ICE1 and ZOU determine the depth of primary seed dormancy in Arabidopsis independently of their role in endosperm development

Dana R. MacGregor1,†, Naichao Zhang1, Mayumi Iwasaki2, Min Chen1,‡, Anuja Dave1, Luis Lopez-Molina2 and Steven Penfield1,§
1Department of Crop Genetics, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK, and
2Department of Plant Biology and Institute for Genetics and Genomics in Geneva (iGE3), University of Geneva, 30, Quai Ernest-Ansermet CH-1211, Geneva 4, Switzerland

Received 3 September 2018; revised 20 November 2018; accepted 12 December 2018; published online 20 December 2018.
*For correspondence (e-mail dana.macgregor@rothamsted.ac.uk).
†Present address: Department of Biointeractions and Crop Protection, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK.
‡Present address: College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China.
§The author responsible for distribution of materials integral to the findings presented in this article is Steven Penfield (steven.penfield@jic.ac.uk).

SUMMARY

Seed dormancy is a widespread and key adaptive trait that is essential for the establishment of soil seed banks and prevention of pre-harvest sprouting. Herein we demonstrate that the endosperm-expressed transcription factors ZHOUPI (ZOU) and INDUCER OF CBF EXPRESSION1 (ICE1) play a role in determining the depth of primary dormancy in Arabidopsis. We show that ice1 or zou increases seed dormancy and the double mutant has an additive phenotype. This increased dormancy is associated with increased ABA levels, and can be separated genetically from any role in endosperm maturation because loss of ABA biosynthesis or DELAY OF GERMINATION 1 reverses the dormancy phenotype without affecting the aberrant seed morphology. Consistent with these results, ice1 endosperms had an increased capacity for preventing embryo greening, a phenotype previously associated with an increase in endospermic ABA levels. Although ice1 changes the expression of many genes, including some in ABA biosynthesis, catabolism and/or signalling, only ABA INSENSITIVE 3 is significantly misregulated in ice1 mutants. We also demonstrate that ICE1 binds to and inhibits expression of ABA INSENSITIVE 3. Our data demonstrate that Arabidopsis ICE1 and ZOU determine the depth of primary dormancy during maturation independently of their effect on endosperm development.

Keywords: ABA, ABI3, endosperm consumption, ICE1, primary dormancy, seed development, ZOU.

INTRODUCTION

After fertilisation, seeds enter a rigid developmental programme which proceeds through embryogenesis to seed maturation, where the basic body plan of the plant is established, desiccation tolerance is gained and primary dormancy is imposed (Baud et al., 2002; Fourquin et al., 2016). The plant hormone abscisic acid (ABA) and a small network of B3-family transcription factors including ABA INSENSITIVE 3 (ABI3), FUSCA3 and LEAFY COTYLEDON 2, otherwise known as the AFL subfamily of B3 transcription factors, induce the seed maturation programme in the embryo and endosperm, as well as seed dormancy (Karssen et al., 1983; Koornneef et al., 1984; Giraudat et al., 1992; Parcy et al., 1994; Nambara et al., 1995; Lopez-Molina et al., 2002).

ABA and ABI3 continue to be important upon seed imbibition when they are required to block the germination of dormant seeds (reviewed in Koornneef et al., 2002; Carbonero et al., 2017; Leprince et al., 2017). After shedding, primary dormancy can be broken by environmental signals such as seasonal changes in temperature or soil nitrate levels or signals of canopy disturbance such as compounds in smoke from forest fires (Finch-Savage and Leubner-Metzger, 2006). In the laboratory, these environmental responses are exploited to create simple dormancy-breaking treatments such as cold stratification or dry afterripening which are often used as methods for comparing the depth of seed dormancy between genotypes.

Depending on the plant species, primary dormancy can either be conferred by the embryo or imposed by the surrounding tissues (Finch-Savage and Leubner-Metzger,
The endosperm is also an important site for ABA signalling in seeds, and ABA transport from the endosperm to the embryo is associated with the prevention of germination in dormant seeds (Lee et al., 2010; Kang et al., 2015; Chahtane et al., 2016). Furthermore, the endosperm may also be the site of perception of environmental signals that regulate seed dormancy and germination. For instance, phytochrome activity in the endosperm is sufficient to regulate germination (Lee et al., 2012a) and the temperature-regulated and dormancy-inducing MOTHER OF FT AND TFL1 (MFT) gene is only expressed in the endosperm during seed development (Vaistij et al., 2013). Furthermore, DELAY OF GERMINATION 1 (DOG1) activity in the endosperm is sufficient for the control of dormancy (Graeber et al., 2014). Taken together, an emerging paradigm is that, at least in the case of Arabidopsis, the endosperm plays a key role in primary control of dormancy. Much of the endosperm is consumed before the switch to seed maturation, making space for the embryo to expand and accumulate storage reserves (Fourquin et al., 2016). Endosperm consumption is triggered by the pressure exerted by the surrounding seed coat, but also requires the activity of a heterodimeric complex of two closely related basic helix-loop–helix transcription factors ZHOUPI (ZOU) and INDUCER OF CBF EXPRESSION1 (ICE1) (Denay et al., 2014; Fourquin et al., 2016). Consistent with the available in silico data (Le et al., 2010), expression analysis shows that ZOU is endosperm-specific (Yang et al., 2008) and ICE1 is expressed in endosperm and to lower levels in embryo and testa, with strong expression in the embryo-surrounding endosperm (Denay et al., 2014). As expected, both ice1 and zou mutants retain an excess of endosperm material at maturity and development of the embryo is restricted, although major embryo tissues differentiate and seeds remain viable (Yang et al., 2008; Denay et al., 2014). The behaviour of ice1 seedlings is not completely normal; Liang and Yang (2015) demonstrated that ice1 mutant seeds exhibit a sugar-dependent seedling growth phenotype and hypersensitivity to ABA and high glucose.

