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RESEARCH ARTICLE

Basic LEUCINE ZIPPER TRANSCRIPTION FACTOR67 Transactivates DELAY OF GERMINATION1 to Establish Primary Seed Dormancy in Arabidopsis

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Short title: bZIP67 regulates DOG1 expression

One-sentence summary: Basic LEUCINE ZIPPER TRANSCRIPTION FACTOR67 acts downstream of LEAFY COTYLEDON1 to induce DELAY OF GERMINATION1 expression during Arabidopsis seed maturation and to establish primary dormancy.

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ABSTRACT

Seed dormancy governs the timing of germination, one of the most important developmental transitions in a plant’s life cycle. The DELAY OF GERMINATION1 (DOG1) gene is a key regulator of seed dormancy and a major quantitative trait locus in Arabidopsis thaliana. DOG1 expression is under tight developmental and environmental regulation, but the transcription factors involved are not known. Here we show that basic LEUCINE ZIPPER TRANSCRIPTION FACTOR67 (bZIP67) acts downstream of the central regulator of seed development, LEAFY COTYLEDON1, to transactivate DOG1 during maturation and help to establish primary dormancy. We show that bZIP67 overexpression enhances dormancy and that bZIP67 protein (but not transcript) abundance is increased in seeds matured in cool conditions, providing a mechanism to explain how temperature regulates DOG1 expression. We also show that natural allelic variation in the DOG1 promoter affects bZIP67-dependent transactivation, providing a mechanism to explain ecotypic differences in seed dormancy that are controlled by the DOG1 locus.

INTRODUCTION

Seed dormancy is a complex life history trait that plays an important role in local adaptation, as well as dispersal (Finch-Savage and Leubner-Metzger 2006). Dormancy prevents seed germination when immediate environmental conditions are suitable, but the longer-term probability of survival is low (Finch-Savage and Leubner-Metzger 2006). In the weedy annual species Arabidopsis thaliana, seed dormancy varies geographically, with strong dormancy being more prevalent where summers are long and dry, and weak
dormancy being associated with short wet summers (Chiang et al., 2011; Kronholm et al., 2012). Alonso-Blanco et al. (2003) mapped several quantitative trait loci (QTL) that control dormancy using a biparental recombinant inbred (RI) population derived from a cross between the highly dormant ecotype Cape Verde Islands (Cvi-0) and a less dormant ecotype Landsberg erecta (Ler-0). The first of these QTL to be cloned was *DELAY OF GERMINATION1 (DOG1)* (Alonso-Blanco et al., 2003; Bentsink et al., 2006). DOG1 is thought to act as a timer for seed dormancy release, since it is modified during after- ripening (Nakabayashi et al., 2012). Its mode of action has been the subject of several studies and remains to be fully elucidated (Nakabayashi et al., 2012; 2015; Graeber et al., 2014; Dekkers et al., 2016; Huo et al., 2016; Née et al., 2017). *DOG1* expression is regulated by key environmental cues that control dormancy, such as temperature (Chiang et al., 2011; Kendall et al., 2011; Nakabayashi et al., 2012), and by allelic variation in *DOG1*, which appears to explain a substantial proportion of the phenotypic variation in dormancy observed in wild populations of Arabidopsis (Alonso-Blanco et al., 2003; Bentsink et al., 2006; Chiang et al., 2011; Kerdaffrec et al., 2016).

Genetic studies have shown that a network of transcriptional master regulators orchestrates the seed maturation program, of which the establishment of primary dormancy is a part (Vicente-Carbajosa and Carbonero 2005; Santos-Mendoza et al., 2008). In Arabidopsis, four transcription factors (TFs) function as positive regulators of this process: *LEAFY COTYLEDON1 (LEC1)*, *ABSCISIC ACID INSENSITIVE3 (ABI3)*, *FUSCA3 (FUS3)* and *LEC2* (Giraudat et al., 1992; Meinke 1992; Keith et al., 1994; West et al., 1994; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001). LEC1 acts at the highest level in the regulatory hierarchy controlling the maturation phase (West et al., 1994; Santos-Mendoza et al., 2008) and encodes a homologue of the mammalian nuclear TF YB (NF-YB) subunit of the trimeric CCAAT-box binding complex (CBC) (Lotan et al., 1998; Lee et al., 2003). ABI3, FUS3 and LEC2 (known collectively as AFL) encode TFs containing a conserved B3 DNA binding domain, which is specific to plants (Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001). *DOG1* is induced during seed maturation (Bentsink et al., 2006; Nakabayashi et al., 2012), and its expression is known to rely indirectly on LEC1 (Pelletier et al., 2017) and to require AFL (Braybrook et al., 2006; Mönke et al., 2012; Wang and Perry 2013; González-Morales et al., 2016). Kendall et al., (2011) also showed that enhanced *DOG1* expression in seeds matured at low temperature requires C-REPEAT BINDING FACTORS from the APETALA2-domain TF family. *DOG1* is also regulated by alternative splicing, alternative polyadenylation, histone modifications
and a cis-acting antisense non-coding transcript (asDOG1) (Bentsink et al., 2006; Müller et al., 2012; Graeber et al., 2014, Cyrek et al., 2016; Fedak et al., 2016).

Despite previous studies on DOG1 regulation, it is not known precisely which TFs bind to the DOG1 promoter and are responsible for driving its expression during embryo maturation, nor is it known how temperature (Chiang et al., 2011; Kendall et al., 2011; Nakabayashi et al., 2012) and allelic variation in the DOG1 promoter (Bentsink et al., 2006; Kerdaffrec et al., 2016) affect their ability to perform this function. We previously carried out a reverse genetic screen on TFs that are induced by LEC1 during Arabidopsis seed maturation and identified basic LEUCINE ZIPPER TRANSCRIPTION FACTOR 67 (bZIP67) as a regulator of several genes involved in seed storage reserve deposition (Mendes et al., 2013). Here, we show that bZIP67 is also a direct regulator of DOG1 expression, specifying LEC1’s action in the establishment of primary dormancy. We also show that temperature regulates bZIP67 at the level of protein abundance and that bZIP67-dependent transactivation of DOG1 is affected by natural variation in the gene promoter (Bentsink et al., 2006; Kerdaffrec et al., 2016).

