The MADS-Domain Transcriptional Regulator AGAMOUS-LIKE15 Promotes Somatic Embryo Development in Arabidopsis and Soybean[1][OA]

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The MADS-domain transcriptional regulator *AGAMOUS-LIKE15* (*AGL15*) has been reported to enhance somatic embryo development when constitutively expressed. Here we report that loss-of-function mutants of *AGL15*, alone or when combined with a loss-of-function mutant of a closely related family member, *AGL18*, show decreased ability to produce somatic embryos. If constitutive expression of orthologs of *AGL15* is able to enhance somatic embryo development in other species, thereby facilitating recovery of transgenic plants, then AGL15 may provide a valuable tool for crop improvement. To test this idea in soybean (*Glycine max*), a full-length cDNA encoding a putative ortholog of AGL15 was isolated from soybean somatic embryos. Subsequently, the corresponding genomic region of the gene was obtained. This gene, designated *GmAGL15*, encodes a protein with highest similarity to AGL15 from Arabidopsis (*Arabidopsis thaliana*) and *Brassica napus* that accumulates to its highest amount in embryos in these species. Like Arabidopsis and *Brassica AGL15*, *GmAGL15* was preferentially expressed in developing embryos. When ectopically overexpressed the soybean protein was able to enhance somatic embryo development in soybean.

Somatic embryo systems provide a more easily accessible tissue for studies on embryogenesis than does zygotic embryo development that occurs embedded within maternal layers and involves small numbers of cells at the early stages. Somatic embryogenesis (SE) is also important as a means to regenerate plants after transformation or to propagate commercially valuable genotypes. However, early events in somatic embryo development remain a mystery and the ability of an explant to form somatic embryos either directly or indirectly depends on many factors including in some cases particular genotype (Vogel, 2005; Rose and Nolan, 2006; Namasivayam, 2007). In most systems, treatment of the explant with auxin, usually the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), is necessary for induction of somatic embryo development (Jiménez, 2005; Vogel, 2005). Whether 2,4-D acts as a hormone or a stressor or both remains open to investigation. Often, but not always, the auxin level must be decreased to allow continued embryo development.

A number of genes have been identified in Arabidopsis (*Arabidopsis thaliana*) that when ectopically expressed promote somatic embryo development. These include the key embryo transcriptional regulators *LEAFY COTYLEDON1* (*LEC1*) and *LEC2* that when ectopically expressed will result in a fraction of seedlings that will then produce somatic embryo tissue without need for any hormone treatment. Likewise, ectopic expression of the B3 domain transcription factor *FUS3* in the L1 layer via the *ML1* promoter causes lateral organs to develop with embryonic features rather than as postgerminative vegetative tissue (Gazzarrini et al., 2004). LEC2 and FUS3 act at least in part by control of GA biosynthesis through regulation of *AtGA3ox2*, the product of which converts inactive GA to biologically active forms. Because LEC2 and FUS3 repress *AtGA3ox2*, less active GA is present, impacting on the GA-to-abscisic acid ratio that in turn determines whether an embryonic or adult leaf develops (Curaba et al., 2004; Gazzarrini et al., 2004; Lumba and McCourt, 2005). LEC1, LEC2, and FUS3 are essential for induction of somatic embryo development (Gaj et al., 2005).

The APETALA2 domain transcription factor *BABY-BOOM* can also promote SE when ectopically expressed (Boutilier et al., 2002) as can the homeodomain transcription factor *WUSCHEL* (*WUS*; Zuo et al., 2002). In some situations the effect of ectopic expression of *WUS* depends on other proteins or hormones: With LEAFY, floral development was induced whereas

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embryogenesis required increased auxin (Gallois et al., 2004). SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) enhances somatic embryo development from the shoot apical region of seedlings that are allowed to complete germination in liquid culture media (Hecht et al., 2001). Likewise, the MADS-domain protein AGAMOUS-LIKE15 (AGL15) promotes somatic embryo development in this system and enhances production of secondary embryonic tissue from cultured zygotic embryos (Harding et al., 2003). *AGL15* was initially identified using differential display of mRNA as an embryo expressed gene as well as during characterization of MADS-box genes in Arabidopsis (Heck et al., 1995; Rounsley et al., 1995). Although the gene is expressed and the protein accumulates to its highest level in developing embryos, *AGL15* is expressed in subsets of cells, generally at lower levels after the completion of germination (Heck et al., 1995; Rounsley et al., 1995; Perry et al., 1996; Fernandez et al., 2000). MADS-domain proteins are a family of transcriptional regulatory factors found in eukaryotic organisms. In plants, MADS-domain proteins are central players in many developmental processes, including control of flowering time, homeotic regulation of floral organogenesis, fruit development, and seed pigmentation (Parenicová et al., 2003, and refs. therein). Interestingly and perhaps relevant for SE, AGL15 has been identified as a component of a SERK1 protein complex (Karlova et al., 2006), and both *SERK1* and *AGL15*, as well as *FUS3*, are expressed in response to auxin treatment (Nolan et al., 2003; Gazzarrini et al., 2004; Zhu and Perry, 2005). Also intriguing are recent results that indicate that LEC2 may directly induce expression of *AGL15* (Braybrook et al., 2006). Like LEC2 and FUS3, AGL15 impacts upon bioactive GA accumulation, but AGL15 mediates its effect at least in part by directly inducing expression of *AtGA2ox6* (At1g02400) that encodes a GA 2-oxidase that catabolizes biologically active GA (Wang et al., 2004). Expression of this GA 2-oxidase affects somatic embryo development from the shoot apical meristem (SAM) of liquid culture grown seedlings in the presence of 2,4-D (Wang et al., 2004). We report here that loss-of-function alleles of *AGL15*, in some cases when combined with loss of function of *AGL18*, a closely related family member that is redundant to *AGL15* in control of flowering time (Adamczyk et al., 2007), significantly impairs the ability to form somatic embryos in two systems in Arabidopsis.

Although immunoreactive proteins could be detected using AGL15-specific antibodies in a variety of embryos or embryonic tissues from angiosperms (Heck et al., 1995; Perry et al., 1996, 1999; S.E. Perry, unpublished data), little is known about the molecular nature of *AGL15* orthologs in higher plants other than Arabidopsis and *Brassica napus*. We report on a putative ortholog of AGL15 from soybean (*Glycine max*) and document that, as found in Arabidopsis, ectopic expression of *GmAGL15* can enhance SE.

