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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.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Peter J. Eastmond (peter.eastmond@rothamsted.ac.uk).

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.

1 ## INTRODUCTION
2 Seed dormancy is a complex life history trait that plays an important role in local
3 adaptation, as well as dispersal (Finch-Savage and Leubner-Metzger 2006). Dormancy
4 prevents seed germination when immediate environmental conditions are suitable, but the
5 longer-term probability of survival is low (Finch-Savage and Leubner-Metzger 2006). In the
6 weedy annual species *Arabidopsis thaliana*, seed dormancy varies geographically, with
7 strong dormancy being more prevalent where summers are long and dry, and weak

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8 dormancy being associated with short wet summers (Chiang et al., 2011; Kronholm et al.,
9 2012). Alonso-Blanco et al. (2003) mapped several quantitative trait loci (QTL) that control
10 dormancy using a biparental recombinant inbred (RI) population derived from a cross
11 between the highly dormant ecotype Cape Verde Islands (Cvi-0) and a less dormant
12 ecotype Landsberg *erecta* (Ler-0). The first of these QTL to be cloned was *DELAY OF*
13 *GERMINATION1 (DOG1*) (Alonso-Blanco et al., 2003; Bentsink et al., 2006). DOG1 is
14 thought to act as a timer for seed dormancy release, since it is modified during after-
15 ripening (Nakabayashi et al., 2012). Its mode of action has been the subject of several
16 studies and remains to be fully elucidated (Nakabayashi et al., 2012; 2015; Graeber et al.,
17 2014; Dekkers et al., 2016; Huo et al., 2016; Née et al., 2017). *DOG1* expression is
18 regulated by key environmental cues that control dormancy, such as temperature (Chiang
19 et al., 2011; Kendall et al., 2011; Nakabayashi et al., 2012), and by allelic variation in
20 *DOG1*, which appears to explain a substantial proportion of the phenotypic variation in
21 dormancy observed in wild populations of Arabidopsis (Alonso-Blanco et al., 2003;
22 Bentsink et al., 2006; Chiang et al., 2011; Kerdaffrec et al., 2016).

23 Genetic studies have shown that a network of transcriptional master regulators
24 orchestrates the seed maturation program, of which the establishment of primary
25 dormancy is a part (Vicente-Carbajosa and Carbonero 2005; Santos-Mendoza et al.,
26 2008). In Arabidopsis, four transcription factors (TFs) function as positive regulators of this
27 process: LEAFY COTYLEDON1 (LEC1), ABSCISIC ACID INSENSITIVE3 (ABI3),
28 FUSCA3 (FUS3) and LEC2 (Giraudat et al., 1992; Meinke 1992; Keith et al., 1994; West et
29 al., 1994; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001). LEC1 acts at the
30 highest level in the regulatory hierarchy controlling the maturation phase (West et al.,
31 1994; Santos-Mendoza et al., 2008) and encodes a homologue of the mammalian nuclear
32 TF YB (NF-YB) subunit of the trimeric CCAAT-box binding complex (CBC) (Lotan et al.,
33 1998; Lee et al., 2003). ABI3, FUS3 and LEC2 (known collectively as AFL) encode TFs
34 containing a conserved B3 DNA binding domain, which is specific to plants (Giraudat et
35 al., 1992; Luerssen et al., 1998; Stone et al., 2001). *DOG1* is induced during seed
36 maturation (Bentsink et al., 2006; Nakabayashi et al., 2012), and its expression is known
37 to rely indirectly on LEC1 (Pelletier et al., 2017) and to require AFL (Braybrook et al., 2006;
38 Mönke et al., 2012; Wang and Perry 2013; González-Morales et al., 2016). Kendall et al.,
39 (2011) also showed that enhanced *DOG1* expression in seeds matured at low temperature
40 requires C-REPEAT BINDING FACTORS from the APETALA2-domain TF family. *DOG1* is
41 also regulated by alternative splicing, alternative polyadenylation, histone modifications

42 and a *cis*-acting antisense non-coding transcript (*asDOG1*) (Bentsink et al., 2006; Müller et
43 al., 2012; Graeber et al., 2014, Cyrek et al., 2016; Fedak et al., 2016).
44 Despite previous studies on *DOG1* regulation, it is not known precisely which TFs
45 bind to the *DOG1* promoter and are responsible for driving its expression during embryo
46 maturation, nor is it known how temperature (Chiang et al., 2011; Kendall et al., 2011;
47 Nakabayashi et al., 2012) and allelic variation in the *DOG1* promoter (Bentsink et al.,
48 2006; Kerdaffrec et al., 2016) affect their ability to perform this function. We previously
49 carried out a reverse genetic screen on TFs that are induced by LEC1 during Arabidopsis
50 seed maturation and identified basic LEUCINE ZIPPER TRANSCRIPTION FACTOR 67
51 (bZIP67) as a regulator of several genes involved in seed storage reserve deposition
52 (Mendes et al., 2013). Here, we show that bZIP67 is also a direct regulator of *DOG1*
53 expression, specifying LEC1's action in the establishment of primary dormancy. We also
54 show that temperature regulates bZIP67 at the level of protein abundance and that
55 bZIP67-dependent transactivation of *DOG1* is affected by natural variation in the gene
56 promoter (Bentsink et al., 2006; Kerdaffrec et al., 2016).
57

58 **RESULTS**

59 ***bZIP67* is required for *DOG1* expression and protein accumulation**

60 We previously characterized two Arabidopsis mutant alleles of *bZIP67* in ecotype Col-0
61 (Mendes et al., 2013). Affymetrix ATH1 microarray experiments indicated that *DOG1*
62 expression may be reduced by as much as 13-fold in whole developing siliques of *bzip67-*
63 *1* (Mendes et al., 2013). To test whether *bZIP67* is required for *DOG1* expression during
64 seed development, we performed quantitative (q)RT-PCR analysis of transcript abundance
65 in wild type (WT), *bzip67-1* and *bzip67-2* plants grown in standard conditions (i.e. 22°C
66 16h light/16°C 8h dark cycle) (Nakabayashi et al., 2012; 2015) (Figure 1). *DOG1* is
67 alternatively spliced, producing five transcript variants, of which epsilon is the major form
68 (Nakabayashi et al., 2015). A noncoding antisense *DOG1* RNA (*asDOG1*) is also
69 expressed independently of the sense transcripts (Fedak et al., 2016). Using a qRT-PCR
70 primer pair that detects all sense transcripts, we determined that total *DOG1* transcript
71 abundance in WT increased over the linear cotyledon (LCOT), mature green (MG) and
72 post mature green (PMG) stages of embryo development (Pelletier et al., 2017) and then
73 declined in freshly harvested dry seeds (DS) (Nakabayashi et al., 2012; 2015) (Figure 1A).
74 However, in *bzip67-1* and *bzip67-2* seeds, total *DOG1* transcript abundance was
75 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