RESULTS

bZIP67 is required for DOG1 expression and protein accumulation

We previously characterized two Arabidopsis mutant alleles of bZIP67 in ecotype Col-0 (Mendes et al., 2013). Affymetrix ATH1 microarray experiments indicated that DOG1 expression may be reduced by as much as 13-fold in whole developing siliques of bzip67-1 (Mendes et al., 2013). To test whether bZIP67 is required for DOG1 expression during seed development, we performed quantitative (q)RT-PCR analysis of transcript abundance in wild type (WT), bzip67-1 and bzip67-2 plants grown in standard conditions (i.e. 22°C 16h light/16°C 8h dark cycle) (Nakabayashi et al., 2012; 2015) (Figure 1). DOG1 is alternatively spliced, producing five transcript variants, of which epsilon is the major form (Nakabayashi et al., 2015). A noncoding antisense DOG1 RNA (asDOG1) is also expressed independently of the sense transcripts (Fedak et al., 2016). Using a qRT-PCR primer pair that detects all sense transcripts, we determined that total DOG1 transcript abundance in WT increased over the linear cotyledon (LCOT), mature green (MG) and post mature green (PMG) stages of embryo development (Pelletier et al., 2017) and then declined in freshly harvested dry seeds (DS) (Nakabayashi et al., 2012; 2015) (Figure 1A). However, in bzip67-1 and bzip67-2 seeds, total DOG1 transcript abundance was significantly (P<0.05) lower, with the largest reduction (>10-fold) detected at the MG and
PMG stages, when expression in WT is strongest (Figure 1A). We also performed immunoblot analysis to quantify DOG1 in DS (Figure 1B), when the protein is most abundant (Nakabayashi et al., 2012). The antibody was raised against an N-terminal peptide (Cyrek et al., 2016) that is conserved in all Col-0 DOG1 isoforms (Nakabayashi et al., 2015). Although we could detect DOG1 protein in WT, it was >7-fold less abundant in bzip67-1 and bzip67-2 (Figure 1B). DOG1 was also absent from the dog1-2 (Nakabayashi et al., 2012) negative control and was recovered to a WT level in the bzip67-1 mutant when complemented with a T-DNA construct expressing GFP-bZIP67 under the control of the bZIP67 promoter (Bensmihen et al., 2005; Mendes et al., 2013) (Figure 1B). These data show that bZIP67 is required for DOG1 expression and protein accumulation in Arabidopsis seeds.

bZIP67 is required for the establishment of primary seed dormancy

To investigate whether disruption of bZIP67 affects primary seed dormancy, we grew WT, bzip67-1 and bzip67-2 plants in standard conditions and scored the percentage of seeds that had germinated at 3 d after imbibition (Nakabayashi et al., 2012; 2015) (Figure 2). Freshly harvested WT seed batches exhibited <20% germination, and an after-ripening period of >1 week was required before >90% germination was achieved (Nakabayashi et al., 2012; 2015) (Figure 2A). By contrast, >90% of fresh bzip67-1 and bzip67-2 seeds germinated before after-ripening (Figure 2A). Seed batches from the ‘complemented’ bzip67-1 mutant containing a T-DNA construct expressing GFP-bZIP67 under the control of the bZIP67 promoter (Bensmihen et al., 2005; Mendes et al., 2013) exhibit a WT dormancy phenotype (Figure 2A). We also grew the Col-0 dog1-2 mutant (Nakabayashi et al., 2012) and a bzip67-1 dog1-2 double mutant as controls and confirmed that they have a similar reduced dormancy phenotype to bzip67-1 (Supplemental Figure 1). These data show that bZIP67 is required for the establishment of primary seed dormancy and raise the possibility that bZIP67 functions within the same pathway as DOG1.

Overexpression of bZIP67 enhances seed dormancy

To determine whether overexpression of bZIP67 might cause a gain-of-function phenotype, we analysed three independent overexpression (OE) lines in which bZIP67 is under the control of the strong embryo maturation-specific glycinin (GLY) promoter (Mendes et al., 2013) (Figure 2B). Disruption of bZIP67 reduced DOG1 expression in freshly harvested seeds (Figure 1A). By contrast, total DOG1 transcript abundance was
significantly ($P<0.05$) enhanced in freshly harvested seeds of the OE lines grown in standard conditions, as compared to WT (Figure 2B). The freshly harvested OE seeds also exhibited deeper dormancy, requiring a longer period of after-ripening than WT to achieve >90% germination (Figure 2C). However, when *bZIP67* was overexpressed in the *dog1-2* background, the enhanced seed dormancy was suppressed (Supplemental Figure 1). These data suggest that *bZIP67* may contribute to the regulation of seed dormancy through the control of *DOG1* expression.

**bZIP67 protein abundance is increased in seeds matured in cool conditions**

When Arabidopsis seeds mature in cool conditions, *DOG1* transcript levels remain high right through to the end of seed desiccation (Kendall et al., 2011), contributing to a deeper state of dormancy (Chiang et al., 2011; Kendall et al., 2011; Nakabayashi et al., 2012; Murphey et al., 2015). To investigate whether *bZIP67* function also affects dormancy in seeds matured in cool conditions, we grew plants to flowering in standard (22°C 16h light / 16°C 8h) conditions and then transferred them to cool (16°C 16h light / 14°C 8h dark) conditions (Nakabayashi et al., 2012) (Figure 3). In ecotype Col-0, *dog1* seeds are dormant when matured in cool conditions, but the level of dormancy is reduced relative to WT (Kendall et al., 2011; Murphey et al., 2015). We observed a similar reduced dormancy phenotype for *bzip67*, where significantly ($P<0.05$) fewer days of seed dry storage (DSDS) than WT were required before >50% of *bzip67* seeds germinated (Murphey et al., 2015) (Figure 3A). Seed batches of *bzip67-1 ProbZIP67:GFP-bZIP67* (Mendes et al., 2013) exhibited WT dormancy levels when matured in cool conditions (Figure 3A). We measured total transcript abundance in the mature seeds of this complemented line and found that, unlike *DOG1* (Kendall et al., 2011; Nakabayashi et al., 2012), there was no significant increase ($P>0.05$) in *bZIP67* expression in cooler conditions (Figure 3B). However, immunoblot analysis performed using an anti-GFP antibody showed that GFP-bZIP67 protein quantity was increased approximately 2-fold in cooler conditions (Figure 3C). These data suggest that bZIP67 is subject to posttranscriptional regulation by temperature; increased bZIP67 abundance could explain why *DOG1* expression is enhanced in seeds matured in cool conditions (Chiang et al., 2011; Kendall et al., 2011; Nakabayashi et al., 2012).