RESULTS

Loss of Function of AGL15 Leads to Decreased Frequency of Somatic Embryo Development

We previously reported that when developing zygotic embryos are removed from the seed at the green bent cotyledon stage and placed into culture on media lacking any exogenous hormones, embryonic foci will appear on the embryos within 2 to 3 weeks (Harding et al., 2003). Embryos from plants carrying a *35S*:*AGL15* transgene produce secondary embryo tissue at higher frequency than wild type (approximately 40% of the cultured *35S*:*AGL15* embryos compared to approximately 18% for Wassilewskija [Ws] wild type), and when embryonic foci are subcultured, *35S*:*AGL15* tissue continues development in embryonic mode for extended periods of time (over 11 years to date), whereas wild-type tissue does not maintain embryonic development. Furthermore, a *35S*:*MIK* transgene that is predicted to function as a dominant negative shows reduced ability to produce secondary embryonic tissue compared to wild type (Harding et al., 2003).

We have now examined effects of insertional mutations into *AGL15* on production of embryonic tissue in this system. Insertional alleles *agl15-3* and *agl15-4* are in the Columbia (Col) ecotype and were compared to wild-type Col and *35S*:*AGL15* introduced into Col for production of secondary embryonic tissue from cultured zygotic embryos. We found that Col wild type was much more efficient at production of secondary embryo tissue than was Ws wild type. For wild-type Col, 47% of cultured embryos had secondary embryonic foci present at 3 weeks of culture (Fig. 1A) compared to 18.2% of Ws as reported by Harding et al. (2003). These numbers represent averages from experiments performed over a number of years and not at the same time (before 2003 for Ws and 2005–2006 for Col). To examine ecotype effect directly, one experiment compared the two ecotypes at the same time and we found that in this particular experiment 50% of wild-type Col produced secondary embryonic tissue compared to only 22% for Ws (data not shown). The fraction of Ws embryos developing secondary foci in this experiment agrees well with that reported in 2003.

Both insertional alleles into *AGL15*, *agl15-3* and *agl15-4*, showed a decreased ability to produce secondary embryo tissue compared to Col wild type that was significant at $P < 0.01$ (Fig. 1A). Production of secondary embryos by the two insertional alleles was not different from one another. An insertional allele in the Ws ecotype, *agl15-2*, was also significantly impaired in production of secondary embryonic tissue (12% of cultured embryos produced secondary embryonic tissue compared to 22% for Ws wild type; this difference is significant at $P < 0.05$, data not shown). Unlike in the Ws ecotype where introduction of a *35S*:*AGL15* transgene significantly enhanced production of embryonic tissue (Harding et al., 2003), the *35S*:*AGL15* transgene in Col did not increase the percentage of

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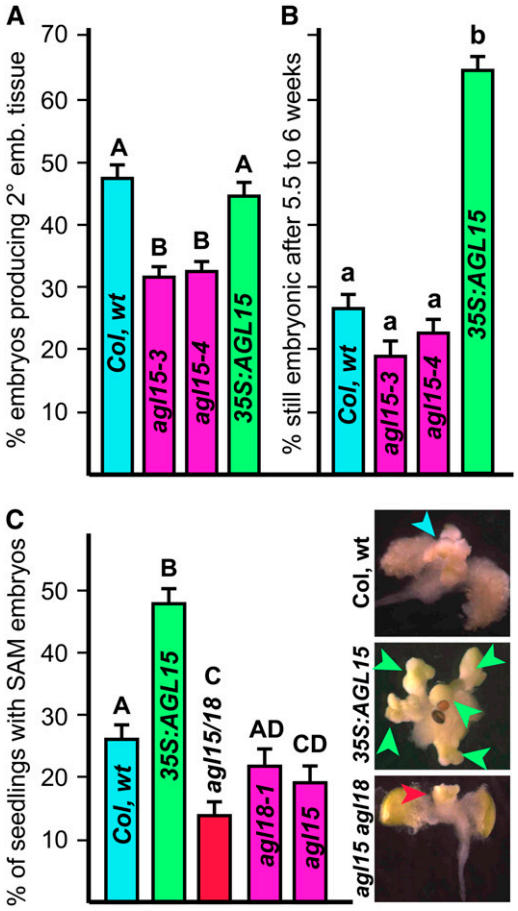

Figure 1. Effect of AGL15/AGL18 accumulation on somatic embryo tissue production. A, Percentage of cultured zygotic embryos of the indicated genotypes that produce secondary embryonic tissue. Results shown are the means and SE of the mean derived from 15 to 16 plates with 81 to 140 embryos per plate (with the exception of *agl15-4* for which there were only six plates). Different letters indicate significance of $P < 0.01$. wt, Wild type. B, Percentage of embryonic foci of the indicated genotypes that were subcultured at approximately 3 weeks and continued to produce embryonic tissue approximately 2.5 to 3 weeks after subculture. Mean and SE of the mean are shown. Different letters indicate significance at $P < 0.01$. C, Percentage of seedlings that showed somatic embryo development from the SAM when seeds of the indicated genotypes were allowed to compete germination in liquid media containing 2,4-D. Mean and SE of the mean are shown. Different letters indicate significance at $P < 0.05$. Images are representative seedlings of the indicated genotypes that have SAM somatic embryo development.

cultured embryos with secondary embryonic foci (Fig. 1A). However, as previously reported in Ws, when embryonic foci are subcultured, the *35S:AGL15* transgene significantly enhances maintenance of embryonic development with 26% of wild type developing embryonic foci continuing development in embryonic mode at 3 weeks after initial subculture (5–6 weeks total in culture) compared to 64% of *35S:AGL15* in the Col background (Fig. 1B). The *agl15* mutant tissue was not significantly different at $P <$ 0.01 than wild type in this regard with 22% of the foci that initiated this development continuing production of embryonic tissue at 3 weeks after the first subculture. *35S:AGL15* in Col has now continuously produced embryonic tissue for over 1 year to date whereas the *35S:AGL15* in Ws that we reported on in 2003 has continued development in embryonic mode for over 11 years.