143 Ectopic expression of *LEC1* in Arabidopsis triggers somatic embryogenesis and activates
144 the transcriptional program for embryo maturation (Lotan et al., 1998; Pelletier et al.,
145 2017). When *LEC1* is expressed, it binds to and activates *bZIP67*, and *lec1* embryos are
146 also deficient in both *bZIP67* and *DOG1* expression (Pelletier et al., 2017). To test whether
147 *LEC1* also induces *DOG1* expression, we transfected Arabidopsis mesophyll protoplasts
148 with a *LEC1* effector plasmid driven by the CaMV 35S promoter or with an empty vector
149 control (EVC) (Yamamoto et al., 2009; Mendes et al., 2013) and measured both *bZIP67*
150 and total *DOG1* transcript abundance over 5 d (Figure 4). *LEC1* expression led to a
151 significant (P<0.05) increase in *bZIP67* transcript abundance by 2 d after transfection, and
152 by 5 d, *bZIP67* expression was >80-fold higher than in the EVC (Figure 4A). A significant
153 (P<0.05) increase in *DOG1* expression was also detected following transfection with the
154 *LEC1* effector plasmid, but it occurred ~1 d later than *bZIP67*, and by 5 d, *DOG1*
155 expression was >25-fold higher than in the EVC (Figure 4A). These data show that *DOG1*
156 is induced by *LEC1* expression and that this occurs after the induction of *bZIP67*.
157

158 **bZIP67 binds to the *DOG1* promoter**

159 To determine whether bZIP67 binds to the *DOG1* promoter, we performed chromatin
160 immunoprecipitation (ChIP)-qPCR experiments on protoplasts from *bzip67-1*
161 *ProbZIP67:GFP-bZIP67* and WT plants transfected with *Pro35S:LEC1* (Mendes et al.,
162 2013). We carried out qPCR using primer pairs corresponding to six regions of *DOG1*
163 (Figure 4B) and also to *ACTIN7* (*ACT7*) as a negative control. Amplicons P2, P3 and P4,
164 spanning 0 to 1 kb upstream of the *DOG1* transcriptional start site (TSS), were significantly
165 (P<0.05) enriched in *bzip67-1 ProbZIP67:GFP-bZIP67* compared to either the *ACT7* or
166 WT controls, and enrichment was strongest at P3 (-400 bp), suggesting that bZIP67 binds
167 to this region of the *DOG1* promoter (Figure 4C). No enrichment was observed using P3
168 when protoplasts were transfected with an empty vector control (EVC) rather than
169 *Pro35S:LEC1* (Figure 4C). To confirm that bZIP67 binds to the *DOG1* promoter *in vivo*, we
170 also performed ChIP-qPCR experiments on developing (MG-PMG stage) seeds of *bzip67-*
171 *1 ProbZIP67:GFP-bZIP67* plants (Mönke et al., 2012; Pelletier et al., 2017) and detected a
172 significant (P<0.05) enrichment using P3 (Supplemental Figure 2).

173 We have previously shown that bZIP67 can bind to G box-like (GBL) *cis*-elements
174 with the core sequence 5′-ACGT-3′ (Mendes et al., 2013). The *DOG1* promoter contains
175 multiple GBL elements (Nakabayashi et al., 2012), but only GBL1 and GBL2 (both 5’-
176 CACGTA-3’) are present in the -400 bp region (Figure 4B and Supplemental Figure 3). To

177 test whether bZIP67 can bind to GBL1 and GBL2, we performed a DNA-protein-interaction
178 enzyme-linked immunosorbent assay (DPI-ELISA) (Brand et al., 2010) (Figure 4D).
179 Epitope-tagged recombinant bZIP67 was incubated with immobilized double-stranded
180 DNA oligonucleotides and binding was determined by immuno-detection (Mendes et al.,
181 2013). When bZIP67 was applied to oligonucleotides containing GBL1 and GBL2, the
182 ELISA signal was >30-fold stronger than when an equal concentration of the
183 corresponding GBL oligonucleotides with mutated (KO) 5′-ACGT-3′ cores (Mendes et al.,
184 2013) were tested (Figure 4D). In competition experiments to define specificity, the
185 addition of free GBL oligonucleotides also significantly reduced the ELISA signal from
186 bound GBLs ($P<0.05$), while the addition of free GBL KO oligonucleotides did not
187 (Supplemental Figure 4). A combination of *in vivo* and *in vitro* experiments therefore
188 suggests that bZIP67 binds to the *DOG1* promoter.
189
190 **Transactivation of *DOG1* by bZIP67 requires *LEC1* expression**
191 Next, we tested whether bZIP67 expression is sufficient to transactivate *DOG1* alone, or
192 whether it requires LEC1 and/or other regulatory factors induced by LEC1 (Pelletier et al.,
193 2017) (Figure 5). We cloned a ~600 bp region of the Col-0 *DOG1* promoter containing
194 GBL1 and GBL2 (Figure 5A) upstream of β-glucuronidase (GUS) and transfected the
195 construct into *bzip67-1* protoplasts in combination with *Pro35S:bZIP67*, *Pro35S:LEC1* or
196 empty vector control (EVC) effector plasmids (Yamamoto et al., 2009; Mendes et al.,
197 2013). Protoplasts from *bzip67-1* were used to prevent induction by endogenous *bZIP67*
198 (Kagaya et al., 2005; Mu et al., 2008) (Figure 4A). Co-transfection of *ProDOG1:GUS* with
199 EVC resulted in minimal GUS reporter activity (Figure 5B), which is consistent with the
200 finding that *DOG1* expression is restricted to seeds (Bentsink et al., 2006; Nakabayashi et
201 al., 2012). Co-transfection with *Pro35S:bZIP67* did not enhance GUS activity significantly
202 ($P>0.05$). Co-transfection with *Pro35S:LEC1* led to a <2-fold increase in GUS activity
203 (Figure 5B). However, when *Pro35S:bZIP67* was co-transfected together with
204 *Pro35S:LEC1*, the level of GUS activity was enhanced by >18-fold (Figure 5B). These data
205 suggest that bZIP67 is required for *DOG1* expression, but is not sufficient, and that
206 transactivation also relies on the expression of *LEC1* (Pelletier et al., 2017). LEC1 induces
207 the expression of several regulators of embryo maturation that may participate in *DOG1*
208 induction, including CBC components LEC1-like (L1L) (Kwong et al., 2003) and NF-YC2
209 (Yamamoto et al., 2009) and AFL (Mu et al., 2008; Pelletier et al., 2017). There is also
210 evidence that bZIPs form ternary complexes with AFL and CBC to regulate transcription

211 during Arabidopsis seed maturation (Nakamura et al., 2001; Yamamoto et al., 2009;
212 Mendes et al., 2013; Baud et al., 2016).
213

214 **Transactivation of *DOG1* by bZIP67 requires GBL and RYL promoter elements**