**Ectopic expression of LEC1 induces bZIP67 and DOG1 expression**
Ectopic expression of *LEC1* in Arabidopsis triggers somatic embryogenesis and activates the transcriptional program for embryo maturation (Lotan et al., 1998; Pelletier et al., 2017). When *LEC1* is expressed, it binds to and activates *bZIP67*, and *lec1* embryos are also deficient in both *bZIP67* and *DOG1* expression (Pelletier et al., 2017). To test whether *LEC1* also induces *DOG1* expression, we transfected Arabidopsis mesophyll protoplasts with a *LEC1* effector plasmid driven by the CaMV 35S promoter or with an empty vector control (EVC) (Yamamoto et al., 2009; Mendes et al., 2013) and measured both *bZIP67* and total *DOG1* transcript abundance over 5 d (Figure 4). *LEC1* expression led to a significant (*P*<0.05) increase in *bZIP67* transcript abundance by 2 d after transfection, and by 5 d, *bZIP67* expression was >80-fold higher than in the EVC (Figure 4A). A significant (*P*<0.05) increase in *DOG1* expression was also detected following transfection with the *LEC1* effector plasmid, but it occurred ~1 d later than *bZIP67*, and by 5 d, *DOG1* expression was >25-fold higher than in the EVC (Figure 4A). These data show that *DOG1* is induced by *LEC1* expression and that this occurs after the induction of *bZIP67*.

### *bZIP67* binds to the *DOG1* promoter

To determine whether *bZIP67* binds to the *DOG1* promoter, we performed chromatin immunoprecipitation (ChIP)-qPCR experiments on protoplasts from *bzip67-1 ProbZIP67:GFP-bZIP67* and WT plants transfected with *Pro35S:LEC1* (Mendes et al., 2013). We carried out qPCR using primer pairs corresponding to six regions of *DOG1* (Figure 4B) and also to *ACTIN7* (*ACT7*) as a negative control. Amplicons P2, P3 and P4, spanning 0 to 1 kb upstream of the *DOG1* transcriptional start site (TSS), were significantly (*P*<0.05) enriched in *bzip67-1 ProbZIP67:GFP-bZIP67* compared to either the *ACT7* or WT controls, and enrichment was strongest at P3 (~400 bp), suggesting that *bZIP67* binds to this region of the *DOG1* promoter (Figure 4C). No enrichment was observed using P3 when protoplasts were transfected with an empty vector control (EVC) rather than *Pro35S:LEC1* (Figure 4C). To confirm that *bZIP67* binds to the *DOG1* promoter *in vivo*, we also performed ChIP-qPCR experiments on developing (MG-PMG stage) seeds of *bzip67-1 ProbZIP67:GFP-bZIP67* plants (Mönke et al., 2012; Pelletier et al., 2017) and detected a significant (*P*<0.05) enrichment using P3 (Supplemental Figure 2).

We have previously shown that *bZIP67* can bind to G box-like (GBL) *cis*-elements with the core sequence 5'-ACGT-3' (Mendes et al., 2013). The *DOG1* promoter contains multiple GBL elements (Nakabayashi et al., 2012), but only GBL1 and GBL2 (both 5'-CAGCTA-3') are present in the -400 bp region (Figure 4B and Supplemental Figure 3). To
test whether bZIP67 can bind to GBL1 and GBL2, we performed a DNA-protein-interaction enzyme-linked immunosorbent assay (DPI-ELISA) (Brand et al., 2010) (Figure 4D). Epitope-tagged recombinant bZIP67 was incubated with immobilized double-stranded DNA oligonucleotides and binding was determined by immuno-detection (Mendes et al., 2013). When bZIP67 was applied to oligonucleotides containing GBL1 and GBL2, the ELISA signal was >30-fold stronger than when an equal concentration of the corresponding GBL oligonucleotides with mutated (KO) 5′-ACGT-3′ cores (Mendes et al., 2013) were tested (Figure 4D). In competition experiments to define specificity, the addition of free GBL oligonucleotides also significantly reduced the ELISA signal from bound GBLs (P<0.05), while the addition of free GBL KO oligonucleotides did not (Supplemental Figure 4). A combination of in vivo and in vitro experiments therefore suggests that bZIP67 binds to the DOG1 promoter.

Transactivation of DOG1 by bZIP67 requires LEC1 expression

Next, we tested whether bZIP67 expression is sufficient to transactivate DOG1 alone, or whether it requires LEC1 and/or other regulatory factors induced by LEC1 (Pelletier et al., 2017) (Figure 5). We cloned a ~600 bp region of the Col-0 DOG1 promoter containing GBL1 and GBL2 (Figure 5A) upstream of β-glucuronidase (GUS) and transfected the construct into bzip67-1 protoplasts in combination with Pro35S:bZIP67, Pro35S:LEC1 or empty vector control (EVC) effector plasmids (Yamamoto et al., 2009; Mendes et al., 2013). Protoplasts from bzip67-1 were used to prevent induction by endogenous bZIP67 (Kagaya et al., 2005; Mu et al., 2008) (Figure 4A). Co-transfection of ProDOG1:GUS with EVC resulted in minimal GUS reporter activity (Figure 5B), which is consistent with the finding that DOG1 expression is restricted to seeds (Bentsink et al., 2006; Nakabayashi et al., 2012). Co-transfection with Pro35S:bZIP67 did not enhance GUS activity significantly (P>0.05). Co-transfection with Pro35S:LEC1 led to a <2-fold increase in GUS activity (Figure 5B). However, when Pro35S:bZIP67 was co-transfected together with Pro35S:LEC1, the level of GUS activity was enhanced by >18-fold (Figure 5B). These data suggest that bZIP67 is required for DOG1 expression, but is not sufficient, and that transactivation also relies on the expression of LEC1 (Pelletier et al., 2017). LEC1 induces the expression of several regulators of embryo maturation that may participate in DOG1 induction, including CBC components LEC1-like (L1L) (Kwong et al., 2003) and NF-YC2 (Yamamoto et al., 2009) and AFL (Mu et al., 2008; Pelletier et al., 2017). There is also evidence that bZIPs form ternary complexes with AFL and CBC to regulate transcription.
during Arabidopsis seed maturation (Nakamura et al., 2001; Yamamoto et al., 2009; Mendes et al., 2013; Baud et al., 2016).

