The BABY BOOM Transcription Factor Activates the LEC1-ABI3-FUS3-LEC2 Network to Induce Somatic Embryogenesis[1][OPEN]

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Somatic embryogenesis is an example of induced cellular totipotency, where embryos develop from vegetative cells rather than from gamete fusion. Somatic embryogenesis can be induced in vitro by exposing explants to growth regulators and/or stress treatments. The BABY BOOM (BBM) and LEAFY COTYLEDON1 (LEC1) and LEC2 transcription factors are key regulators of plant cell totipotency, as ectopic overexpression of either transcription factor induces somatic embryo formation from Arabidopsis (*Arabidopsis thaliana*) seedlings without exogenous growth regulators or stress treatments. Although LEC and BBM proteins regulate the same developmental process, it is not known whether they function in the same molecular pathway. We show that BBM transcriptionally regulates *LEC1* and *LEC2*, as well as the two other *LAFL* genes, *FUSCA3* (*FUS3*) and *ABSCISIC ACID INSENSITIVE3* (*ABI3*). LEC2 and ABI3 quantitatively regulate BBM-mediated somatic embryogenesis, while FUS3 and LEC1 are essential for this process. BBM-mediated somatic embryogenesis is dose and context dependent, and the context-dependent phenotypes are associated with differential *LAFL* expression. We also uncover functional redundancy for somatic embryogenesis among other Arabidopsis BBM-like proteins and show that one of these proteins, PLETHORA2, also regulates *LAFL* gene expression. Our data place BBM upstream of other major regulators of plant embryo identity and totipotency.

Plant cells show a high degree of developmental plasticity and can be induced readily to regenerate new tissues or organs (pluripotency) and even embryos (totipotency) from in vitro-cultured explants. Somatic embryogenesis is a type of plant totipotency in which embryos are induced to form on vegetative explants, usually in response to exogenous hormones, especially auxins, and/or stress treatments (Fehér, 2015). Somatic embryogenesis is used extensively as a clonal propagation tool in biotechnology applications (Lelu-Walter et al., 2013; Sharma et al., 2013b; Park and Paek, 2014), and while the tissue culture requirements for somatic embryo induction are well known (Gaj, 2004), only a few of the molecular components that drive this process have been described (Elhiti et al., 2013).

A number of plant transcription factors have been identified that can convert somatic cells into embryogenic, totipotent cells. One of these transcription factors, *Brassica napus* BABY BOOM (BBM), encodes an AINTEGUMENTA-LIKE (AIL) APETALA2/ethylene-responsive element-binding factor (AP2/ERF; Boutilier et al., 2002). In Arabidopsis (*Arabidopsis thaliana*), *AIL* genes form a small, eight-member clade within the AP2/ERF transcription factor family, which, in addition to BBM, comprises *AINTEGUMENTA* (*ANT*), *AIL1*, *PLETHORA1* (*PLT1*), *PLT2*, *AIL6/PLT3*, *CHOTTO1* (*CHO1*)/*EMBRYOMAKER* (*EMK*)/*AIL5/PLT5*, and *PLT7*. *AIL* genes are expressed in dividing tissues, including root, shoot, and floral meristems, where they act in a redundant manner to maintain a meristematic state (Horstman et al., 2014). Single *AIL* knockout mutants show no or only minor defects, but double or triple mutants have stronger phenotypes related to reduced cell proliferation or altered cell identity, including

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smaller floral organs with partial loss of identity (Krizek, 2009, 2015), embryo arrest (Galinha et al., 2007), and impaired root and shoot meristem maintenance (Aida et al., 2004; Galinha et al., 2007; Mudunkothge and Krizek, 2012). BBM is expressed in the embryo and root meristem, where it regulates cell identity and growth together with other AIL proteins (Aida et al., 2004; Galinha et al., 2007). In line with their loss-of-function phenotypes, overexpression of Arabidopsis AIL transcription factors induces pluripotency, totipotency, and/or cell proliferation, with different functions being assigned to specific AIL proteins (Krizek, 1999; Nole-Wilson et al., 2005; Galinha et al., 2007; Tsuwamoto et al., 2010; Krizek and Eaddy, 2012). However, unlike other *AIL* genes, the genetic pathways in which *BBM* functions have not been well characterized (Horstman et al., 2014), and it is not known how this single protein controls both pluripotent and totipotent growth.

The overexpression of native or heterologous BBM genes also induces regeneration in other species (Morcillo et al., 2007; Deng et al., 2009; El Ouakfaoui et al., 2010; Heidmann et al., 2011; Lutz et al., 2011; Bandupriya et al., 2014; Yang et al., 2014; Florez et al., 2015; Lowe et al., 2016) and, therefore, is used as a biotechnology tool to improve plant transformation in model and crop species (Deng et al., 2009; Heidmann et al., 2011; Lutz et al., 2011; Florez et al., 2015; Lowe et al., 2016).

Other transcription factors also induce somatic embryogenesis when expressed ectopically in seedlings, including LEAFY COTYLEDON1 (LEC1), which encodes subunit B of a nuclear factor Y protein (NF-YB), and the B3 domain protein LEC2 (Lotan et al., 1998; Stone et al., 2001). LEC1 and LEC2, together with LEC1-LIKE (L1L) and the B3 domain proteins ABSCISIC ACID-INSENSITIVE3 (ABI3) and FUSCA3 (FUS3), are collectively referred to as the LAFL network (for LEC1/L1L, ABI3, FUS3, and LEC2; Jia et al., 2014). LAFL proteins function throughout embryogenesis, where they redundantly regulate early developmental processes such as embryo identity and later processes such as embryo maturation (storage product accumulation and desiccation tolerance) and dormancy (Jia et al., 2013). Although FUS3 and ABI3 do not induce somatic embryogenesis when overexpressed, they do confer embryo traits to seedlings (Parcy et al., 1994; Parcy and Giraudat, 1997; Gazzarrini et al., 2004). LEC1 and LEC2 directly regulate the expression of seed maturation and auxin response and biosynthesis genes (Lotan et al., 1998; Braybrook et al., 2006), and both of these functions could play a role in inducing a totipotent state (Braybrook and Harada, 2008). Moreover, LEC2 directly activates the MADS box transcription factor gene *AGAMOUS-LIKE15* (*AGL15*), which enhances somatic embryogenesis from immature zygotic embryos when overexpressed (Harding et al., 2003; Braybrook et al., 2006). *LAFL* gene expression is controlled by the chromatin remodeler PICKLE (PKL) and by B3 domain-containing VIVIPAROUS1/ABI3-LIKE (VAL)/HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE (HSI) transcription factors. Mutations in *PKL* or *VAL* genes lead to increased *LAFL* gene expression and maintain embryo identity in seedlings (Ogas et al., 1999; Rider et al., 2003; Henderson et al., 2004; Suzuki et al., 2007).