215 To determine whether transactivation of *DOG1* requires the GBL1 and GBL2 *cis*-elements
216 in the promoter, we mutated the 5'-ACGT-3' core sequences in the *ProDOG1:GUS*
217 construct (Mendes et al., 2013) and tested its ability to drive GUS expression in *bzip67-1*
218 protoplasts when co-transfected together with *Pro35S:bZIP67* and *Pro35S:LEC1*.
219 Disruption of GBL1 and GBL2 each reduced GUS reporter activity by >70%, and
220 mutations in both blocked transactivation of *GUS* completely (Figure 5C). ChIP
221 experiments have suggested that LEC1 does not bind *DOG1* (Pelletier et al., 2017) and
222 that CCAAT-box motifs that may be bound directly by CBC containing LEC1 or L1L
223 (Gnesutta et al., 2017) are absent from the -600 bp *DOG1* promoter. However, an
224 overlapping RY-like (RYL) element (5′-GCATGC-3′) repeat exists between GBL1 and
225 GBL2 (Figure 4A and Supplemental Figure 3). This putative *cis*-element could be bound
226 by AFL (Braybrook et al., 2006; Baud et al., 2016). AFL are induced by LEC1 (Pelletier et
227 al., 2017) and are required for *DOG1* expression (Braybrook et al., 2006; Mönke et al.,
228 2012; Wang and Perry 2013; González-Morales et al., 2016; Pelletier et al., 2017).
229 Furthermore, ChIP experiments suggested that AFL FUS3 binds to *DOG1 in vivo* (Wang
230 and Perry 2013). Mutating the core sequence of the repeated RYL *cis*-element (Baud et
231 al., 2016) eliminated GUS reporter activity driven by *Pro35S:bZIP67* and *Pro35S:LEC1*
232 (Figure 5C). These data suggest that bZIP67-dependent transactivation of *DOG1*, induced
233 by LEC1, relies on GBL and RYL *cis*-elements.
234

235 **Natural variation in the *DOG1* promoter affects bZIP67-dependent transactivation**

236 *DOG1* was originally cloned by QTL mapping by exploiting the natural variation in
237 dormancy observed between ecotypes Cvi-0 and Ler-0, and expression analysis revealed
238 a positive correlation between *DOG1* transcript abundance and dormancy in these (and
239 many other) Arabidopsis accessions (Bentsink et al., 2006; Chiang et al., 2011; Kerdaffrec
240 et al., 2016). Although allelic variation at the *DOG1* locus explains a substantial proportion
241 of phenotypic variation in seed dormancy, it is not known how *cis*-variation affects *DOG1*
242 expression (Bentsink et al., 2006; Chiang et al., 2011; Kerdaffrec et al., 2016). We
243 therefore created *ProDOG1:GUS* constructs using -600 bp promoter regions from Cvi-0
244 and Ler-0 and tested their ability to drive *GUS* reporter expression when co-transfected

245 together with *Pro35S:bZIP67* and *Pro35S:LEC1* in *bzip67-1* protoplasts (Figure 6). GUS
246 reporter activity driven by the Ler-0 promoter (Figure 6A) was >5-fold lower than when
247 driven by that of Cvi-0 or Col-0 (Figure 6B). The intergenic region upstream of *DOG1* in
248 Ler-0 and Cvi-0 contains 15 sequence variants, including three within the -600 bp
249 promoter region (Bentsink et al., 2006). None of these variants lie in GBL1, GBL2 or RYL,
250 but a 285 bp insertion/deletion (INDEL) is present at -328 bp, situated between these *cis*-
251 elements and the TSS (Figure 6A and Supplemental Figure 3).
252 Deletion of this 285 bp insertion from the Ler-0 *DOG1* promoter led to a >5-fold
253 increase in GUS reporter activity, and conversely, its insertion into either the Cvi-0 or Col-0
254 *DOG1* promoters led to a >5-fold decrease in GUS reporter activity (Figure 6B). These
255 data suggest that the INDEL may be responsible for the allele-specific difference in *DOG1*
256 transcript abundance observed between Ler-0 and Cvi-0 (Bentsink et al., 2006). It is not
257 clear precisely how the INDEL affects *DOG1* expression. It might act by introducing
258 repressive *cis*-elements into the promoter or by changing its length/context, i.e., the
259 distance between existing *cis*-elements and the core promoter region most proximal to the
260 TSS (Liu et al., 2014). To help distinguish between these possibilities, we replaced the
261 Ler-0 insertion with an unrelated 285 bp intergenic sequence from Arabidopsis and found
262 that this also suppressed bZIP67-dependent GUS reporter activity (Figure 6B). This result
263 suggests that the INDEL could simply acts as a spacer (Liu et al., 2014). Although we
264 cannot discount the possibility that *cis*-elements might also exist in the insertion, they do
265 not appear to be required to attenuate *DOG1* expression.
266
267 **Natural variation in the *DOG1* promoter and GBL elements affect dormancy**
268 To test whether the INDEL and GBLs affect *DOG1* function *in vivo*, we transformed the
269 non-dormant *dog1-2* mutant with a ~5 kb genomic construct containing Col-0 *DOG1*
270 (Nakabayashi et al., 2015) and also with variants of this construct containing either the 285
271 bp Ler-0 insertion or mutations in GBL1 and GBL2 (Figure 7). Analysis of freshly harvested
272 seed batches from multiple homozygous transgenic lines grown in standard conditions
273 showed that the Col-0 *DOG1* genomic clone could complement *dog1-2* (Nakabayashi et
274 al., 2015), whereas the variant clones with either the 285 bp Ler-0 insertion or GBL1 and
275 GBL2 mutations failed to restore WT levels of seed dormancy (Figure 7A). We also
276 measured *DOG1* expression in freshly harvested seeds of the transgenic lines using a
277 qRT-PCR primer pair selective for the WT allele (Nakabayashi et al., 2012). Total *DOG1*
278 transcript abundance in *dog1-2* seeds containing the Col-0 *DOG1* genomic construct was

279 >6-fold higher than in seeds containing the variant clones with either the 285 bp Ler-0
280 insertion or GBL1 and GBL2 mutations (Figure 7B). These data confirm the notion that
281 GBL1 and GBL2 are necessary for *in vivo DOG1* expression, and they indicate that the
282 285 bp INDEL also modifies *DOG1* expression, and consequently, the strength of primary
283 dormancy (Bentsink et al., 2006; Nakabayashi et al., 2012).
284

285 **DISCUSSION**

286 In this study, we showed that the bZIP67 TF induces *DOG1* expression during Arabidopsis
287 seed development by binding to GBL *cis*-elements in the promoter of this gene and that it
288 operates downstream of LEC1 and likely in concert with other central regulators of seed
289 maturation from the CBC and AFL TF families (Figure 8). Models based on temporal and
290 spatial transcriptional profiling have previously placed bZIP67 within the regulatory circuitry
291 that governs seed maturation (Belmonte et al., 2013). *bZIP67* expression relies on LEC1
292 and AFL (Kagaya et al., 2005; Braybrook et al., 2006; Mu et al., 2008; Mönke et al., 2012;
293 Wang and Perry 2013; González-Morales et al., 2016; Pelletier et al., 2017), and there is
294 evidence that CBC and AFL form ternary complexes with bZIPs to transactivate seed
295 maturation genes (Nakamura et al., 2001; Yamamoto et al., 2009; Baud et al., 2016). It
296 remains to be determined whether CBC or AFL are direct regulators of *DOG1* expression.
297 Whole-genome ChIP experiments suggested that FUS3 binds to *DOG1* (Wang and Perry
298 2013), while Pelletier et al., (2017) and Mönke et al., (2012) did not detect *DOG1* binding
299 by LEC1 and ABI3, respectively. Unlike mutants in many of these central regulators of
300 seed maturation, *bzip67* lacks a morphological phenotype (Bensmihen et al., 2005;
301 Belmonte et al., 2013). However, we previously found that *DOG1* is one of just a few seed
302 maturation-associated genes that are strongly downregulated in developing *bzip67*
303 siliques (Mendes et al., 2013), and we have shown here that, because bZIP67 is required
304 for *DOG1* expression in developing seeds, it is also required for the establishment of
305 primary seed dormancy. It is noteworthy that *bZIP67* maps adjacent to two dormancy
306 genes (*DOG6* and *REDUCED DORMANCY1*) whose identities are unclear, although they
307 are unlikely to be synonymous, based on their contrasting phenotypes (Léon-Kloosterziel
308 et al., 1996; Peeters et al., 2002; Alonso-Blanco et al., 2003).