**Transactivation of ****DOG1** by **bZIP67** requires **GBL** and **RYL** promoter elements
To determine whether transactivation of **DOG1** requires the **GBL** and **GBL2** **cis-elements** in the promoter, we mutated the 5'-ACGT-3' core sequences in the **ProDOG1:GUS** construct (Mendes et al., 2013) and tested its ability to drive GUS expression in bzip67-1 protoplasts when co-transfected together with **Pro35S:bZIP67** and **Pro35S:LEC1**.
Disruption of **GBL1** and **GBL2** each reduced GUS reporter activity by >70%, and mutations in both blocked transactivation of **GUS** completely (Figure 5C). ChIP experiments have suggested that **LEC1** does not bind **DOG1** (Pelletier et al., 2017) and that CCAAT-box motifs that may be bound directly by CBC containing **LEC1** or **L1L** (Gnesutta et al., 2017) are absent from the -600 bp **DOG1** promoter. However, an overlapping **RY**-like (**RYL**) element (5'-GCATGC-3') repeat exists between **GBL1** and **GBL2** (Figure 4A and Supplemental Figure 3). This putative **cis-element** could be bound by **AFL** (Braybrook et al., 2006; Baud et al., 2016). **AFL** are induced by **LEC1** (Pelletier et al., 2017) and are required for **DOG1** expression (Braybrook et al., 2006; Mönke et al., 2012; Wang and Perry 2013; González-Morales et al., 2016; Pelletier et al., 2017). Furthermore, ChIP experiments suggested that **AFL** **FUS3** binds to **DOG1** **in vivo** (Wang and Perry 2013). Mutating the core sequence of the repeated **RYL** **cis-element** (Baud et al., 2016) eliminated GUS reporter activity driven by **Pro35S:bZIP67** and **Pro35S:LEC1** (Figure 5C). These data suggest that **bZIP67-dependent transactivation** of **DOG1**, induced by **LEC1**, relies on **GBL** and **RYL** **cis-elements**.

**Natural variation in the ****DOG1** promoter affects **bZIP67-dependent transactivation**
**DOG1** was originally cloned by QTL mapping by exploiting the natural variation in dormancy observed between ecotypes Cvi-0 and Ler-0, and expression analysis revealed a positive correlation between **DOG1** transcript abundance and dormancy in these (and many other) Arabidopsis accessions (Bentsink et al., 2006; Chiang et al., 2011; Kerdaffrec et al., 2016). Although allelic variation at the **DOG1** locus explains a substantial proportion of phenotypic variation in seed dormancy, it is not known how **cis**-variation affects **DOG1** expression (Bentsink et al., 2006; Chiang et al., 2011; Kerdaffrec et al., 2016). We therefore created **ProDOG1:GUS** constructs using -600 bp promoter regions from Cvi-0 and Ler-0 and tested their ability to drive **GUS** reporter expression when co-transfected
together with Pro35S:bZIP67 and Pro35S:LEC1 in bzip67-1 protoplasts (Figure 6). GUS reporter activity driven by the Ler-0 promoter (Figure 6A) was >5-fold lower than when driven by that of Cvi-0 or Col-0 (Figure 6B). The intergenic region upstream of DOG1 in Ler-0 and Cvi-0 contains 15 sequence variants, including three within the -600 bp promoter region (Bentsink et al., 2006). None of these variants lie in GBL1, GBL2 or RYL, but a 285 bp insertion/deletion (INDEL) is present at -328 bp, situated between these cis-elements and the TSS (Figure 6A and Supplemental Figure 3).

Deletion of this 285 bp insertion from the Ler-0 DOG1 promoter led to a >5-fold increase in GUS reporter activity, and conversely, its insertion into either the Cvi-0 or Col-0 DOG1 promoters led to a >5-fold decrease in GUS reporter activity (Figure 6B). These data suggest that the INDEL may be responsible for the allele-specific difference in DOG1 transcript abundance observed between Ler-0 and Cvi-0 (Bentsink et al., 2006). It is not clear precisely how the INDEL affects DOG1 expression. It might act by introducing repressive cis-elements into the promoter or by changing its length/context, i.e., the distance between existing cis-elements and the core promoter region most proximal to the TSS (Liu et al., 2014). To help distinguish between these possibilities, we replaced the Ler-0 insertion with an unrelated 285 bp intergenic sequence from Arabidopsis and found that this also suppressed bZIP67-dependent GUS reporter activity (Figure 6B). This result suggests that the INDEL could simply acts as a spacer (Liu et al., 2014). Although we cannot discount the possibility that cis-elements might also exist in the insertion, they do not appear to be required to attenuate DOG1 expression.

Natural variation in the DOG1 promoter and GBL elements affect dormancy
To test whether the INDEL and GBLs affect DOG1 function in vivo, we transformed the non-dormant dog1-2 mutant with a ~5 kb genomic construct containing Col-0 DOG1 (Nakabayashi et al., 2015) and also with variants of this construct containing either the 285 bp Ler-0 insertion or mutations in GBL1 and GBL2 (Figure 7). Analysis of freshly harvested seed batches from multiple homozygous transgenic lines grown in standard conditions showed that the Col-0 DOG1 genomic clone could complement dog1-2 (Nakabayashi et al., 2015), whereas the variant clones with either the 285 bp Ler-0 insertion or GBL1 and GBL2 mutations failed to restore WT levels of seed dormancy (Figure 7A). We also measured DOG1 expression in freshly harvested seeds of the transgenic lines using a qRT-PCR primer pair selective for the WT allele (Nakabayashi et al., 2012). Total DOG1 transcript abundance in dog1-2 seeds containing the Col-0 DOG1 genomic construct was
>6-fold higher than in seeds containing the variant clones with either the 285 bp Ler-0 insertion or GBL1 and GBL2 mutations (Figure 7B). These data confirm the notion that GBL1 and GBL2 are necessary for in vivo DOG1 expression, and they indicate that the 285 bp INDEL also modifies DOG1 expression, and consequently, the strength of primary dormancy (Bentsink et al., 2006; Nakabayashi et al., 2012).