We also previously reported that a *35S:AGL15* transgene enhanced production of somatic embryo tissue from SAMs of seedlings in liquid culture (Harding et al., 2003). When seeds are allowed to complete germination in liquid media containing the synthetic auxin 2,4-D, the shoot apical region can produce somatic embryo tissue and mutants in which the SAM is enlarged are more efficient at producing this tissue (Mordhorst et al., 1998). Because a very young SAM is one place where nuclear accumulation of AGL15 can be detected, but this detectable accumulation is transient (Fernandez et al., 2000; Harding et al., 2003), we tested whether accumulation of AGL15, in terms of level or persistence, would impact on the ability to form somatic embryos. Previous work demonstrated that a *35S:AGL15* transgene in the Ws ecotype of Arabidopsis or in the *amp1* mutant that has an enlarged meristem increased the fraction of seedlings with somatic embryo tissue compared to the wild-type/*amp1* background alone (Harding et al., 2003). However, because neither Ws nor *amp1* efficiently produced somatic embryos in our hands, we were unable to test loss of function.

Because many of the insertional mutants are in the Col ecotype, including two of the *agl15* alleles, we tested the effect of accumulation of AGL15 on SAM SE in the Col ecotype. As shown in Figure 1C, for experiments performed with several seed lots and over a 2-year period, an average of 26% of Col wild-type seedlings had somatic embryo tissue at the apex in this system (7,427 seedlings total, $n = 29$). While over all experiments, *agl15-3* and *agl15-4* (5,114 seedlings total scored, $n = 19$) did show a significant reduction in production of SAM somatic embryos at $P < 0.05$ with 19% of seedlings showing this development, whether the difference was significant or not in individual experiments varied and the difference was not significant at $P < 0.01$. Because *AGL18* is the closest family member to *AGL15* and is expressed in overlapping developmental context (Lehti-Shiu et al., 2005), we tested an *agl15 agl18* double mutant in this system. While *agl18-1* did not significantly decrease production of SAM somatic embryos (2,162 seedlings scored, $n = 13$), the double mutant showed significant reduction compared to wild type (14% of *agl15-3 agl18-1* and *agl15-4 agl18-1* seedlings had SAM somatic embryo tissue, 4,916 seedlings, $n = 27$; this is significant at $P < 0.01$; Fig. 1C). Conversely, prolonged or increased accumulation of AGL15 as provided by the *35S:AGL15* transgene increased the fraction of seedlings with SAM somatic embryo development (48% of 2,666 seedlings scored, $n = 19$; Fig. 1C). Not only did

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an increased fraction of seedlings have somatic embryo tissue, but the extent of somatic embryo tissue development was also on average greater than found for Col wild type (inset images in Fig. 1C).

Isolation of an Ortholog of AGL15 from Soybean

Would ectopic expression of *AGL15* enhance SE in other species, perhaps providing a tool to facilitate recovery of transgenic plants via SE? To address this question, we cloned a soybean MADS-box gene from somatic embryo tissue. Isolation of sequences encoding *GmAGL15* was initiated by searching the GenBank EST database for possible candidates. One entry (accession no. AW756465) was found annotated as ''similar to…AGL15.'' BLASTX program (http://www.ncbi.nlm.nih.gov/blast) confirmed this EST represented the N-terminal 37 amino acid residues of the conserved MADS domain. To obtain the full-length cDNA, RNA was isolated from a soybean somatic embryo culture (Reddy et al., 2001). Oligonucleotide primers were designed based on the EST sequence, and 3′-RACE PCR was performed as described by Ausubel et al. (1998). After sequencing this partial cDNA, additional primers were designed for 5′-RACE PCR to recover the full-length cDNA. This gene, designated *GmAGL15*, had highest sequence similarity to the previously published *AGL15*s.

The longest cDNA clone (GenBank accession no. AY370659) consisted of an approximately 270-bp 5′-untranslated region (UTR), an approximately 260-bp 3′-UTR plus polyA tail, and an open reading frame of 708 bp, which encodes a protein of 235 amino acid residues. A BLASTP search was performed using the protein sequence. The highest scoring matches were to Arabidopsis AGL15 (AtAGL15), *Brassica* type I and type II AGL15 (BnAGL15-1 and -2; accession nos. NP_196883, Q39295, and AAB03807, respectively), and to proteins from cotton (*Gossypium hirsutum*), petunia (*Petunia hybrida*), and grape (*Vitis vinifera*) that when used to search the Arabidopsis AGI protein database had highest similarity to AGL15. Pairwise comparison of GmAGL15 protein with the other three AGL15s revealed an identity of approximately 50%. Multiple sequence alignment showed a moderate conservation among these proteins (Fig. 2A). This was not unexpected, because even between Arabidopsis and *B. napus*, two closely related species, considerable divergence in AGL15 exists. Nevertheless, the soybean sequence displayed overall homology to the AGL15 proteins, including divergent domains outside the conserved MADS domain. In addition, the soybean protein contained several signature sequences that are rarely found in MADS-domain proteins other than AGL15, such as the C-terminal LENETLRRQI/VE/QELR and LGLP motifs. Therefore, it is likely that GmAGL15 is the soybean ortholog of AGL15.

The 5.8-kb genomic region of *GmAGL15* (accession no. AY370660) was amplified from 'Jack' soybean genomic DNA, using primers corresponding to the UTRs of the cDNA. An alignment of the genomic and cDNA sequences revealed that *GmAGL15* contained eight exons and seven introns (Fig. 2B). The introns were longer than found in Arabidopsis. Nevertheless, the exon-intron boundary locations appeared to be identical between the two species, as often observed among evolutionarily conserved orthologs. This further suggested *GmAGL15* was the soybean counterpart of *AGL15*.

A phylogenetic tree was constructed using protein sequences of GmAGL15, BnAGL15s, and all 39 Arabidopsis MIKC-type MADS-domain proteins (Parenicová et al., 2003). GmAGL15 was grouped more closely to the AGL15s (Fig. 2C).

Expression of *GmAGL15* in Soybean

To investigate the expression pattern of *GmAGL15*, semiquantitative reverse transcription (RT)-PCR was performed on RNAs isolated from various tissues. As shown in Figure 3, *GmAGL15* transcript was not detected in the vegetative tissues (leaves, stems), or in the open flowers. *GmAGL15* mRNA was not detectable at very early stages of seed pod development, but was present in young developing embryos, and the level declined after maturation. This pattern was consistent with that previously reported for *AGL15* from Arabidopsis and *Brassica* (Heck et al., 1995; Rounsley et al., 1995). Notably, the highest level of *GmAGL15* mRNA accumulation was detected in the somatic embryo culture. AGL15-specific antiserum (Perry et al., 1999) detected immunoreactive protein in nuclear extracts prepared from soybean somatic embryos (data not shown). Additionally, reaction of the antiserum against the product of the cloned *GmAGL15* was tested by expressing the soybean gene in *Escherichia coli*. The AGL15-specific antiserum recognized the *E. coli* produced protein (Fig. 5B).

