AGAMOUS-LIKE67 Cooperates with the Histone Mark Reader EBS to Modulate Seed Germination under High Temperature

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Seed germination is a vital developmental process that is tightly controlled by environmental signals, ensuring germination under favorable conditions. High temperature (HT) suppresses seed germination. This process, known as thermoinhibition, is achieved by activating abscisic acid and inhibiting gibberellic acid biosynthesis. The zinc-finger protein SOMNUS (SOM) participates in thermoinhibition of seed germination by altering gibberellic acid/abscisic acid metabolism, but the underlying regulatory mechanism is poorly understood. In this study, we report that SOM binds to its own promoter and activates its own expression in Arabidopsis (Arabidopsis thaliana) and identify the MADS-box transcription factor AGAMOUS-LIKE67 (AGL67) as a critical player in SOM function, based on its ability to recognize CArG-boxes within the SOM promoter and mediate the transcription of SOM under HTs. In addition, AGL67 recruits the histone mark reader EARLY BOLTING IN SHORT DAY (EBS), which recognizes H3K4me3 at SOM chromatin. In response to HTs, AGL67 and EBS are highly enriched around the SOM promoter. The AGL67-EBS complex is also necessary for histone H4K5 acetylation, which activates SOM expression, ultimately inhibiting seed germination. Taken together, our results reveal an essential mechanism in which AGL67 cooperates with the histone mark reader EBS, which bridges the process of H3K4me3 recognition with H4K5 acetylation, thereby epigenetically activating SOM expression to suppress seed germination under HT stress.
ABA levels (Matakiadis et al., 2009). Similarly, seeds from mutant lines that are impaired in GA biosynthesis (e.g., the ga requiring [gal] mutants gal–ga5) do not germinate well in the absence of exogenous GA (Koornneef and van der Veen, 1980; Talon et al., 1990; Sun and Kamiya, 1994; Xu et al., 1995), whereas mutants with defects in the GA2 OXIDASE (GA2OX) gene, which lack GA catabolism, show high endogenous seed GA content and reduced seed dormancy (Ogawa et al., 2003; Yamaguchi, 2008).

In addition to the ABA/GA biosynthetic pathways, the components associated with the transduction of the ABA/GA signal, such as the transcription factors ABA INSENSITIVE3 (ABI3), ABI4, and ABI5 or the DELLA-type transcriptional repressors (mainly GA INSENSITIVE [GAI], REPRESSOR OF ga1–3 [RGA], and RGA-LIKE2 [RGL2]), also affect seed germination or dormancy (Lee et al., 2002; Penfield et al., 2006; Park et al., 2011; Dai et al., 2013; Lim et al., 2013). Furthermore, these components reciprocally affect seed germination by interfering with ABA/GA biosynthesis; for example, RGL2 binds to the ABI5 promoter to induce ABI5 expression, and thus ABA biosynthesis, which in turn prevents seed germination (Piskurewicz et al., 2008; Hu et al., 2019). The balance between GA and ABA levels therefore plays a crucial role in determining whether seed dormancy is maintained or seed germination is initiated.

Light and temperature are the two main environmental cues controlling seed germination and dormancy (Quail, 2002; Rajjou et al., 2012). In Arabidopsis, members of the phytochrome (phy) photoreceptor family perceive and respond to light irradiation in the red/far-red portion of the light spectrum (between 660 and 730 nm). Red light converts phy molecules into their photoactivated Pfr form and induces seed germination. By contrast, far-red light exposure converts phy back to their inactive Pr form, which suppresses seed germination (Quail, 2002; Mathews, 2006). Active phytochrome B (PhyB) interacts with the downstream basic helix-loop-helix transcription factor PHYTOCHROME-INTERACTING FACTOR1 (PIF1) to promote PIF1 degradation, modulate the expression of genes associated with ABA/GA metabolism, and initiate seed germination (Gu et al., 2017; Majee et al., 2018). The CCCH-type zinc-finger protein SOMNUS (SOM) was identified as a negative regulator of seed germination that acts downstream of PhyB. A loss-of-function som mutant germinates irrespective of the light conditions, even when all PhyB is inactivated by irradiation with far-red light (Kim et al., 2008; Park et al., 2011; Lim et al., 2013). Further characterization of the role of SOM in seed germination revealed that SOM activates ABA biosynthesis while repressing GA biosynthesis. Thus, som mutant seeds contain lower ABA but higher GA levels than wild-type seeds, conditions that promote seed germination (Chang et al., 2018).

Inhibition of seed germination by unfavorable ambient temperatures, or thermoinhibition by supraoptimal conditions, is a common limitation of many winter annual or biennial species that prevents germination under adverse environments (Toh et al., 2008; Auge et al., 2015). High temperatures (HTs) induce the expression of ABA biosynthetic genes or signaling components, such as NCED4, NCED9, and ABI5, and inhibit seed germination in Arabidopsis and lettuce (Lactuca sativa) seeds. Thermoinhibition of seed germination can, however, be alleviated by treating seeds with ABA biosynthetic inhibitors or providing them with exogenous GA (Argyris et al., 2008; Toh et al., 2008; Schwembrer and Bradford, 2010; Huo et al., 2013). By contrast, seed germination of aba2 and abi3 mutants is not disrupted by HT stress (Toh et al., 2008; Yang et al., 2019). HTs also activate SOM expression, which prevents seed germination by increasing ABA seed content and reducing GA biosynthesis. This step requires functional ABA and GA biosynthetic pathways, as the transcription factors ABI3, ABI5, and DELLA can target the SOM promoter and induce SOM expression during HT stress (Lopez-Molina et al., 2003; Lim et al., 2013; Yang et al., 2019). We recently demonstrated that ABI5 BINDING PROTEIN2 (AFP2) enhanced HT tolerance with respect to seed germination by interfering with the ABI5 signal (Garcia et al., 2008; Chang et al., 2018). In addition to affecting transcription, HTs disturb the activity of the epigenetic factor POWERDRESS (PWR) and modify histone H3 deacetylation levels and deposition of the histone H2A variant H2A.Z at the SOM locus, ultimately activating ABI3-dependent SOM expression and thereby blocking seed germination (Yang et al., 2019). These data suggest that SOM, as an essential negative regulator of seed germination, is strictly controlled by HTs at multiple points; however, the underlying mechanism has yet to be fully elucidated.

Apart from seed germination and dormancy, tolerance to seed desiccation is a central adaptive trait for terrestrial plants that allows plant seeds to remain viable in a dry state for several years, sometimes even centuries, and to resume germination after rehydration. Integrative genomics, bioinformatics, and metabolomics analyses highlighted the role of two specific transcriptional subnetworks in this process. The transcription factors PLATZ1 and PLATZ2 (for plant AT-rich sequence- and zinc-binding protein) and AGAMOUS-LIKE67 (AGL67) are key nodes in these networks; their respective mutants exhibited lower seed desiccation tolerance, whereas overexpression of their encoding genes enhanced the desiccation tolerance of the strong abi3–5 mutant, demonstrating their function in seed desiccation tolerance (González-Morales et al., 2016).

AGL67 also regulates seed dormancy, as the agl67 mutant shows reduced seed dormancy. Genetic analysis indicated that AGL67 interacts with EARLY BOLTING IN SHORT DAY (EBS), and both genes may belong to a common pathway to control seed dormancy (Narro-Diego et al., 2017). EBS possesses a bivalent bromo-adjacent homology (BAH)-plant homeodomain (PHD) and functions as a reader of histone marks that binds specifically to H3K4me2 and H3K4me3 in vitro.
(Piñeiro et al., 2003; López-González et al., 2014; Yang et al., 2018). These observations suggest that EBS may control seed dormancy at the epigenetic level, but whether the AGL67-EBS module controls the thermoinhibition of seed germination through SOM has not been determined.