While both BBM and LEC promote totipotency, it is not known whether they function in the same developmental pathways. To gain insight into the signaling pathways regulated by BBM, we performed a global analysis of BBM DNA-binding sites in somatic embryo tissue (Horstman et al., 2015). Here, we show that BBM induces cell totipotency during seed germination through transcriptional activation of the *LAFL* network and that BBM induces somatic embryogenesis in a dose- and context-dependent manner.

RESULTS

BBM Binds and Transcriptionally Activates *LAFL* Genes

To understand BBM regulatory networks during embryogenesis, we identified genes that were bound by BBM in somatic embryo cultures using chromatin immunoprecipitation of BBM-YFP (*BBM*:*BBM-YFP*) and BBM-GFP (*35S*:*BBM-GFP*) fusion proteins followed by next-generation sequencing (ChIP-seq; Horstman et al., 2015). BiNGO analysis (Maere et al., 2005) of the top 1,000 potential target genes in these chromatin immunoprecipitation (ChIP) experiments was performed to identify overrepresented Gene Ontology categories (Supplemental Data Set S1; a selection of which is shown in Supplemental Table S1). Genes involved in auxin biosynthesis, transport, and signaling, as well root development, meristem initiation, and maintenance, were overrepresented in the ChIP-seq data sets, as expected from BBM's function in the root and in line with studies of other AIL proteins (Horstman et al., 2014; Santuari et al., 2016). Genes involved in adaxial/abaxial polarity specification and shoot development also were overrepresented among BBM-bound genes (Supplemental Data Set S1; Supplemental Table S1). Notably, BBM bound to the promoter regions of genes with known functions in embryo identity and maturation, including the *LAFL* genes *LEC1*, *LEC2*, *ABI3*, and *FUS3* (but not *L1L*), the MADS box transcription factor gene *AGL15*, which enhances somatic embryogenesis and functions in a positive feedback network with *LEC2* (Harding et al., 2003; Braybrook et al., 2006; Zheng et al., 2009), and *NF-YA9*, which also induces somatic embryogenesis when overexpressed (Mu et al., 2013; Fig. 1A; Supplemental Data Set S1; Supplemental Table S1). Here, we focus our efforts on characterization of the interaction between BBM and members of the *LAFL*/*AGL15* network. We confirmed BBM binding to the promoters of *LEC1*, *LEC2*, *ABI3*, and *AGL15* by independent ChIP-qPCR experiments in somatic embryos (Supplemental Fig. S1A). We did not observe BBM binding to the *FUS3* promoter, which is consistent with