309 Temperature is a key environmental regulator of *DOG1* expression and seed
310 dormancy in Arabidopsis (Chiang et al., 2011; Kendall et al., 2011; Nakabayashi et al.,
311 2012). We demonstrated that cool conditions during seed maturation enhance bZIP67
312 protein (but not transcript) abundance and that overexpression of *bZIP67* can increase

313 *DOG1* expression and cause dormancy to deepen. bZIP67 is one of four bZIPs from clade
314 A, which are expressed in Arabidopsis seeds (Bensmihen et al., 2005). The best-
315 characterised of these bZIPs is ABSCISIC ACID INSENSITIVE5 (ABI5), which is also
316 bZIP67's closest homologue (Bensmihen et al., 2005). ABI5 functions in ABA signalling
317 and regulates seed germination and early seedling growth in response to abiotic stress
318 (Finkelstein et al., 2000; Lopez-Molina et al., 2001; Skubacz et al., 2016). *ABI5* is
319 expressed later in seed development than *bZIP67* (Bensmihen et al., 2002; 2005) and
320 does not appear to be required for primary dormancy (Finkelstein 1994), although it is
321 regulated by *DOG1* (Dekkers et al., 2016). Interestingly, ABI5 is subject to extensive
322 posttranslational regulation (Skubacz et al., 2016), and key phosphorylation, ubiquitination,
323 and *s*-nitrosylation sites identified in ABI5 are also within regions conserved in bZIP67
324 (Supplemental Figure 5). Group A bZIPs are also thought to form heterodimers
325 (Deppmann et al., 2004). They can bind to similar (or identical) GBL *cis*-elements
326 (Finkelstein 1994; Carles et al., 2002; Deppmann et al., 2004) and have negative, as well
327 as positive, regulatory functions (Finkelstein 1994; Finkelstein and Lynch 2000). Further
328 work will therefore be required to determine precisely how bZIP67 is regulated by factors
329 such as temperature (Figure 8) and whether additional clade A bZIPs are also involved in
330 *DOG1* expression.

331 DOG1 also plays important roles in secondary dormancy and dormancy cycling
332 (Finch-Savage et al., 2007; Footitt et al., 2011; Finch-Savage and Footitt 2017). *DOG1*
333 expression is enhanced in secondary dormant Cvi-0 seed (Cadman et al., 2006; Finch-
334 Savage et al., 2007). *DOG1* expression correlates with other dormancy marker genes over
335 the course of an annual cycle in the seed soil bank (Footitt et al., 2011), and the principal
336 QTL for timing of emergence from the soil in a Cvi-0/Burren (Bur-0) RI mapping population
337 also co-locates with *DOG1* (Finch-Savage and Footitt 2017). However, *bZIP67* does not
338 appear to be expressed in secondary dormant Cvi-0 seed, based on published microarray
339 data (Cadman et al., 2006; Finch-Savage et al., 2007). Therefore, it is possible that
340 bZIP67's role in transactivating *DOG1* is restricted to seed maturation and that other TFs
341 may control *DOG1* (and *asDOG1*) expression post-imbibition and through dormancy
342 cycles in the seed soil bank (Finch-Savage and Footitt 2017).

343 Functional allelic variation in *DOG1* is believed to be widespread and to have
344 considerable adaptive significance (Alonso-Blanco et al., 2003; Bentsink et al., 2006;
345 Bentsink L, et al., 2010; Chiang et al., 2011; Kerdaffrec et al., 2016). Nakabayashi et al.,
346 (2015) previously identified a non-synonymous substitution in *DOG1* that affects protein

347 function, but *DOG1* expression also differs greatly between Arabidopsis accessions
348 (Bentsink et al., 2006; Chiang et al., 2011). *Cis*-regulation of gene expression is common
349 in Arabidopsis (Keurentjes et al., 2007), and Gan et al., (2011) previously reported that
350 potential *cis*-acting sequence variants, associated with ecotypic differences in gene
351 expression, are concentrated in the promoter regions, which are also hotspots for meiotic
352 recombination (Choi et al., 2015). Here we showed that variation in promoter length,
353 caused by a 285 bp INDEL, affects bZIP67-dependent transactivation of *DOG1* (Figure 8),
354 providing a molecular mechanism to explain how the *DOG1* QTL contributes to the
355 phenotypic difference in seed dormancy observed between ecotypes Cvi-0 and Ler-0
356 (Alonso-Blanco et al., 2003; Bentsink et al., 2006). Transcriptional activation is known to
357 be modulated by promoter context, as well as response element-dependent specificity
358 (Nagpal et al., 1992; Sanguedolce et al., 1997). Liu et al. (2014) previously showed that
359 natural variation in *FLOWERING LOCUS T* (*FT*) promoter length, resulting from INDELs,
360 is widespread in Arabidopsis and modulates the photoperiodic response of the floral
361 transition.

362 The 285 bp insertion in the Ler-0 *DOG1* promoter that confers low expression
363 appears to be a duplication of an intergenic region from Chromosome 3, corresponding to
364 9,981,927 to 9,982,211 bp in Col-0. It is noteworthy that Ler-0 carries the *erecta* mutation,
365 which is a product of x-ray mutagenesis (Zapata et al., 2016). This raises the following
366 question: is the 285 bp INDEL a natural polymorphism? The insertion is present in the
367 chromosome-level assembly of the Ler-0 genome (Zapata et al., 2016), but it is not listed
368 in Polymorph 1001 (http://tools.1001genomes.org/polymorph), which contains variants
369 from *de novo* assembly of short-read sequencing of 1135 Arabidopsis accessions (The
370 1001 Genomes Consortium 2016). When we mapped the short-read data for 1135
371 accessions (The 1001 Genomes Consortium 2016) to Ler-0 *DOG1*, we found that the 285
372 bp insertion is also present in two accessions that are phylogenetically distinct from Ler-0.
373 These accessions are Landsberg-0 (La-0) and Kazakhstan-13 (Kz-13). The 285 bp
374 insertion is therefore a natural polymorphism, but it appears to be rare. However, *DOG1*
375 expression is also known to vary amongst ecotypes that lack the 285 bp insertion
376 (Bentsink et al., 2006), and other *cis*-acting polymorphisms in non-coding regions of the
377 gene are most likely to be causal in these instances (Bentsink et al., 2006; Kerdaffrec et
378 al., 2016). Further work will be required to identify the mode of action of these
379 polymorphisms, but it is likely that many also affect the efficiency of bZIP67-dependent
380 transactivation.

