5g61590-2* (SALK_015182C and SALK_009249C, respectively, which are confirmed homozygous knockout lines [Alonso et al., 2003]; please note that SERF already refers to an uncharacterized protein family in The Arabidopsis Information Resource and therefore is not used as a designation for At5g61590). Both knockout lines showed significant decreases in the production of SAM SE to the level found in the *agl15 agl18* mutant. One of the knockout alleles, *at5g61590-1*, was crossed into the *35Spro*:*AGL15* background, and a subsequent generation was bred to reestablish the homozygous knockout allele in the *35Spro*:*AGL15* segregating background (the homozygous *35Spro*:*AGL15* plants are sterile, necessitating use of the hemizygous *35Spro*:*AGL15*). The *at5g61590-1* background significantly reduced the ability of *35Spro*:*AGL15* to enhance SAM SE compared with *35Spro*:*AGL15* in the *At5g61590* background. This indicates that up-regulation of *At5g61590* by ectopic expression/overexpression of *AGL15* is at least partially responsible for the increased SAM SE in response to *35Spro*:*AGL15*. Reduction of SAM SE in *35Spro*:*AGL15 at5g61590-1* was not to *at5g61590-1* or *at5g61590-2* levels, probably because AGL15 regulates other targets involved in SE (Wang et al., 2004; Zheng et al., 2009).

Ethylene Biosynthesis and Response Genes, Including Potential Orthologs of *MtSERF1*/*At5g61590*, Are Up-Regulated by GmAGL15 in Soybean

To extend our findings to a crop plant, a gene encoding an ortholog of AGL15 was isolated from soybean (*Glyma11g16105*; hereafter referred to as *GmAGL15*; Thakare et al., 2008). When expressed via the *35S* promoter, increased numbers of transformants were recovered compared with the control, perhaps because *35Spro*:*GmAGL15* may enhance regeneration by SE (Thakare et al., 2008). Transgenic plants were recovered, and the ability to form somatic embryos from immature cotyledon explants isolated from embryos that were 4 to 5 mm was assessed compared with nontransgenic controls. The *35Spro*:*GmAGL15* transgene significantly increased somatic embryo production from explants (Q. Zheng and S.E. Perry, unpublished data).

To better understand the mechanism for the enhancement of SE by *GmAGL15*, we performed

transcriptome analysis comparing tissue from *35Spro*:*GmAGL15* and the cv Jack wild type at 0, 3, and 7 d after culture (dac) on D40 medium that induces SE. Overall results from the microarray analysis are reported by Q. Zheng and S.E. Perry (unpublished data) with a focus on genes related to ethylene reported here. Lists of genes significantly ($P < 0.05$) up-regulated (*35Spro*:*GmAGL15*/nontransgenic > 1.5) or down-regulated (*35Spro*:*GmAGL15*/nontransgenic < 0.67) for at least one time point were generated. Putative Arabidopsis orthologs were identified using information from Soybase Annotation AffyChip version 3 and The Arabidopsis Information Resource 8. Genes for which a putative Arabidopsis ortholog could be identified were analyzed for overrepresented Gene Ontology categories using DAVID (Huang et al., 2009), revealing an enrichment of genes involved in ethylene biosynthesis ($P = 4.4e-1$), response to ethylene stimulus ($P = 2.8e-5$), and the ethylene signaling pathway ($P = 8.6e-05$). Those for which the putative ortholog in Arabidopsis also responded to AGL15 are shown in Tables I and II. Many of these genes encode transcription factors, and interestingly, some are also involved in auxin, GA, and ABA response.

We found a number of genes encoding products involved in ethylene biosynthesis, including a 1-aminocyclopropane-1-carboxylic-acid (ACC) synthase (ACS) that catalyzes the production of ACC from *S*-adenosyl-Met and an ACC oxidase (ACO) that subsequently generates ethylene from ACC, responded to GmAGL15 accumulation. Gene products involved in ethylene signaling and response were also differentially regulated, with some putative orthologs responding in a similar manner in soybean and Arabidopsis (e.g. a putative ortholog of Arabidopsis *ERF1*; Table I). We confirmed results for select genes by qRT-PCR (Fig. 3A). *Glyma05g37410*, which encodes an ACS closest to Arabidopsis At3g61510 (ACS1) and At4g11280 (ACS6), was significantly up-regulated by *35Spro*:*GmAGL15* at 0 dac (uncultured embryo tissue) as well as at 7 dac under conditions to induce SE. There was a slight increase in transcript accumulation at 3 dac. At least one *ACO* (*Glyma02g43560*; for which the closest Arabidopsis ortholog is encoded by *At1g05010/EFE/ACO4*) was also positively regulated by increased/ectopic GmAGL15, but another, *Glyma05g36310* (*At2g19590*; *ACO1*), was significantly repressed in response to GmAGL15 at 0 dac compared with the nontransgenic control, which was set to 1 (Fig. 3A). ERF1 is a transcription factor that is downstream of the signaling pathway from CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), ETHYLENE INSENSITIVE2 (EIN2) and EIN3 in Arabidopsis (Stepanova and Alonso, 2009), and a gene encoding a putative ortholog of ERF1 in soybean was also significantly induced by GmAGL15 at all stages tested (Fig. 3A). Although *EIN3* was not tested by qRT-PCR, it was also expressed in response to AGL15 in both the soybean and the Arabidopsis somatic embryo systems (Table I).

The protein sequence encoded by *MtSERF1* (*Jf357924*; *Medtr1g059490*) was used to search the Phytozome database (http://www.phytozome.net/search.php?show=blast; Goodstein et al., 2012). The best matches in soybean were encoded by *Glyma20g16920* (referred to as *GmSERF1* after the nomenclature for the *M. truncatula* gene) and *Glyma10g23460* (*GmSERF2*), with 68% to 63% identity (78%–74% similarity) to the *M. truncatula* protein. The relationship between the proteins encoded by these genes, *MtSERF1*, *At5g61590*, and related genes is shown in Figure 3B. *GmSERF2* was not represented on the Affymetrix soybean array, but *GmSERF1* showed an increased transcript accumulation at 3 dac in *35Spro*:*GmAGL15* compared with the wild type (1.7), although at $P = 0.18$. We performed qRT-PCR for both of these genes and found that transcript from *GmSERF1* was significantly increased in response to *35Spro*:*GmAGL15*, and the difference between overexpressor and wild-type tissue increased with time in culture (Fig. 3A). Transcript accumulation from *GmSERF2* in *35Spro*:*GmAGL15* tissue compared with the control was also significantly increased in both the isolated explant tissue and after culture (Fig. 3A).

To ascertain whether GmAGL15 may directly regulate *GmSERF1* and *GmSERF2*, as AtAGL15 does *At5g61590*, regulatory regions 5′ of the start codons of the soybean genes were examined for the presence of binding sites for AGL15. The soybean genes both have a C-8-G type CArG motif (binding site for MADS such as AGL15) within about 700 bp of the start codon. ChIP was performed on immature soybean embryos expressing the *GmAGL15* transgene and using anti-AGL15 serum that was raised against *Brassica napus* AGL15 (Perry et al., 1996) and primers designed to amplify regions with the C-8-G suspected to be bound by GmAGL15. As shown in Figure 3C, regulatory regions of both genes were associated with GmAGL15 compared with a nonbound control (*GmTUB2*; *Glyma03g15020*; the DSO of the targets was larger than the *GmTUB2* control of 1.0).