ICE1 has multiple functions in plants including regulation of cold acclimation and stomatal lineage development (Chinnusamy et al., 2003; Agarwal et al., 2006; Miura et al., 2007; Zhu et al., 2011; Kim et al., 2015). The target genes of ICE1 in cold signalling, the C-REPEAT BINDING FACTORS (CBFs), are also necessary for normal seed dormancy but are not temperature-regulated in seeds (Kendall et al., 2011). In contrast ZOU, also known as RETARDED GROWTH OF EMBRYO1 (RGE1) is only expressed in the endosperm, where it regulates the expression of genes necessary for endosperm breakdown and embryonic surface formation (Kondou et al., 2008; Yang et al., 2008; Xing et al., 2013; Moussu et al., 2017).

Here we show that the ice1 and zou mutants have increased dormancy accompanied by increased ABA levels in the mature seeds. During late embryogenesis and in mature seeds, ICE1, which is present in the endosperm, inhibits expression of the transcription factor ABA INSENSITIVE 3, which itself is a central player in the formation of dormant seeds (Giraudat et al., 1992) and prevention of germination (Giraudat et al., 1992; Nambara et al., 1992). Our data therefore show that, in Arabidopsis, ICE1 and ZOU act during maturation to determine the depth of primary dormancy independently of their effect on endosperm development.

RESULTS

Loss of ice1 or of zou clearly led to reduced germination of newly produced seeds (Figure 1). These phenotypes were robust and the differences between the mutants and wild type were seen across multiple experiments, as demonstrated in Figure S1 in the online Supporting Information; this statement is supported by the statistical analysis in Table S2. To demonstrate that this phenotype indeed increased dormancy levels, we determined what effect dormancy-breaking treatments would have on the wild type and ice1 and zou mutants. Stratification promoted the germination of two alleles of ice1 and two alleles of zou (Figure 1a,b). As ZOU and ICE1 are known to form hetero- and homo-dimers (Denay et al., 2014), we investigated dormancy in the ice1-2 zou-4 double mutant. The latter was more dormant than either ice1-2 or zou-4 single mutants (Figure 1a). Although 7 days of cold stratification was sufficient to significantly promote germination of all four mutants, the application of exogenous potassium nitrate only promoted the germination of the two alleles of zou but not the ice1 alleles (Figure 1b). Furthermore, the application of exogenous gibberellic acid (GA3), which is a hormone that is able to promote germination of most dormant Arabidopsis seeds, was sufficient to promote germination of freshly harvested ice1 or zou seeds, as was after-ripening (Figure S2). These data suggest that, in addition to the morphological phenotype, ICE1 and ZOU have a role in control of seed dormancy.

The increased dormancy effect of ice1 was complemented when we crossed ICE-GFP under its own promoter (pICE1:ICE1-GFP; Figure 1c; Denay et al., 2014) into the ice1-2 background (ice1-2 pICE1:ICE1-GFP Figure 1c). As predicted from in silico data (Le et al., 2010) and previous expression (Denay et al., 2014) and localisation studies (Kanaoka et al., 2008), we observed the GFP signal in the stomata of leaves and the endosperm of developing seeds.
ICE1 and ZOU determine primary seed dormancy depth

Figure 1. Loss of ice1 or zou from the endosperm results in increased dormancy, where homodimers as well as heterodimers may both play a role.

(a) The germination frequency for seeds of the wild type (Col-0, blue diamonds), ice1-2 (red squares), ice1-3 (green triangles), zou-3 (purple crosses), zou-4 (cyan asterisks) and the ice1-2/zou-4 double mutant (orange circles) matured at 22°C without or with stratification at 4°C for the given times.

(b) The germination frequency of freshly harvested seeds of ice1-2, ice1-3, zou-3 and zou-4 matured at 22°C (green bars) compared with stratification at 4°C for 7 days (yellow bars) or without stratification but with 10 mM potassium nitrate included in the water agar (blue bars).

(c) The germination frequency for seeds of the wild type (Col-0, blue diamonds), ice1-2 (red squares), wild type expressing ICE1-GFP under its own promoter (Col pICE1:ICE1-GFP, green diamonds) or ice1-2 expressing ICE1-GFP under its own promoter (ice1-2 pICE1:ICE1-GFP, purple crosses).

(d) The germination frequency for seeds of the wild type (Col-0 blue bars), ice1-2 (red bars) and reciprocal crosses with wild-type maternal crossed by ice1-2 pollen (green bars) or ice1-2 maternal crossed by wild-type paternal (purple), without or with stratification for 3 days at 4°C.

(e) The germination frequency of freshly harvested seeds matured at 16°C of the wild type (Col-0, blue diamonds), ice1-2 (red squares) and zou-3 (green triangles) without or with stratification at 4°C for the given times.

For (a), (b), (c) and (e), data are averages of five biological replicate seed batches with at least 45 seeds per batch ± SE. For (d), data are averages of five or more biological replicates of Col-0 or ice1-2, respectively, with at least 20 seeds per batch or six Col, ice1-2♂ or eight ice1-2♀ Col♂ individual siliques with an average of 15 seeds per siliqure ± SE. For all, significant differences by Student’s t-test on arcsine-transformed germination data are shown where *P < 0.05; **P < 0.01.

in ice1-2 expressing pICE1:ICE1-GFP (Figure S3). The ice1 dormancy phenotype is not inherited maternally, as the heterozygotes demonstrate a wild-type phenotype regardless of whether the ice1 is of maternal or paternal origin (Figure 1d). Therefore, we concluded that ICE1 activity in the Arabidopsis endosperm was necessary for normal control of seed dormancy and that both paternal and maternal copies contributed to this process.