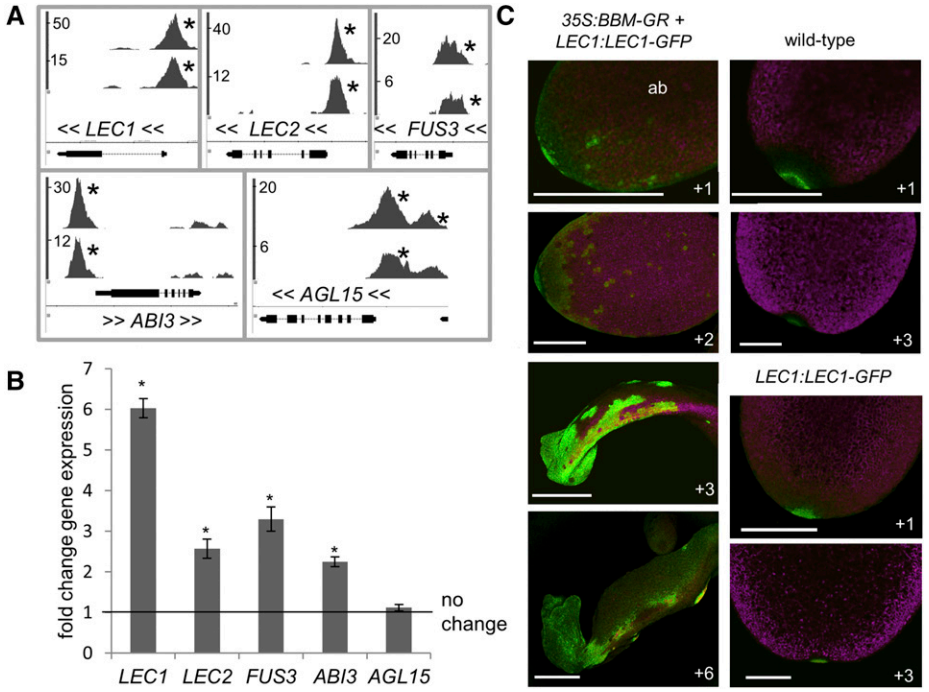

Figure 1. BBM binds and transcriptionally regulates *LAFL* genes. A, ChIP-seq BBM-binding profiles for embryo-expressed genes: *35S:BBM-GFP* (top profiles) and *BBM:BBM-YFP* (bottom profiles). *x* axis, Nucleotide positions (TAIR 10 annotation; black bars indicate exons, and lines indicate introns); *y* axis, ChIP-seq score (fold enrichment of the BBM-GFP/YFP ChIP to the control ChIP), as calculated by the CSAR package in Bioconductor; <<, direction of gene transcription; *, peaks with scores that are considered statistically significant (false discovery rate < 0.05). B and C, Transcriptional regulation of *LAFL* genes. Relative expression was determined by quantitative real-time reverse transcription-PCR (qPCR) in *35S:BBM-GR* and Columbia-0 (Col-0) seeds 1 d after seed plating. Samples were treated for 3 h with dexamethasone (DEX) and cycloheximide (CHX; both at 10 μM). B, Error bars indicate SE values of the three biological replicates. Statistically significant differences (asterisks) between DEX+CHX-treated *35S:BBM-GR* and DEX+CHX-treated Col-0 were determined using Student's *t* test ($P < 0.01$). C, *LEC1:LEC1-GFP* regulation by BBM. Samples were treated with 10 μM DEX 1 d after plating and imaged on subsequent days as indicated. The images show the adaxial sides of cotyledons, unless indicated (ab, abaxial side). The green signal in Col-0 and *LEC1:LEC1-GFP* cotyledon tips is autofluorescence. Bars = 250 μm.

its lower and atypically shaped BBM ChIP-seq peak (Supplemental Fig. S1).

ANT/AIL DNA-binding motifs were determined previously in vitro by SELEX and electrophoretic mobility shift assay (Nole-Wilson and Krizek, 2000; Yano et al., 2009; O'Malley et al., 2016; Santuari et al., 2016). MEME analysis (Bailey and Elkan, 1994) of in vivo BBM-bound regions identified an overrepresented sequence motif that resembles the ANT/AIL DNA-binding motif (Supplemental Fig. S1B; Santuari et al., 2016). The BBM-bound region in *LEC1*, *LEC2*, and *FUS3* contains this BBM-binding motif, while the *ABI3*-bound region contains a slightly degenerate version thereof (Supplemental Fig. S1C). The BBM-binding motif was not found in the region bound by BBM in the *AGL15* gene, which suggests that BBM binds *AGL15* using a different motif or via an intermediate protein.

We determined whether BBM regulates *LAFL/AGL15* gene expression during somatic embryo induction from imbibed seeds using a steroid (DEX)-regulated *35S::BBM-GR* line in combination with qRT-PCR and reporter gene analysis. qRT-PCR analysis in the presence of DEX and the translational inhibitor CHX showed that *LEC1*, *LEC2*, *FUS3*, and *ABI3* expression, but not *AGL15* expression, was up-regulated after BBM-GR activation in imbibed seeds (Fig. 1B). *LAFL/AGL15* genes were not differentially regulated upon BBM-GR activation in the same material that was used for ChIP (2,4-dichlorophenoxyacetic acid [2,4-D]-induced embryo cultures; data not shown). An explanation for this lack of transcriptional response could be the use of different promoters to drive BBM in the two experiments. BBM might only bind and activate *LAFL/AGL15* genes during the induction/early stages of somatic embryogenesis (*pBBM:BBM-YFP* used for ChIP is expressed during early embryogenesis; Horstman et al., 2015) but not at later stages of somatic embryogenesis (*p35S:BBM-GR* used for transcription analysis [qRT-PCR] is expressed during late embryogenesis; Johnson et al., 2005).

Next, a *LEC1:LEC1-GFP* reporter (Li et al., 2014) was used to chart the dynamics of *LEC1* expression during BBM-induced somatic embryogenesis (Fig. 1C). *35S:BBM-GR* seedlings initially form somatic embryos on

the cotyledon tip and later from the shoot apical meristem and cotyledon margins. LEC1-GFP was observed 1 d after BBM-GR activation, in small patches of cells on the abaxial side of the cotyledon (Fig. 1C, +1), and 1 d later at the cotyledon tip and in patches of cells on the adaxial cotyledon blade (Fig. 1C, +2). LEC1-GFP became stronger in the cotyledon tip and margin at the time when the tip began to swell (Fig. 1C, +3) and could be found later in the somatic embryos that formed at the cotyledon tip and at the margin (Fig. 1C, +6).

Our results demonstrate that BBM overexpression can ectopically activate *LAFL* gene expression during seed germination.

LAFL Proteins Modulate BBM-Induced Embryogenesis

We investigated the genetic relationship between BBM and its *LAFL/AGL15* gene targets. Since outcrossing BBM overexpression lines silences the BBM phenotype (Supplemental Fig. S2), we introduced the *35S:BBM-GR* construct into the *lec1-2*, *lec2-1*, *fus3-3*, *agl15-3*, and *abi3* (three alleles) mutant backgrounds by transformation (Fig. 2). The *lec1-2* and *fus3-3* seeds are desiccation intolerant (Meinke et al., 1994); therefore, heterozygous mutants (*lec1-2/+* and *fus3-3/+*) were used for transformation.

In wild-type Arabidopsis, 6% to 7% of the primary (T1) *35S:BBM-GR* transformants were embryogenic when grown on DEX (Fig. 2). Transformation of the *lec1-2/+*, *lec2-1*, *fus3-3/+*, and *agl15-3* mutants with the *35S:BBM-GR* construct resulted in significantly reduced percentages of embryogenic seedlings compared with transformed wild-type seedlings (Fig. 2). We determined the genotype of the few embryogenic seedlings that were generated after transformation of *35S:BBM-GR* to the *lec1-2/+* and *fus3-3/+* backgrounds. Only one of the embryogenic *lec1-2/+* progeny contained the *lec1-2* mutant allele, while none of the embryogenic *fus3-3/+* progeny contained the *fus3-3* mutant allele (Supplemental Table S2). To determine whether BBM-GR activation can induce somatic embryogenesis in a homozygous *lec1-2* background, we rescued immature zygotic embryos from the single embryogenic *lec1-2/+ 35S:BBM-GR* line to bypass the *lec1-2* desiccation-intolerant phenotype and cultured them on DEX-containing medium. Embryos were separated phenotypically into *lec1-2* homozygous mutant and combined *lec1-2* heterozygous/wild-type classes. Somatic embryos formed in *lec1-2* heterozygous/wild-type seedlings but not in the homozygous *lec1-2* seedlings (Supplemental Table S2).