**DISCUSSION**

In this study, we showed that the bZIP67 TF induces DOG1 expression during Arabidopsis seed development by binding to GBL cis-elements in the promoter of this gene and that it operates downstream of LEC1 and likely in concert with other central regulators of seed maturation from the CBC and AFL TF families (Figure 8). Models based on temporal and spatial transcriptional profiling have previously placed bZIP67 within the regulatory circuitry that governs seed maturation (Belmonte et al., 2013). bZIP67 expression relies on LEC1 and AFL (Kagaya et al., 2005; Braybrook et al., 2006; Mu et al., 2008; Mönke et al., 2012; Wang and Perry 2013; González-Morales et al., 2016; Pelletier et al., 2017), and there is evidence that CBC and AFL form ternary complexes with bZIPs to transactivate seed maturation genes (Nakamura et al., 2001; Yamamoto et al., 2009; Baud et al., 2016). It remains to be determined whether CBC or AFL are direct regulators of DOG1 expression. Whole-genome ChIP experiments suggested that FUS3 binds to DOG1 (Wang and Perry 2013), while Pelletier et al., (2017) and Mönke et al., (2012) did not detect DOG1 binding by LEC1 and ABI3, respectively. Unlike mutants in many of these central regulators of seed maturation, bzip67 lacks a morphological phenotype (Bensmihen et al., 2005; Belmonte et al., 2013). However, we previously found that DOG1 is one of just a few seed maturation-associated genes that are strongly downregulated in developing bzip67 siliques (Mendes et al., 2013), and we have shown here that, because bZIP67 is required for DOG1 expression in developing seeds, it is also required for the establishment of primary seed dormancy. It is noteworthy that bZIP67 maps adjacent to two dormancy genes (DOG6 and REDUCED DORMANCY1) whose identities are unclear, although they are unlikely to be synonymous, based on their contrasting phenotypes (Léon-Kloosterziel et al., 1996; Peeters et al., 2002; Alonso-Blanco et al., 2003).

Temperature is a key environmental regulator of DOG1 expression and seed dormancy in Arabidopsis (Chiang et al., 2011; Kendall et al., 2011; Nakabayashi et al., 2012). We demonstrated that cool conditions during seed maturation enhance bZIP67 protein (but not transcript) abundance and that overexpression of bZIP67 can increase
**DOG1** expression and cause dormancy to deepen. bZIP67 is one of four bZIPs from clade A, which are expressed in Arabidopsis seeds (Bensmihen et al., 2005). The best-characterised of these bZIPs is ABSCISIC ACID INSENSITIVE5 (ABI5), which is also bZIP67’s closest homologue (Bensmihen et al., 2005). ABI5 functions in ABA signalling and regulates seed germination and early seedling growth in response to abiotic stress (Finkelstein et al., 2000; Lopez-Molina et al., 2001; Skubacz et al., 2016). *ABI5* is expressed later in seed development than *bZIP67* (Bensmihen et al., 2002; 2005) and does not appear to be required for primary dormancy (Finkelstein 1994), although it is regulated by *DOG1* (Dekkers et al., 2016). Interestingly, ABI5 is subject to extensive posttranslational regulation (Skubacz et al., 2016), and key phosphorylation, ubiquitination, and S-nitrosylation sites identified in ABI5 are also within regions conserved in bZIP67 (Supplemental Figure 5). Group A bZIPs are also thought to form heterodimers (Deppmann et al., 2004). They can bind to similar (or identical) GBL cis-elements (Finkelstein 1994; Carles et al., 2002; Deppmann et al., 2004) and have negative, as well as positive, regulatory functions (Finkelstein 1994; Finkelstein and Lynch 2000). Further work will therefore be required to determine precisely how bZIP67 is regulated by factors such as temperature (Figure 8) and whether additional clade A bZIPs are also involved in *DOG1* expression.

**DOG1** also plays important roles in secondary dormancy and dormancy cycling (Finch-Savage et al., 2007; Footitt et al., 2011; Finch-Savage and Footitt 2017). **DOG1** expression is enhanced in secondary dormant Cvi-0 seed (Cadman et al., 2006; Finch-Savage et al., 2007). **DOG1** expression correlates with other dormancy marker genes over the course of an annual cycle in the seed soil bank (Footitt et al., 2011), and the principal QTL for timing of emergence from the soil in a Cvi-0/Burren (Bur-0) RI mapping population also co-locates with **DOG1** (Finch-Savage and Footitt 2017). However, bZIP67 does not appear to be expressed in secondary dormant Cvi-0 seed, based on published microarray data (Cadman et al., 2006; Finch-Savage et al., 2007). Therefore, it is possible that bZIP67’s role in transactivating **DOG1** is restricted to seed maturation and that other TFs may control **DOG1** (and as**DOG1**) expression post-imbibition and through dormancy cycles in the seed soil bank (Finch-Savage and Footitt 2017).

Functional allelic variation in **DOG1** is believed to be widespread and to have considerable adaptive significance (Alonso-Blanco et al., 2003; Bentsink et al., 2006; Bentsink L, et al., 2010; Chiang et al., 2011; Kerdaffrec et al., 2016). Nakabayashi et al., (2015) previously identified a non-synonymous substitution in **DOG1** that affects protein...
function, but DOG1 expression also differs greatly between Arabidopsis accessions (Bentsink et al., 2006; Chiang et al., 2011). Cis-regulation of gene expression is common in Arabidopsis (Keurentjes et al., 2007), and Gan et al., (2011) previously reported that potential cis-acting sequence variants, associated with ecotypic differences in gene expression, are concentrated in the promoter regions, which are also hotspots for meiotic recombination (Choi et al., 2015). Here we showed that variation in promoter length, caused by a 285 bp INDEL, affects bZIP67-dependent transactivation of DOG1 (Figure 8), providing a molecular mechanism to explain how the DOG1 QTL contributes to the phenotypic difference in seed dormancy observed between ecotypes Cvi-0 and Ler-0 (Alonso-Blanco et al., 2003; Bentsink et al., 2006). Transcriptional activation is known to be modulated by promoter context, as well as response element-dependent specificity (Nagpal et al., 1992; Sanguedolce et al., 1997). Liu et al. (2014) previously showed that natural variation in FLOWERING LOCUS T (FT) promoter length, resulting from INDELs, is widespread in Arabidopsis and modulates the photoperiodic response of the floral transition.