Ectopic Expression of *GmAGL15* Enhances SE in Soybean

To test whether ectopic expression of *AGL15* via the *35S* promoter would enhance SE in soybean, biolistic transformation of 'Jack' soybean somatic embryo tissue was performed with *35S*:*gGmAGL15* (includes introns), *35S*:*cGmAGL15* (lacks introns), *35S*:*AtAGL15* (Arabidopsis AGL15 containing the first three introns), or empty vector control. After recovery for 7 d, the bombarded tissue was cultured for 10 weeks in selective media that contained hygromycin, changing media at 7- to 8-d intervals. After 10 weeks, putative transformants were identified by their green color and characteristic shape and subcultured individually in six-well plates (schematic showing steps involved is shown in Fig. 4A). The number of putative transformants per bombardment was scored and is shown in Figure 4B. Transformation with either the *35S*:*gGmAGL15* and *35S*:*cGmAGL15* transgenes showed enhanced recovery of putative transformants compared to the

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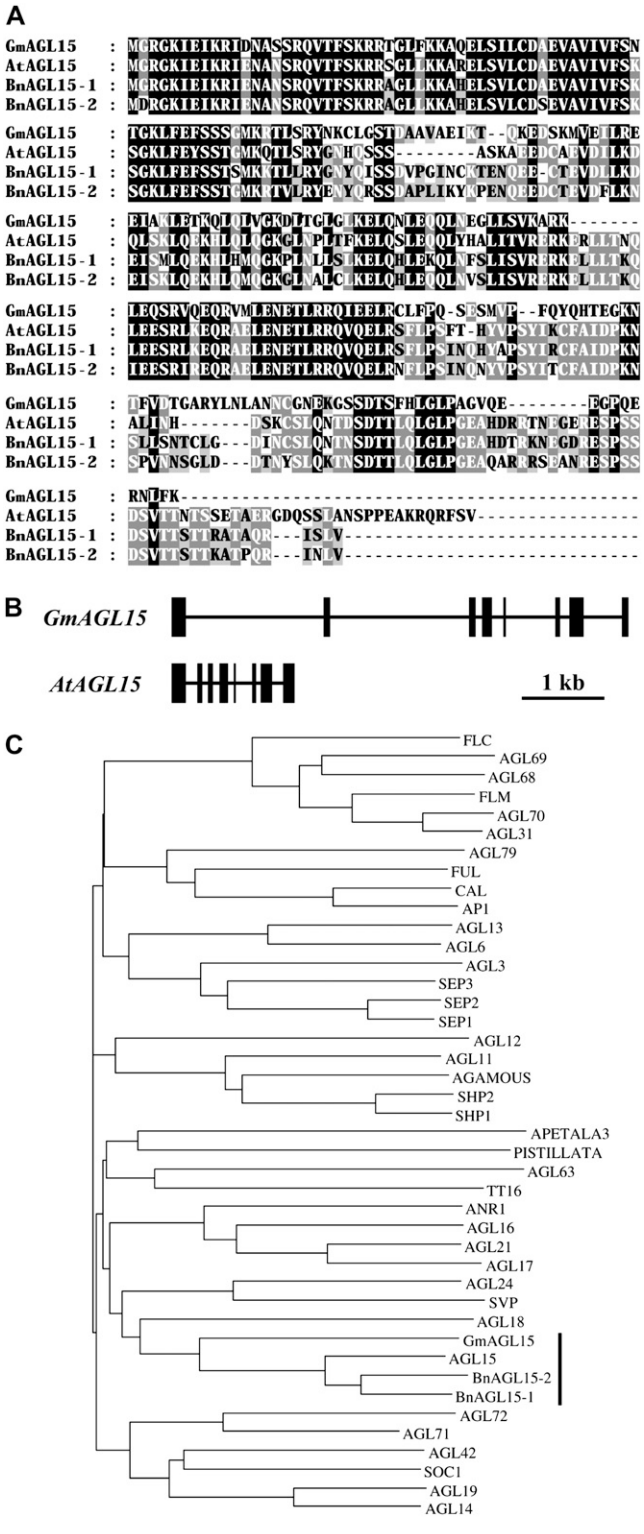

Figure 2. A, GeneDoc (www.pac.edu/biomed/genedoc) sequence alignment between GmAGL15, AtAGL15, BnAGL15-1, and BnAGL15-2. The shade levels represent conservation degrees. B, Schematic representation of the gene structures of *GmAGL15* and *AtAGL15*. The numbers and positions of the introns/exons were conserved between these two species. Boxes, exons; lines, introns. C, A phylogenetic tree generated from GmAGL15, BnAGL15s, and all 39 MIKC-type Arabidopsis MADS-domain proteins. Neighbor-joining method with a bootstrap number of 1,000 was used (ClustalX 1.81, http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX). AGL15 group is indicated.

empty vector control (16.6 and 14.0 putative transformants moved to individual subculture for the genomic and for the cDNA transformations, respectively, compared to 8.0 putative transformants for the empty vector). Transformation with a *35S*:*AtAGL15* transgene did not significantly increase the number of potential transformants recovered at 10 weeks compared to the empty vector control (Fig. 4B).

Constitutive expression of *GmAGL15* also impacted on survival in culture. After individual transformants were subcultured in six-well plates, they were allowed to continue development for an additional 2.5 months under selection. Some putative transformants will proliferate, whereas others die during this period. Individual putative transformants were scored for survival after approximately 2.5 months. For the empty vector control, 21% survived, compared to 33% for *35S*:*cGmAGL15*, 26% for *35S*:*gGmAGL15*, and 25% for *35S*:*AtAGL15*. Promotion of SE and survival in culture thus resulted in a net of 1.7 transformants per bombardment progressing to maturation media for the empty vector control compared to 4.6 for *35S*:*cGmAGL15*, 4.3 for *35S*:*gGmAGL15*, and 2.1 for *35S*:*AtAGL15*.

Regeneration of Transgenic Soybean Plants

35S:*AGL15* in Arabidopsis cultured embryos leads to long-term (over 11 years) maintenance of development as embryo tissue (Harding et al., 2003; data not shown). Although no attempt was made in Arabidopsis to switch the tissue from embryonic to postgerminative development, a concern for use of orthologs of AGL15 in other species such as soybean may be that overexpression of *AGL15* via a constitutive promoter would not allow for maturation and plantlet development of the transgenic somatic embryos. However, Arabidopsis seed containing *35S*:*AGL15* undergoes maturation, desiccation, and can complete germination to give a seedling.