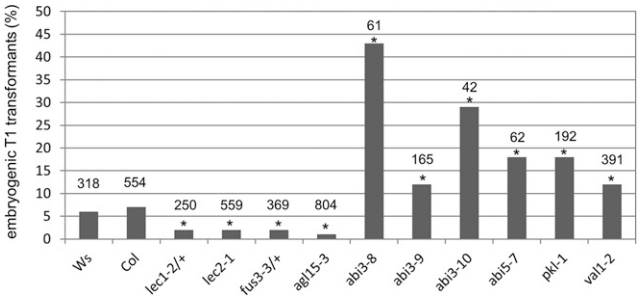

Figure 2. BBM-induced embryogenesis is modulated by *LAFL* genes. The percentage of primary embryogenic transformants obtained is shown after transformation of the *35S:BBM-GR* construct to the wild-type Wassilewskija (Ws) or Col-0 background or the indicated mutant lines. Statistically significant differences (asterisks) in the number of embryogenic transgenic lines between the mutant and the corresponding wild-type line were determined using Pearson's χ^2 test ($P < 0.05$). The total number of transformants per line is indicated above each bar. Somatic embryo formation was not observed in any of the individual mutant backgrounds alone or in mutant + *35S:BBM-GR* backgrounds in the absence of BBM-GR activation.

These results suggest that LEC1, LEC2, and FUS3 are positive regulators of BBM-mediated somatic embryogenesis and that LEC1 and FUS3 are essential for this process. Surprisingly, we found that *AGL15* also is a positive regulator of BBM-induced somatic embryogenesis, even though it is not transcriptionally regulated by BBM at the start of somatic embryo induction; *AGL15* might be transcriptionally regulated by BBM at a later time point.

In contrast to the results obtained with the *fus3*, *lec*, and *agl15* mutants, transformation of the *35S:BBM-GR* construct to three different *abi3* mutants enhanced the number of embryogenic seedlings (Fig. 2). *abi3* is the only *LAFL* mutant that is insensitive to abscisic acid (ABA), and overexpression of *ABI3* does not induce somatic embryogenesis (Parcy et al., 1994; Parcy and Giraudat, 1997). To separate the effects of ABA insensitivity and other embryo defects of *abi3* mutants on the BBM phenotype, we tested another ABA-insensitive mutant, *abi5-7*, which does not show mutant embryo phenotypes other than ABA insensitivity (Nambara et al., 2002). As with the *abi3* mutants, the *abi5-7* mutant also enhanced the frequency of the BBM phenotype (Fig. 2), suggesting that BBM-mediated totipotency is suppressed by ABA signaling rather than by other functions of the ABI3/ABI5 proteins.

A number of regulatory proteins repress *LAFL* gene expression during the transition from seed to postembryonic growth. Seedlings with loss-of-function mutations in these proteins ectopically express *LAFL* genes and retain embryo identity (Jia et al., 2014). We determined whether loss-of-function mutants for two of these proteins, the CHD3 chromatin remodeler PKL and the B3 domain protein VAL1/HSI2, influence the penetrance of BBM-induced embryogenesis (Fig. 2). The *pkl-1* and *val1-2* (*hsi2-5*) mutants improved the efficiency of BBM-mediated somatic embryogenesis, as measured by the higher percentage of embryogenic primary transformants.

Together, the data show that members of the *LAFL* network, as well as their upstream negative regulators, are important direct and indirect components of the BBM signaling pathway.

AIL/PLT Proteins Promote Totipotency and Regulate *LAFL* Gene Expression

BBM is expressed in the embryo and the root meristem, where it regulates cell identity and growth along with other AIL proteins (Aida et al., 2004; Galinha et al., 2007). *PLT1* and *PLT2* induce spontaneous root organogenesis (Aida et al., 2004) and have roles in hormone-mediated regeneration (Kareem et al., 2015), while *BBM* and *CHO1/EMK/AIL5/PLT5* (Tsuwamoto et al., 2010) are the only genes reported to induce somatic embryogenesis. We generated *35S:AIL* overexpression lines for the six *AIL* genes that have not been reported to induce somatic embryogenesis when overexpressed, namely *ANT*, *AIL1*, *PLT1*, *PLT2*, *PLT3/AIL6*, and *PLT7*, and found that overexpression of all these genes except the phylogenetically distinct *ANT* and *AIL1* (Kim et al., 2006) induced somatic embryogenesis in the primary transformants (Supplemental Fig. S3, A and B). A PLT2-GR fusion protein directly activated *LEC1*, *LEC2*, and *FUS3* gene expression but not *ABI3* and *AGL15* expression (Supplemental Fig. S3C). Together, these data suggest extensive overlap in AIL protein function.

BBM- and PLT2-Mediated Somatic Embryogenesis Is Dose Dependent

Ectopic AIL expression induces spontaneous adventitious growth, including somatic embryos, ectopic shoots and roots, and callus formation (Boutilier et al., 2002). These phenotypes are correlated with the amount of nucleus-localized BBM protein in DEX-treated *35S:BBM-(GFP)-GR* lines (Figs. 3 and 4A). A relatively low BBM dose induced the formation of small seedlings with epinastic cotyledons and leaves (Figs. 3B and 4B) that showed reduced cell differentiation (Fig. 4, C and D). Intermediate DEX concentrations also induced ectopic trichome-bearing protrusions or ectopic leaves on their cotyledon petioles (Figs. 3, C–E, and 4B), and ectopic roots appeared after longer exposure to DEX (Fig. 3, F and G). A low frequency of seedlings with somatic embryos on their cotyledons (Fig. 3H) also was observed at intermediate DEX concentrations, and this phenotype became highly penetrant at the highest effective DEX concentration (Fig. 4B). PLT2 also directs the same dose-dependent overexpression phenotypes (Supplemental Fig. S4) as BBM overexpression, although ectopic root formation was evident earlier in the PLT2-GR lines than in the BBM-GR lines. These data suggest that AIL protein dose drives the developmental fate of regenerating tissues.