The 285 bp insertion in the Ler-0 DOG1 promoter that confers low expression appears to be a duplication of an intergenic region from Chromosome 3, corresponding to 9,981,927 to 9,982,211 bp in Col-0. It is noteworthy that Ler-0 carries the erecta mutation, which is a product of x-ray mutagenesis (Zapata et al., 2016). This raises the following question: is the 285 bp INDEL a natural polymorphism? The insertion is present in the chromosome-level assembly of the Ler-0 genome (Zapata et al., 2016), but it is not listed in Polymorph 1001 (http://tools.1001genomes.org/polymorph), which contains variants from de novo assembly of short-read sequencing of 1135 Arabidopsis accessions (The 1001 Genomes Consortium 2016). When we mapped the short-read data for 1135 accessions (The 1001 Genomes Consortium 2016) to Ler-0 DOG1, we found that the 285 bp insertion is also present in two accessions that are phylogenetically distinct from Ler-0. These accessions are Landsberg-0 (La-0) and Kazakhstan-13 (Kz-13). The 285 bp insertion is therefore a natural polymorphism, but it appears to be rare. However, DOG1 expression is also known to vary amongst ecotypes that lack the 285 bp insertion (Bentsink et al., 2006), and other cis-acting polymorphisms in non-coding regions of the gene are most likely to be causal in these instances (Bentsink et al., 2006; Kerdaffrec et al., 2016). Further work will be required to identify the mode of action of these polymorphisms, but it is likely that many also affect the efficiency of bZIP67-dependent transactivation.
METHODS

Plant material and growth conditions

The Arabidopsis thaliana bzip67 T-DNA insertion mutants (Mendes et al., 2013) were originally identified on the SIGnAL T-DNA Express web page (http://signal.salk.edu/cgi-bin/tdnaexpress) and seeds were obtained from the European Arabidopsis Stock Centre (University of Nottingham, UK). The dog1-2 mutant and ProBZIP67:GFP-bZIP67 reporter line used in this study have been described previously (Bensmihen et al., 2005; Nakabayashi et al., 2012). For plant growth, seeds were sown on moist Levington F2 compost in 7 cm² pots. The pots were stored in the dark at 4°C for 4 d before being transferred to a Gallenkamp 228 growth cabinet with T5 49 watt fluorescent tubes set to a ‘standard’ 22°C 16h light (PPFD = 150 µmol m⁻² s⁻¹) / 16°C 8 h dark regime (Nakabayashi et al., 2012) and 70% relative humidity. In some experiments, plants were then transferred at the onset of flowering to a ‘cool’ 16°C 16h light (PPFD = 150 µmol m⁻² s⁻¹) / 14°C 8 h dark regime (Nakabayashi et al., 2012). During seed set, plants were monitored every day and seeds were harvested from individual siliques on the primary raceme, as soon as they became dehiscent. The seeds were used immediately for germination assays or were after-ripened by dry storage at 22°C in the dark at 70% relative humidity.

Germination assays

Approximately 50 freshly harvested or after ripened seeds from each individual plant were sown directly onto a 0.8% (w/v) agar plate prepared using deionized water and the plate was placed in a Gallenkamp 228 growth cabinet set to 22°C, 70% relative humidity (16h light/8h dark; PPFD = 150 µmol m⁻² s⁻¹). After-ripened WT and mutant seed batches exhibited >90% germination after 3 d of imbibition, and so this time point was used routinely for germination assays (Nakabayashi et al., 2012; 2015). Germination was scored as radicle emergence and was observed under a dissecting stereomicroscope. To determine DSDS₅₀ (the number of days of seed dry storage required before seeds germinated at >50%), we carried out germination assays on seed batches every 7 days, until >90% germination was achieved (Murphey et al., 2015).

Gene expression analysis and immunoblotting

DNase-treated total RNA was isolated from seeds at different morphological stages of development (Pelletier et al., 2017) and from protoplasts using the RNeasy kit from Qiagen.
Ltd. (Crawley, West Sussex, UK), except that for seeds, the method was modified (Mendes et al., 2013). The synthesis of single-stranded cDNA was carried out using SuperScript™ II RNase H− reverse transcriptase from Invitrogen Ltd. (Paisley, UK). Quantitative (q)-PCR was performed as described previously (Mendes et al., 2013), except that DOG1 or bZIP67 expression levels were normalized to the geometric mean of three reference genes. The reference genes (UBQ5, EF-1α and ACT8) were selected owing to their stable expression over the course of seed development (Gutierrez et al., 2008) and at different temperatures (Chiang et al., 2011; Nakabayashi et al., 2012). The primer pairs used for qPCR are listed in Supplemental Table 1. For analysis of DOG1 protein, seeds were homogenized in 50 mM Tris-HCl buffer (pH 6.8) and the total protein was denatured and concentrated using chloroform/methanol precipitation (Wessel and Flügge 1984). Protein quantification, SDS-PAGE, and immunoblotting were then performed as described previously (Craddock et al., 2015), except that anti-DOG1 (AS15 3032, Agrisera AB), anti-3-ketoacyl-CoA thiolase (KAT2) (Germain et al., 2001) or anti-GFP antibodies (Roche) and anti-IgG-HRP (Invitrogen) were used as primary and secondary antibodies at 1 in 1000 and 1 in 10,000 dilutions, respectively, and HRP was detected using either an Enhanced Chemiluminescence kit (Perkin-Elmer) or colorimetric kit (Bio-Rad). Images were scanned, and band intensity was quantified using Image J (https://imagej.nih.gov/ij/).