Although *35S*:*GmAGL15* transgenes enhanced recovery of putative transformants, perhaps by promoting somatic embryo production, the somatic embryos obtained in soybean underwent maturation and differentiation, and produced plantlets that were transplanted to soil. The plants continued development and appeared relatively normal considering the culture process. Of 17 separate events that were recovered as plantlets and subsequently tested, 88% were verified as containing the transgene by PCR. We tested a number of plants for expression and verified transcript accumulation from the transgenes by RT-PCR (using a *GmAGL15* forward primer and an oligonucleotide primer to the C-terminal c-myc tag as a reverse primer). As shown in Figure 5A, transcript from the transgene was detected in young leaf tissue

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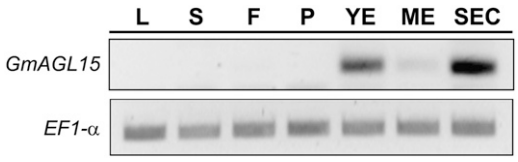

Figure 3. Expression pattern of *GmAGL15*. Semiquantitative RT-PCR was performed on RNA derived from various tissues of 'Jack' soybean. The coding region of *EF1-α* was amplified for normalization. L, Young leaves; S, stems; F, open flowers; P, seed pods containing very young embryos; YE, young embryos (average length 2 mm); ME, mature green embryos; SEC, somatic embryo culture. The color of the image is inverted for clarity.

of potential transgenics but not in untransformed 'Jack' soybean or in the empty vector transgenics.

To determine if transgenic plants accumulated AGL15, nuclei were isolated from open flowers, extract prepared and analyzed by SDS-PAGE, transfer to membrane and probing with anti-AGL15 serum. As shown in Figure 5B, the different transgenics exhibited a range of AGL15 accumulation from no detectable accumulation from the transgene (data not shown), low levels of accumulation (gGm, 8983-21A), to high levels of accumulation (cGm, 8981-17C; and gGm, 8984-9A). The slightly lower band in 'Jack' and in the empty vector control likely represents endogenous AGL15 that is expressed, at least in Arabidopsis, at low levels in flowers (Fernandez et al., 2000).

DISCUSSION

Previous work demonstrated that ectopic expression of *AGL15* could enhance production of somatic embryos from cultured zygotic embryos and from the SAMs of seeds that complete germination in liquid media that contains 2,4-D (Harding et al., 2003). However, at the time, no loss of function of *AGL15* was available. In lieu of a knockout allele of *AGL15*, a form of AGL15 missing the C-terminal domain (*35S:MIK*) and predicted to function as a dominant negative was tested. Although a decrease in production of secondary embryos from cultured zygotic embryos was observed, it was not possible to judge the effect on the SAM because wild-type seedlings of the Arabidopsis ecotype Ws did not produce somatic embryos efficiently in this system (Harding et al., 2003).

The Arabidopsis ecotype Col was more efficient at somatic embryo production than was Ws in both the cultured embryo and the SAM somatic embryo systems. Other work has demonstrated differences between ecotypes in the ability to produce somatic embryos. Specifically, Luo and Koop (1997) found that while Col was moderately efficient at producing somatic embryos from zygotic embryos cultured in liquid media with 2,4-D, Ws was the one ecotype tested that did not form somatic embryos. Ecotype effects on stress-induced SE have also been reported (Ikeda-Iwai et al., 2003). The increased competence for SE in wild-type Col, combined with the fact that *agl15* and *agl18* insertional alleles in Col were available (Alonso et al., 2003), allowed us to test the loss of function of these proteins on somatic embryo production.

Two different loss-of-function alleles of *AGL15* in the Col ecotype significantly decreased secondary embryo production when zygotic embryos were cultured on media lacking exogenous hormones (Fig. 1A). A loss-of-function allele of *AGL15* (*agl15-2*) in the Ws ecotype also significantly decreased the fraction of cultured zygotic embryos producing secondary embryo tissue in this system (data not shown). Although the double mutant of *agl15* with the closest related family member *agl18* (Col) was not tested in this system, the *agl18* single mutant alone did not show a decrease in fraction of cultured zygotic embryos that produce secondary embryo tissue compared to wild type (data not shown). However, expression of a transgene that produces a form of AGL15 that is predicted to function as a dominant negative (*35S:MIK*), and could interfere with not only AGL15 but other MADS or proteins that

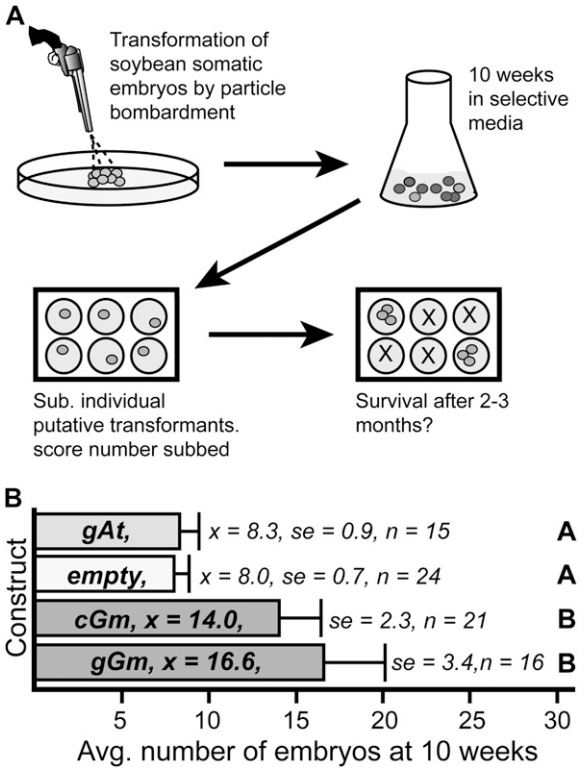

Figure 4. Effects of overexpression of *AGL15* on SE in soybean. A, Schematic of the transformation and scoring scheme for soybean. Please see "Materials and Methods" for further details. B, Transformation of soybean with soybean genomic (gGm) or cDNA (cGm) versions of *GmAGL15* driven by the *35S* cauliflower mosaic virus promoter produced more potential transformants that were individually subcultured than did the empty vector control. However, expression of Arabidopsis *AGL15* did not result in an increase in potential transformants. Different letters indicate significant difference at $P < 0.05$.