BBM Promotes Context-Specific Embryogenesis

Previously, we identified direct BBM target genes in 4-d-old *35S:BBM-GR* seedlings using microarray analysis (Passarinho et al., 2008). *LAFL/AGL15* genes were not identified as BBM target genes in this study, and in general, there was little overlap between these seedling microarray-derived targets and the top 1,000 ChIP-seq-derived targets identified in somatic embryos (Supplemental Table S3). This discrepancy might be explained by the different tissues that were used in each study. Therefore, we examined the relationship between the developmental competence for BBM-mediated regeneration and *LAFL* transcription.

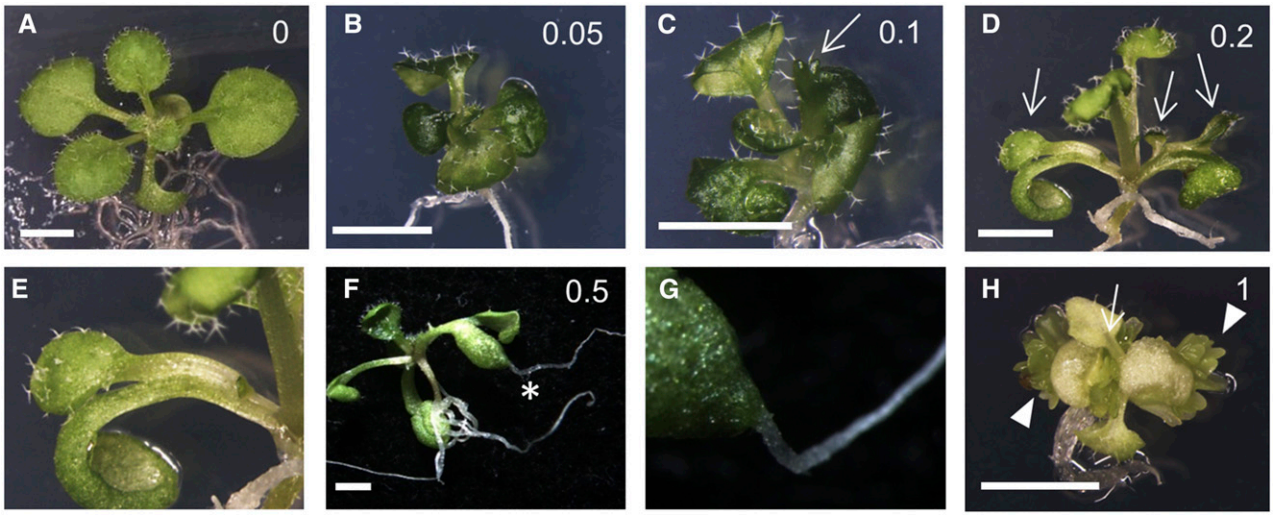

Figure 3. BBM overexpression phenotypes are dose dependent. Phenotypes are shown for *35S:BBM-GR* seedlings grown for 2 weeks (A–E and H) or 3 weeks (F and G) on the DEX concentration (μM) indicated in each image. A, A phenotypically normal seedling. B, A small seedling with epinastic leaves and cotyledons. C, A small, epinastic seedling with a trichome-bearing ectopic leaf (arrow) on the cotyledon petiole. D, A seedling with ectopic leaves on the petioles of both cotyledons (arrows). E, A magnified view of the ectopic leaf in D. F, A *35S:BBM-GR* seedling with an ectopic root (asterisk). G, A magnified view of the ectopic root in F. H, A seedling with somatic embryos on the cotyledon margins (arrowheads). Bars = 2.5 mm.