Lowering the temperature during seed maturation is sufficient to increase levels of seed dormancy (MacGregor et al., 2015). ICE1 has been implicated in the response to and propagation of the cold signalling response (Chinnusamy et al., 2003; Miura et al., 2007; Kim et al., 2015). We therefore determined whether ICE1 or ZOU were required for the response to low temperatures during seed maturation. Both ice1-2 and zou-3 responded to this decrease in maturation temperature and, like the wild type, exhibited increased dormancy (Figure 1e). Therefore, increased dormancy in response to decreased temperature is independent of ICE1 and ZOU.

ice1 and zou exhibit abnormal seed development including arrest of the endosperm developmental programme at the fully cellularised stage and the resultant mechanical restriction of embryo development (Denay et al., 2014). We therefore considered whether the alterations to dormancy we observed were an indirect consequence of these changes. For instance, retarded embryo development and a larger endosperm to penetrate could cause the germination programme to run slowly or not at all. To determine whether the seeds were truly dormant or simply slow to
germinate, we assessed germination of ice1 or zou for 30 days in seeds with or without cold stratification treatments. In these extended germination experiments we observed little or no extra ice1 or zou mutant seed germination after 7 days without stratification (Figure 2a). This shows that the mutant embryos are not defective in the germination process itself but rather germinate to low levels due to an increase in seed dormancy levels. Because of the morphological retardation of embryo development in ice1 and zou we tested whether ice1 seeds had acquired an additional morphological dormancy that was released by stratification. We found that stratification caused no change to ice1 mutant embryo morphology or development, but was sufficient to release dormancy, demonstrating that the increased dormancy in ice1 is physiological (Figure 2b,c).

To further test whether seed dormancy in ice1 and zou is physiological we crossed ice1-2 to the abscisic acid deficient 2 (aba2-1) mutant and to dog1-2, noting that DOG1 activity in the endosperm is sufficient to confer seed dormancy (Graeber et al., 2014). Both the ice1-2 aba2-1 and ice1-2 dog1-2 double mutants showed high germination frequencies, reversing the stronger dormancy of the ice1-2 mutant (Figure 3a,b). Although non-dormant, the double

**Figure 2.** The altered germination frequency of ice1 and zou are not an indirect consequence of retarded embryo morphology that can be rectified by long germination periods or cold stratification.

(a) The germination frequency for freshly harvested wild-type (Col-0), ice1-2 and zou-3 seeds matured at 22°C without (red circles) or with stratification for 1 (green squares), 3 (blue triangles) or 7 (purple diamonds) days. Data are averages of five or more biological replicate seed batches with at least 20 seeds per batch ± SE.

(b) Morphology of wild-type (Col-0) or ice1-2 embryos dissected from seeds with 0, 1 or 3 days of stratification.

(c) The germination frequency for freshly harvested wild-type (Col-0, blue diamonds) or ice1-2 (red squares) from seeds out of which the embryos in (b) were dissected. (b). Data are averages of five or more biological replicate seed batches with at least 15 seeds per batch ± SE.
mutant seeds between aba2 or dog1 and ice1 still exhibited the darker shrivelled seed phenotype and altered embryo morphology characteristic of ice1 (Figure 3c,d). These data further support the conclusion that the failure of ice1 mutant seeds to germinate is not directly related to the defect in embryo development, because seeds exhibiting the ice1/zou morphological phenotype are capable of normal germination rates. Taken together, our data show that ICE1 is necessary for normal seed dormancy and acts in the endosperm in a manner dependent on both ABA and DOG1 to affect the germination of primary dormant seeds. This effect is genetically separable from the role in endosperm developmental transitions.

Production of ABA by the endosperm is known to be a critical step in repressing the germination of dormant seeds upon their imbibition (Lee et al., 2010; Kang et al., 2015) and ice1-2 mutants showed an ABA-dependent increased seed dormancy phenotype (Figure 3). To determine if there were altered levels of ABA in the ice1 and zou mutants, we measured the ABA content of mature seeds (Figure 4a). Consistent with the increase in seed dormancy, both mutants had a higher ABA content in the mature seed compared with the wild type (Figure 4a). To test whether the increase in seed ABA was being produced by the endosperm, we used a previously described seed coat bedding assay (SCBA) (Lee et al., 2010; Figure 4c). Wild-type and ice1-2 embryos were slower to green on a bed of ice1-2 endosperms than on an equivalent bed of wild-type endosperms (Figure 4c). Furthermore, the greening rates of wild-type and ice1-2 embryos were similar, suggesting that embryo ABA content and signalling were not substantially dissimilar between the two genotypes. Taken together, our results suggest that ICE1 activity affects seed dormancy through endospermic ABA production.