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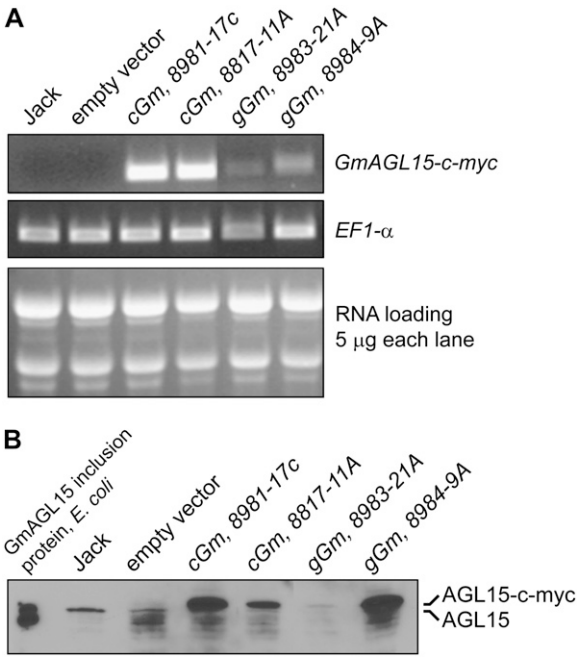

Figure 5. Evaluation of expression of *35S:AGL15* in soybean. A, RT-PCR was performed using a *GmAGL15* forward primer and a *c-myc* reverse primer on RNA extracted from young leaves. RT-PCR with *EF1-α* was performed as a normalization control. Total RNA was also analyzed on an agarose gel to evaluate integrity. B, Protein gel-blot analysis to evaluate accumulation of AGL15 in transgenic soybean. Protein extracts were prepared from flowers of the indicated genotypes and proteins were separated on a polyacrylamide gel, blotted to membrane, and probed using anti-AGL15 serum. Inclusion protein containing GmAGL15 (without a c-myc tag) was obtained by expression in *E. coli* and used as a positive control. All lanes within a section are from the same gel or blot.

interact with MADS factors, had a more severe effect on production of secondary embryos than did the *agl15-2* mutant in the Ws background (2.4% compared to 12%; Harding et al., 2003; data not shown). This may indicate redundancy of AGL15 with other factors, perhaps including AGL18. Recent work has indicated that AGL15 can bind not only to its own regulatory regions but also to regulatory regions of *AGL18*, and can repress *AGL18* expression (Hill et al., 2008). Therefore, although the single *agl18* mutant did not decrease somatic embryo formation, it may still have an effect when tested as a higher order mutant with *agl15*. The *35S:AGL15* transgene did not cause an increase in the fraction of cultured embryos that initially produce secondary embryo tissue in the Col ecotype compared to wild-type Col, as previously reported for Ws (Fig. 1A; Harding et al., 2003). However, the *35S:AGL15* transgene still promoted continued development as embryo tissue for extended time periods in Col as previously found for Ws (Fig. 1B; Harding et al., 2003). It would be interesting to determine whether there are differences in accumulation of AGL15 in later embryo development in these two ecotypes to perhaps explain the differences observed during the early culture period.

There exist systems by which Arabidopsis cultured embryos are used to generate somatic embryo tissue in the presence of 2,4-D and sometimes other hormones (Wu et al., 1992; Pillon et al., 1996; Luo and Koop, 1997; Mordhorst et al., 1998; Ikeda-Iwai et al., 2002). However, *35S:AGL15* embryos can produce secondary embryo tissue in the absence of any exogenous hormone and then maintain development as embryo tissue for very extended time periods (Harding et al., 2003; this study). Interestingly, expression of *AGL15* is induced by auxin, both 2,4-D and indole-3-acetic acid (Zhu and Perry, 2005). *AGL18* is also induced somewhat by auxin treatment (S.E. Perry, unpublished data).

In the SAM SE system, where seeds are allowed to complete germination in liquid media with 2,4-D, the single *agl15* mutant alleles did not show a completely consistent significant reduction in production of SAM somatic embryos for every experiment. However, in some experiments there was such a reduction and when the data collected over more than a 2-year period is considered as a whole, the single *agl15* mutants are significantly different from wild type in production of SAM somatic embryos. The single *agl18* mutant showed some reduction in frequency of SAM SE compared to wild type but this was not significant. Data as a whole shows significant reduction of SAM SE for the *agl15 agl18* double mutant compared to wild type at $P < 0.01$, and in almost all cases individual experiments showed a significant reduction. It is interesting to note that often the tips of the cotyledons for the double mutant remained green and uncallused (Fig. 1C). This was also observed in the Ws ecotype containing the dominant negative construct *35S:MIK* (Harding et al., 2003). This could reflect an altered response to auxin. Because the SAM SE system, unlike the cultured embryo system, requires auxin treatment for embryo formation, other factors responsive to auxin must be involved.

To expand our work to plants that are agriculturally relevant, we cloned a gene and genomic region encoding a potential AGL15 ortholog from soybean. Sequence similarity, gene structure, and expression pattern support *GmAGL15* as the likely soybean ortholog to previously reported *AGL15*s from Arabidopsis and *B. napus*. In addition, ectopic expression of either the cDNA or the intron-containing version of *GmAGL15* via the *35S* promoter enhanced recovery of transformants via SE. In the Arabidopsis Ws ecotype, ectopic expression of *AGL15* promoted the initial production of secondary foci and then enhanced continued development in this mode. Similarly, in soybean, more potential transformants were recovered at 10 weeks, and then, at least for the cDNA version of the transgene, there was a significant enhancement in survival after subculturing the individual putative transformants. However, it was possible to switch the tissue out of embryogenic mode and recover plantlets. Although we have not attempted purposefully to switch the Arabidopsis embryogenic culture tissue that has been developing in embryonic mode for

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over 11 years, into a postembryonic state, it is interesting that we have observed increased leaf-like development when plates are not subcultured for lengthy times and begin to dry (S.E. Perry, unpublished data). The recovered transgenic soybean plants constitutively expressing *AGL15* appear relatively normal, with some plants showing perhaps increased branching or darker green color combined with reduced seed set and some meristem abnormalities. All of these potential phenotypes would be consistent with phenotypes observed with ectopic expression of *AGL15* in Arabidopsis (Fernandez et al., 2000; Wang et al., 2004). However, because the plants thus far are from tissue culture, it is premature to make any conclusions about effects of the transgene on plant morphology or development at this time. Transgenic plants were found to accumulate AGL15 and the slightly higher mass compared to endogenous GmAGL15 is due to a c-myc tag on the carboxyl-terminal end of the transgenic form (Fig. 5B). It is interesting to note that when AGL15-c-myc accumulates, there may be a reduction in the endogenous AGL15. This could indicate that AGL15 may negatively autoregulate its own expression in soybean as has been reported in Arabidopsis (Zhu and Perry, 2005).