381

382 **METHODS**

383 **Plant material and growth conditions**

384 The *Arabidopsis thaliana bzip67* T-DNA insertion mutants (Mendes et al., 2013) were
385 originally identified on the SIGnAL T-DNA Express web page (http://signal.salk.edu/cgi-
386 bin/tdnaexpress) and seeds were obtained from the European Arabidopsis Stock Centre
387 (University of Nottingham, UK). The *dog1-2* mutant and *ProbZIP67:GFP-bZIP67* reporter
388 line used in this study have been described previously (Bensmihen et al., 2005;
389 Nakabayashi et al., 2012). For plant growth, seeds were sown on moist Levington F2
390 compost in 7 cm^2 pots. The pots were stored in the dark at 4^oC for 4 d before being
391 transferred to a Gallenkamp 228 growth cabinet with T5 49 watt fluorescent tubes set to a
392 'standard' 22^oC 16h light (PPFD = 150 μmol m^{-2} s^{-1}) / 16^oC 8 h dark regime (Nakabayashi
393 et al., 2012) and 70% relative humidity. In some experiments, plants were then transferred
394 at the onset of flowering to a 'cool' 16^oC 16h light (PPFD = 150 μmol m^{-2} s^{-1}) / 14^oC 8 h
395 dark regime (Nakabayashi et al., 2012). During seed set, plants were monitored every day
396 and seeds were harvested from individual siliques on the primary raceme, as soon as they
397 became dehiscent. The seeds were used immediately for germination assays or were
398 after-ripened by dry storage at 22^oC in the dark at 70% relative humidity.

399

400 **Germination assays**

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

411

412 **Gene expression analysis and immunoblotting**

413 DNase-treated total RNA was isolated from seeds at different morphological stages of
414 development (Pelletier et al., 2017) and from protoplasts using the RNeasy kit from Qiagen

415 Ltd. (Crawley, West Sussex, UK), except that for seeds, the method was modified
416 (Mendes et al., 2013). The synthesis of single-stranded cDNA was carried out using
417 SuperScriptTM II RNase H- reverse transcriptase from Invitrogen Ltd. (Paisley, UK).
418 Quantitative (q)-PCR was performed as described previously (Mendes et al., 2013), except
419 that *DOG1* or *bZIP67* expression levels were normalized to the geometric mean of three
420 reference genes. The reference genes (*UBQ5*, *EF-1α* and *ACT8*) were selected owing to
421 their stable expression over the course of seed development (Gutierrez et al., 2008) and at
422 different temperatures (Chiang et al., 2011; Nakabayashi et al., 2012). The primer pairs
423 used for qPCR are listed in Supplemental Table 1. For analysis of DOG1 protein, seeds
424 were homogenized in 50 mM Tris-HCl buffer (pH 6.8) and the total protein was denatured
425 and concentrated using chloroform/methanol precipitation (Wessel and Flügge 1984).
426 Protein quantification, SDS-PAGE, and immunoblotting were then performed as described
427 previously (Craddock et al., 2015), except that anti-DOG1 (AS15 3032, Agrisera AB), anti-
428 3-ketoacyl-CoA thiolase (KAT2) (Germain et al., 2001) or anti-GFP antibodies (Roche) and
429 anti-IgG-HRP (Invitrogen) were used as primary and secondary antibodies at 1 in 1000
430 and 1 in 10,000 dilutions, respectively, and HRP was detected using either an Enhanced
431 Chemiluminescence kit (Perkin-Elmer) or colorimetric kit (Bio-Rad). Images were scanned,
432 and band intensity was quantified using Image J (https://imagej.nih.gov/ij/).
433
434 **Transient expression in Arabidopsis protoplasts**
435 The -600 bp promoter region of *DOG1* was amplified from Col-0, Cvi-0 and Ler-0 genomic
436 DNA using primer pairs listed in Supplemental Table 1. The products were cloned into the
437 entry vector pENTR/D-TOPO and then transferred to the destination vector pBGWFS7
438 (Karimi et al., 2002) using the Gateway LR Clonase enzyme mix from Invitrogen Ltd.,
439 following the manufacturer's instructions. Additional versions of the *DOG1* promoter
440 containing GBL (5′-ACGT-3′ to 5′-AAGG-3′) and RYL (5′-CATG-3′ to 5′-CAAC-3′) *cis*-
441 element mutations (Figure 6A; Mendes et al., 2013) and INDELs (Figure 7A) were created
442 by gene synthesis and cloned into pBGWFS7 using the same procedure. Arabidopsis
443 mesophyll protoplasts were prepared from the leaves of WT, *bzip67-1 ProbZIP67:GFP-*
444 *bZIP67* and *bzip67-1* plants and transfected with effector plasmids as described previously
445 (Yamamoto et al., 2009; Mendes et al., 2013). After PEG-calcium transfection with plasmid
446 DNA carrying reporter gene constructs (*ProDOG1:GUS* and *Pro35S:LUC*) and/or
447 combinations of effector plasmids (*Pro35S:bZIP67* and *Pro35S:LEC1*), the cells were
448 cultured for up to 5 d (Kim and Somers 2010) before performing qPCR analysis of

449 transcript abundance or luciferase (LUC) and β-glucuronidase (GUS) activity assays using
450 methods described previously (Yamamoto et al., 2009; Mendes et al., 2013).
451
452 **Chromatin Immunoprecipitation and protein-DNA binding assays**
453 Chromatin immunoprecipitation (ChIP) assays were performed using leaf mesophyll
454 protoplasts (Mendes et al., 2013) and developing seeds (Mönke et al., 2012) from *bzip67-*
455 *1 ProbZIP67:GFP-bZIP67* and WT plants. Protoplasts were transfected with a
456 *Pro35S:LEC1* effector plasmid or an empty vector control (EVC) as described previously
457 (Yamamoto et al., 2009; Kim and Somers 2010; Mendes et al., 2013). After 5 d, the
458 protoplasts were harvested, and ChIP-qPCR assays were carried out following the
459 procedures described previously (Mendes et al., 2013). Chromatin isolation from MG-PMG
460 stage seeds (Pelletier et al., 2017) was performed by following the method described by
461 Junker et al., (2012) and as adapted by Mönke et al., (2012). After chromatin was isolated,
462 it was extensively sheared by sonication to obtain fragment sizes between 300-400 bp.
463 Rat anti-GFP monoclonal antibodies (Roche) and Dynabeads Protein G magnetic beads
464 (Invitrogen) were used to immunoprecipitate the genomic fragments. qPCR was performed
465 on the immunoprecipitated DNA from *bzip67-1 ProbZIP67:GFP-bZIP67* and WT plant
466 material as described previously (Mendes et al., 2013) using primer sets corresponding to
467 six regions of the *DOG1* gene and to *ACT7* as a control (Supplemental Table 1) and were
468 corrected for their individual PCR amplification efficiencies (Mendes et al., 2013). Protein-
469 DNA binding assays were performed using the DPI-ELISA method (Brand et al., 2010) as
470 described previously (Mendes et al., 2013). Biotinylated complementary oligonucleotides
471 for GBL1 and GBL2 *cis*-elements in the *DOG1* promoter are listed in Supplemental Table
472 1.
473
474 **Complementation of *dog1***
475 A ~5kb region of Col-0 *DOG1,* including ~2.2 kb upstream and ~1 kb downstream of the
476 coding region, was amplified by PCR (Nakabayashi et al., 2015) and cloned into the entry
477 vector pENTR/D-TOPO and then transferred to destination vector pEarlyGate 301 (Earley
478 et al., 2006). Versions of Col-0 *DOG1* containing the 285 bp Ler-0 insertion or GBL1 and
479 GBL2 mutations were also created by overlap extension PCR (Heckman and Pease
480 2007), using the Ler-0 or mutated Col-0 *DOG1* promoter and Col-0 *DOG1* genomic
481 constructs as templates. Heat shock was used to transform the plasmids into
482 *Agrobacterium tumefaciens* strain GV3101, and Arabidopsis transformation was then