Ethylene Production Is Increased in Soybean in Response to GmAGL15

Although some genes involved in ethylene biosynthesis were up-regulated in response to GmAGL15, others were repressed, at least at some stages. Because there can be complex feedback mechanisms, and we measured transcript accumulation that does not necessarily translate into protein accumulation or activity, up- or down-regulation of particular genes involved in ethylene synthesis does not automatically mean more or less ethylene production. Soybean provides an opportunity to isolate sufficient embryo tissue without wounding the embryo to assess ethylene production. Therefore, we tested whether tissue with the *35Spro*:*GmAGL15* construct produced more ethylene than the wild type using gas chromatography-flame ionization detection. As shown in Figure 4, *GmAGL15* overexpression lines in the soybean cultivars Jack (line 8981) and Williams 82 (line 1041) produced significantly increased

Table I. *Soybean genes involved in ethylene metabolism, signaling, or response that show increased transcript accumulation in 35Spro:GmAGL15 tissue compared with the wild type for at least one time point and for which a putative Arabidopsis ortholog was identified that is also responsive to AGL15*

Italicized boldface numbers indicate opposite regulation in Arabidopsis compared with soybean. $*P < 0.05$, $**P < 0.01$.

Soybean Affy Chip Probe	Soybean Gene	Arabidopsis Ortholog	Gene Name (Arabidopsis)	*35Spro:GmAGL15*/ Nontransgenic cv Jack			Arabidopsis Microarray (Zheng et al., 2009)	
				Day 0	Day 3	Day 7	*agl15agl18*/ Col	*35S:AGL15*/ Col
GmaAffx.132.1.S1_at	Glyma13g30720.1	AT3G23240	Ethylene-responsive transcription factor1, AtERF1	2.03*	0.94	1.05	1.99	7.59*
GmaAffx.53033.1.S1_at	Glyma10g33060.1	AT3G23240	Ethylene-responsive transcription factor1, AtERF1	2.98*	2.03	1.25	1.99	7.59*
GmaAffx.84566.1.S1_s_at	Glyma09g12230.1 DQ822967	AT3G47600	Myb domain protein94	1.77*	1.03	0.89	0.92	2.03*
GmaAffx.84566.1.S1_at	Glyma09g12230.1 DQ822967	AT3G28910	Myb domain protein30	2.23*	1.16	0.83	1.26	1.93**
GmaAffx.84566.1.S1_x_at	Glyma18g49360.1 DQ822967	AT3G28910	Myb domain protein30	2.20*	1.13	0.84	1.26	1.93**
GmaAffx.92676.1.S1_s_at	Glyma02g07310.1	AT5G07310	Ethylene-responsive transcription factor ERF115	5.14*	0.87	0.98	0.78	1.73*
Gma.487.1.S1_at	Glyma18g43750.1	AT3G11020	Dehydration-responsive element-binding protein2B	3.08*	1.03	0.94	1.07	1.69*
GmaAffx.65829.1.A1_at	Glyma17g15480.1 AK245452	AT5G47220	Ethylene-responsive transcription factor2, AtERF2	3.02*	1.57	0.97	1.35	1.65*
GmaAffx.84566.3.S1_x_at	Glyma09g37340.1 DQ822980	AT5G62470	Myb domain protein96	2.29**	1.26	1.11	0.49*	1.32
GmaAffx.84566.3.S1_s_at	Glyma09g37340.1 DQ822980	AT5G62470	Myb domain protein96	2.48*	1.54	0.78	0.49*	1.32
GmaAffx.84566.3.S1_at	Glyma09g37340.1 DQ822980	AT5G62470	Myb domain protein96	2.30**	1.51	1.25	0.49*	1.32
GmaAffx.93218.1.S1_at	Glyma02g44220.1	AT3G20770	Ethylene insensitive3, AtEIN3	1.79*	1.83**	0.87	0.33**	0.89
GmaAffx.87134.1.S1_at	BM094926	AT5G61890	Ethylene-responsive transcription factor ERF114	5.52*	0.58	0.93	***1.71****	***0.41****
Gma.3202.1.S1_at	Glyma01g39540.1 AK244754	AT2G23340	Ethylene-responsive transcription factor ERF008, DEAR3, DREB and ear motif protein3	3.10*	0.81	0.99	***1.10***	***0.56****
Gma.4155.2.S1_s_at	Glyma03g42450.1 EU681278	AT1G53910	Ethylene-responsive transcription factor RAP2-12	1.58*	1.21	0.92	***1.68****	***1.00***
GmaAffx.29929.1.S1_at	Glyma20g29410.1 EF551167	AT4G25480	Dehydration-responsive element-binding protein1A	3.09**	1.07	1.04	***1.71****	***1.33***
Gma.3189.1.S1_at	Glyma09g30300.1	AT1G73500	MAP kinase kinase9, AtMKK9	3.77*	2.90*	1.32	***3.03****	***1.90***
Gma.3189.1.S1_a_at	Glyma07g11910.1	AT1G73500	MAP kinase kinase9, AtMKK9	2.33*	2.57*	1.26	***3.03****	***1.90***
GmaAffx.15015.1.S1_at	Glyma10g00980.1	AT3G23240	Ethylene-responsive transcription factor1, AtERF1	1.00	2.53**	1.19	1.99	7.59*
GmaAffx.90574.1.S1_at	Glyma02g44220.1, Glyma14g04550.1	AT3G20770	Ethylene insensitive3, AtEIN3	1.41	1.88*	1.01	0.33**	0.89
GmaAffx.93218.1.S1_at	Glyma02g44220.1	AT3G20770	Ethylene insensitive3, AtEIN3	1.79	1.83**	0.87	0.33**	0.89
GmaAffx.90574.1.S1_s_at	Glyma02g44220.1	AT3G20770	Ethylene insensitive3, AtEIN3	1.36	1.66*	0.96	0.33**	0.89
GmaAffx.13724.1.S1_s_at	Glyma14g04550.1	AT3G20770	Ethylene insensitive3, AtEIN3	1.09	1.62*	0.81	0.33**	0.89
Gma.10608.1.S1_at	Glyma02g44220.1	AT3G20770	Ethylene insensitive3, AtEIN3	1.39	1.51**	1.07	0.33**	0.89
GmaAffx.67456.1.S1_at	Glyma02g09600.1	AT2G28550	Ethylene-responsive transcription factor RAP2-7	1.07	2.10**	1.01	***0.73***	***0.48****
GmaAffx.27137.2.S1_at	Glyma02g09600.1	AT2G28550	Ethylene-responsive transcription factor RAP2-7	1.03	1.77**	0.95	***0.73***	***0.48****