**Transient expression in Arabidopsis protoplasts**

The -600 bp promoter region of DOG1 was amplified from Col-0, Cvi-0 and Ler-0 genomic DNA using primer pairs listed in Supplemental Table 1. The products were cloned into the entry vector pENTR/D-TOPO and then transferred to the destination vector pBGWFS7 (Karimi et al., 2002) using the Gateway LR Clonase enzyme mix from Invitrogen Ltd., following the manufacturer’s instructions. Additional versions of the DOG1 promoter containing GBL (5′-ACGT-3′ to 5′-AAGG-3′) and RYL (5′-CATG-3′ to 5′-CAAC-3′) cis-element mutations (Figure 6A; Mendes et al., 2013) and INDELs (Figure 7A) were created by gene synthesis and cloned into pBGWFS7 using the same procedure. Arabidopsis mesophyll protoplasts were prepared from the leaves of WT, bzip67-1 ProbZIP67:GFP-bZIP67 and bzip67-1 plants and transfected with effector plasmids as described previously (Yamamoto et al., 2009; Mendes et al., 2013). After PEG-calcium transfection with plasmid DNA carrying reporter gene constructs (ProDOG1:GUS and Pro35S:LUC) and/or combinations of effector plasmids (Pro35S:bZIP67 and Pro35S:LEC1), the cells were cultured for up to 5 d (Kim and Somers 2010) before performing qPCR analysis of
transcript abundance or luciferase (LUC) and β-glucuronidase (GUS) activity assays using methods described previously (Yamamoto et al., 2009; Mendes et al., 2013).

Chromatin Immunoprecipitation and protein-DNA binding assays
Chromatin immunoprecipitation (ChIP) assays were performed using leaf mesophyll protoplasts (Mendes et al., 2013) and developing seeds (Mönke et al., 2012) from bzip67-1 ProbZIP67:GFP-bZIP67 and WT plants. Protoplasts were transfected with a Pro35S:LEC1 effector plasmid or an empty vector control (EVC) as described previously (Yamamoto et al., 2009; Mendes et al., 2013). After 5 d, the protoplasts were harvested, and ChIP-qPCR assays were carried out following the procedures described previously (Mendes et al., 2013). Chromatin isolation from MG-PMG stage seeds (Pelletier et al., 2017) was performed by following the method described by Junker et al., (2012) and as adapted by Mönke et al., (2012). After chromatin was isolated, it was extensively sheared by sonication to obtain fragment sizes between 300-400 bp. Rat anti-GFP monoclonal antibodies (Roche) and Dynabeads Protein G magnetic beads (Invitrogen) were used to immunoprecipitate the genomic fragments. qPCR was performed on the immunoprecipitated DNA from bzip67-1 ProbZIP67:GFP-bZIP67 and WT plant material as described previously (Mendes et al., 2013) using primer sets corresponding to six regions of the DOG1 gene and to ACT7 as a control (Supplemental Table 1) and were corrected for their individual PCR amplification efficiencies (Mendes et al., 2013). Protein-DNA binding assays were performed using the DPI-ELISA method (Brand et al., 2010) as described previously (Mendes et al., 2013). Biotinylated complementary oligonucleotides for GBL1 and GBL2 cis-elements in the DOG1 promoter are listed in Supplemental Table 1.

Complementation of dog1
A ~5kb region of Col-0 DOG1, including ~2.2 kb upstream and ~1 kb downstream of the coding region, was amplified by PCR (Nakabayashi et al., 2015) and cloned into the entry vector pENTR/D-TOPO and then transferred to destination vector pEarlyGate 301 (Earley et al., 2006). Versions of Col-0 DOG1 containing the 285 bp Ler-0 insertion or GBL1 and GBL2 mutations were also created by overlap extension PCR (Heckman and Pease 2007), using the Ler-0 or mutated Col-0 DOG1 promoter and Col-0 DOG1 genomic constructs as templates. Heat shock was used to transform the plasmids into Agrobacterium tumefaciens strain GV3101, and Arabidopsis transformation was then
carried out using the floral-dip method (Clough and Bent 1998). Herbicide resistance was used to select more than 40 T1 primary transgenic lines per construct, and multiple homozygous T3 lines were subsequently recovered and analyzed.

**Statistical analysis**

All experiments were carried out using either three or five biological replicates, and the data are presented as the mean values ±SE. We used analysis of variance (ANOVA) to assess differences between genotypes or treatments (Supplemental Table 2). Following significant (P<0.05) F-test results, means were compared using the appropriate least significant difference (LSD) value at the 5% (P=0.05) level of significance, on the corresponding degrees of freedom. The GenStat (©VSN International Ltd., Hemel Hempstead, UK) statistical system was used for these analyses.

**Accession numbers**

The TAIR accession numbers for the sequences of major genes mentioned in this study are as follows: bZIP67 (At3g44460), DOG1 (At5g45830), LEC1 (At1g21970), ABI3 (At3g24650), FUS3 (At3g26790), LEC2 (At1g28300), L1L (At5g47670), NF-YC2 (At1g56170), ABI5 (At2g36270) and FT (At1g65480).

**Supplemental data**

Supplemental Figure 1. Comparison of seed dormancy in bzip67-1 and dog1-2.
Supplemental Figure 2. Detection of DOG1 promoter binding by bZIP67 in seeds.
Supplemental Figure 3. Sequence alignment of DOG1 promoter regions showing cis-elements and 285 bp INDEL present in the promoter.
Supplemental Figure 4. Binding specificity of bZIP67 to GBL1 and GBL2.
Supplemental Figure 5. Sequence alignment of clade A bZIPs from Arabidopsis that are expressed in seeds.
Supplemental Table 1. Primers used in study.
Supplemental Table 2. ANOVA Tables.