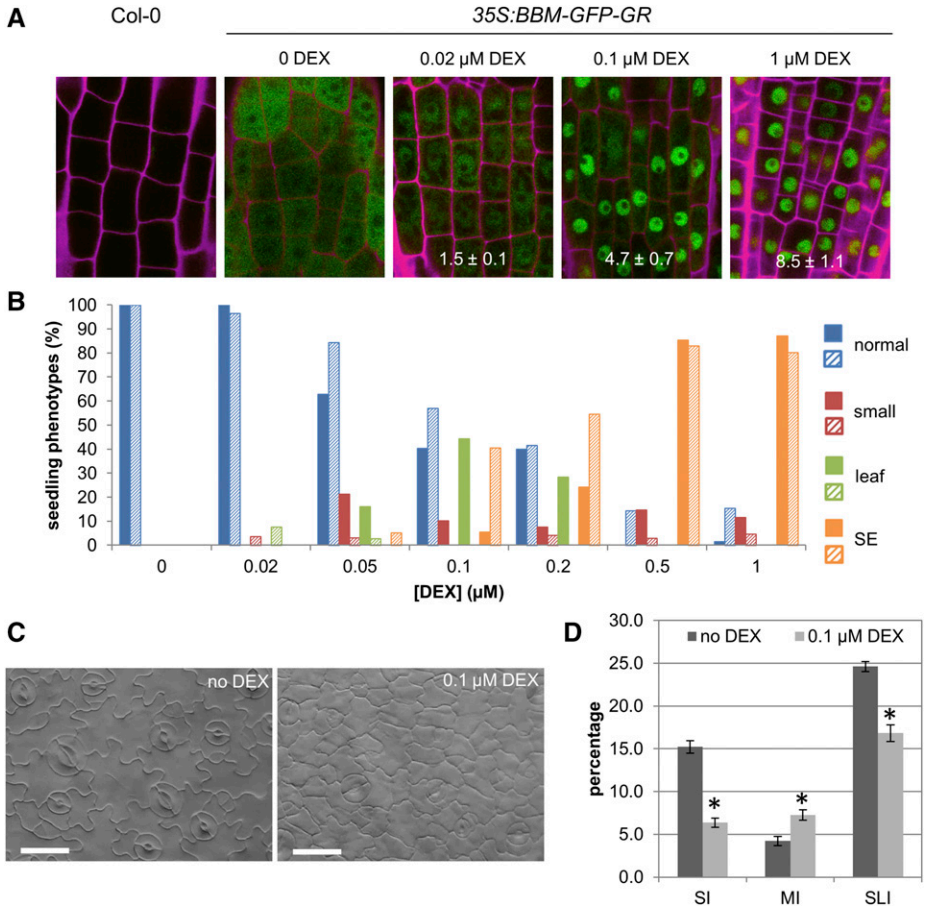

Figure 4. Quantification of BBM dose-dependent phenotypes. A, BBM-GFP-GR nuclear localization increases with increasing DEX concentration. The effect of DEX on BBM localization in roots of *35S:BBM-GFP-GR* seedlings grown for 7 d in medium containing the indicated DEX concentration is shown. Non-DEX-treated (Col-0) roots are shown as a GFP-negative control. Subcellular BBM-GFP-GR localization was quantified for each DEX concentration by calculating the average nuclear-cytoplasmic GFP ratio (63–133 cells of five to eight roots per DEX concentration), which is indicated on the bottom of the images (±SE). The average ratios are significantly different from each other ($P < 0.01$, Student's *t* test). Green, GFP; magenta, propidium iodide. B, Frequency of phenotypes observed in *35S:BBM-GR* seedlings from two independent transgenic lines (solid and hatched bars) grown for 2 weeks on medium containing different DEX concentrations ($n = 100$–350 seedlings). Leaf, Ectopic leaves; SE, somatic embryos. Seedlings that showed both ectopic shoots and somatic embryos were scored as SE. SE refers to both embryogenic tissue (smooth, swollen, bright green in color, and lacking trichomes) and cotyledons as well as histodifferentiated embryos. C, A relatively low BBM dose induces smaller and less-lobed leaf pavement cells compared with the control. The abaxial sides of cleared first leaves of 9-d-old *35:BBM-GR* seedlings grown on medium without DEX (left) or with 0.1 µM DEX (right) are shown. Bars = 25 µm. D, Stomatal differentiation in DEX-treated *35:BBM-GR* seedlings is reduced compared with untreated seedlings. In DEX-treated *35S::BBM-GR* seedlings, fewer cells are committed to stomatal development, as reflected by the lower stomatal lineage index (SLI). Also, fewer mature stomata were found in leaves of DEX-treated seedlings (stomatal index [SI]), while the number of stomatal meristemoids was increased (meristemoid index [MI]). Error bars indicate SE. Asterisks indicate statistically significant differences compared with the non-DEX-treated control (*, $P < 0.05$, Student's *t* test).

We activated BBM-GR at different time points before and after germination. When *35S:BBM-GR* seeds were placed directly in DEX-containing medium before or during germination (days 0–2), 100% of the seedlings formed somatic embryos directly on their cotyledons after approximately 1 week (Fig. 5A; Supplemental Fig. S5A). By contrast, postgermination DEX treatment (days 3–4) induced callus formation on the adaxial side of the cotyledons of approximately 40% of the seedlings from which somatic embryos eventually developed (Fig. 5B; Supplemental Fig. S5A). We obtained similar results when we activated PLT2-GR before and after germination (Supplemental Fig. S5, B and C). Thus, AIL proteins induce somatic embryogenesis in two ways depending on the developmental stage of the explant: directly from cotyledons in a narrow window before germination, and indirectly via a callus phase after germination. In agreement with our previous microarray results, neither *LEC1*, *LEC2*, *FUS3*, nor *ABI3* was expressed when BBM-GR (or PLT2-GR) was activated

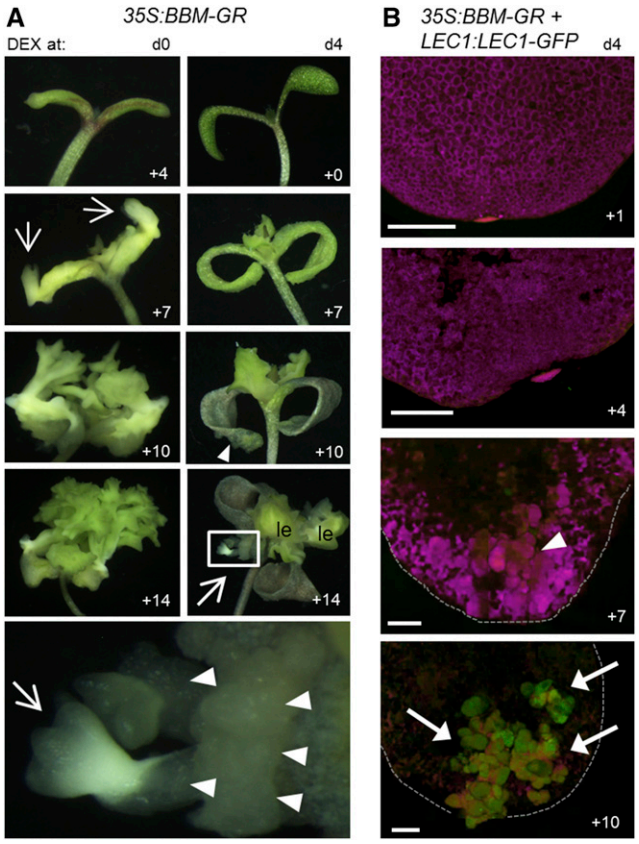

Figure 5. BBM-induced embryogenesis is context dependent. A, *35S:BBM-GR* plants were cultured with 10 μM DEX starting at the dry seed stage (d0) or after germination (d4). The culture time after DEX application is indicated on the bottom right of each image. Arrowheads, Callus; arrows, somatic embryos/embryogenic tissue; le, callused leaf tissue. The bottom image is a magnification of the boxed region in the d4 +14 image. B, *LEC1:LEC1-GFP* regulation by BBM. Seedlings were treated with 10 μM DEX after germination (d4) and imaged on subsequent days as indicated. The images show the adaxial sides of cotyledons. Arrowhead, Callus on the distal end of the cotyledon blade; arrows, GFP-positive embryo clusters. The outline of the cotyledon margins is shown with dashed lines. Autofluorescence (magenta) was used to delineate the tissue. Bars = 250 μm.

in postgermination seedlings, although *AGL15* expression was up-regulated slightly under these conditions (Supplemental Fig. S5D). LEC1-GFP was only detected in this indirect pathway 10 d after DEX induction (Fig. 5B), when it was localized to globe-like embryos that emerged from the callus. The desiccation intolerance combined with the lack of or weak embryogenesis phenotypes of the *lec1/fus3 35S:BBM-GR* lines (see above) complicated further genetic analysis of the role of LEC1 and FUS3 in this indirect pathway.