GmAGL15, like Arabidopsis AGL15, appears to promote development as embryonic tissue and may be a valuable tool for recovery of transgenics in this and other species recalcitrant to regeneration by SE. However, ectopic expression of *AtAGL15* did not enhance embryogenesis in soybean. The *35S*:*GmAGL15* constructs were also stably transformed into Arabidopsis and the transgenic plants recovered did not show phenotypes typical of overexpression of *AtAGL15* in this plant (i.e. lack of abscission and senescence of floral organs after fertilization; Fernandez et al., 2000). Although it is possible that there may be differences in protein accumulation in the heterologous system, codon usage in Arabidopsis and soybean is similar (Wang and Roossinck, 2006). Recently we found that a LxLxL motif that is conserved between Arabidopsis and *B. napus* AGL15s and that is also found in a number of potential orthologs of AGL15 identified in other species, acts to repress gene expression and the mechanism by which this occurs may be via recruitment of SAP18 (for SIN3-associated polypeptide of 18 kD) and subsequently histone deacetylase components (Hill et al., 2008). Intriguingly, the first Leu in this motif is replaced by a Phe in GmAGL15. Perhaps differences in protein-protein interactions may be responsible for the lack of effect of expression of *AGL15* in a heterologous system. An EST encoding a probable SAP18 ortholog is in the EST database (EV274062) that is 76% identical and 87% similar to AtSAP18 (At2g45640). It will be intriguing to test interactions between AGL15 and SAP18 between and within species.

The experiments performed in this report utilized the cultivar 'Jack' that has been reported as highly embryogenic as well as uniformly responsive to induction of SE across diverse locations (Meurer et al., 2001). It would be interesting to determine if transformation of a less consistently embryogenic cultivar such as 'Stonewall' or 'Defiance' would enhance subsequent SE in these cultivars, thereby facilitating transformation with other constructs. If so, this could address a priority outlined in the Soybean Genomic Research Strategic Plan for 2008 to 2012 to expand the number of genotypes that can undergo SE (Specht et al., 2007).

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) ecotypes Ws and Col wild type, loss-of-function mutants with insertional alleles (*agl15-2* in Ws; *agl15-3*, *agl15-4*, and *agl18-1* in Col), and transgenic plants were sown on germination medium (GM; Murashige and Skoog, 1962) with 50 μg mL^{-1} kanamycin for transgenic seed, chilled for 2 to 3 d at 4°C, and transferred to a growth room with a 23°C to 24°C, 16-h-light/8-h-dark regime. The loss-of-function insertional mutants are described by Lehti-Shiu et al. (2005). After 7 to 10 d, seedlings were transplanted to potting mix (ProMix BX; Premier Brands, Inc.) and grown under a 16-h-light (20°C)/8-h-dark (18°C) cycle in a growth chamber. To stage siliques, flowers were tagged on the day that they opened.

Soybean (*Glycine max* Merr. 'Jack') plants were grown in greenhouse at a temperature of approximately 27°C and 15-h-light regime as described by Parrott et al. (1989) in a steam-sterilized mixture of 2:2:1 of soil:Promix:sand and fed weekly with Peters 20:20:20 (The Scotts Company).

Arabidopsis Somatic Embryo Systems

The embryo culture system has been described previously (Harding et al., 2003). Briefly, developing embryos were removed at 10 to 12 d after flowering (green bent cotyledon stage) and placed into culture on GM. After about 3 weeks, embryos were scored for presence of secondary embryo tissue that was then subcultured on GM and scored again after an additional 2.5 to 3 weeks for continued development in embryo mode.

The SAM somatic embryo system is as described by Mordhorst et al. (1998). Briefly, seed was surface sterilized as done by Harding et al. (2003), chilled for 2 d at 4°C, and put into liquid culture media containing 2,4-D (Mordhorst et al., 1998). Cultures were incubated at 23°C to 24°C on a rotary shaker under a 23-h-light/1-h-dark regime. Cultures were scored at 21 d.

Plasmid Constructs

Two different versions of the cDNA and of the genomic *GmAGL15* constructs were generated. One version was untagged, while the other contained a c-myc tag at the carboxyl-terminal end. For the untagged versions, the coding region lacking (cDNA) or containing introns (gDNA) were inserted downstream of the cauliflower mosaic virus *35S* promoter in the pBIMC vector (gift from Dr. D. Falcone, University of Massachusetts Lowell). A nopaline synthase (*Nos*) terminator was present after the stop codon. The *35S-AGL15-Nos* expression cassettes were subsequently cloned into pCambia 1301 that also constitutively expresses a *GUS* gene and confers hygromycin B resistance.

To add a c-myc tag onto the C-terminal end of cGm and gGmAGL15, the respective forms of *AGL15* were PCR amplified from the original pCambia 1301-based plasmid constructs, using oligonucleotides that introduced restriction sites *Spe*I at the 5′ end and *Sac*I at the 3′ end. The reverse 3′-oligonucleotide corresponded to the sequence before the stop codon and engineered in sequences encoding a c-myc epitope. The sequence of the forward and reverse oligonucleotides was 5′-GACTAGTCCATGGGTCGAGGGAAAATCGAG-3′ and 5′-GAGCTC**TCA***CAGGTCCTCCTCTGAGATCAGCTTCTGCTCCTC*TTTGAAAAGGTTTCTTTCTTGGGGGCC-3′, respectively. The *Spe*I and *Nco*I (forward) and *Sac*I (reverse) sites are underlined, the sequence encoding the c-myc epitope is in italics, and the engineered stop codon is in bold. For amplification, high-fidelity Taq polymerase (*Ex Taq*;

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Takara Bio Inc.) was used following the manufacturer's instructions. The PCR-amplified fragments were gel purified and cloned into the pGEM-T Easy vector system (Promega Corporation) for sequence verification. For cloning into a binary vector system, the *Spe*I/*Sac*I fragment was recovered from pGEM-T and cloned into pBIMC such that the AGL15-c-myc sequences were flanked by the *35S* promoter and *Nos* terminator. The *35S:cGmAGL15-cmyc:NOS* cassette was then restricted with *Eco*RI/*Hind*III and the purified fragment was ligated into the pC1301 vector resulting in vector *pcGmAGL15-cmyc*. To transfer *gGmAGL15-cmyc* from pBIMC, the plasmid was restricted with *Sal*I and *Sac*I and the purified insert was ligated with vector obtained by digestion of *pcGmAGL15-cmyc* with the same restriction enzymes. The finished vector *35S:gGmAGL15-cmyc:NOS* cassette, i.e. *pgGmAGL15-cmyc*, was confirmed by sequencing and used for biolistic transformation of soybean.