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Soybean Affy Chip Probe	Soybean Gene	Arabidopsis Ortholog	Gene Name (Arabidopsis)	*35Spro:GmAGL15*/ Nontransgenic cv Jack			Arabidopsis Microarray (Zheng et al., 2009)	
				Day 0	Day 3	Day 7	*agl15agl18*/ Col	*35S:AGL15*/ Col
GmaAffx.17289.1.S1_at	Glyma04g21340.1	AT1G14920	DELLA protein GAI	0.68	1.52*	0.87	***1.58****	***1.18***
Gma.15127.1.A1_at	Glyma18g04500.1	AT2G01570	DELLA protein RGA	0.83	1.74**	1.14	***1.55****	***1.27***
Gma.3189.1.S1_at	Glyma09g30300.1	AT1G73500	MAP kinase kinase9, AtMKK9	3.77	2.90*	1.32	***3.03****	***1.90***
Gma.3189.2.S1_at	Glyma07g11910.1	AT1G73500	MAP kinase kinase9, AtMKK9	1.89	2.63*	1.27	***3.03****	***1.90***
Gma.3189.1.S1_a_at	Glyma07g11910.1	AT1G73500	MAP kinase kinase9, AtMKK9	2.33	2.57*	1.26	***3.03****	***1.90***
Gma.17730.1.A1_at	Glyma09g34300.1	AT1G31930	Extra-large GTP-binding protein3, XLG3	1.31	1.319	1.59*	0.57**	0.95

amounts of ethylene compared with the nontransgenic controls.

Ethylene Perception and Signaling, and Somatic Embryogenesis

Ethylene was demonstrated to enhance SE from leaf explants in *M. truncatula* (Mantiri et al., 2008b), but in general, the roles for ethylene in SE are not well studied. Will ethylene enhance SE from the shoot apical region of seedlings in Arabidopsis or from cotyledon explants of soybean? As shown in Figure 5A, a variety of chemicals can be used to inhibit particular steps in ethylene biosynthesis or response. As shown in Figure 5B, 25 μM ACC, a precursor to ethylene (Cameron et al., 1979), significantly increased SAM SE compared with no ACC addition (40.8% at 25 μM compared with 24.8% without exogenous ACC).

To test the effect of ACC on soybean somatic embryo development from immature cotyledon explants, 10 μM ACC was added to the D40 medium,

Table II. *Soybean genes involved in ethylene metabolism, signaling, or response that show decreased transcript accumulation in 35Spro:GmAGL15 tissue compared with the wild type for at least one time point and for which a putative Arabidopsis ortholog was identified that is also responsive to AGL15*

Italicized boldface numbers indicate opposite regulation in Arabidopsis compared with soybean. $*P < 0.05$, $**P < 0.01$.

Soybean Affy Chip Probe	Soybean Gene	Arabidopsis Ortholog	Gene Name	*35S:GmAGL15*/ Nontransgenic cv Jack			Arabidopsis Microarray (Zheng et al., 2009)	
				Day 0	Day 3	Day 7	*agl15agl18*/ Col	*35S:AGL15*/ Col
GmaAffx.55264.1.S1_at	Glyma14g06290.1	AT2G40220	Ethylene-responsive transcription factor ABI4	0.62*	1.30	1.31	1.97**	3.52
GmaAffx.19343.1.S1_at	Glyma04g01460.1 BT097041	AT4G34460	Guanine nucleotide-binding protein subunit β	0.59**	1.04	0.97	1.58**	1.06
GmaAffx.31202.1.S1_at	Glyma06g23940.1	AT1G14920	DELLA protein GAI	0.61*	1.16	1.14	1.58*	1.18
GmaAffx.58365.1.S1_at	Glyma18g04500.1	AT2G01570	DELLA protein RGA	0.67*	1.07	0.97	1.55*	1.27
GmaAffx.86317.1.S1_at	Glyma01g00510.1	AT1G19220	Auxin response factor19	0.66**	0.93	0.80	***0.21****	***1.00***
Gma.6846.1.S1_at	Glyma07g15640.1	AT1G19220	Auxin response factor19	0.64**	0.87	1.10	***0.21****	***1.00***
Gma.4207.1.S1_at	Glyma06g00630.1 DQ822882	AT4G34990	Transcription factor MYB32	0.35**	0.74	0.78	***0.56****	***1.44***
Gma.12924.1.A1_at	Glyma15g00660.1	AT5G25190	Ethylene-responsive transcription factor ERF003	0.54*	0.43**	0.64	***1.07***	***1.95****
GmaAffx.2962.1.S1_at	Glyma15g04930.1	AT2G28550	Ethylene-responsive transcription factor RAP2-7	0.81	0.45*	0.87	0.73	0.48**
Gma.5405.1.S1_at	Glyma19g36200.1	AT2G28550	Ethylene-responsive transcription factor RAP2-7	0.64	0.44*	0.51	0.73	0.48**
Gma.15862.1.S1_at	Glyma11g15650.1	AT2G28550	Ethylene-responsive transcription factor RAP2-7	1.02	0.61**	1.09	0.73	0.48**
Gma.612.1.A1_at	Glyma04g05080.1	AT4G37750	AP2-like ethylene-responsive transcription factor ANT	1.02	0.55**	0.69**	***0.47****	***1.02***
Gma.12924.1.A1_at	Glyma15g00660.1	AT5G25190	Ethylene-responsive transcription factor ERF003	0.54*	0.43**	0.64	***1.07***	***1.94****