483 carried out using the floral-dip method (Clough and Bent 1998). Herbicide resistance was
484 used to select more than 40 T1 primary transgenic lines per construct, and multiple
485 homozygous T3 lines were subsequently recovered and analyzed.
486
487 **Statistical analysis**
488 All experiments were carried out using either three or five biological replicates, and the
489 data are presented as the mean values ±SE. We used analysis of variance (ANOVA) to
490 assess differences between genotypes or treatments (Supplemental Table 2). Following
491 significant (P<0.05) F-test results, means were compared using the appropriate least
492 significant difference (LSD) value at the 5% (P=0.05) level of significance, on the
493 corresponding degrees of freedom. The GenStat (©VSN International Ltd., Hemel
494 Hempstead, UK) statistical system was used for these analyses.
495
496 **Accession numbers**
497 The TAIR accession numbers for the sequences of major genes mentioned in this study
498 are as follows: *bZIP67* (At3g44460), *DOG1* (At5g45830), *LEC1* (At1g21970), *ABI3*
499 (At3g24650), *FUS3* (At3g26790), *LEC2* (At1g28300), *L1L* (At5g47670), *NF-YC2*
500 (At1g56170), *ABI5* (At2g36270) and *FT* (At1g65480).
501
502 **Supplemental data**
503 Supplemental Figure 1. Comparison of seed dormancy in *bzip67-1* and *dog1-2*.
504 Supplemental Figure 2. Detection of *DOG1* promoter binding by bZIP67 in seeds.
505 Supplemental Figure 3. Sequence alignment of *DOG1* promoter regions showing *cis*-
506 elements and 285 bp INDEL present in the promoter.
507 Supplemental Figure 4. Binding specificity of bZIP67 to GBL1 and GBL2.
508 Supplemental Figure 5. Sequence alignment of clade A bZIPs from Arabidopsis that are
509 expressed in seeds.
510 Supplemental Table 1. Primers used in study.
511 Supplemental Table 2. ANOVA Tables.
512
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AUTHOR CONTRIBUTIONS

P.J.E designed research; F.M.B. and P.J.E. performed research; F.M.B., D.H., K.H-P. and P.J.E. analyzed data; and P.J.E. wrote the paper.

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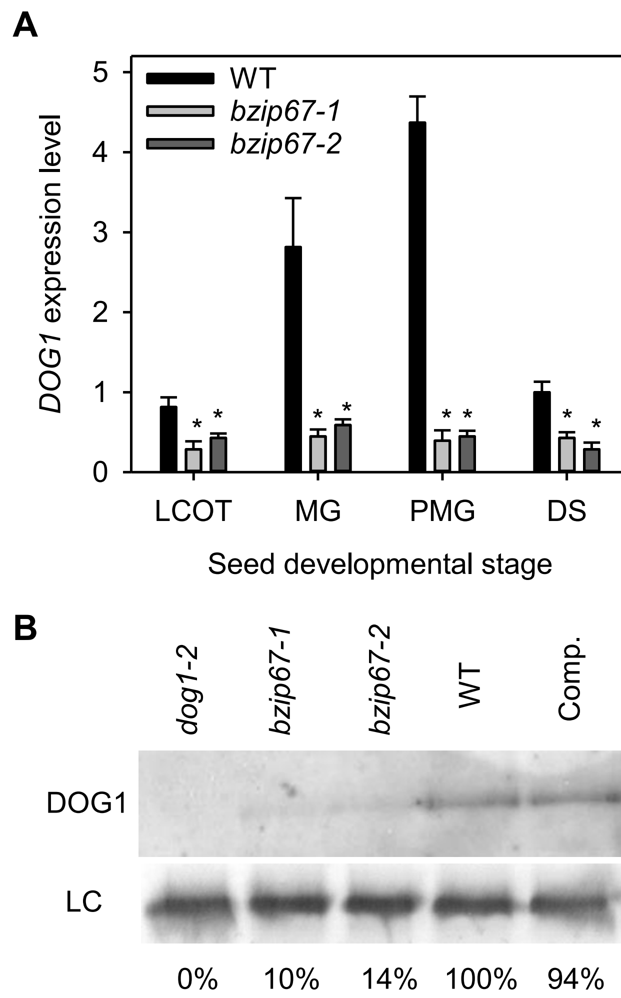

Figure 1. Effect of bZIP67 disruption on *DOG1* expression and protein abundance in Col-0 seeds. **(A)** *DOG1* transcript abundance in developing seeds of wild type (WT), *bzip67-1* and *bzip67-2*. qRT-PCR was performed on linear cotyledon (LCOT), mature green (MG), postmature green (PMG) and dry seed (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.

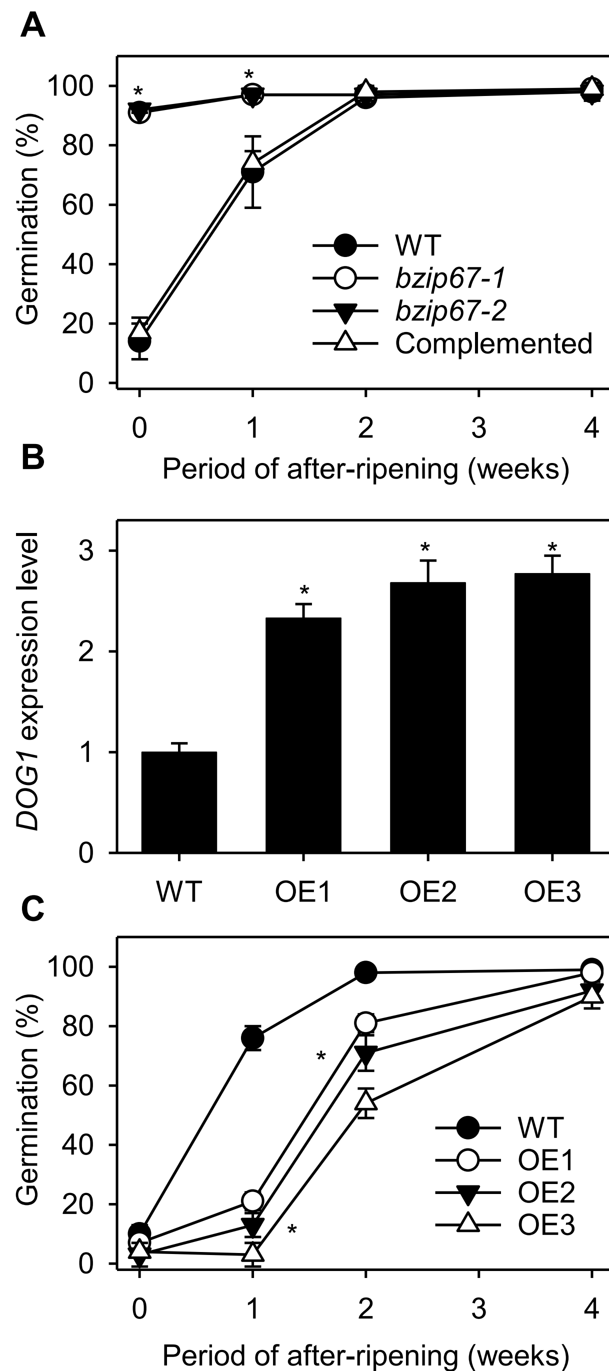

Figure 2. Effect of bZIP67 disruption and overexpression on the dormancy of Col-0 seeds. **(A)** Effect of dry storage period on germination of seed batches harvested from wild type (WT), *bzip67-1*, *bzip67-2* and *bzip67-1 ProbZIP67:GFP-bZIP67* (Complemented). **(B)** *DOG1* expression level in freshly 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).