ICE1 is a basic helix-loop-helix transcription factor and has been shown to bind to promoter elements and alter gene expression (Chinnusamy et al., 2003; Agarwal et al., 2006; Zhu et al., 2011). To investigate the mechanism(s) through which ICE1 regulates ABA responses, we examined the expression levels of relevant genes in developing seeds of ice1 compared with the wild type. Understanding how transcripts are regulated by ICE1 in whole seeds is complicated by the fact that ice1 not only has a potentially direct effect on regulation of gene expression but also, because of the aberrant endosperm consumption that occurs after the heart stage (Denay et al., 2014), the embryo to endosperm ratio is altered in these mutants. Therefore, it is reasonable to expect a general over-representation of endosperm-expressed transcripts in ice1 mutant seeds. Thus, we first examined the expression of endosperm- and embryo-specific markers in wild-type and ice1-2 mutant seeds (Figure 5). The transcripts of endosperm-expressed ZOU (Kondou et al., 2008; Yang et al., 2008) and MYB118 (Barthole et al., 2014) were more highly expressed in ice1-2 during the early stages of development (Figure 5a,b). The development of wild-type and ice1 seeds is visually comparable until the heart stage of development (Denay et al., 2014), so these data suggest that ICE1 affects the transcript levels of both genes. Conversely, the embryo-expressed genes At2g23230 (Le et al., 2010) and ABSCISIC ACID INSENSITIVE4 (ABI4) (Penfield, 2006) were expressed at a similar level in wild-type and ice1-2 mutant seeds until the cotyledon stage, at which point expression was lower in ice1-2 (Figure 5c,d). These expression patterns are consistent with the reduced embryo-endosperm ratio in ice1 in the later developmental stage and suggest that indirect effects of ICE1 on transcription caused by alterations in seed development are only likely to be observed after the torpedo stage of seed development in our analysis.

The genes ABI3 and ABI5 encode transcription factors with key roles in ABA signalling in seeds (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). In the wild type both genes are expressed in the embryo and endosperm (Penfield, 2006). Loss of ICE1 does not have a significant effect on the expression of ABI5 (Figure 5e), but ice1-2 exhibits increased ABI3 expression compared with the wild type at all stages after the heart stage (Figure 5f). Because ABI3 is an important dormancy-inducing protein, unlike ABI5, the increase in expression observed in ice1 may be important for the observed changes in dormancy, especially as they are accompanied by changes in ABA levels (Figure 4).

The two 9-cis-epoxycarotenoid dioxygenases NCED6 and NCED9 are required for the catalysis of the first step of ABA biosynthesis from carotenoids (Iuchi et al., 2001; Lefebvre et al., 2006). In wild-type seeds, NCED6 is expressed in the endosperm during seed development (Lefebvre et al., 2006), although more recent transcriptome analysis shows NCED6 mRNA to be present mainly in the seed coat (Le et al., 2010). Although NCED9 is present in the peripheral layers of both the endosperm and the embryo, its expression during early stages of development is in the outer integument layer 1 of the testa and is confined to epidermal cells of the embryo after mid-development (Le et al., 2010; Frey et al., 2012). The loss of ICE1 does not affect NCED6 expression (Figure 5g) while NCED9 is increased in ice1 during the later stages of development (Figure 5h). This increase in the ABA-biosynthetic NCED9 is consistent with the increased ABA content observed in ice1 seeds (Figure 4).

We also investigated two ABA 8′-hydroxylases, CYP707A1 and CYP707A2. Of the major transcripts encoding enzymes with roles in ABA metabolism, CYP707A1 is the only one predominantly expressed in wild-type endosperm tissue during mid-maturation (Okamoto et al., 2018). The Plant Journal published by John Wiley & Sons Ltd and Society for Experimental Biology, The Plant Journal, (2019), 98, 277–290
In the wild type, CYP707A2 is expressed in the embryo and the endosperm during late maturation through to germination and is responsible for the regulation of ABA levels during late maturation to germination (Okamoto et al., 2006). Expression of both CYP707A1 and CYP707A2 was higher in ice1-2 than the wild type at all time points (Figure 5i,j). This is not consistent with this effect being associated with dormancy change in ice1-2, because high CYP707A expression is associated with low dormancy in wild-type seeds (see, for example, Kendall et al., 2011). This is instead consistent with the fact that the expression of these genes is induced by ABA (Kushiro et al., 2004) and ice1-2 seeds have elevated ABA levels (Figure 4). We therefore concluded that this effect must be secondary to the elevated ABA content rather than due to a direct effect of ice1.

ICE1 is a transcriptional activator with demonstrated DNA-binding capabilities and has been shown to bind to
Figure 4. Mature ice1 and zou seeds contain more ABA and the ice1 endosperm is necessary and sufficient to slow the greening of excised embryos.

(a) Measurements of ABA from freshly harvested seed from four or more biological replicates of wild-type (Col-0), ice1-2 or zou-4 seeds matured at 22°C or the wild type (Col-0) matured at 16°C. Significant differences by Student’s t-test are shown where *P < 0.05; **P < 0.01.

(b) Seed coat bedding assay using wild-type (Col-0) or ice1-2 embryos on water agar, Col-0 endosperm or ice1-2 endosperm photographed every 24 h for 72 h. Intact seeds of each genotype sown on water agar are shown for reference.