In this study, we aimed to further elucidate the molecular mechanism of SOM function during thermoinhibition of seed germination. A series of biochemical methods revealed that SOM interacted with AGL67 and EBS as a complex to epigenetically activate SOM expression through reading the H3K4me3 mark and H4K5 acetylation. Genetic and physiological analyses showed that the AGL67-EBS module coordinated SOM activity and downstream GA/ABA metabolism, ultimately suppressing seed germination under HT. Thus, our study extends previous knowledge of AGL67 function and provides insight into the molecular mechanism that couples a transcription factor, a reader of histone methylation, and a change in chromatin structure through histone acetylation during thermoinhibition of seed germination.

RESULTS

SOM Binds to Its Own Promoter to Facilitate Its Transcription

To investigate the transcriptional machinery upstream of SOM, we initially screened for putative trans-acting factors in the promoter region of SOM by a yeast one-hybrid (Y1H) assay. With this goal in mind, we first wished to identify a minimal SOM promoter that would fully complement the som-2 mutant when driving the expression of SOM. To this end, we fused the coding region of SOM to a FLAG tag and placed the gene under the control of a 2.4-kb fragment upstream of its start codon as the native promoter (SOMpro:SOM-FLAG, abbreviated as SOM-FLAG). We detected a strong signal in individual transgenic seeds expressing SOM-FLAG by immunoblot analysis (Supplemental Fig. S1A). We then introduced SOM-FLAG into the som-2 mutant to generate complementation lines. HT treatment of 32°C blocked seed germination in wild-type Columbia-0 (Col-0) as well as SOMpro:SOM-FLAG but not som-2 (Fig. 1, A and B), demonstrating effective complementation of som-2 by the SOM-FLAG construct in the context of germination thermostolerance. These results also indicate that the 2.4-kb SOM promoter fragment bears sufficient cis-acting elements to drive adequate SOM expression and rescue som-2. Furthermore, HT induced SOM-FLAG protein accumulation in transgenic SOM-FLAG seeds during the first 24 h of HT exposure, followed by a slight drop after 36 h (Fig. 1C). These results agree with the published effects of HT on SOM expression (Lim et al., 2013).

To identify the trans-acting factors that control SOM expression, we used this 2.4-kb fragment as a bait in a Y1H assay to screen an Arabidopsis normalized cDNA library. One of the positive clones isolated during the screen was SOM itself, suggesting that SOM might bind to its own promoter and control its own expression. We independently confirmed the strong binding of SOM to this 2.4-kb promoter fragment by retransforming yeast cells. We also tested each half of the 2.4-kb SOM promoter for SOM binding and only saw evidence of binding between SOM and the proximal 1.2-kb fragment (P-SOM) but not the distant 1.2-kb fragment (D-SOM; Fig. 1D).

The SOM promoter is A/T-rich, and tandem zinc finger (TF) proteins like SOM preferentially bind to A/T-rich elements in the 3′ untranslated regions of mRNAs to trigger their degradation (Laity et al., 2001; Jang, 2016). We reasoned that SOM, also known as TZF4, might also bind these A/T-rich regions (Kim et al., 2008). Using a chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) assay, we established that the C3 and C4 fragments within the proximal fragment of the SOM promoter were dramatically enriched after pull-down with an anti-FLAG antibody (Fig. 1E). Thus, these data suggest that SOM binds to its own promoter, somewhere along the proximal 1.2 kb immediately upstream of the ATG.

We also generated transgenic SOMpro:GUS reporter lines that placed the GUS gene under the control of the 2.4-kb SOM promoter. We detected a strong GUS signal in Col-0 seeds bearing the SOMpro:GUS reporter (Fig. 2A) but a much reduced GUS signal when SOMpro:GUS was introduced into the som-2 mutant background (SOMpro:GUS/som-2). The transgenic lines that overexpressed SOM as a GFP fusion under the control of the cauliflower mosaic virus 35S promoter (35Spro:SOM-GFP, referred to as SOM-GFP; Supplemental Fig. S1B) was generated, and the SOMpro:GUS reporter was introduced into SOM-GFP lines by genetic crossing to obtain SOMpro:GUS SOM-GFP double transgenic lines. Reverse transcription (RT)-qPCR analysis revealed that GUS expression was lower in SOMpro:GUS som-2 seeds but much higher in SOMpro:GUS SOM-GFP double transgenic seeds (Fig. 2B). Thus, these results confirm that SOM activates its own expression.

AGL67 Binds to the SOM Promoter and Activates SOM Expression

During our Y1H screen, we also established that the MADS-box transcription factor AGL67 associates with the SOM promoter, specifically to the proximal 1.2-kb SOM promoter fragment (P-SOM; Supplemental Data Set S1; Supplemental Fig. S2, A and B). To infer the function of AGL67, we assessed the expression pattern of AGL67 in the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). AGL67 is only and strongly expressed in dry seeds or during late seed development (Supplemental Fig. S3). Consistent with these in silico data, the GUS staining pattern of AGL67pro:GUS transgenic lines (expressing GUS under the control of the AGL67 promoter) was strong in
siliques, germinated seeds, and mature embryos but weaker in young seedlings and mature leaves (Fig. 2C), indicating its potential role in seed germination.

To determine whether AGL67 is required for SOM expression, we obtained two T-DNA insertion mutants, **agl67-1** and **agl67-2**. In **agl67-1**, the T-DNA had inserted into the first intron of **AGL67**, while **agl67-2** carried the T-DNA in the third exon of **AGL67**. Both alleles abolished the accumulation of a functional **AGL67** transcript (Supplemental Fig. S4). We then crossed **agl67-1** and **agl67-2** to our **SOMpro:GUS** reporter line. GUS staining was clearly lower in **SOMpro:GUS agl67-1** or **SOMpro:GUS agl67-2** hydrated seeds compared with **SOMpro:GUS** seeds in the Col-0 background (Fig. 2D). RT-qPCR analysis showed that HT stress gradually increased the expression of **AGL67** and **SOM** (Fig. 2E; Supplemental Fig. S5A), but the HT-induced increase of **SOM** transcript levels was compromised in the **agl67-1** or **agl67-2** mutants (Fig. 2E). Collectively, these data suggest that AGL67 binding to the **SOM** promoter is necessary to activate **SOM** expression.

To identify which region of the **SOM** promoter is necessary for AGL67 binding, we divided the 1.2-kb **SOM** promoter into a series of smaller fragments and tested their interaction with AGL67 by Y1H analysis. As shown in Supplemental Figure S2C, the C3 region of the **SOM** promoter is necessary for AGL67 binding.
fragment was the smallest fragment to interact with AGL67 in yeast cells. In plants, MADS-box transcription factors recognize the cis-element CArG (C-[A/T]rich-G) within the promoter of target genes to regulate their expression (Castelán-Muñoz et al., 2019). Bioinformatic analysis of the 1.2-kb proximal SOM promoter fragment revealed 22 candidate CArG-box motifs (labeled as cis-a to cis-v in Supplemental Fig. S2C). To establish which CArG-box(es) contribute to AGL67 binding in planta, we performed a ChIP assay using transgenic seeds that overexpress AGL67-FLAG from the 35S promoter (35Spro:AGL67-FLAG, termed AGL67-FLAG; Supplemental Fig. S1C) and assessed several regions spanning different CArG motifs. The P3 region, which is included within the C3 fragment identified earlier through Y1H screening, was highly enriched in the pull-down with an anti-FLAG antibody (Fig. 3A). Thus, the P3 region is important for the association of AGL67 with the SOM promoter.