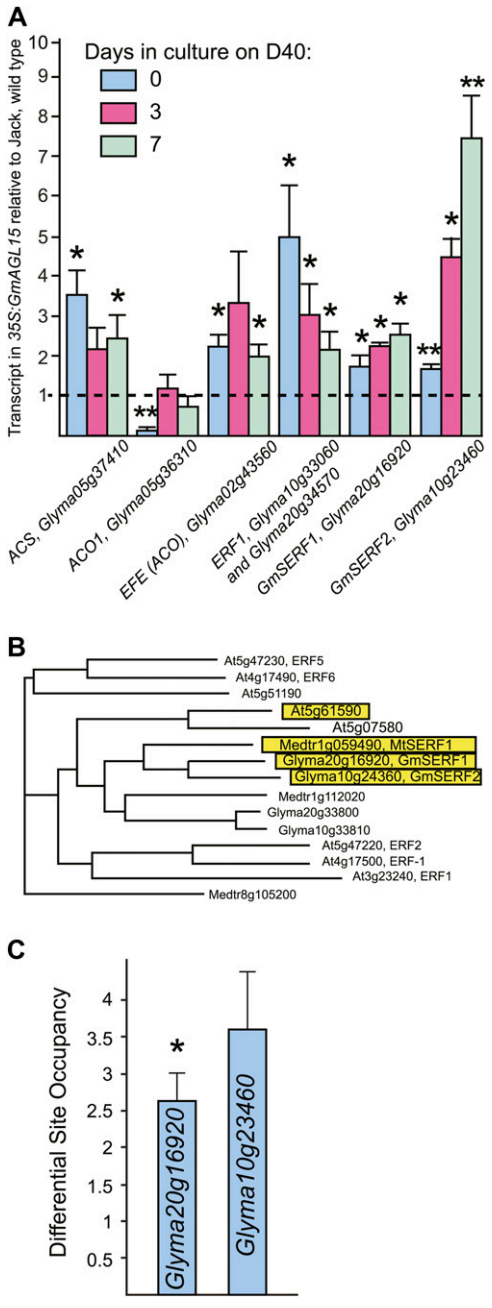

Figure 3. Transcripts encoding proteins involved in ethylene biosynthesis and response respond to increased GmAGL15 accumulation. A, qRT-PCR to assess transcript accumulation from genes involved in ethylene biosynthesis (*ACS*, *ACO1*, *EFE*) and response (*ERF1*, *GmSERF1*, *GmSERF2*) in soybean immature cotyledon explants carrying a *35Spro:GmAGL15* construct relative to nontransgenic tissue. Transcript was assessed from the isolated explants (0 d in culture) and after 3 and 7 d on the D40 medium that induces somatic embryogenesis. Results shown are means and SE for at least three biological replicates. The asterisks signify significant differences from nontransgenic tissue (set to 1) as follows: $*P < 0.05$, $**P < 0.01$. B, Phylogram generated with ClustalW2 showing the relationship between the predicted proteins encoded by *MtSERF1* and the genes under study from Arabidopsis and soybean (highlighted) and other closely related genes from *M. truncatula* (*Medtr*), soybean (*Glyma*), and Arabidopsis (*At*). C, DSO calculations from qPCR on three independent ChIP populations. Recovery of target fragments by coimmunoprecipitation with anti-AGL15 serum was compared with recovery of a nonbound control DNA fragment (*GmTUB2*) in the same immune precipitation. The asterisk signifies a significant difference from the nonbound control fragment at $P < 0.05$. [See online article for color version of this figure.]

and genotypes Williams 82 and Defiance that are recalcitrant for SE were used. Approximately 21 to 25 explants were placed on a plate and scored as described by Meurer et al. (2001), where individual explants were scored after 30 d in culture 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 on an explant. The average was calculated per plate and then among all plates of a genotype. As shown in Figure 5C, the addition of ACC significantly increased somatic embryo production for cv Defiance and had a greater effect on cv Williams 82.

In both the Arabidopsis SAM SE system and the soybean cotyledon explant system, addition of aminoethoxyvinylglycine (AVG), which inhibits ACC synthase (Yu and Yang, 1979), reduced the number of somatic embryos produced (Fig. 5, B and D). In soybean, this was true for both cv Williams 82 and the highly embryogenic cv Jack. In both the Arabidopsis SAM SE and soybean SE systems, the addition of ACC with AVG at least partially rescued the inhibition of SE caused by AVG alone (Fig. 5, B and E). For Arabidopsis, the recovery in SAM SE production was to the level found in the no-addition control, although SAM SE was still less than with ACC alone (Fig. 5B). These results indicate that at least part of the effect of the AVG may be due to a block in ethylene accumulation. Images of the soybean explants at 31 dac on AVG and on AVG plus ACC are shown in Supplemental Figure S1. ACC did not significantly increase SE in cv Jack compared with the no-addition control, as found for the less embryogenic genotypes (Fig. 5E). In Arabidopsis, addition of $AgNO_3$, which blocks ethylene perception (Beyer, 1976), or $CoCl_2$, which acts as an inhibitor of ACO (Yu and Yang, 1979), also reduced Arabidopsis SAM SE (Fig. 5B).

Gene Expression in Response to Ethylene Perturbations

SE and ethylene accumulation and perception are positively correlated, as shown in Figures 4 and 5. We determined the consequences of culture additions that impact ethylene on the expression of genes involved in SE. As shown in Figure 6, *At5g61590* and *GmSERF1* showed significantly increased transcript accumulation in response to ACC addition to the medium. Conversely, addition of $AgNO_3$ (Fig. 6A) or AVG

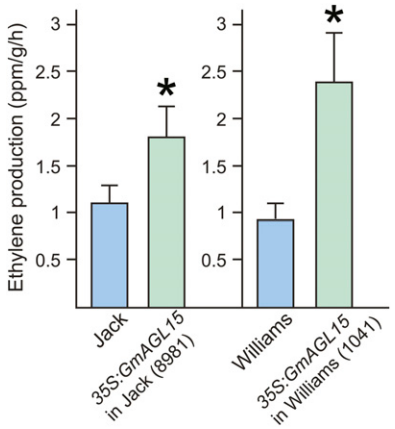

Figure 4. Increased expression of *GmAGL15* leads to increased ethylene production. Ethylene production by isolated developing embryos (4–5 mm) of nontransgenic soybean cultivars Jack and Williams was compared with transgenic lines constitutively expressing *GmAGL15* via a *35S* promoter (line number). Data shown are means and SE for at least five independent experiments. The asterisks indicate significant differences in ethylene production within a cultivar between *35Spro*:*GmAGL15* and nontransgenic tissue at $P < 0.05$ (determined using Student's *t* test). [See online article for color version of this figure.]

(Fig. 6) led to a decrease in transcript accumulation from *At5g61590/GmSERF1*. Addition of ACC with AVG rescued transcript accumulation of *At5g61590/GmSERF1* to at least control (no additions to medium) levels (Fig. 6). Both the Arabidopsis and soybean genes were also responsive to biologically active GA, with the addition of GA_3 leading to reduced transcript while addition of the GA biosynthetic inhibitor paclobutrazol increased transcript abundance (Fig. 6).

Prior work demonstrated that AtAGL15 directly upregulates *ABI3* and *FUS3*, two transcriptional regulators important for embryogenesis (Zheng et al., 2009). Likewise, GmAGL15 impacts the transcript accumulation of genes important for embryo development (Q. Zheng and S.E. Perry, unpublished data). We tested whether perturbations in ethylene impacted the transcript accumulation of select genes with roles in embryogenesis, and as shown in Figure 7, ACC significantly increased transcript accumulation from *GmAGL15* and *GmFUS3* (*Glyma16g05480*) compared with the control medium at 3 dac. Conversely, AVG treatment for 3 dac resulted in significantly decreased transcript from *GmAGL15*, *GmAGL18* (*Glyma02g33040*), *GmABI3* (*Glyma08g47240*), and *GmFUS3*. The addition of ACC with AVG increased transcript accumulation from these genes compared with AVG alone (Fig. 7). These results indicate a complex network of not only the regulation of genes encoding embryo transcription factors (Zheng et al., 2009; Q. Zheng and S.E. Perry, unpublished data) and ethylene biosynthesis/response genes by GmAGL15 but also the regulation cross talk between ethylene and embryo transcription factors.