© 2018 The Authors. The Plant Journal, (2019), 98, 277–290
MYC recognition sites (5'-CANNTG-3') found in the CBF3/ DREB1A and BON1-ASSOCIATED PROTEIN1 promoters (Chinnusamy et al., 2003; Lee et al., 2005; Agarwal et al., 2006; Zhu et al., 2011). Therefore, we wanted to determine if there was any evidence for direct binding of ICE1 to the ABA genes investigated above. We searched the promoters of these genes for putative ICE1-binding sites and found several candidate locations in the ABI3 promoter (Yilmaz et al., 2010; File S1). Chromatin immunoprecipitation (ChIP) on endosperm-enriched fractions of mature ice1-2 pICE1:ICE1-GFP or wild-type (Col-0) seeds were used to test for evidence of the association of ICE1 with the ABI3 promoter. As a control, we analysed the ABI5 promoter because ABI5 expression in seeds was not affected by ice1-2 (Figure 5). No evidence was found for GFP enrichment at the promoter of ABI5 or with the other negative controls (Figure 6). We also found no evidence for enrichment at putative ICE1-binding sites in the promoters of CYP707A2, CYP707A1, NCED6 or NCED9 (Figure S4). However, the ice1-2 pICE1:ICE1-GFP line demonstrated enrichment over the wild type at the CBF3 promoter, as expected from Chinnusamy et al. (2003), as well as at three locations.
in the promoter of *ABI3* (Figure 6). This area is approximately 2 kb upstream of the *ABI3* translation start site and coincides with a cluster of putative cis-elements that strongly resemble those previously identified as ICE1-binding sites (Chinnusamy *et al.*, 2003; Kim *et al.*, 2015). ICE1 is enriched at the *ABI3* promoter in a region containing the sequence of previously described cis-elements that are bound by the ICE1 protein *in vitro*. Loss of ICE1 leads to high levels of *ABI3* transcript, so we therefore conclude that ICE1 represses *ABI3* transcription. Because the AFL transcription factors directly upregulate ABA synthesis in Arabidopsis seeds (Gazzarrini *et al.*, 2004), our data suggest that ICE1 promotes dormancy through modulation of the levels of AFL transcription factor in the endosperm.

**DISCUSSION**

The acquisition of seed dormancy has allowed plants to establish seed banks and correctly time their germination according to seasonal cues. We demonstrate herein that loss of function of *ICE1* and/or *ZOU* results in seeds with increased primary dormancy and elevated accumulation of ABA (Figures 1, 2 and 4). The characterisation of the dormancy effects of ICE1 and ZOU is complicated by the co-occurrence of the effects on seed development caused by the failure of endosperm consumption. However, we show that the two processes can be separated. The aberrant endosperm consumption alone is insufficient to explain the dormancy phenotype, because in the *aba2* and *dog1* mutant backgrounds normal germination is restored without an effect on seed morphology (Figure 3). Our data show that the increase in dormancy is associated with an increase in seed ABA levels and that this ABA is probably present in the endosperm (Figure 4). The SCBA data demonstrate that mature *ice1* endosperm works more efficiently to arrest embryonic growth (Figure 4), which is consistent with the idea that this is a mature endosperm that has higher ABA levels. This view is further supported by the fact that both *ICE1* and *ZOU* are expressed in the endosperm of seeds and bolsters the increasing body of evidence demonstrating that the endosperm is the primary site of control of dormancy and germination in Arabidopsis. Our data show that the AFL transcription factor gene *ABI3* is a direct target of ICE1 in seeds (Figure 6) and *ABI3* transcript levels are higher in *ice1* seeds than in the wild type (Figure 5). A similar effect of *ICE1* on *ABI3* levels has been observed in seedlings on high-sugar media (Liang and Yang, 2015). Transcript levels of some endosperm-expressed AFL target genes such as *MYB118* (Barthole *et al.*, 2014) are also
increased in ice1 (Figure 5). Our data are therefore consistent with a model in which ICE1 and ZOU are inhibitors of the seed maturation programme in the endosperm via control of AFL activity, as well as promoters of endosperm consumption and biogenesis of embryonic cuticle via ABNORMAL LEAF-SHAPE 1 (ALE1; Denay et al., 2014). This role is very similar to that described previously for MYB118. This transcription factor, which is closely related to MYB115 (Wang et al., 2009), functions in the endosperm and is essential for biosynthesis of omega-7 monounsaturated fatty acids via transcription of two Δ9 acyl-ACP desaturases, AAD2 and AAD3 (Troncoso-Ponce et al., 2016), and inhibits AFL gene activity, thus delaying the seed maturation programme (Barthole et al., 2014; Figure 7).

Mature angiosperm seeds display considerable morphological diversity, and this is accompanied by a range of dormancy-inducing mechanisms. For instance, in morphological dormancy seed dormancy is initiated by an arrest of embryo development before maturation, such that further development is necessary after shedding before the seed can germinate. There are also examples of seeds displaying two distinct types of dormancy, especially combining morphological dormancy with physiological dormancy, each of which may be responsive to distinct environmental signals (Baskin and Baskin, 2004). These variations in dormancy programmes appear to be able to evolve independently multiple times, but it is unclear whether or how seed development and physiological dormancy evolve separately or by a common process.

According to the classification of Baskin and Baskin (2014), seeds whose embryos are differentiated but underdeveloped and which exhibit physiological dormancy are classed as having morphophysiological dormancy. The ice1 and zou mutant embryos clearly meet the morphological definition (Denay et al., 2014) and the phenotypes of these mutant embryos strongly resemble those from many gymnosperm seeds. During germination, embryo growth takes place before emergence of the shoot and before and during emergence of the root. However, lack of germination is not simply due to delayed embryo growth because prolonged incubation of ice1-2 or zou-4 seeds does not result in increased levels of germination (Figure 2). Therefore, although ice1 and zou seeds have increased dormancy and altered morphology, they do not exhibit morphological dormancy. To qualify as seeds with morphophysiological dormancy, embryo growth must be a pre-requisite for either root or shoot emergence and this growth can be promoted by a separate signal from that which breaks the physiological dormancy. We showed that cold does not promote the growth of ice1-2 embryos during stratification (Figure 2). In this case, cold is required to break the increased physiological dormancy of ice1 and embryo growth resumes only after seeds are placed in the warm lit conditions. This behaviour resembles a morphophysiological dormancy state described as ‘non-deep simple’ (Baskin and Baskin, 2014). Thalictrum mirabile (Ranunculaceae) exhibits non-deep simple dormancy and the seeds require cold stratification followed by warm temperatures which allow embryo growth to resume as the seeds germinate (Walck et al., 2011). Regardless of whether ice1 and/or zou seeds exhibit complete morphophysiological dormancy, this raises the prospect that single mutations in key genes can couple physiological dormancy with morphological changes to the embryo in the mature seed, suggesting mechanisms through which the evolution of seed dormancy can occur.