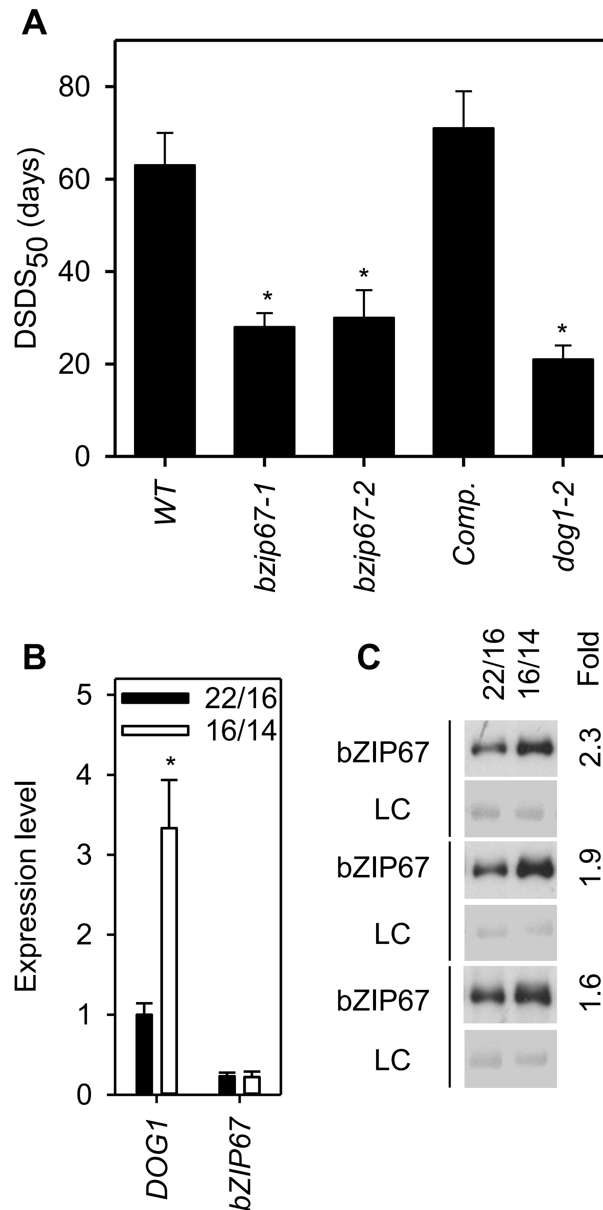

Figure 3. Effect of cool conditions during seed maturation on bZIP67 function. **(A)** Number of days of seed dry storage required to reach >50% germination ($DSDS_{50}$). Wild type (WT), *bzip67-1*, *bzip67-2*, *bzip67-1 ProbZIP67:GFP-bZIP67* (Complemented [Comp.].) and *dog1-2* seeds were matured in 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 verses 22/16.

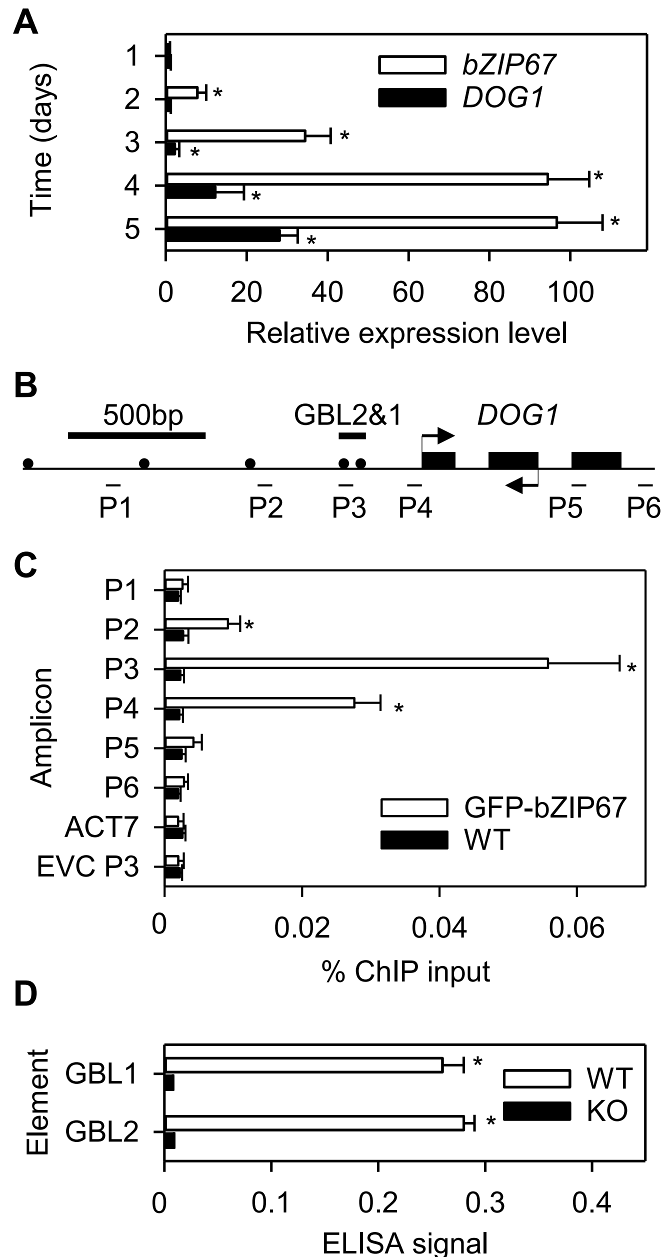

Figure 4. Induction of *DOG1* by *LEC1* expression and *DOG1* promoter binding by bZIP67. **(A)** A time course of *bZIP67* and *DOG1* expression in WT protoplasts following transfection with *Pro35S:LEC1*. Values are the mean ±SE of measurements on three biological replicates (i.e. three separate protoplast 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.