There are two canonical CArG cis-elements (termed cis-j and cis-k) within the P3 region. An electrophoretic mobility shift assay (EMSA) revealed that AGL67 formed a complex with labeled probes containing the cis-j or cis-k element, as evidenced by a shift in mobility (Fig. 3B). The interaction was specific to the CArG motif, as (1) the shift in mobility was gradually competed by the addition of cold probes and (2) mutated labeled probes without a CArG motif did not associate with AGL67 (Fig. 3B). AGL67 therefore binds to the SOM promoter by recognizing the cis-j and cis-k CArG motifs.

To assess the effect of AGL67 on SOM expression in vivo, we performed transient expression assays in Arabidopsis protoplasts. The effector consisted of AGL67 or SOM under the control of the 35S promoter, while the reporters contained the firefly (Photinus pyralis) luciferase gene under the control of a 2.4-kb SOM promoter (SOMpro:LUC) or promoter variants lacking various CArG motifs (Fig. 3, C and D). AGL67 activated the expression of the SOMpro:LUC reporter in Arabidopsis protoplasts, and SOM cooperated with AGL67 to further enhance the expression of the SOMpro:LUC reporter. AGL67 also activated the expression of SOMproP4:LUC (driving LUC under the control of the proximal SOM promoter) or SOMproPΔP4:LUC (driving LUC under the control of the proximal SOM

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**Figure 2.** Expression pattern of SOM and AGL67. A, GUS staining of freshly harvested SOMpro:GUS transgenic seeds in the Col-0 and som-2 backgrounds. Nontransgenic Col-0 was used as a control. All seeds were hydrated for 12 h before GUS staining. The top row shows intact seeds, and the bottom row shows embryos after the removal of seed coat and endosperm. B, RT-qPCR analysis of GUS transcripts in SOMpro:GUS hydrated seeds in Col-0, som-2 mutant, and SOM-GFP backgrounds. All seeds were hydrated in water for 12 h. PROTEIN PHOSPHATASE2A (PP2A) was used for normalization. Values are means ± SD of three biological replicates; bars labeled with different letters are significantly different at P < 0.05 (Tukey’s test). C, GUS staining of the AGL67pro:GUS line in wild-type seeds. D, Effects of AGL67 genotype on SOMpro:GUS staining pattern in the seed embryo. Freshly harvested seeds of transgenic SOMpro:GUS Col-0 and agl67-1 and agl67-2 mutants were hydrated for 12 h before GUS staining. The seed coat and endosperm were removed before capturing photographs of embryos. E, RT-qPCR analysis of SOM transcript levels in hydrated seeds of SOMpro:GUS in wild-type Col-0, agl67-1, and agl67-2 backgrounds. All seeds were hydrated at 32°C for 0 to 24 h. PP2A was used for normalization. Values are shown as means ± SD from three biological replicates.
promoter with a deletion of the P4 region) but not when the P3 region was deleted from the proximal SOM promoter (SOMproPΔP3: LUC). These observations further confirmed that AGL67 specifically recognizes the cis-j and cis-k elements in the P3 region to activate SOM expression in planta.

SOM Interacts with AGL67 in Vitro and in Vivo

Considering that SOM and AGL67 bind to the same region of the SOM promoter, we speculated that SOM might form a complex with AGL67 to coordinate SOM expression. To test this hypothesis, we first confirmed the interaction between AGL67 and SOM in a yeast two-hybrid (Y2H) assay (Fig. 4A). Pull-down analysis also showed that His-SOM (SOM with an N-terminal His tag) was coimmunoprecipitated by GST-AGL67 (AGL67 with an N-terminal GST tag) but not by GST alone in vitro (Fig. 4B).

We then performed a bimolecular fluorescence complementation (BiFC) analysis to test the interaction of SOM and AGL67 in planta. As shown in Figure 4D, strong YFP fluorescence was observed in the nuclei of epidermal cells transiently coexpressing AGL67-nYFP and SOM-cYFP in Nicotiana benthamiana leaves. We did not detect YFP fluorescence in the control experiments, in which AGL67-nYFP was transiently coexpressed with empty cYFP or SOM-cYFP was transiently coexpressed with empty nYFP. As confirmation, we performed a coimmunoprecipitation (Co-IP) assay using a total protein extract from AGL67-GFP SOM-FLAG or GFP SOM-FLAG hydrated seeds with an anti-GFP antibody. In agreement with the BiFC results, SOM-FLAG was pulled down with AGL67-GFP but not with GFP alone (Fig. 4C). Taken together, these results
demonstrate that AGL67 interacts with SOM in vitro and in vivo. Using the transgenic AGL67-GFP lines, we examined the localization of AGL67. We observed strong GFP fluorescence in the nucleus as well as a weaker signal at the plasma membrane (Fig. 4E). Similarly, we observed strong GFP fluorescence in nuclei of SOM-GFP transgenic lines (Fig. 4E). AGL67 and SOM colocalized to the nucleus of N. benthamiana leaves transiently coexpressing AGL67-CFP and SOM-mCherry under the control of the 35S promoter (Fig. 4F). These results all suggest that AGL67 and SOM physically interact in the nucleus.

AGL67 Negatively Controls Seed Germination through SOM under HT Stress

Having established that AGL67 interacts with SOM, we reasoned that AGL67 might control seed germination by modulating SOM expression. To test this hypothesis, we measured seed germination rates in Col-0, agl67 mutants, and the AGL67-FLAG line. Gradually increasing ambient temperature suppressed the germination of Col-0 seeds, whereas agl67 mutant seeds exhibited a higher germination rate at the same temperatures. Conversely, the germination rate of AGL67-FLAG was lower than that of Col-0 seeds under the same HT conditions (Fig. 5A). Thus, we propose that AGL67 negatively regulates seed germination thermotolerance.

To explore the genetic relationship between AGL67 and SOM, we next crossed the AGL67-FLAG line with the som-2 mutant to generate AGL67-FLAG som; we also crossed the agl67-1 mutant with SOM-GFP to generate SOM-GFP agl67 (Supplemental Fig. S5, B and C). Under HT stress at 32°C, the seed germination rates of SOM-GFP and AGL67-FLAG were lower than those of som-2 or agl67-1 (Fig. 5B). By contrast, AGL67-FLAG som and som-2 seeds both showed relatively higher and similar germination rates. Although the agl67-1 mutant germinated better than Col-0 under HT (Fig. 5A), both SOM-GFP and SOM-GFP agl67 had even lower germination rates than those observed for Col-0 (Fig. 5B). As
loss of AGL67 (as in *agl67-1*) had no effect on the germination rate of *SOM-GFP* under HT stress, these data suggest that SOM acts downstream of AGL67. Thus, SOM is epistatic to AGL67 and AGL67 targets SOM to control seed germination under HT stress.