Our results highlight the existence of a narrow developmental window of competence for direct embryogenesis and suggest that tissues outside this window require more extensive reprogramming (e.g. callus formation) before embryogenesis can be induced. *LAFL* genes are transcriptionally silenced after germination (Zhang et al., 2012; Zhou et al., 2013); therefore, these loci might only become transcriptionally accessible after redifferentiation of cotyledon cells to callus.

DISCUSSION

BBM-Mediated Embryogenesis Requires *LAFL* Genes

An increasing number of proteins are being identified that regulate cell totipotency in vivo and in vitro, including members of the LAFL network and AIL proteins (Horstman et al., 2014; Fehér, 2015). *LAFL* transcription is regulated at the chromatin level and by extensive transcriptional feedback loops between LAFL proteins. The interactions between LAFL proteins and other regulators of cell totipotency are less well known. Our data now place BBM/AIL proteins directly upstream of the *LAFL/AGL15* genes. There is some evidence that LAFL proteins might act upstream of AILs: a *Phaseolus vulgaris* ABI3-like factor (PvALF) binds and activates Arabidopsis *CHO1/EMK/AIL5/PLT5* (Sundaram et al., 2013), and Arabidopsis FUS was shown to bind *BBM*, *PLT2*, *AIL6/PLT3*, and *PLT7*, although transcriptional regulation was not investigated (Wang and Perry, 2013). In contrast, the lack of *AIL* deregulation after inducible overexpression of LEC1, ABI3, FUS3, or LEC2 (Braybrook and Harada, 2008; Yamamoto et al., 2010; Junker et al., 2012; Mönke et al., 2012) suggests that there is no direct feedback of LAFLs on *AILs*. *BBM* expression is reduced in *lafl* mutant seeds (Supplemental Fig. S6), but this genetic interaction could be indirect. Although BBM proteins appear to directly activate *LAFL* genes, the data are inconclusive regarding whether there are additional direct transcriptional feedback loops in the AIL-LAFL cell totipotency network.

LAFL/AGL15 proteins are required for BBM function, as BBM overexpression in *lec1*, *lec2*, *fus3*, and *agl15* mutants either reduced or eliminated the capacity of seedlings to form somatic embryos. The reduced competence for somatic embryogenesis in these mutants could be explained in two ways: (1) the developmental defects in the mutants change the physiological state of the mature embryo/seedling in such a way that it is no longer responsive for BBM; or (2) BBM-induced embryogenesis relies on transcriptional activation of the *LEC1*, *LEC2*, *FUS3*, and *AGL15* genes. Several lines of evidence support the latter scenario. First, we observed a reduced responsiveness to BBM in segregating *lec1* and *fus3* populations, which contained wild-type and heterozygous plants. However, the few embryogenic transformants in these populations were mainly wild types, suggesting that the *lec1* and *fus3* mutations affect BBM function in the heterozygote state. Heterozygous *lec1* and *fus3* mutants do not show obvious growth defects, suggesting that reduced LEC1 or FUS3 level (dose) in the heterozygous mutants, rather than a change in the physiological state of the tissue, reduces the response to BBM overexpression. Second, the *abi3* mutant shows overlapping maturation defects with the

other *LAFL* mutants (To et al., 2006; Roscoe et al., 2015), yet the *abi3* mutations had the opposite effect on BBM-induced embryogenesis. Therefore, we hypothesize that the inability of BBM to induce *LEC* and *FUS* gene expression reduces the capacity for embryogenic growth in these mutants. This hypothesis is further strengthened by our observations that mutations in *LAFL* repressors enhance BBM-mediated embryogenesis, possibly by facilitating elevated *LAFL* gene expression.

Embryo Induction Is a Dose-Dependent Phenotype

It was suggested previously that the PLT2 protein regulates root meristem size and maintenance through a protein concentration gradient, with high, intermediate, and low AIL concentrations instructing stem cell fate, cell division, and differentiation, respectively (Galinha et al., 2007). AIL6/PLT3 overexpression also induces dose-dependent phenotypes in floral organs (Krizek and Eaddy, 2012). We showed that a high BBM/PLT2 dose induces embryogenesis, a lower dose induces organogenesis, and the lowest dose inhibits differentiation. Our overexpression data, therefore, support a general dose-dependent AIL output in plant tissues. This dose dependence complicates functional complementation studies of AILs, as even complementation with endogenous promoters (Galinha et al., 2007; Santuari et al., 2016) can lead to differences in expression levels among transformants and a range of developmental outcomes.

How dedifferentiation, organogenesis, and somatic embryogenesis are induced at successively higher AIL doses is not clear, but it likely reflects the endogenous roles of AIL proteins during embryo, organ, and meristem development. Mechanistically, AIL dose-dependent phenotypes could result from dose-dependent expression levels of the same set of target genes and/or from dose-specific activation of specific target genes. A transcription factor gradient can regulate the different sets of target genes through differences in binding site number and affinity (Rogers and Schier, 2011). Target genes with many or high-affinity binding sites are activated by low levels of the transcription factor, whereas genes with few or low-affinity binding sites are activated only at high transcription factor levels. Specificity also might be determined at the level of protein-protein interactions (Horstman et al., 2015). Defining the overlapping and unique target genes for each AIL transcription factor at different doses and the protein complexes in which they function will shed light on how the AIL dose directs specific developmental fates.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) *lec2-1* (CS3868), *lec1-2* (CS3867), *fus3-3* (CS8014), *agl15-3* (CS16479), and *pkl-1* (CS3840) mutants were obtained from the Nottingham Arabidopsis Stock Centre. The *val1-2* (*hsi2-5*; Sharma et al., 2013a), *abi3-8*, *abi3-9*, *abi3-10*, and *abi5-7* (Nambara et al., 2002) mutants and the *LEC1*:*LEC1-GFP* marker (Li et al., 2014) were described previously. All mutants are in the Col-0 background, except *lec1-2* and *lec2-1*, which are in the Wassilewskija background.