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**Figure 1.** Effect of bZIP67 disruption on DOG1 expression and protein abundance in Col-0 seeds. (A) DOG1 transcript abundance in developing seeds of wild type (WT), bzip67-1 and bzip67-2. qRT-PCR was performed on linear cotyledon (LCOT), mature green (MG), postmature green (PMG) and dry seed (DS) stages, using a primer pair that detects all sense transcripts. Values are the mean ±SE of measurements on five biological replicates (i.e. batches of seeds from separate plants) and are normalized to the geometric mean of three reference genes and expressed relative to WT DS. Asterisk denotes a significant difference from WT (P<0.05). (B) DOG1 protein content in DS of dog1-2, bzip67-1, bzip67-2, WT and bzip67-1 ProbZIP67:GFP-bZIP67 (Comp.). The top panel is an immunoblot using anti-DOG1 and the bottom using anti-KAT2 as a loading control (LC). DOG1 abundance was measured by densitometry, normalized to LC, and is given below as a percentage of WT.
Figure 2. Effect of bZIP67 disruption and overexpression on the dormancy of Col-0 seeds. (A) Effect of dry storage period on germination of seed batches harvested from wild type (WT), bzip67-1, bzip67-2 and bzip67-1 ProbZIP67:GFP-bZIP67 (Complemented). (B) DOG1 expression level in freshly harvested seeds of WT and three ProGLY:bZIP67 overexpressing (OE) lines. (C) Effect of dry storage period on germination of seeds overexpressing bZIP67. Values are the mean ±SE of measurements on five biological replicates (i.e. batches of seeds from separate plants) and in B are normalized to the geometric mean of three reference genes and expressed relative to WT. Asterisk denotes a significant difference from WT ($P<0.05$).
Figure 3. Effect of cool conditions during seed maturation on bZIP67 function. (A) Number of days of seed dry storage required to reach >50% germination (DSDS₅₀). Wild type (WT), bzip67-1, bzip67-2, bzip67-1 ProbZIP67::GFP-bZIP67 (Complemented [Comp.]) and dog1-2 seeds were matured in a cool 16°C 16h light/14°C 8h dark (16/14) regime and germinated after increasing periods of dry storage. (B) GFP-bZIP67 and DOG1 transcript and (C) GFP-bZIP67 protein abundance in freshly harvested Comp. seeds matured in a standard (22/16) or a cool (16/14) regime. In A and B values are the mean ±SE of measurements on three biological replicates (i.e. batches of seeds from separate plants) and in B are normalized to the geometric mean of three reference genes and expressed relative to DOG1 22/16. Asterisk denotes a significant difference from WT in A and 22/16 in B (P<0.05). In C the top panel is an immunoblot using anti-GFP and the bottom using anti-KAT2 as a loading control (LC). Immunoblots are shown for three biological replicates (i.e. batches of seeds from separate plants). GFP-bZIP67 abundance was measured by densitometry, normalized to LC, and is given on the right as a fold increase in 16/14 verss 22/16.
**Figure 4.** Induction of DOG1 by LEC1 expression and DOG1 promoter binding by bZIP67. (A) A time course of bZIP67 and DOG1 expression in WT protoplasts following transfection with Pro35S:LEC1. Values are the mean ±SE of measurements on three biological replicates (i.e. three separate protoplast preparations) and are normalized to the geometric mean of three reference genes and expressed relative to the empty vector control (EVC). (B) A diagram of the DOG1 locus showing the positions of amplicons (P1-6) used for ChIP-qPCR. GBL elements are marked as circles. (C) Detection of DOG1 promoter binding by bZIP67 in protoplasts using ChIP-qPCR. Protoplasts from bzip67-1 ProbZIP67:GFP-bZIP67 and WT plants were transfected with Pro35S:LEC1 (or EVC) and anti-GFP antibodies were used for ChIP. ACT7 was used as a negative control. Values are expressed as a percentage of the input and are the mean ±SE of measurements on three biological replicates (i.e. three separate protoplast preparations). (D) DPI-ELISA assays quantifying in vitro bZIP67 binding to an equimolar concentration of wild type (WT) verses mutant (KO) GBL1 and GBL2 oligonucleotides. Values are the mean ±SE of five biological replicates (i.e. separate incubations). Asterisk denotes a significant (P<0.05) difference from time zero in A, ACT7 in C and WT in D.
Figure 5. Transactivation of DOG1 in bzip67-1 protoplasts by co-expression of bZIP67 and LEC1. (A) A diagram of the -600 bp DOG1 promoter showing the positions of GBL1, GBL2 and RYL elements. (B) Effect of bZIP67 and LEC1 expression on ProDOG1:GUS reporter activity. EVC is empty vector control. (C) Effect of GBL and RYL element mutations on ProDOG1:GUS reporter activity. Values are the mean ±SE of measurements on three biological replicates (i.e. three separate protoplast preparations) and are expressed as a ratio with Pro35S:LUC. Asterisk denotes a significant (P<0.05) difference from EVC in B and WT promoter in C.
Figure 6. Effect of promoter length on bZIP67-dependent transactivation of DOG1. (A) A diagram of natural and synthetic DOG1 promoter variants showing the position of the 285 bp INDEL, GBL (closed circle) and RYL (open circles) elements relative to the TSS. (B) Effect of the INDEL (+ or – I) on ProDOG1:GUS reporter activity in bzip67-1 protoplasts co-transfected with Pro35S:bZIP67 and Pro35S:LEC1. R is the insertion of 285 bp of a randomly selected intergenic sequence from Arabidopsis. Values are the mean ±SE of measurements on three biological replicates (i.e. three separate protoplast preparations) and are expressed as a ratio with Pro35S:LUC. Asterisk denotes a significant (P<0.05) difference from Ler-0.
Figure 7. Effect of the 285 bp INDEL and GBL1 and 2 on seed dormancy and DOG1 expression. (A) Germination and (B) DOG1 expression in freshly harvested dog1-2 seed from three homozygous transgenic lines (a-c) containing either a Col-0 DOG1 genomic construct (Col-0) or a variant with either the Ler-0 285 bp insertion (Col-0+I) or GBL1 and GBL2 mutated (Col-0-GBL). Values are the mean ±SE of measurements on five biological replicates (i.e. batches of seeds from separate plants) and in B are normalized to the geometric mean of three reference genes and expressed relative to WT. Asterisk denotes a significant \( P<0.05 \) difference from dog1-2 in A and WT in B.
Figure 8. A model for transcriptional regulation of DOG1 expression during seed maturation. Expression of LEC1 is necessary and sufficient for the induction of bZIP67 and AFL (ABI3, FUS3 and LEC2), and ALF are also necessary for bZIP67 expression (Pelletier et al., 2017). Our data suggest that LEC1 expression also induces DOG1 in a bZIP67-dependent manner and that bZIP67 binds to GBL cis-elements in the DOG1 promoter, which are necessary for expression. Other LEC1-inducible proteins are also necessary for DOG1 expression, but it is not known whether AFL bind to RYL cis-elements in DOG1 directly. However, loss and gain of bZIP67 function in seeds is sufficient to reduce and increase DOG1 expression (and dormancy), respectively, and bZIP67 protein abundance is also increased by cool conditions during seed maturation that promote DOG1 expression (and dormancy). Our data also suggest that a 285 bp INDEL situated between GBL and the transcriptional start site is responsible for the difference in DOG1 expression found in ecotypes Ler-0 and Cvi-0.