Considering that SOM regulates seed germination by altering GA/ABA metabolism (Kim et al., 2008; Lim et al., 2013; Chang et al., 2018), we next examined whether AGL67 affects GA/ABA levels after exposure to HT stress. The seed content of the bioactive form GA4 decreased in all genotypes (Col-0, *agl67*, *SOM-GFP*, and *SOM-GFP agl67*) when subjected to HT stress, but this reduction was not as pronounced in *som-2*, *agl67 som* double mutants, or *AGL67-FLAG som* (Fig. 5C). By contrast, the ABA content of Col-0, *SOM-GFP*, and *SOM-GFP agl67* seeds increased after HT stress, although the same HT stress only modestly raised the ABA content in *som-2*, *agl67 som*, and *AGL67-FLAG som* seeds (Fig. 5D). Consistent with this pattern, transcript levels of two GA biosynthesis genes (*GA3ox1* and *GA3ox2*) and one ABA catabolic gene (*CYP707A2*) were higher in *agl67*, *som-2*, and *AGL67-FLAG som* relative to Col-0 or *AGL67-FLAG* after HT stress. Conversely, transcript levels of the GA catabolic gene (*GA2ox2*) and ABA biosynthesis genes (*ABA1*, *NCED6*, and *NCED9*) were relatively lower in *agl67*, *som-2*, and *AGL67-FLAG som* than in Col-0 or *AGL67-FLAG* seeds (Supplemental Fig. S6). These data collectively support our conclusion that AGL67 does affect GA/ABA metabolism, possibly through SOM.

**Figure 5.** Regulation of seed germination thermotolerance by AGL67 and SOM. A, Seed germination rates in Col-0, *agl67* mutants, and *AGL67-FLAG* lines. Seeds were surface sterilized and plated on solid one-half-strength Murashige and Skoog medium before being released at different high temperatures as indicated. Seed germination rates were recorded after 5 d of treatment. For each biological replicate, we tested the seeds (>100) from the same batch, and three technical replicates were conducted for statistical analysis. Values represent means ± SD from three biological replicates. Asterisks indicate significant difference by Student’s *t* test (**P < 0.01 and *P < 0.05). B, SOM is epistatic to AGL67 in the control of seed germination inhibition at high temperatures. Seeds from Col-0, *som*, *agl67 som*, *AGL67-FLAG som*, *SOM-GFP*, and *SOM-GFP agl67* were surface sterilized and plated on solid one-half-strength Murashige and Skoog medium before being released at 22°C or 32°C. Seed germination rates were recorded after 5 d of treatment. Values are means ± SD of three biological replicates; bars labeled with different letters are significantly different at *P < 0.05 (Tukey’s test). C and D, GA4 and ABA contents in hydrated seeds. All seeds were germinated either at 22°C or 32°C, and the content of GA4 or ABA was measured after 3 d of treatment. Values are means ± SD of three biological replicates; bars labeled with different letters are significantly different at *P < 0.05 (Tukey’s test).
The Histone Reader EBS Negatively Controls Seed Germination under HT by Activating SOM Expression

EBS controls primary seed dormancy and interacts genetically with AGL67 in this process (Narro-Diego et al., 2017), raising the question of a potential function for EBS in controlling seed germination under HT. To test this possibility, we first characterized an ebs loss-of-function allele, in which the T-DNA was inserted into the first exon and severely suppressed EBS transcript accumulation (Supplemental Fig. S7). The ebs mutant displayed a higher germination rate than Col-0 specifically under HT. Indeed, ebs seeds even germinated at a rate of 10.2% under HT stress at 34°C, a condition that completely abolished seed germination in wild-type Col-0 (Fig. 6A).

Next, we generated ebs complementation lines by transforming the ebs mutant with a construct driving the expression of EBS-FLAG under the control of the EBS promoter (EBSpro:EBS-FLAG ebs, termed c-EBS; Supplemental Fig. S7D). As expected, the EBSpro:EBS-FLAG complementation construct restored c-EBS seeds to a wild-type germination rate under HT (Fig. 6A). This result validated our hypothesis that higher germination of the ebs mutant under HT was indeed due to the loss of EBS function.

Published microarray data demonstrate that SOM transcript levels are reduced in the ebs mutant (Narro-Diego et al., 2017). To evaluate the effect of EBS on SOM expression, we first examined SOM expression in wild-type Col-0, ebs, and c-EBS seeds by RT-qPCR. HT treatment caused an up-regulation of SOM transcripts in Col-0 and c-EBS seeds but not in ebs mutant seeds (Supplemental Fig. S8A), suggesting that EBS is required for HT-mediated induction of SOM expression. In line with these results, ebs som double mutant seeds and c-EBS som seeds germinated at the same rate as the som-2 mutant under HT (Fig. 6B). Furthermore, ebs, som-2, and c-EBS som seeds contained higher GA4 levels and lower ABA levels than Col-0 or c-EBS seeds after exposure to HT stress for 24 h (Supplemental Fig. S8B). We also detected higher GA20 and GA3 levels in the seeds of som-2, agl67, and ebs single mutants compared with wild-type Col-0 after HT stress (Supplemental Fig. S8C). These data suggest that EBS controls the threonine inhibition of seed germination by activating SOM expression and subsequently altering downstream GA/ABA metabolism.

We next tested whether EBS binds to the SOM promoter to induce transcription in hydrated c-EBS seeds. ChIP-qPCR revealed that the P3 region within the SOM promoter was highly enriched in pull-downs with the anti-FLAG antibody (used to tag EBS) and that HT treatment further enhanced this enrichment (Fig. 6C). The P3 region of the SOM promoter and the E1 region (in the exon region) were also enriched, although HT had no additional effect. These data suggest that EBS mainly deposits to the same region as AGL67 in the promoter of SOM.

Because EBS is a histone reader that recognizes H3K4me2 or H3K4me3 modifications in vitro (Yang et al., 2018), we next examined whether EBS binding to the SOM promoter might depend on the chromatin H3K4 methylation status at the SOM locus. To this end, we introduced c-EBS into the atx1-2 (Arabidopsis homolog of Trithorax) mutant, which is deficient in H3K4 methyltransferase activity (Alvarez-Venegas et al., 2003), to generate c-EBS atx1 lines (Supplemental Fig. S9A). ChIP analysis using an anti-H3K4me3 antibody demonstrated that the atx1-2 mutant had reduced H3K4me3 levels in most SOM genomic regions compared with wild-type Col-0, while overexpression of ATX1-GFP in the atx1-2 background (35Spro:ATX1-GFP atx1-2, abbreviated as ATX1-GFP/atx1) increased H3K4me3 levels over the entire SOM locus (Supplemental Fig. S9B). Using ATX1-GFP seeds, ChIP analysis with an anti-GFP antibody uncovered a strong enrichment at most regions of the SOM promoter and exons (Supplemental Fig. S9D). These results suggest that ATX1 targets SOM chromatin to mediate H3K4me3 modification at this locus.

Furthermore, EBS enrichment at the SOM promoter similarly decreased in c-EBS atx1 seeds, as measured by ChIP with an anti-FLAG antibody, and did not respond to HT stress either (Supplemental Fig. S9C). We also generated transgenic plants that expressed ATX1, cloned in frame with the glucocorticoid receptor gene (GR), under the control of the 35S promoter (35Spro:ATX1-GR, termed as ATX1-GR). Dexamethasone (DEX) treatment induces the translocation of ATX1-GR from the cytoplasm to the nucleus to modulate target gene expression (Jing et al., 2019). DEX treatment increased H3K4me3 levels at the SOM promoter in ATX1-GR seeds as well as SOM expression itself (Supplemental Fig. S10A and B). Phenotype analysis showed that the seed germination rate was relatively higher in atx1-2, but lower in the transgenic ATX1-GFP line, compared with Col-0 under HT stress. Additional DEX also suppressed the seed germination of ATX1-GR under HT stress (Supplemental Fig. S10C). Together, these data support a model in which EBS reads ATX1-mediated H3K4me3 histone marks at the SOM promoter that are required for the activation of SOM expression.