Seeds were sterilized with liquid bleach as described previously (Boutilier et al., 2002; Passarinho et al., 2008) and germinated on one-half-strength Murashige and Skoog medium containing 1% Suc and vitamins. DEX and CHX (both Sigma) were added to the medium as described in the text. Solid and liquid (rotary shaker, 60 rpm) cultures were kept at 21°C and 25°C, respectively (16-h-light/8-h-dark regime). *lec1-2 35S:BBM-GR* mutant embryos were rescued by excising them from sterilized siliques and allowing them to germinate on solid one-half-strength Murashige and Skoog medium containing 1% Suc and vitamins with DEX and kanamycin (for selection of the *BBM* transgene).

Vector Construction and Transformation

The *35S:BBM-GR* construct was described previously (Passarinho et al., 2008). The *ANT*, *PLT3/AIL6*, *PLT7*, and *PLT1* protein-coding regions were amplified from Arabidopsis Col-0 genomic DNA and the *PLT2* protein-coding region from cDNA using the primers listed in Supplemental Table S4. *35S:AIL* ectopic overexpression constructs were made using the Gateway binary vector pGD625 (Chalfun-Junior et al., 2005). The *35:BBM-GFP-GR* construct was made using the Gateway-compatible destination vector pARC146 (Danisman et al., 2012). *BBM-GFP* used in the *35:BBM-GFP-GR* construct was amplified from a *BBM:BBM-GFP* plasmid (Horstman et al., 2015). Constructs were introduced into wild-type or mutant lines by floral dip transformation (Clough and Bent, 1998).

Confocal Microscopy

Confocal imaging was performed as described previously (Soriano et al., 2014). Propidium iodide (10 μg mL^{-1}) counterstaining (*35S:BBM-GFP-GR* roots) and autofluorescence (*LEC1:LEC1-GFP* cotyledons) were used to delineate the tissue. Both fluorophores were excited with a 532-nm laser and detected at 600 to 800 nm.

To quantify the subcellular BBM-GFP-GR localization in root, confocal images were made of the meristematic region of roots of *35S:BBM-GFP-GR* seedlings grown for 7 d in medium containing different DEX concentrations. ImageJ was used to calculate the ratio of nuclear to cytoplasmic fluorescence by comparing the average fluorescence intensity in the nucleus with the average fluorescence intensity of an area of equal size in the cytoplasm.

Leaf Imaging and Quantification of Stomatal Development

The first leaf pairs of 9-d-old seedlings were placed overnight in 70% ethanol at 4°C, then transferred to 85% ethanol for 6 h, and subsequently to 3% bleach overnight or until imaging. Leaves were mounted in HCG solution (80 g of chloral hydrate, 10 mL of glycerol, and 30 mL of water) prior to imaging with a Nikon Optiphot microscope.

To calculate SI, eight images from the abaxial sides of cleared first leaves of 9-d-old *35:BBM-GR* plants grown with or without DEX were analyzed (n = 125 and 350 cells per image). The SI, MI, and SLI were calculated as described previously (Peterson et al., 2013). SI = (number of stomata/(total number of stomata + nonstomatal epidermal cells)) × 100. For the SI, only mature stomata with a pore were counted. MI = (number of meristemoids/(total number of stomata + nonstomatal epidermal cells)) × 100. SLI = (number of stomata and stomata precursors/(total number of stomata + nonstomatal epidermal cells)) × 100.

ChIP-Seq

The previously published ChIP-seq data and data analysis (Horstman et al., 2015) can be downloaded from the Gene Expression Omnibus (GSE52400). The ChIP-seq experimental setup has been described (Horstman et al., 2015). Briefly, the experiments were performed using somatic embryos from either 2,4-D-induced *BBM:BBM-YFP* cultures (with *BBM:NLS-GFP* as a control) or a *35S:BBM-GFP* overexpression line (with *35S:BBM* as a control). Two independent ChIP-qPCR experiments on 2,4-D-induced *BBM:BBM-YFP* cultures were performed to validate the ChIP-seq results, using the same protocol as described previously (Horstman et al., 2015). A fold change was calculated by comparison with an unbound genomic control region (*ARR6*), and statistically significant differences between BBM-bound regions and a second unbound genomic

control region (*HSF1*) were determined using Student's *t* test ($P < 0.05$). The DNA primers are shown in Supplemental Table S4.

Expression Analysis

cDNA from Col-0 and *35S:BBM-GR* seeds or seedlings (three biological replicates of each) were treated as described in the text and used for qPCR. qPCR was performed using the BioMark HD System (Fluidigm). The data were normalized against the *SAND* gene (Czechowski et al., 2005), and relative gene expression was calculated by comparison with similarly treated wild-type Col-0 or untreated *35S::BBM-GR* (Livak and Schmittgen, 2001). The DNA primers are shown in Supplemental Table S4.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. BBM binds to an ANT-like DNA-binding motif.

Supplemental Figure S2. Outcrossing BBM overexpression lines silences the BBM phenotype.

Supplemental Figure S3. Ectopic overexpression of AIL proteins induces somatic embryogenesis and activates *LAFL* expression.

Supplemental Figure S4. PLT2 ectopic overexpression induces dose-dependent phenotypes.

Supplemental Figure S5. AIL proteins induce context-dependent somatic embryogenesis.

Supplemental Figure S6. *BBM* expression is reduced in *lafl* mutant seeds.

Supplemental Table S1. BBM target genes.

Supplemental Table S2. Effects of the *lec1* and *fus3* mutant backgrounds on BBM-mediated somatic embryogenesis.

Supplemental Table S3. Overlap between BBM targets obtained using microarray and ChIP-seq analyses.

Supplemental Table S4. Oligonucleotide primers used in this study.

Supplemental Data Set S1. BBM target genes.

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