DISCUSSION

Genes Involved in Ethylene Biosynthesis and Response Are Regulated by AGL15

Genes responsive to AGL15 accumulation in soybean for which an Arabidopsis ortholog was identified were found to be enriched for genes involved in ethylene biosynthesis and response. A subset of these genes also responded to AGL15 accumulation in Arabidopsis, in some cases in a consistent but in others in an opposite manner (Tables I and II). While genes involved in ethylene biosynthesis were regulated in response to GmAGL15 accumulation in soybean (e.g. *ACS*, *ACO1*, *EFE*; for the qRT-PCR verification, see Fig. 3), the closest ortholog in Arabidopsis did not show significant changes. None of the *ACS* or *ACO* genes in Arabidopsis appeared to be up-regulated in response to AGL15 accumulation, and two putative *ACO* genes may be indirectly repressed (data set from Zheng et al., 2009). The differences between regulation by AGL15 in these two species may be due to several factors. First, the tissue contexts were quite different between Arabidopsis and soybean. In Arabidopsis, mature dry seeds were used to initiate the SAM SE cultures, whereas in soybean, explants from immature zygotic embryos were cultured. This latter situation involved wounding, because the cotyledon explants were excised from the rest of the embryo. Wounding can lead to ethylene production (for review, see Karami and Saidi, 2010). Both systems included the synthetic auxin 2,4-D, although at different concentrations (4.5 compared with 181 μM for Arabidopsis and soybean, respectively), but tissue was submerged in one situation and placed on solid medium in the other. Different 2,4-D concentrations can lead to different patterns of gene regulation, including genes associated with ethylene (Raghavan et al., 2006). Tissue was exposed to 2,4-D for different amounts of time, and it may be notable that for *ACS* and *ACO*, the degree of increase of the transcript in *35Spro*:*GmAGL15* compared with the nontransgenic soybean tissue decreased with time in culture. However, the extent of *GmSERF* transcript overaccumulation increased with time for the *35Spro*:*GmAGL15* tissue compared with nontransgenic tissue, possibly reflecting the potential role of these gene products in actually potentiating embryogenesis rather than the more immediate response to the induction.

Whether 2,4-D acts directly as an auxin, or induces endogenous auxin synthesis, or acts as a stressing agent, increased ethylene production would be expected (Raghavan et al., 2006; Karami et al., 2009). Although the gene regulation is complex, the net result was that soybean embryos with the *35Spro*:*GmAGL15* transgene produced more ethylene than wild-type control tissue (Fig. 4). Care was taken to ensure that the embryos were not wounded during isolation for these experiments. Also, both systems showed an increase of somatic embryos in response to additions that enhance ethylene production (addition of ACC)

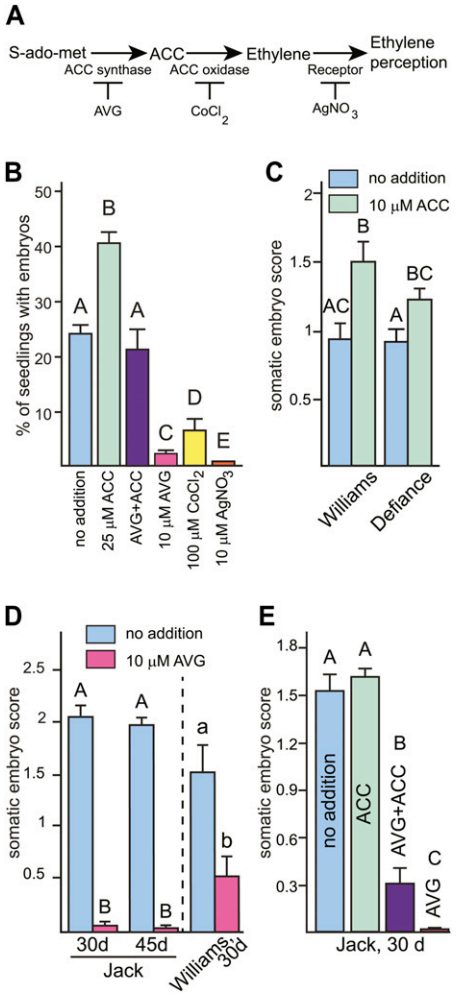

Figure 5. Ethylene accumulation and perception impact somatic embryogenesis in the Arabidopsis SAM SE and soybean cotyledon explant systems. A, Steps at which the various additions to the culture medium enhance or inhibit ethylene production/signaling. B, ACC, the precursor to ethylene, promotes SAM SE, while inhibition of ethylene perception ($AgNO_3$) or biosynthesis (AVG or $CoCl_2$) leads to decreased SAM SE. The addition of ACC with AVG rescues SAM SE to no-addition control levels. Means and SE from at least three independent experiments with independently generated seed lots are shown. Different letters indicate significant differences in the percentage of seedlings with SAM SE development comparing the different treatments at $P < 0.05$. C, Addition of ACC to D40 medium significantly increases somatic embryo production from immature cotyledon explants of the poorly embryogenic soybean cultivars Williams 82 and Defiance. Tissue was scored after 30 d in culture. Different letters indicate significant differences in SE production by the genotypes and treatments at $P < 0.05$. D, Addition of AVG significantly inhibits somatic embryo formation on cotyledon explants of soybean. Different letters indicate significant reductions by AVG at $P < 0.0001$ (Jack) or $P < 0.05$ (Williams) compared with medium without AVG. E, Inhibition of somatic embryo formation on soybean cotyledon explants by AVG can be at least partially rescued by ACC (both added at 10 μM). Different letters indicate significant differences in SE score in response to the culture additions at $P < 0.05$. Student's t test was used for B to E to determine significance. [See online article for color version of figure.]

and a decrease with perturbations in ethylene production or response (AVG, $AgNO_3$ and *at5g61590-1*, *at5g61590-2*).

We have found that adding ACC with AVG could significantly rescue SE production in Arabidopsis and soybean compared with AVG alone (Fig. 5). While AVG caused significant reduction in transcript accumulation from genes related to embryogenesis, the addition of ACC with AVG could recover or at least partially recover transcript accumulation (Figs. 6 and 7). This indicates that at least part of the effect of AVG may be through perturbations in ethylene production. However, all of the pharmacological agents have other effects. AVG and $AgNO_3$ affect auxin biosynthesis, response, and transport (Strader et al., 2009; Soeno et al., 2010), while ACC has ethylene-independent effects on cell wall stress responses (Xu et al., 2008; Tsang et al., 2011). In addition, interactions between hormones are quite complex, and ethylene and auxin both impact each other's accumulation and interact in development in what has been described as "a highly entangled cooperative manner" (Vandenbussche et al.,