Figure 7. Model summarising how repression of the AFL transcription factor ABI3 by ICE1 and ZOU will regulate ABA metabolism in the endosperm. In endosperm, ICE1 is enriched at the ABI3 promoter and represses its expression. The AFL transcription factors, which are maximally expressed in the developing endosperm (Le et al., 2010), upregulate ABA synthesis in Arabidopsis seeds. ABA is necessary and sufficient to repress germination. The AFL transcription factors act by regulating each other’s expression and are necessary for dormancy establishment. ZOU and ICE1 are also involved in regulating ALE1 and therefore embryonic cuticle formation (Denay et al., 2014). This parallels the activity of MYB115/MYB118, which in addition to regulating fatty acid biosynthesis through the Δ9 acyl-ACP desaturases AAD2 and AAD3 (Troncoso-Ponce et al., 2016) also inhibit endosperm maturation via the ALF transcription factor LEC2 (Barthole et al., 2014). LEC2 is also a transcriptional activator of MYB118 (Barthole et al., 2014).
EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh ecotype Columbia (Col-0) was used in this study. ice1-2 (SALK-003155; Kanaoka et al., 2008) was a kind gift from Keiko Torii. zou-4, ice1-2 zou-4 double mutant and the pICE1::ICE1-GFP in Columbia (Denay et al., 2014) were kind gifts from Gwyneth Ingram. dog1-2 (Nakabayashi et al., 2012) was a kind gift from Wim Sompe. aba2-1 (MacGregor et al., 2008) was a kind gift from Jocelyn Malamy. Segregating populations of ice1-3 (SALK_003426, N503426, not previously characterised) and zou-3 (WiscDelox64565F, N857109, (Zhang et al., 2016) were obtained from the Nottingham Arabidopsis Stock Centre and homozygous plants were isolated using standard PCR methods and the primers presented in Table S1. zou-3 is in the Col background, despite what is stated elsewhere (Yang et al., 2008).

Plants were sown, grown and harvested as per the methods described by MacGregor et al. (2015). Great care was taken to ensure that for each figure the controls and mutants were grown together under conditions that were as uniform as possible (e.g. at the same time, in the same tray, on the same shelf, within the same cabinet) so that comparisons between the lines could be made. Dry sterile seeds were sown out and stratified at 4°C for 2–4 days on MS agar plates (4.4 g L−1 MS basal salt mixture, Sigma Aldrich, cat. no. R0901) or the appropriate solvent (Sigma Aldrich, cat. no. A1296, http://www.sigmaaldrich.com/) after 7 days of exposure to a Leica LAS X software. The stage of development was verified by chloral hydrate clearing of seeds after microscopy.

Dormancy assays

Mature dry seeds set under the conditions above were harvested and poorly filled seeds excluded using a 250-µm sieve (Fisher Scientific, cat. no. 11542153, http://www.thermofisher.com/). These sieved seeds were sown directly onto water-agar (0.9%; Sigma Aldrich, cat. no. A1296) and cold-stratified at 4°C in the dark using a Panasonic MIR-154 incubator (Panasonic, https://www.pchd.com/global/biomedical/) for the desired length and/or put directly into 12-h:12-h light:dark light regimen at 22°C under standard long days using fluorescent white light at 80–100 µmol m−2 sec−1 until bolting or anthesis of the first flowers. Once flowering, plants were transferred to growth cabinets running the same conditions, but with the indicated seed maturation temperatures, and left to set seed until dehiscence began.

Seed coat bedding assays

Seed coat bedding assays were performed using freshly harvested seeds that had been stored at −80°C until analysis according to the protocols in Lee and Lopez-Molina (2013).

Phytohormone assays

Abscisic acid was quantified from five biological replicate batches of 100 mg of freshly harvested dry seeds that were flash frozen in liquid nitrogen and stored at −80°C until analysis. Quantification of hormones was performed by ultraperformance liquid chromatography-mass spectrometry analysis of acidified isopropanol (1% acetic acid) extracts as described previously (Dave and Graham, 2012).

Analysis of gene expression

Three biological replicates of developing seeds at the stages indicated were dissected out of siliques of wild-type or ice1-2 plants grown at 22°C under conditions above directly into RNAlater (Sigma Aldrich, cat. no. R9001), which was subsequently removed before the seeds were flash frozen in liquid nitrogen and stored at −80°C until required for analysis. The RNA was extracted from these seeds as described previously (Penfield et al., 2005) and purified via the clean-up protocol of the RNeasy Plant RNA isolation kit (Qiagen, cat. no. 74904, http://www.qiagen.com/) according to the manufacturer’s protocol. First-strand cDNA was synthesised with 1 µg of total RNA in 20-µl reactions using Superscript III Reverse Transcriptase (Invitrogen, cat. no. 18080-044, http://www.invitrogen.com/) and Oligo(dT)12-18 (Sigma Aldrich, cat. no. 18418-012) according to the manufacturer’s instructions. Then 180 µl of...
water was added before the quantitative PCR step. Gene expression levels were determined in a Bio-Rad CFX96 instrument (http://www.bio-rad.com/) using the primers indicated in Table S1 and Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, cat. no. 600883, http://www.agilent.com/) according to both manufacturer’s protocols.