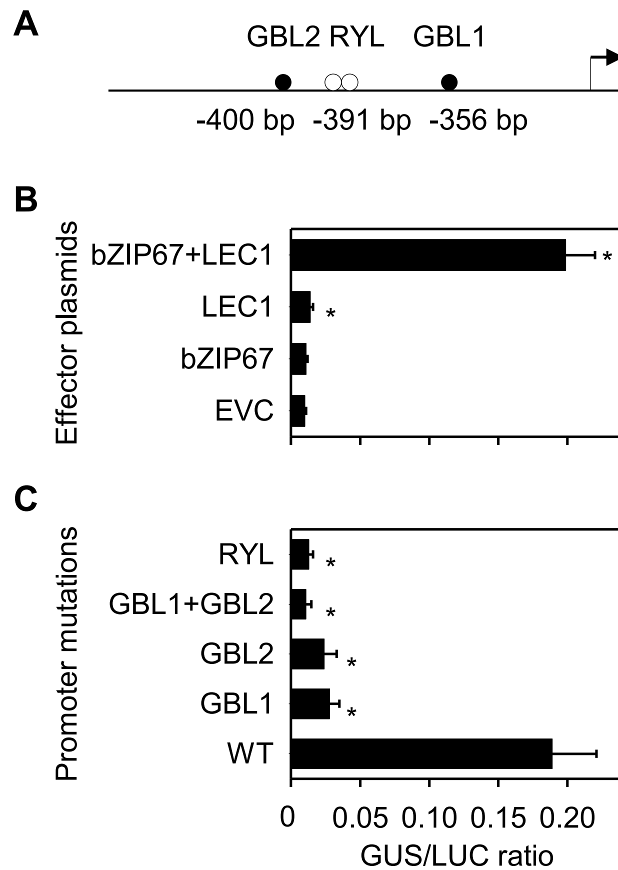

Figure 5. Transactivation of *DOG1* in *bzip67-1* protoplasts by co-expression of bZIP67 and LEC1. **(A)** A diagram of the -600 bp *DOG1* promoter showing the positions of GBL1, GBL2 and RYL elements. **(B)** Effect of *bZIP67* and *LEC1* expression on *ProDOG1:GUS* reporter activity. EVC is empty vector 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.

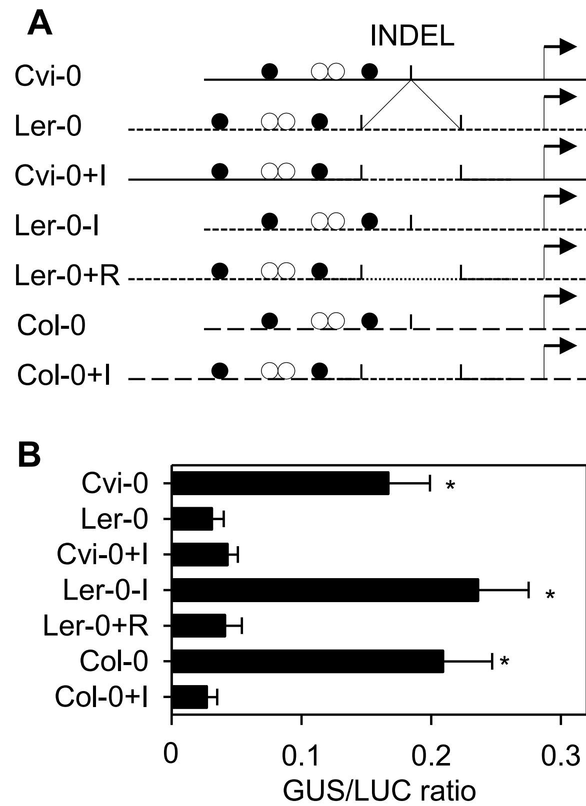

Figure 6. Effect of promoter length on bZIP67-dependent transactivation of *DOG1*. **(A)** A diagram of natural and synthetic *DOG1* promoter variants showing the position of the 285 bp INDEL, GBL (closed circle) and RYL (open circles) elements relative to the TSS. **(B)** Effect of the INDEL (+ or – 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.

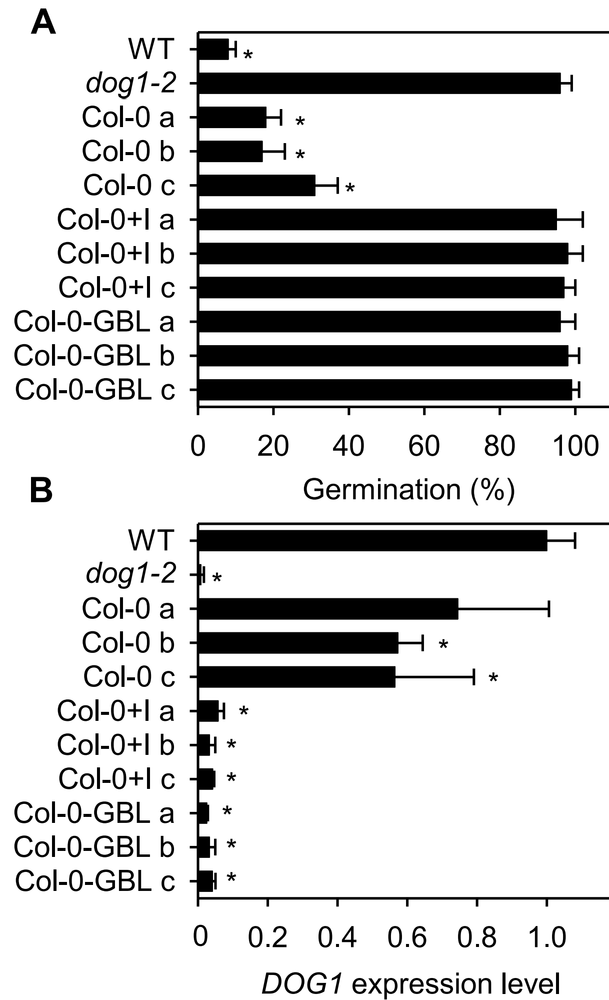

Figure 7. Effect of the 285 bp INDEL and GBL1 and 2 on seed dormancy and *DOG1* expression. **(A)** Germination and **(B)** *DOG1* expression in freshly harvested *dog1-2* seed from three homozygous transgenic lines (a-c) containing either a Col-0 *DOG1* genomic construct (Col-0) or a variant with either 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.

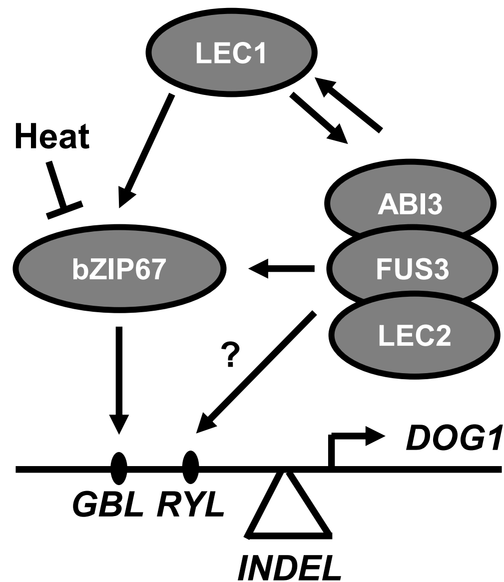

Figure 8. A model for transcriptional regulation of *DOG1* expression during seed maturation. Expression of *LEC1* is necessary and sufficient for the induction of *bZIP67* and *AFL* (*ABI3*, *FUS3* and *LEC2*), and *ALF* are also necessary for *bZIP67* expression (Pelletier et al., 2017). Our data suggest 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.

Basic LEUCINE ZIPPER TRANSCRIPTION FACTOR 67 transactivates DELAY OF GERMINATION 1 to establish primary seed dormancy in Arabidopsis
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