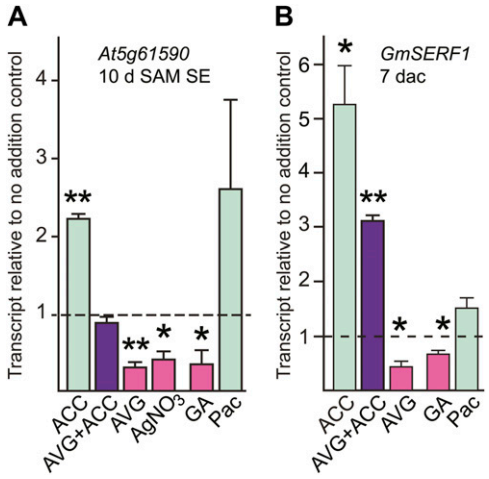

Figure 6. Transcript accumulation of *At5g61590/GmSERF1* in response to culture supplements correlates with somatic embryogenesis. A, Transcript accumulation of *At5g61590* in 10-d SAM SE cultures was evaluated by qRT-PCR. Results with culture supplements found to enhance (ACC, paclobutrazol [Pac]) or inhibit (AVG, $AgNO_3$, GA) SAM SE were compared with the untreated control tissue (set to 1). ACC was added to the AVG treatment to test whether gene expression could be rescued compared with AVG alone. Means and SE are shown for three independent biological replicates. B, qRT-PCR to measure transcript abundance in immature cotyledon explants on D40 medium or D40 medium with additions to stimulate (ACC) or inhibit (AVG) ethylene production, or the addition of biologically active GA or of paclobutrazol to inhibit bioactive GA synthesis. ACC and AVG were also added together to the medium and compared with the unsupplemented control. Results with treatment at 7 d of culture are compared with the D40 control. Means and SE are shown for at least three independent biological replicates. Significance is indicated as follows: $^*P < 0.05$ and $^{**}P < 0.01$ as determined using Student's t test. [See online article for color version of this figure.]

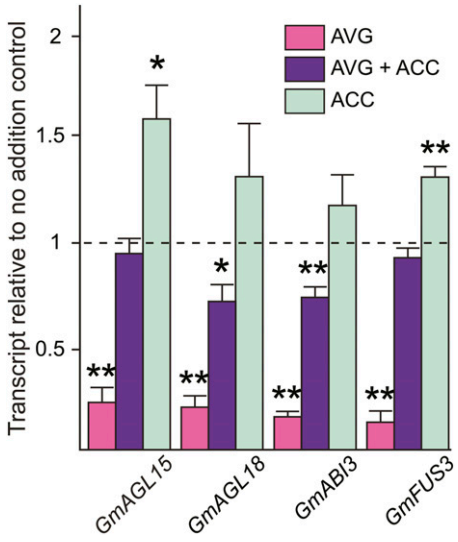

Figure 7. Transcripts from genes involved in the control of embryogenesis respond to perturbations in ethylene production. qRT-PCR was used to measure transcript abundance in immature cotyledon explants on D40 medium or D40 medium with additions to stimulate (ACC) or inhibit (AVG) ethylene production. ACC and AVG were added together to D40 medium to test whether ACC could rescue any changes caused by AVG. Results with treatment at 3 d of culture are compared with the D40 control (set to 1). Means and SE are shown for at least three independent biological replicates. Asterisks indicate significant differences as follows: $*P < 0.05$, $**P < 0.01$. [See online article for color version of figure.]

2012). There are also interactions with other hormones, such as GA, that also impact SE in both the Arabidopsis and soybean systems (Wang et al., 2004; Q. Zheng, unpublished data; see below for further discussion). However, the combined observations of the effects of additions to culture medium, correlation between increased SE for *35Spro*:*GmAGL15* and increased ethylene production from this tissue compared with the wild type, and decreased SAM SE for loss of function in a member of the ERF family suggest a role for ethylene in the promotion of SE.

Genes involved in ethylene signaling were often regulated in a consistent manner in response to AGL15 in soybean and Arabidopsis. Ethylene stabilizes EIN3 and EIN3-like1, which are positive regulators of ethylene signaling (An et al., 2010). Based on the microarray results, *EIN3* transcript increased in response to GmAGL15 at 0 and 3 dac and was also potentially expressed in response to AGL15 in Arabidopsis, as significantly decreased transcript abundance was observed for the *agl15 agl18* mutant compared with the wild type (Table I). A target of EIN3 is *ERF1*, which activates genes involved in the ethylene response, and this gene also showed increased transcript in *GmAGL15* or *AtAGL15* overexpressors (Table I). Constitutive expression of *ERF1* in Arabidopsis phenocopies ethylene overproduction (Solano et al., 1998). Based on ChIP-tiling array results from Arabidopsis, both *EIN3* and *ERF1* appear to be indirect targets of AGL15, with no significant peaks (assessed using CisGenome) indicating binding by AGL15 near these genes (data set from Zheng et al., 2009). A number of other genes regulated by (Gm)AGL15 are also involved in other hormone or stress signaling pathways besides ethylene. These include the DELLA proteins RGA and GAI, auxin response factors, and Myb domain proteins involved in ABA, jasmonate, auxin, salicylic acid, GA, and stress responses (Tables I and II).

Putative Orthologs of *MtSERF* Are Downstream Targets of AGL15 in Arabidopsis and Soybean

At5g61590, a member of the ERF family, was identified as a directly up-regulated downstream target of AGL15 using ChIP-chip and expression array analysis (Zheng et al., 2009). This result was confirmed here with qPCR enrichment tests and qRT-PCR to verify changes in transcript abundance in SAM SE and to extend findings to developing seeds. In addition, we have confirmed a CArG within the bound region as important to the regulation of this gene in response to AGL15. Expression of *At5g61590* is relevant to SE, with two loss-of-function alleles showing reduced SAM SE, and *35Spro*:*AGL15* required *At5g61590* to increase SAM SE significantly above the wild-type control (Fig. 2). Other targets of AGL15 are likely involved in explaining why SAM SE of *35Spro*:*AGL15 at5g61590-1* was not reduced to the level of *at5g61590-1*. For example, the directly expressed AGL15 targets *GA2OX6* and *INDOLE-3-ACETIC ACID INDUCIBLE30* (*IAA30*) have been found to be involved in SAM SE development (Wang et al., 2004; Zheng et al., 2009).

Two potential soybean orthologs of *MtSERF1*/*At5g61590* were found to be up-regulated during somatic embryo induction in response to GmAGL15. Transcript accumulation from these genes was also increased in response to ACC and decreased in response to an inhibitor of ethylene biosynthesis, consistent with other *ERF*s (e.g. *ERF1*; Solano et al., 1998). A binding site for AGL15 of the form preferentially bound in vitro (C-8-G) is directly upstream of the 5′ UTR and within the Arabidopsis DNA fragment isolated by ChIP was found to be important for the regulation of this gene. The soybean genes both have a C-8-G type CArG motif (binding site for MADS such as AGL15) within about 700 bp of the start codon, and regions including these motifs were associated with GmAGL15 in vivo (Fig. 3C). *MtSERF1* has been proposed to be regulated by WUS due to the presence of binding sites for WUS in regulatory regions as well as the involvement of these two gene products in SE (Mantiri et al., 2008a). Interestingly, ectopic expression of *WUS* appears to only produce SE in root tissue if the tissue is also treated with auxin, conditions under which *AGL15* is expressed (Gallois et al., 2004).