Chromatin immunoprecipitation

Freshly harvested seeds from wild-type and ice1-1 pICE1:ICE1-GFP or wild-type Col-0 plants were grown under standard long-day greenhouse conditions, surface-sterilised for 3 min in bleach and washed at least four times with sterile water. Sterile seeds were plated onto filter paper in Petri dishes containing 20 μL paclobutrazol (Sigma Aldrich, cat. no. 46046). The Petri dishes were sealed with micropore tape and incubated in a 12-h:12-h light (80 trazol (Sigma Aldrich, cat. no. 46046). The Petri dishes were sealed with micropore tape and incubated in a 12-h:12-h white light (80°C) and then transferred to a growth cabinet (Panasonic) for 24 h. Glass microscope slides were used to extract seeds until the embryos were forced from the endosperm and seed coat, all of which were collected in a 50-ml tube. A fraction enriched in endosperm and seed coat was obtained by spinning these mechanically disrupted seeds at 3000 g for 10 min in 40% sucrose (w/v), which separates embryos from endosperm and/or seed coat and intact seeds. Embryos were discarded and the endosperm-enriched fractions were rinsed with sterile distilled water to remove the sucrose and fixed in 1% formaldehyde for 10 min under a vacuum. Fixed tissues were quenched with a final concentration of 125 mM glycine under a vacuum for 5 min and rinsed at least three times with sterile distilled water before being flash frozen in liquid nitrogen. Isolation and shearing of chromatin and immunoprecipitation of GFP-enriched fractions were all performed as described elsewhere (Keily et al., 2013) using primers described in Table S1.

ACCESSION NUMBERS AND PRIMER SEQUENCES

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases using the accession numbers AB2A2 (AT1G52340), ABI3 (AT3G24650), ABI4 (AT2G40220), ABI5 (AT2G36270), AT2G23230, CACS (At5g46630), CYP707A1 (AT4G19230), CYP707A2 (AT2G29090), DOG1 (AT5G45830), ICE1 (AT3G26744), MYB118 (AT3G27785), NCE6D (AT3G24220), NCE9D (AT1G78390) and ZOU (AT1G49770). The primer sequences used are detailed in Table S1. Primers that have not been previously published elsewhere were designed by hand or using dCaps Finder (http://helix.wustl.edu/dcaps/dcaps.html), QuantPrime (Arvidsson et al., 2008) or Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012).

AUTHOR CONTRIBUTIONS

DRM designed and performed the research, analysed the data and wrote the paper. NZ performed the research and analysed the data. MI designed and performed the research and analysed the data. MC performed the research and analysed the data. AD designed and performed the research and analysed the data. LLM designed the research and wrote the paper. SDP designed the research and wrote the paper.

ACKNOWLEDGEMENTS

We thank Keiko Torii, Gwyneth Ingrain, Wim Soppe and Jocelyn Malamy for their kind gifts of seeds. We thank Rebecca Wilsbury and Kate Le Coq for their technical assistance during this project. This work was funded by BBSRC research grant number BB/L003198/2 to DRM and SDP. DRM is currently funded on the Smart Crop Protection Industrial Strategy Challenge Fund (BBS/OS/CP/000001) at Rothamsted Research. Work in LLM’s laboratory was supported by grants from the Swiss National Science Foundation (31003A_152660) and by the State of Geneva.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

SUPPLEMENTARY INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The dormancy phenotypes of ice1 and zou are repeatable and robust.

Figure S2. The increased dormancy of ice1 or zou can be rescued by exogenous gibberellin in a concentration dependent manner or by after-ripening.

Figure S3. ICE1-GFP is located in the nuclei of both stomata in true leaves and endosperm of developing seeds.

Figure S4. Chromatin immunoprecipitation using endosperm-enriched fractions of ice1-1 pICE1:ICE1-GFP shows no evidence for enrichment at putative ICE1-binding sites in the promoters of CYP707A2, CYP707A1, NCE6D or NCE9D.

File S1. Putative ICE1-binding sites in the targets in Figure 5.

Table S1. Primers used herein.

Table S2. Testing the significance of the ice1-2 and zou-4 dormancy phenotypes over multiple experiments.

REFERENCES

Adams, S., Manfield, I., Stockley, P. and Carré, I. (2015) Revised morning loops of the Arabidopsis circadian clock based on analyses of direct regulatory interactions. PLoS ONE 10, e0143943.

Agarwal, M., Hao, Y., Kapoor, A., Dong, C.-H., Fujii, H., Zheng, X. and Zhu, J.-K. (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. J. Biol. Chem. 281, 37636–37645.

Arvidsson, S., Kvasničeský, M., Riano-Pachon, D. and Mueller-Roeber, B. (2008) QuantPrime - a flexible tool for reliable high-throughput primer design for quantitative PCR. BMC Bioinformatics, 9, 465.

Barthole, G., To, A., Marchive, C., Brunaud, V., Soulignou-Taconnat, L., Berger, N., Dubreucq, B., Lepiniec, L. and Baud, S. (2014) MYB118 represses endosperm maturation in seeds of Arabidopsis. Plant Cell, 26, 3519–3537.

Baskin, J. and Baskin, C. (2004) A classification system for seed dormancy. Seed Science Research, 14, 1–16. https://doi.org/10.1076/SSR2003150

Baskin, C. and Baskin, J. (2014) Seeds: ecology, biogeography, and evolution of dormancy and germination.