To examine whether EBS bridges H3K4me3 modification with histone acetylation for the activation of SOM expression after HT, we measured histone acetylation levels for histone H4 at the SOM locus in wild-type Col-0 and ebs mutant seeds before and after HT stress by ChIP. Only the P3 promoter fragment was enriched with an antibody recognizing acetylated H4K5 (anti-H4K5ac) and further enhanced by HT treatment. However, enrichment at the SOM locus was lost in ebs mutant seeds (Fig. 6D). These data indicate that EBS reads the H3K4me3 marker at the SOM locus and then initiates histone acetylation for increased chromatin accessibility and thus SOM expression.

AGL67 Physically Interacts with EBS to Coordinate EBS-Dependent Histone Acetylation at the SOM Locus

EBS interacts genetically with AGL67 to regulate seed dormancy (Narro-Diego et al., 2017), indicating that
EBS and AGL67 function in the same pathway in the context of seed dormancy. To determine whether EBS and AGL67 also work together in response to HT, we introduced c-EBS into the agl67-1 mutant. Seeds from ebs, agl67-1, and c-EBS agl67 germinated better than Col-0 seeds under HT, whereas c-EBS lines had a germination rate even lower than that of Col-0 seeds (Fig. 7A). In agreement with these observations, HT failed to fully

**Figure 6.** EBS regulates thermoinhibition of seed germination through SOM. A, Hydrated seeds for Col-0, ebs, and c-EBS were incubated at different temperatures, as indicated, for 5 d, when the germination rate was calculated. Values are means ± so of three biological replicates. B, Col-0, som-2, ebs, c-EBS, c-EBS som, and ebs som seeds were incubated at 22°C or 32°C for 5 d, and the germination rate was calculated. Values are means ± so of three biological replicates; bars labeled with different letters are significantly different at P < 0.05 (Tukey’s test). C, ChIP-qPCR analysis of the enrichment of EBS at the promoter (P) and exon (E) fragments of the SOM locus under normal conditions (22°C) or HT conditions (32°C) for 24 h. Wild-type Col-0 and c-EBS seeds were used for the analysis, with an anti-FLAG antibody. Top, Schematic diagram of the SOM promoter showing the positions of the CArG-boxes (blue rectangles) and three regions (P2, P3, and P5) covering various cis-elements for ChIP-qPCR amplification; E1 and E2 are located at the beginning and the end of the exon. Bottom, Fold enrichment of five amplified fragments, quantified by qPCR with chromatin isolated from Col-0 and c-EBS. ACTIN2 served as an internal control. Enrichment values were normalized to the level of input DNA. Values are shown as means ± so from three biological replicates. Asterisks indicate significant difference by Student’s t test (**P < 0.01). D, ChIP-qPCR analysis of histone H4K5 acetylation levels in the promoter and exon fragments of the SOM locus in Col-0 or c-EBS seeds under normal conditions at 22°C or HT conditions at 32°C for 24 h. An anti-H4K5ac antibody was used for immunoprecipitation. Fold enrichment of five amplified fragments was quantified by qPCR with chromatin isolated from Col-0 and c-EBS. ACTIN2 served as an internal control, and enrichment values were normalized to the level of input H4. Values are shown as means ± so of three biological replicates. Asterisks indicate significant difference by Student’s t test (**P < 0.01).
induce SOM expression in agl67-1, ebs, and c-EBS agl67 seeds (Supplemental Fig. S11). These results are consistent with AGL67 being required for EBS-mediated SOM expression under HT treatment. Furthermore, EBS and AGL67 physically interact, as evidenced in a Y2H assay (Fig. 7, B and C). EBS-FLAG coimmunoprecipitated with AGL67-GFP in a Co-IP using an anti-GFP antibody and total protein extracts from hydrated AGL67-GFP EBS-FLAG seeds (Fig. 7C), further confirming that EBS and AGL67 interact in planta.

To explore whether AGL67 recruits EBS to the SOM promoter and thereby mediates histone acetylation, we examined the binding of EBS at the SOM locus in hydrated c-EBS or c-EBS agl67 seeds. ChIP-qPCR exposed the enhanced binding of EBS to the P3 fragment of the SOM promoter in response to HT treatment for 24 h, which was partially counteracted by the loss of AGL67 function in c-EBS agl67-1 seeds (Fig. 7D). These data indicate that AGL67 facilitates EBS binding to the SOM promoter upon exposure to HT stress.

In agreement with this conclusion, ChIP-PCR analysis showed that HT treatment increased H4K5ac levels at the P3 fragment within the SOM locus, but this was abolished in the agl67-1 mutant (Fig. 7E). These data are consistent with the model from our genetic analysis: AGL67 and EBS promote SOM expression. They further establish that AGL67 recruits EBS to enhance H4 acetylation levels at the SOM locus to induce SOM expression, which results in therminhibition of seed germination. EBS function may require HISTONE ACETYLTRANSFERASE OF THE MYST FAMILY (HAM) members HAM1 and HAM2, previously reported to be recruited by the histone mark reader MORF RELATED GENES MRG1 and MRG2 for H4K5 acetylation modification (Xu et al., 2014b).
DISCUSSION

AGL67 Controls Seed Germination Thermotolerance through SOM

We had previously shown that the negative regulator of ABA signaling, AFP2, influences the trans-activation activity of ABI5 for SOM expression, thereby enhancing thermotolerance of seed germination (Chang et al., 2018). In this study, we identified a 2.4-kb fragment upstream of the SOM start codon that was sufficient to drive SOM expression under HT (Fig. 1C) and fully rescued the som mutant phenotype (Fig. 1, A and B). We then demonstrated that SOM can bind to its own promoter and activate its own expression (Fig. 1, D and E). Zinc-finger proteins (of which SOM is one) stabilize RNA by binding to A/U-rich 3’ untranslated regions (Laity et al., 2001; Jang, 2016); the SOM promoter is A/T rich and therefore constitutes a likely target for SOM binding. This strategy may allow SOM to quickly drive the accumulation of its own encoded protein and thus enact its biological function, as for example in response to HT stress. Indeed, other regulatory factors involved in salicylic acid, ABA, or light signaling, such as NONEXPRESSER OF PR GENES1, ABI5, and ELOGATED HYPOCOTYL5, bind to their own promoters for feedback activation of their own expression (Abbas et al., 2014; Xu et al., 2014a; Chen et al., 2019b).

Previous data showed that ABI3 interacts with ABI5 and DELLAs to form a complex that binds to the RY motif in the SOM promoter and control its expression under HT stress (Lim et al., 2013). Here, we identified the MADS-box transcription factor AGL67 as an important player that associates with the SOM promoter (Fig. 3; Supplemental Fig. S2). AGL67 was reported to be an important regulator of seed desiccation tolerance: overexpressing AGL67 partially rescued the low germination rate of the strong (and desiccation-sensitive) allele abi3-5 after dry treatment during seed storage (González-Morales et al., 2016). AGL67 also regulates primary seed dormancy (Narro-Diego et al., 2017).