Two other AP2/ERF domain proteins have more obvious roles in SE: BBM (At5g17430; Boutilier et al., 2002) and EMBRYOMAKER (also AIL5, PLT5, CHO1; At5g57390; Tsuwamoto et al., 2010). However, these two genes are assigned to the AP2 family based on the presence of two AP2/ERF domains, and both show highest transcript accumulation in developing seeds (based on results summarized in The Bio-Array Resource for Plant Biology; Winter et al., 2007, using data from Schmid et al., 2005). *At5g61590* is assigned to the ERF family (Nakano et al., 2006) and shows transcript accumulation throughout Arabidopsis tissue types and developmental stages. During seed development, the transition from the heart stage to the torpedo-embryo stage, and into cotyledon stage and dry seed, has the highest levels of *At5g61590* transcript accumulation. The expression pattern in seed development is similar to *AGL15*, although *AGL15* transcript decreases as the seed matures (Perry et al., 1996). Using Genevestigator (Zimmermann et al., 2004), *At5g61590* was found to show increased expression in contexts where SE is promoted, such as in *LEC1* overexpression and uniconazol (a GA biosynthesis inhibitor) treatment of the *pkl* mutant (which produces embryos from the root and does so at a greater frequency with uniconazol treatment). Uniconazol results in the up-regulation of *At5g61590* in wild-type tissue. In fact, other information in Genevestigator indicates a responsiveness of *At5g61590* to GA, including increased transcript abundance in the *ga1-3* mutant (defective in GA biosynthesis), and in response to the induction of *rga-Δ17*, which represses GA signaling. Conversely, treatment of leaf discs with GA for 1 h led to a reduction of transcript from this gene. We have confirmed an inverse relationship between *At5g61590/GmSERF1* transcript and biologically active GA accumulation (Fig. 6).

Stress, Ethylene, and Somatic Embryogenesis

In soybean, a number of genes involved in ethylene biosynthesis and response were up-regulated in response to AGL15, while others were repressed. However, the net outcome of the complex gene regulation was increased ethylene production from tissue with a *35Spro*:*GmAGL15* transgene compared with nontransgenic tissue (Fig. 4). In both soybean and Arabidopsis, SE appears correlated with ethylene production/signaling.

Roles for ethylene in SE are not well understood, but at least in *M. truncatula* cultures (Mantiri et al., 2008b) and in this paper for Arabidopsis and soybean, additions to medium at the initiation of culture that increased ethylene resulted in enhanced somatic embryo development. However, contrary to the culture results reported by Mantiri et al. (2008b), another study assessing gene expression in the embryogenic genotype of *M. truncatula* (2HA) compared with the non-embryogenic cv Jemalong found genes involved in ethylene biosynthesis and response to be down-regulated in 2HA, including *EIN3* (Imin et al., 2008). An *ACO* was previously reported as up-regulated during SE, although transcript accumulation was higher on the abaxial side than the adaxial side that forms embryos (Thibaud-Nissen et al., 2003). In other systems, ethylene has been shown to have a role in cotyledon development of *B. napus* microspore embryos (Hays et al., 2000) and in the development and conversion of alfalfa somatic embryos (Kepczynska et al., 2009; Kepczynska and Zielinska, 2011). In some situations, a suppression of embryogenesis with ethylene is observed (for review, see Jiménez and Thomas, 2006), and a number of systems show enhanced SE by the addition of $AgNO_3$ to medium (for review, see Kumar et al., 2009). Thus, ethylene effects on SE vary with species and culture system.

Ethylene is also considered a stress hormone, and stress is a major factor in inducing SE. This stress can take different forms depending on the species, but temperature, nutrient, osmotic, and dehydration are some stress treatments used. Wounding and the 2,4-D commonly used in somatic embryo systems may also act as stressors. Ethylene is up-regulated in response to stress, including wounding and auxin (Karami and Saidi, 2010). Given the expression array results as a whole, it appears that AGL15 overexpression may lead to the activation of stress-responsive pathways, at least during the early stages of SE (Q. Zheng and S.E. Perry, unpublished data).

Hormone Interactions, Somatic Embryogenesis, and AGL15

Complicating the understanding of hormones and development is the extensive cross talk between hormones. A gene encoding a protein involved in GA catabolism was found to be a directly expressed target in Arabidopsis and soybean, and biologically active GA was inversely correlated with SE (Wang et al., 2004; Q. Zheng and S.E. Perry, unpublished data). AGL15 was found to directly express an *IAA* (*IAA30* in Arabidopsis) with effects on SE (Zheng et al., 2009). Furthermore, AGL15 appears to repress the expression of a gene encoding an auxin receptor (TIR1) in both Arabidopsis and soybean (Q. Zheng, Y. Zheng, and S.E. Perry, unpublished data). Auxin promotes GA biosynthesis/signaling by a couple of mechanisms. First, some auxin response factors induce the expression of GA biosynthetic genes and/or repress the expression of GA catabolic genes, leading to increased GA accumulation (Weiss and Ori, 2007a). *Aux/IAAs* are generally rapidly expressed in response to auxin, and AUX/IAA proteins inhibit gene regulation by auxin response factors until auxin leads to degradation of the AUX/IAA, allowing the regulation of downstream genes by the auxin response factor (Mockaitis and Estelle, 2008). Interestingly, Aux/IAAs also stabilize DELLA proteins that repress GA signaling until

GA perception leads to their proteolysis (Weiss and Ori, 2007b). If AGL15 is reducing the response to auxin by up-regulating *IAA30* (Zheng et al., 2009) and repressing *TIR1* (Q. Zheng, Y. Zheng, and S.E. Perry, unpublished data), it may further contribute to reduction in biologically active GA and the promotion of development in embryo mode. The GA-ABA ratio has been found to determine developmental mode, with low GA-ABA ratio promoting the embryo mode of development (Gazzarrini et al., 2004). Intriguingly, it has been reported that although auxin is a common inducer for SE, the cells that appear to be auxin resistant are those that form the embryos (Emons, 1994). AGL15 also directly controls genes encoding transcription factors involved in embryo development, such as FUS3 and ABI3 in Arabidopsis and soybean (Zheng et al., 2009; Q. Zheng and S.E. Perry, unpublished data), and these gene products also control hormone biosynthesis and response as well as being impacted by hormones.

Here, we report on AGL15 and ethylene biosynthesis and response. Ethylene and auxin impact each other's biosynthesis (Tang and Perry, 2003; Stepanova et al., 2008), and ethylene can act cooperatively or antagonistically with GA depending on the context (Weiss and Ori, 2007a, 2007b). Results in SE indicate a potential antagonist interaction. A mechanism of antagonistic interaction is the stabilization of DELLA proteins by ethylene via signaling through EIN3 and CTR1. Besides potential stabilization of the proteins, genes encoding the DELLA proteins RGA and GAI were up-regulated at some stages of SE induction (Tables I and II). Therefore, several mechanisms may contribute to decreased GA signaling in response to increased AGL15, including direct regulation of GA catabolic genes, and interactions via auxin and ethylene pathways as well as the regulation of other components of GA signaling. The addition of biologically active GA to medium reduced SE from both cv Jack and *35Spro*:*GmAGL15* (Q. Zheng and S.E. Perry, unpublished data). Further work will be needed to determine the mechanisms of interaction of these hormones in embryogenesis.