Baud, S., Boutin, J.-P., Miquel, M., Lepiniec, L. and Rochat, C. (2002) An integrated overview of seed development in Arabidopsis thaliana ecotype WS. Plant Physiol. Biochem. 40, 151–160.

Bethke, P., Llibouret, I., Aoyama, N., Chung, Y.-Y., Still, D. and Jones, R. (2007) The Arabidopsis aleurone layer responds to nitric oxide, gibberellin, and abscisic acid and is sufficient and necessary for seed dormancy. Plant Physiol. 143, 1173–1188.

Carbonero, P., Iglesias-Fernández, R. and Vicente-Carbajosa, J. (2017) The AFL subfamily of B3 transcription factors: evolution and function in angiosperm seeds. J. Exp. Bot. 68, 871–880.
Frey, A., Effroy, D., Lefebvre, V., Seo, M., Perreau, F., Berger, A., Sechet, J., Kendall, S., Hellwege, A., Marriot, P., Whalley, C., Graham, I. and Finkelstein, R. and Lynch, T.

Karssen, C.M., Brinkhorst-van der Swan, D.L.C., Breekland, A.E. and Koornneef, M., Yim, S., Choi, H., Kim, A., Lee, K., Lopez-Molina, L., Martinoia, E., Kanaoka, M., Pillitteri, L., Fujii, H., Yoshida, Y., Bogenschutz, N., Takabayashi, J., Zhu, J.-K.

Proceedings of the National Academy of Sciences

Plant Cell

7 (2003) ICE1: a regulator of cold-induced transcriptome and parallel regulation of DOG1 and hormone metabolism by low temperature and CBP transcription factors. Plant Cell, 23, 2569–2590.

© 2018 The Authors.
The Plant Journal published by John Wiley & Sons Ltd and Society for Experimental Biology., The Plant Journal (2019), 98, 277–290.
dormancy release in Arabidopsis is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. *Plant Cell*, 24, 2826–2838.

Nambara, E., Naito, S. and McCourt, P. (1992) A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new abf3 allele. *Plant J.*, 2, 435–441.

Nambara, E., Nambara, E., McCourt, P. and Naito, S. (1995) A regulatory role for the ABI3 gene in the establishment of embryo maturation in Arabidopsis thaliana. *Development*, 121, 629–636.

Penfield, S., Josse, E.-M., Kannangara, R., Gilday, A., Halliday, K. and Grahn, M. (2016) Arabidopsis ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. *Plant Physiol.*, 141, 97–107.

Pary, F., Valon, C., Raynal, M., Gautier-Comella, P., Delseny, M. and Giraudat, J. (1994) Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. *Plant Cell*, 6, 1567–1582.

Penfield, S. (2006) Arabidopsis ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. *Plant Cell*, 18, 1887–1899.

Penfield, S., Josse, E.-M., Kannangara, R., Gilday, A., Halliday, K. and Graham, I. (2005) Cold and light control seed germination through the bHLH transcription factor SPATULA. *Plant Mol. Biol.*, 561. https://doi.org/10.1007/s11103-016-0487-2

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. and Rozen, S.G. (2012) Primer3-new capabilities and interfaces. *Nucleic Acids Res.*, 40, e115–e115.

Vaistij, F.E., Gan, Y., Penfield, S., Gilday, A.D., Dave, A., He, Z., Josse, E.M., Choi, G., Halliday, K.J. and Graham, I.A. (2013) Differential control of seed primary dormancy in Arabidopsis ecotypes by the transcription factor SPATULA. *Proc. Natl Acad. Sci. USA*, 110, 10866–10871.

Walc, J., Hidayati, S., Dixon, K., Thompson, K. and Poschloid, P. (2011) Climate change and plant regeneration from seed. *Glob. Change Biol.*, 17, 2145–2161.

Wang, X., Niu, Q.-W., Teng, C., Li, C., Mu, J., Chua, N.-H. and Zuo, J. (2009) Overexpression of PGA37/MYB118 and MYB115 promotes vegetative-to-embryonic transition in Arabidopsis. *Cell Res.*, 19, 224–235. https://doi.org/10.1038/cr.2008.276.

Xing, Q., Gref, A., Waters, A., Tanaka, H., Goodrich, J. and Ingram, G. (2013) ZHOUI controls embryonic cuticle formation via a signalling pathway involving the subtilisin protease ABNORMAL LEAF-SHAPE1 and the receptor kinases GASSHO1 and GASSHO2. *Development*, 140, 770–779.

Yang, S., Johnston, N., Talideh, E., Mitchell, S., Jeffree, C., Goodrich, J. and Ingram, G. (2008) The endosperm-specific ZHOUI gene of Arabidopsis thaliana regulates endosperm breakdown and embryonic epidermal development. *Development*, 135, 3501–3509.

Yilmaz, A., Mejia-Guerra, M.K., Kurz, K., Liang, X., Welch, L. and Grotewold, E. (2010) AGRIS: the Arabidopsis gene regulatory information server, an update. *Nucleic Acids Res.*, 39, D1118–D1122.

Zhang, Y., Li, X., Goodrich, J., Wu, C., Wei, H., Yang, S. and Feng, X. (2016) Reduced function of the RNA-binding protein FPA rescues a T-DNA insertion mutant in the Arabidopsis ZHOUI gene by promoting transcriptional read-through. *Plant Mol. Biol.*, 91, 549–561. https://doi.org/10.1007/s11103-016-0487-2

Zhu, Y., Yang, H., Mang, H.-G. and Hua, J. (2011) Induction of BAP1 by a moderate decrease in temperature is mediated by ICE1 in Arabidopsis. *Plant Physiol.*, 159, 589–588.