In this study, we report that aGL67 mutant seeds have a higher germination rate, whereas AGL67-overexpressing seeds have a lower germination rate, when compared with Col-0 seeds after HT stress (Fig. 5A). These results suggest that AGL67 negatively controls seed germination under HT. Genetic analysis further confirmed that SOM is epistatic to AGL67 in thermoinhibition of seed germination, because both aGL67-ox som and aGL67 som seeds showed a higher germination rate that was similar to that of the som-2 mutant (Fig. 5B). GUS staining in SOMpro:GUS lines and RT-qPCR and transient transformation analyses further revealed that AGL67 transactivates SOM expression (Figs. 2 and 3). In particular, EMSA and ChIP analysis demonstrated that AGL67 specifically binds to the proximal region of the SOM promoter to activate its expression by recognizing the cis- and cis-elements containing CArG motifs. Deleting this motif compromised AGL67-mediated activation of SOM expression (Fig. 3, C and D).

Not only do AGL67 and SOM bind to the SOM promoter, but they also colocalized to the nucleus (Fig. 4, E and F). Furthermore, ChIP analysis showed that both of AGL67 and SOM bind to the same P3 region within the SOM promoter (Figs. 1E and 3A). Their interaction was confirmed by Y2H assay, in vitro pulldown, in vivo BiFC, and Co-IP experiments (Fig. 4). Transient transfection of protoplasts demonstrated that expressing SOM enhanced the activation conferred by AGL67 on SOM expression (Fig. 3, C and D). Based on these data, we hypothesize that the interaction of SOM and AGL67 intensifies the trans-activation activity of AGL67 at the SOM promoter under HT.

AGL67 Recruits the Histone Reader EBS to Epigenetically Activate SOM for Thermoinhibition of Seed Germination

In a previous study, EBS was shown to mediate the break of seed primary dormancy; SOM expression was also lower in the ebs mutant based on microarray analysis (Narro-Diego et al., 2017), consistent with a role for EBS in controlling seed germination through SOM. In support of this hypothesis, we established that both ebs and som-2 mutants shared a high germination rate after HT stress, in contrast to wild-type Col-0 seeds (Fig. 6, A and B). SOM transcript levels remained low in ebs mutant seeds after HT stress, but this was fully complemented by introducing the EBSpro:EBS-FLAG construct (expressing EBS-FLAG from the EBS promoter) into the ebs mutant background (Fig. 6A), suggesting that EBS positively regulates SOM expression after HT stress. Seeds of both the ebs som double mutant and the c-ebs som line displayed higher germination rates than Col-0 seeds (Fig. 6B). As a result, EBS controls seed germination through activating SOM expression and thus altering downstream ABA/GA metabolism (Supplemental Fig. S8).

EBS was reported to act as a bivalent histone mark reader via its BAH domain, which recognizes trimethylated Lys-27 of histone H3 (H3K27me3), as well as via the PHD, itself recognizing trimethylation of Lys-4 (H3K4me3; Yang et al., 2018). In yeast and animals, proteins with PHDs bind to H3K4me3 and act as a molecular bridge between local changes in histone acetylation or methylation levels and activation of gene expression (Wysocka et al., 2006). In Arabidopsis, ORIGIN OF REPLICATION COMPLEX1 is a PHD that specifically binds to H3K4me3 and stimulate target gene expression by increasing histone H4 acetylation and H4K20 trimethylation (de la Paz Sanchez and Gutierrez, 2009). EBS exhibits a higher affinity for H3K27me3 than H3K4me3; however, removing the C-terminal extension of EBS increased its affinity for H3K4me3 (Yang et al., 2018).

In this study, we show that EBS specifically binds to the SOM promoter within the P3 region, containing functional cis-/-K CArG-box elements (Fig. 6C). The recruitment of EBS to the SOM promoter depends on ATX1-mediated trimethylation at H3K4 (Supplemental...
Histone marker readers, such as MRG1/2, bind to H3K4 or H3K36me3 via their chromodomains and recruit H4-specific acetyltransferases, such as HAM1 and HAM2, which catalyze H4K5 acetylation and promote target gene expression (Xu et al., 2014b). Other proteins, such as CONSTANS or PIF7, have been shown to interact with MRG1/2 and modulate their recognition of H3K4me3 or H3K36me3 around target genes, ultimately controlling plant photoperiodic flowering (for CONSTANS) or plant shade avoidance (for PIF7; Bu et al., 2014; Peng et al., 2018). As is the case for MRG1/2, EBS bridges H3K4me3 and H4K5 acetylation for transcriptional activation of SOM during HT stress based on the observation that the ebs mutation reduced the positive effect of HT on H4K5 acetylation at the SOM locus (Fig. 6D). EBS and its putative paralog SHORTLIFE read H3K27me3 H3 histone marks through the BAH domain to form a complex with EMBRYONIC FLOWER1 (EMF1) and play a Polycomb Repressive Complex1-like repressive role (Li et al., 2018). Furthermore, the EMF1 complex interacts with the MADS-box transcription factors FLOWERING LOCUS C and FLOWERING LOCUS M to suppress FLOWERING LOCUS T expression and prevent flowering (Wang et al., 2014). Our Y2H and Co-IP results show that the MADS-box protein AGL67 also interacts with EBS (Fig. 7, B and C). The germination rate of ebs or agl67 under HT stress was clearly higher than that of Col-0 or c-EBS seeds. In addition, the introduction of c-EBS into the agl67-1 background resulted in the same germination rate as the agl67-1 mutant under HT stress (Fig. 7A) and was accompanied by lower SOM expression under HT stress (Supplemental Fig. S10). These data suggest that EBS depends on AGL67 to activate SOM expression under HT.

In agreement with our findings, AGL67 and SOM expression was low in freshly harvested ebs mutant seeds, as determined by a microarray analysis (Narro-Diego et al., 2018). Furthermore, the EMF1 complex interacts with the MADS-box transcription factors FLOWERING LOCUS C and FLOWERING LOCUS M to suppress FLOWERING LOCUS T expression and prevent flowering (Wang et al., 2014). Our Y2H and Co-IP results show that the MADS-box protein AGL67 also interacts with EBS (Fig. 7, B and C). The germination rate of ebs or agl67 under HT stress was clearly higher than that of Col-0 or c-EBS seeds. In addition, the introduction of c-EBS into the agl67-1 background resulted in the same germination rate as the agl67-1 mutant under HT stress (Fig. 7A) and was accompanied by lower SOM expression under HT stress (Supplemental Fig. S10). These data suggest that EBS depends on AGL67 to activate SOM expression under HT.

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We note that HT treatment increased the affinity of EBS to the P3 region in the SOM promoter in Col-0 seeds but not in agl67 mutant seeds (Fig. 7D), suggesting that EBS requires AGL67 to bind to the SOM P3 promoter region. It is possible that HT increases the transcription rate of AGL67 (Supplemental Fig. S5A), thereby recruiting more EBS to H3K4me3 marks at the SOM promoter and ultimately initiating H4K5 acetylation and opening the chromatin at the SOM locus. In support of this hypothesis, HT stress did not increase H4K5 acetylation at the SOM locus in agl67 mutant seeds (Fig. 7E) and resulted in lower SOM expression (Supplemental Fig. S11). Because HTs increased the protein level of SOM, our data also showed that SOM synergistically enhances the trans-activation effect of AGL67 on SOM expression (Fig. 3D). It is possible that the interaction of SOM and AGL67 collaboratively intensifies the recruitment of EBS to the SOM promoter, ensuring rapid activation of SOM expression following exposure to HT stress. Thus, the SOM-AGL67-EBS module forms a positive feedback loop that efficiently and rapidly magnifies the inhibitory effect of SOM on seed germination, guaranteeing seed dormancy under harsh HT conditions. Future research should compare the effect of SOM on the deposition of EBS to the SOM promoter between Col and som-2 mutant seeds.