CONCLUSION

AGL15 directly regulates genes related to the *M. truncatula* SE gene *MtSERF1* in Arabidopsis and soybean. At5g61590 has a role in the promotion of SE as assessed using loss-of-function mutants. In soybean, two orthologs are expressed in response to GmAGL15 and in response to induction of SE in culture, and they may be directly controlled by GmAGL15. Furthermore, increased GmAGL15 leads to increased ethylene production that may, in part, be involved in the enhancement of SE by this protein. The SERF-related genes are induced by ethylene but repressed by GA, correlative with the effects of these hormones on SE in both Arabidopsis and soybean.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) Columbia (Col) wild type, insertional loss-of-function alleles *agl15-4* and *agl18-1*, the *agl15-4 agl18-1* double mutant (from Dr. D. Fernandez), *At5g61590-1* (SALK_015182), and *At5g61590-2* (SALK_009249; from the Arabidopsis Biological Resource Center) were grown on germination 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 5.6 to 5.8. The same conditions were used but with 50 μg mL^{-1} kanamycin for *35Spro*:*AGL15*. All seeds were chilled for 4 d at 4°C and transferred to a growth room with a 16-h-light/8-h-dark cycle. At about 10 d, seedlings were transferred to potting mix (ProMix BX; Premier Brands) and grown in a chamber with a 16-h-light (20°C)/8-h-dark (18°C) cycle. Dry seeds were collected for the SAM SE system described by Mordhorst et al. (1998). After 10 d of culture, seedlings were collected from the SAM SE system and flash frozen in liquid nitrogen for RNA extraction for qRT-PCR. No SAM SE development is observed at 10 d. After 21 d, the frequency of somatic embryos at the apex of the callused seedlings was determined. Developing seeds were staged by tagging flowers on the day that they opened, collecting seed at the appropriate time point, and flash freezing in liquid nitrogen for RNA extraction. Under our growth conditions, 5- to 6-d seed would contain heart to young torpedo stage embryos, 7- to 8-d seed would contain torpedo stage embryos, and 9- to 10-d seed would contain green bent-cotyledon embryos.

The Arabidopsis embryonic culture system has been described previously (Harding et al., 2003), and the tissues from this system were used for ChIP (Zheng et al., 2009). The embryo culture tissues were from developing zygotic embryos of two genotypes, *35Spro*:*AGL15* with or without a C-terminal TAP tag (Zheng et al., 2009).

For soybean (*Glycine max*), the identification of *GmAGL15* and the generation of the *35Spro*:*GmAGL15* construct were as described (Thakare et al., 2008), where we reported the transformation of cv Jack. Subsequent transformations of a related construct that included a 10× c-myc tag were performed using cv Jack and Williams 82 (obtained from the U.S. Department of Agriculture Soybean Germplasm Collection; http://www.ars-grin.gov/npgs/orders.html) as described previously. Transgenic and wild-type control soybeans were grown in a greenhouse at approximately 27°C with 15 h of light in a mixture of 2:2:1 soil:Promix:sand and fed weekly as described (Thakare et al., 2008). The cotyledon explant culture and scoring strategy were as described by Meurer et al. (2001). Tissue was frozen at appropriate time points for RNA extraction. Cultures were scored at 30, 45, and 60 dac. In the soybean system, the embryos first become apparent as green smooth globular tissue by 21 dac for nontransgenic tissue and at about 17 dac for *35S*:*GmAGL15* (line 8981). They may elongate or proliferate with time. The tissue that is best for proliferation for transformation resembles green clusters of grapes (rather than elongated green structures).

ChIP, Enrichment Test, qPCR, and qRT-PCR

The details of ChIP, the enrichment test, and qPCR have been described previously (Zheng et al., 2009). Modifications for ChIP in soybean are as described (Wang and Perry, 2013). RNA isolation, reverse transcription, qRT-PCR, and data analysis are as described (Q. Zheng and S.E. Perry, unpublished data). The specific primers for qPCR and qRT-PCR are listed in Supplemental Table S1.

Analysis of GUS Activity

The reporter gene construct (*pAt5g61590*:*GUS*) fused the *At5g61590* promoter to the reporter gene *GUS*. The sequence of the *At5g61590* promoter including the 5′ UTR is about 1,100 bp and was the intergenic region upstream of the *At5g61590* gene. The *At5g61590* promoter sequence was obtained from cetyltrimethylammonium bromide-isolated genomic Arabidopsis DNA by PCR amplification with the forward primer 5′-AAAAGCTTATCTAAACACTTCATATCTGCTAA-3′ and the reverse primer 5′-TTGGATCCTTCTTCCTCTCTCTTTTTTCTTTA-3′, incorporating *Hin*dIII and *Bam*HI restriction enzyme sites. PCR was performed in an MJ Thermocycler on 5 μL of genomic DNA added to a reaction containing 0.25 mM deoxyribonucleotide triphosphates, 0.5 μM of each oligonucleotide, 5 μL of 10× ExTaq buffer, 2.5 units of TaKaRa Taq, and 32 μL of sterilized distilled water (2 min at 95°C; followed by 30 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 1.5 min; and a final

72°C for 5 min). The PCR product was gel purified (Seaplaque GTG Agarose and Qiagen Mini Elute kit) and cloned into pGEM-T (Promega) for transformation and blue/white selection. Plasmid DNA and the transformation vector pBI101 (Clontech) were digested by *Hin*dIII and *Bam*HI sequentially, and the products were purified by the Qiagen kit and ligated. After confirmation in *Escherichia coli*, the construct was transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into Arabidopsis by using the floral dip transformation system (Clough and Bent, 1998).

For generation of the mutant CArG motif in the promoter region of At5g61590, the approach is the same as with the *pAt5g61590*:*GUS* construct except that the CArG motif was changed by the use of two oligonucleotides, 5′-GGATTCCTTTCTCTCTCTAGCCAACAATATCATC-3′ and 5′-GATGATATTGTTGGCTAGAGAGAGAAAGGAATCC-3′, and the QuickChange site-directed mutagenesis kit (Stratagene).

The confirmed homozygous transgenic lines with a single insert were crossed to *35Spro*:*AGL15* hemizygous plants. The F1 siblings were stained for GUS activity as described (Wang et al., 2002). After staining, seedlings with or without the *35Spro*:*AGL15* transgene were separated visually, with the *35Spro*:*AGL15* seedlings having reduced hypocotyl length and cotyledon epinasty (Fernandez et al., 2000).

Ethylene Measurements

For ethylene production determination, several 4- to 5-mm immature embryos were isolated from developing seeds, taking care to avoid any injury. The embryos were sealed in a 5-mL tube with an air-tight cork at room temperature for approximately 24 h, withdrawing 1 mL of head-space gas, and injecting it into a gas chromatograph (model 910; Buck Scientific) fitted with a flame ionization detector and an activated alumina column. The ethylene standard curve was created with different ethylene concentrations: 0.125, 0.25, 0.5, 1.0, and 1.5 μg mL^{-1}, with equation $y = 0.086x + 0.005$ and $r^2 = 0.9952$.

Supplemental Data

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

Supplemental Figure S1. Partial rescue of inhibition of soybean SE by AVG and simultaneous addition of ACC to the D40 medium.

Supplemental Table S1. Primers used in this study.

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