Transformation of Soybean

Somatic embryos were generated from the cultivar 'Jack' (soybean Merr.). Immature pods (approximately 15–20 d after anthesis) were harvested, rinsed with distilled water containing a few drops of liquinox, and surface sterilized by immersing for 30 s in 70% isopropanol, 12 min in 25% Clorox solution with a few drops of liquinox, and washing twice for 5 min with sterile distilled water. Immature zygotic embryos of 4.0 ± 1 mm were aseptically excised and the embryonic axis removed by cutting through both cotyledons. About 25 individual cotyledons were cultured abaxial side down toward the media, on D40 induction media plates under diffused light (Murashige and Skoog salt containing B5 vitamins, 3% Suc, 40 mg L^{-1} 2,4-D, 0.2% phytagel [Sigma], pH 7.0). After 6 to 8 weeks of incubation, the embryos induced on the cotyledons from D40 medium were transferred to the D20 media (Murashige and Skoog salt containing B5 vitamins, 3% Suc, 20 mg L^{-1} 2,4-D, 0.2% phytagel, pH 5.8). Good quality proliferating embryos clusters were subcultured after every 4 weeks on fresh D20 media plates for no more than four cycles.

Four to five days before transformation, equal amounts of somatic embryo tissue was placed at the center of a plate containing D20 media. Gold microcarriers (5 mg of 0.6 μm; Bio-Rad) were washed with ethanol (100%, 10 s), incubated on ice for 30 s, centrifuged at approximately 2,300*g*, and the liquid removed. Ethanol (35 μL of 100%) was added, microcarriers gently vortexed for 1 min, and pelleted (approximately 2,000*g*, 10 s). One milliliter of water was added and carriers pelleted at 400*g*, 5 min. The supernatant was removed, and DNA in the order as follows (3 μg at 0.5 μg μL^{-1}, 220 μL sterile water, 250 μL of 2.5 M $CaCl_2$, and 100 μL of 0.1 M spermidine) were added while mixing gently (vortex speed 3), incubated on ice for 2 min, and then mixed gently by vortex, 10 min at 0°C to 4°C. Carriers were pelleted by centrifugation 5 min, approximately 100*g*, liquid removed, and carriers washed by adding ethanol (600 μL, 100%) and gentle vortexing, 1 min. Wash was removed by pelleting carriers as before, followed by resuspension in 36 μL ethanol and incubation on ice for 1 h. Microcarriers were resuspended by pipetting and 10 μL used per macrocarrier (Bio-Rad, catalog no. 1652335), for bombardment using a DuPont BioListic particle delivery system (model PSD-1000; Bio-Rad), and 1,100-psi rupture discs (Inbio Gold). Bombardment of embryos with each construct was performed in triplicate for each experiment.

After bombardment, plates were sealed with parafilm and tissue let recover by inverting plates and incubating at room temperature under diffused light for 7 d. Tissue was then transferred to 25 mL of FN-Lite (Finner and Nagasawa, 1988) media containing 20 mg L^{-1} hygromycin B (Invitrogen) in a 125-mL flask and incubated at room temperature, 200 rpm, and under diffused light. The media was changed periodically after every week for 8 to 10 weeks. After 10 weeks of culturing the potential transformants per bombardment were scored by green color and morphology and were transferred to individual wells of six-well tissue culture plates (MidSci) containing FN-Lite media supplemented with hygromycin for an additional month. Surviving cultures were transferred to 125-mL flasks containing 25 mL of FN-Lite media without hygromycin and allowed to continue development for additional 2 months, replacing the media at 1 month and separating the larger embryonic masses into several pieces. The numbers of surviving green calli for each construct were also counted after 2 months.

Transgenic plants were recovered by disruption of tissue and transfer to soybean histodifferentiation and maturation media as described by Schmidt et al. (1995), for 4 to 5 weeks with constant agitation (200 rpm), followed by desiccation in petri plates sealed with parafilm for 7 to10 d and then transfer to MSO lite media (Murashige and Skoog salts, B5 vitamins, 1.5% Suc, 0.2% phytagel, pH 5.8) in 100- × 20-mm petri dishes at room temperature to allow shoot and root development. Healthy plants (branched roots and elongating shoots) were transferred to 0.5× B5 media in phyta-trays (Sigma). These plantlets were then allowed to grow for 2 to 3 weeks and subsequently transplanted to soil.

RT-PCR

For detection of accumulation of c-myc-tagged *AGL15* transcript in soybean transgenic plants, RNA was extracted from leaf tissue using Trizol (Invitrogen Life Technologies). The AMV RT system (Promega) was used for cDNA synthesis following the manufacturer's protocol. The primers for PCR were 5′-GGTCCCATTCCAATACCAAC-3′ corresponding to *GmAGL15* and 5′-CAGGTCCTCCTCGTAGATCAGCTT-3′ corresponding to c-myc epitope tag. Control primers amplified *EF1-α* (5′-ACGCTCTACTTGCTTTCACC-3′ and 5′-GCACCGTTCCAATACCACC-3′).

Protein Gel Blot

Crude nuclear extracts were prepared from approximately 75 mg of open flowers following the protocol described by Busk and Pagès (1997), except 50 μL of low salt buffer and 25 μL of high salt buffer were used per sample and the proteinase inhibitors aprotinin, leupeptin, and pepstatin (1 mM each) were added to buffers. After extraction by vortexing, the proteins (60 μg per lane) were separated on a 12% polyacrylamide gel, blotted to Immobilon polyvinylidene difluoride (Millipore), and probed with anti-AGL15 antiserum as described by Heck et al. (1995).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AtAGL15, At5g13790; GmAGL15, AY370659 (cDNA); and AY370660 (genomic).

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