In summary, our study uncovers a mechanism that couples a transcription factor, a reader of histone methylation, and chromatin structure reprogramming through histone acetylation during thermoinhibition of seed germination (Fig. 8). Under normal conditions, low levels of SOM and AGL67 cannot induce SOM expression due to the presence of a suppressor complex such as PWR at the SOM locus (Yang et al., 2019). Under these conditions, seeds accumulate more GA and less ABA, ensuring seed germination. When subjected to thermoinhibitory temperatures, HT induces the accumulation of AGL67, which interacts with and recruits EBS to the SOM promoter by reading H3K4me3 marks. Subsequently, H4K5 at the SOM locus is acetylated, which activates SOM expression and ultimately up-regulates ABA biosynthesis and GA degradation, inhibiting seed germination. Concurrently, the interaction of SOM and AGL67 reinforces the trans-activation effect of AGL67, possibly preventing a suppressor complex from binding to the SOM promoter. However, inactivation of AGL67 or EBS decreases the extent of histone acetylation, allowing the suppressor complex to move back onto the promoter, thus silencing SOM expression during seed germination under HT. Together, our findings reveal AGL67 as an important regulator of the thermoinhibition of seed germination and highlight the critical role of EBS in linking H3K4 methylation reading and H4K5 acetylation modifications to the activation of SOM in response to HT.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

- All Arabidopsis (Arabidopsis thaliana) T-DNA insertion mutants, including agl67-1 (SAIL_124_F12), agl67-2 (SALK-144818), ebs (CS906904), and atx1-2 (SALK_140002), were obtained from the Arabidopsis Biological Resource Center. som-2 was generously provided by Gilbsu Choi. ATXI-GFP and ATX1-GR were reported previously (Jing et al., 2019). Double mutants or lines carrying two different transgenes were generated by crossing individual lines and selecting homozygous progeny. Seeds were surface sterilized and sown on 0.8% (w/v) agar (pH 5.7) plates under white light (50 μmol m⁻² s⁻¹). Adult plants were grown in soil with vermiculite (3:1) at 22°C under long-day (16 h of light/8 h of darkness) conditions for 6 to 8 weeks, and seeds were harvested at the same time in each batch for germination or dormancy assays.

**Plasmid Construction and Transgenic Plants**

To generate the SOM-FLAG construct, the 2-kb promoter fragment upstream of the start codon was fused with the SOM coding region by PCR amplification with PrimeSTAR enzyme (Takara) and cloned into the p35Spro fragment with the resulting PCR product. Similarly, a genomic fragment containing an approximately 1-kb upstream region and the coding region of EBS was PCR amplified and cloned into the prRI101-6FLAG vector, removing the 35Spro fragment to generate the EBSpro-EBS-FLAG construct. To generate the 35Spro:AGL67-FLAG construct, the coding region of AGL67 was inserted at the NdeI/Sall sites of the prRI101-6FLAG vector and placed under the control of the 35S promoter. The 2.4-kb SOM promoter fragment was also inserted at the HindIII/BamHI sites of pBII101 to generate the SOMpro:GUS reporter. These constructs were introduced into Arabidopsis Col-0 plants by Agrobacterium tumefaciens-mediated transformation (Clough and Bent, 1998). Primers used for cloning are listed in Supplemental Table S1.

**Seed Germination Assays**

Seeds were harvested and dried for 3 to 5 weeks at room temperature, and seed germination assays were performed as previously described (Chen et al., 2019a; Yang et al., 2019). In brief, seeds were surface sterilized in a 5% (v/v) hypochlorite and 0.02% (v/v) Triton X-100 solution for about 10 min and then rinsed several times with sterile water before being plated on germination medium consisting of one-half-strength Murashige and Skoog salts with 1% (w/v) agar (pH 5.7) plates under white light (50 μmol m⁻² s⁻¹) supplemented with phosphatase inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Proteins were cleared by centrifugation at 15,000g for 10 min at 4°C. Protein concentration was measured using Bradford Quantitative Reagent (Bio-Rad). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes, which were then probed with the appropriate primary anti-FLAG (1:3,000; Sigma-Aldrich), anti-GFP (1:3,000; Clontech), or anti-actin (1:1,000; Sigma-Aldrich) antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:3,000; Promega). Signals were detected using a ONE-HOUR IP-Western Kit (Genscript).

**Protein Extraction and Immunoblots**

We extracted total protein from hydrated seeds using extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8, 0.1% [v/v] Triton X-100, 10 mM NaF, and 5% [v/v] glycerol) supplemented with phosphatase inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Proteins were cleared by centrifugation at 15,000g for 10 min at 4°C. Protein concentration was measured using Bradford Quantitative Reagent (Bio-Rad). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes, which were then probed with the appropriate primary anti-FLAG (1:3,000; Sigma-Aldrich), anti-GFP (1:3,000; Clontech), or anti-actin (1:1,000; Sigma-Aldrich) antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:3,000; Promega). Signals were detected using a ONE-HOUR IP-Western Kit (Genscript).

**Y1H and Y2H Assays**

The Matchmaker GoldY1H Library Screening system (Clontech) was used for Y1H and Y2H assays. The construct carrying the 2-kb SOM promoter fragment was linearized, and the bait-reporter yeast (Saccharomyces cerevisiae) strain was obtained by integrating the linearized construct into the YHGold strain genome. The cDNA library was constructed with mRNAs isolated from wild-type hydrated seeds. The bait-reporter strain and cDNA library were used to screen for putative SOM regulators. Positive colonies were isolated on selective medium (synthetic dextrose/-Leu) with 100 ng mL⁻¹ aureobasidin A. Positive prey fragments were identified by PCR and DNA sequencing and BLAST. For the Y2H assay, the full-length cDNAs of AGL67, SOM, and EBS were cloned into the pGBKTO prey vector and the pGADT7 prey vector using the
In-Fusion cloning system (Clontech). Two-hybrid screening was performed using the mating proto-Co-I described in the Matchmaker Gold Yeast Two-Hybrid user manual (Clontech).

**In Vitro Pull-Down Assays**

The coding regions for AGL67 and SOM were cloned into the pGEX-4T-1 (Pharmacia) and pET28a (Merck) vectors to generate the pGST-AGL67 and pHis-SOM constructs, respectively. The primers used for construction are listed in Supplemental Table S1. For prokaryotic protein expression, the constructs were transformed into the Escherichia coli Rosetta strain, and protein accumulation was induced by isopropyl β-D-1-thiogalactopyranoside. Soluble GST-AGL67 protein was extracted and immobilized onto Glutathione Sepharose beads (GE Healthcare), while the soluble His-SOM was extracted and immobilized onto Ni-NTA agarose beads (Qiagen). For pull-down assays, 2 μg of His-SOM was incubated with GST alone or GST-AGL67 in binding buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.1% [v/v] Triton X-100) at 4°C overnight. Pulldown proteins were extensively washed with buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.6% [v/v] Triton X-100) before the samples were resolved on 8% SDS-PAGE gels and analyzed by immunoblot analysis using anti-His antibody (Abmart) or anti-GST antibody (Abmart) followed by a mouse secondary antibody (1:5,000; Promega).

**CoIP**

Plants containing the transgenes AGL67-GFP and SOM-FLAG or AGL67-GFP and EBS-FLAG were generated by crossing AGL67-GFP with SOM-FLAG or EBS-FLAG, respectively. We also crossed a line expressing GFP to SOM-FLAG and a line expressing a GFP-GFP fusion to EBS-FLAG to generate suitable negative control genotypes. For the in vivo Co-IP using AGL67-GFP as bait, hydrated AGL67-GFP SOM-FLAG or AGL67-GFP EBS-FLAG transgenic seeds were ground to a fine powder in liquid nitrogen. Total proteins were extracted in MOPS buffer (100 mM MOPS, pH 7.6, 150 mM NaCl, 0.1% [w/v] Nonidet P-40, 40 μg mL−1 ethidium bromide, 2 μg mL−1 aprotinin, 5 μg mL−1 leupeptin, 1 μg mL−1 pepstatin, 2× Complete Protease Inhibitor Cocktail, and PhosStop Cocktail from Roche), centrifuged at 13,000 rpm at 4°C for 10 min, and filtered through two layers of Miracloth. Supernatant (1 mL) was incubated with GFP-Trap (Chromotek) overnight under gentle rotation at 4°C. Beads were washed four times with wash buffer (50 μL Tris, pH 8, 150 mM NaCl, and 0.1% [v/v] Triton X-100). The supernatants were sequentially passed through preconditioned tandem columns of 1 mL of 10% (v/v) acetonitrile and sub- followed by the addition of 50 μL of 10% (v/v) acetonitrile and sub-

**EMSA**

EMSA was performed as previously described (Hu et al., 2014). Briefly, oligonucleotide probes were synthesized, annealed, and labeled using the Biotin-DNA labeling kit (Pierce). E. coli BL21 cells that had been transformed with pGST-AGL67 were induced with the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside at 16°C when the OD600 reached 1.5. Cells were lysed in lysate buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.1% [v/v] Triton X-100), and the proteins were eluted at 95°C for 10 min in 2× loading buffer (100 mM Tris-HCl, pH 6.8, 200 μM DTT, 2% [w/v] SDS, 20% [v/v] glycerol, and 0.2% [w/v] Bromophenol Blue) and analyzed by immunoblotting with anti-FLAG (1:3,000; Sigma-Aldrich) or anti-GFP (1:3,000; Clontech) monoclonal antibodies.

**RT-qPCR Analysis**

Total RNA was extracted from hydrated seeds using TRIzol reagent (Invitrogen). RT-qPCR was performed as described (Hu et al., 2014). Briefly, first-strand cDNA was synthesized from 1.5 μg of DNase-treated RNA in a 20 μL reaction volume using Moloney murine leukemia virus reverse transcriptase (Fermentas) with oligo(dT)18 primer. The cDNA samples were diluted to 2 to 10 ng μL−1, and qPCR was performed in the presence of SYBR Green 1 Master Mix on a Roche LightCycler 480 real-time PCR machine according to the manufacturer’s instructions. All RT-qPCR experiments were independently performed in triplicate, and representative results are shown. PPA2 was used as an internal control. The primer pairs for RT-qPCR are listed in Supplemental Table S1.

**Protoplast Transient Expression Assay**

For the transient expression assay, a 2.4-kb SOM promoter fragment was inserted into the pGreenII 0800-LUC vector to generate a series of SOMpro::LUC reporter constructs. The coding sequences of AGL67 and SOM were inserted into the pGreenII 62-SK vector and placed under the control of the 35S promoter. For analysis of the truncated SOM promoter activity, the corresponding CArG-box in the SOM promoter was simultaneously removed to generate different SOM promoter variants (pSOMΔP3 and pSOMΔM4), as described in the text. The variants of the SOM promoter were also inserted into the pGreenII 0800-LUC vector to generate the reporter constructs. All primers used for these constructs are listed in Supplemental Table S1. After protoplast preparation and subsequent transfection, firefly luciferase and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), and the LUC/REN ratio was presented.

**ChIP-qPCR Analysis**

Chromatin affinity purification was performed as described previously (Hu et al., 2014). Seeds were cross-linked with a 1% (v/v) formaldehyde solution under a vacuum for 1 h. The chromatin was extracted and sheared to an average length of 300 to 500 bp by sonication and then immunoprecipitated with specific antibodies, including anti-GFP (catalog no. 6795; Sigma-Aldrich), anti-FLAG M2 gel (catalog no. A2220; Sigma-Aldrich), anti-histone H4 (catalog no. 04-858; Millipore), and anti-acetyl-histone H4K5 (catalog no. 07-327; Millipore). The cross-linking was then reversed, and the amount of each immunoprecipitated DNA fragment was determined by qPCR using gene-specific primers (Supplemental Table S1).
For ABA measurement, seeds were ground in liquid nitrogen, and 45 pmol of [H]ABA internal standard was added to 200 μg of powder. The samples were extracted with 2 mL of methanol at 20°C overnight. After centrifugation at 4°C for 15 min at 18,000 rpm, the supernatant was dried under nitrogen gas and dissolved in 1 mL of 5% (v/v) ammonia solution. Crude extracts were purified on a preconditioned Oasis MAX strong anion-exchange column (Waters), and the samples were eluted with 4 mL of methanol containing 5% (v/v) formic acid. The eluent was dried under nitrogen gas and dissolved in 200 μL of 80% (v/v) methanol and then subjected to ultra-performance liquid chromatography–tandem mass spectrometry analysis.

GUS Staining

Seeds from different genetic backgrounds and homoygous for the SOM-proGUS reporter were hydrated in water for 3 h and then plated on 0.8% (w/v) agar (pH 5.7) for 24 h at normal conditions (22°C) or HT (32°C). After heat treatment, seeds were incubated in 0.1 mM sodium phosphate buffer containing 50 mM K3Fe(CN)6, 50 mM K4Fe(CN)6, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide at 37°C for 12 h. GUS staining was examined with a stereo-microscope, and images were captured with a digital camera (Zeiss).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: ABA1, AT5G67030; ATX1, AT2G31650; AGL67, AT1G77950; CYP707A2, AT2G29090; EBS, AT4G22140; and SOM, AT1G03790.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Validation of SOM-FLAG, 35Spro:SOM-GFP, and 35Spro:Agl7-FLAG by immunoblot analysis.

Supplemental Figure S2. Agl67 binds to the SOM promoter in a Y1H analysis.

Supplemental Figure S3. Expression pattern of Agl67 in different tissues or developmental stages.

Supplemental Figure S4. Identification of T-DNA insertion mutants in Agl67.

Supplemental Figure S5. Genotype analysis of the crossed lines of agl67 and SOM-GFP.

Supplemental Figure S6. HT regulates transcript levels of GA and ABA metabolic genes.

Supplemental Figure S7. Identification of a T-DNA insertion mutant of EBS.

Supplemental Figure S8. EBS regulates SOM expression and alters downstream GA/ABA metabolic genes.

Supplemental Figure S9. ATX1 influences the binding of EBS to the SOM promoter.

Supplemental Figure S10. ATX1 mediates the HT-induced increase of H3K4me3 level at the SOM locus, thus activating SOM expression to suppress seed germination.

Supplemental Figure S11. SOM transcript levels in Col-0, c-ebs, and ebs mutant seeds before and after HT stress.

Supplemental Table S1. List of primers used in this study.

Supplemental Data Set S1. Sequence information of the SOM promoter and its truncated versions.

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