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

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Running title

ICE1 & ZOU determine primary seed dormancy depth

Keywords

ABA, ABI3, endosperm consumption, ICE1, primary dormancy, seed development, ZOU

## Abstract

Seed dormancy is a widespread and key adaptive trait that is essential for the establishment of soil seed banks and prevention of preharvest sprouting. Herein we demonstrate that the endosperm-expressed transcription factors *ZHOUPI* (*ZOU*) and *INDUCER OF CBF EXPRESSION1* (*ICE1*) play a role in determining primary dormancy depth in Arabidopsis. We show that *ice1* or *zou* increases seed dormancy and the double mutant has an additive phenotype. The increased dormancy is associated with increased ABA levels, and can be separated genetically from their 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 the *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.

## Abbreviations

9-cis-epoxycarotenoid dioxygenase (NCED)
Abscisic Acid (ABA)
Abscisic Acid 8'-hydroxylase (CYP707A)
*ABSCISIC ACID DEFICIENT 2* (*ABA2*)
*ABSCISIC ACID INSENSITIVE3* (*ABI3*)
*ABSCISIC ACID INSENSITIVE4* (*ABI4*)
*ABSCISIC ACID INSENSITIVE5* (*ABI5*)
Chromatin immunoprecipitation (ChIP)
Columbia (Col)
*C-REPEAT BINDING FACTORS* (*CBFs*)
*DELAY OF GERMINATION 1* (*DOG1*).
Gibberellin (GA)
*INDUCER OF CBF EXPRESSION1* (*ICE1*)
*AFL* transcription factors (*ABSCISIC ACID INSENSITIVE 3, FUSCA3 and LEAFY COTYLEDONS 2*),
*ZHOUPI* (*ZOU*)

## Introduction

After fertilisation seeds enter a rigid developmental program 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 where 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 (Cadman et al 2006). In the laboratory, these environmental responses are exploited to create simple dormancy-breaking treatments such as cold stratification or dry after-ripening which are often used as methods for comparing seed dormancy depth between genotypes.

Depending on the plant species, primary dormancy can be conferred either by the embryo or imposed by the surrounding tissues (Finch-Savage and Leubner-Metzger, 2006). The latter is known as coat-imposed dormancy, and is prevalent in the *Brassicaceae* including Arabidopsis. Coat-imposed dormancy requires properties of both the seed coat and endosperm in Arabidopsis (Debeaujon et al., 2000; Bethke et al., 2007; Doherty and Kay, 2010; Lee et al., 2010; Lee et al., 2012b; Piskurewicz and Lopez-Molina, 2016; Fedi et al., 2017).

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 regulating 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 dormancy control (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 dormancy control. Before the switch to seed maturation much of the endosperm is consumed, 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 *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 ICE1-target genes in cold signalling, the *C-REPEAT BINDING FACTORS* (*CBF*s) also are 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 show 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 germination prevention (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 lead 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 Supplemental Figure 1; this statement is supported by the statistical analysis in Supplemental Table 2. To demonstrate that this phenotype was indeed increased dormancy levels, we determined what effect dormancy breaking treatments would have on wild type, *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 seven 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 ($GA_3$), 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 (Supplemental Figure 2). These data suggest that in addition to the morphological phenotype, *ICE1* and *ZOU* have a role in seed dormancy control.

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 predicated 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 in *ice1-2* expressing *pICE1:ICE1-GFP* (Supplemental Figure 3). 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 seed dormancy control, and that both paternal and maternal copies contributed to this process.

Lowering the temperature during seed maturation is sufficient to increase seed dormancy levels (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 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 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 program to run slowly or not at all. To determine whether the seeds were truly

dormant or simply slow to germinate, we assessed *ice1* or *zou* germination 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 seven 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 3 A, B). Although non-dormant, the double mutant seeds between *aba2* or *dog1* and *ice1* still exhibited the darker shrivelled seed phenotype and altered embryo morphology characteristic of *ice1* (Figure 3 C, D). These data further support the conclusion that the germination failure of *ice1* mutant seeds 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.

ABA production by the endosperm is known to be a critical step to repress 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 have a higher ABA content in the mature seed compared to 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 was 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 is regulating ABA responses, we examined the expression levels of relevant genes in developing seeds of *ice1* compared to wild-type. Understanding how transcripts are regulated by ICE1 in whole seeds is complicated by the fact that *ice1* not only potentially directly affects gene expression regulation, but 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 5 A&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 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 cotyledon stage, at which point expression was lower in *ice1-2* (Figure 5 C&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 *ABI3* and *ABI5* genes 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 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 to 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 dormancy changes, 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 testa outer integument layer 1 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 Abscisic Acid 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., 2006). In wild type, *CYP707A2* is expressed in the embryo and the endosperm during late maturation through 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 wild type at all time points (Figure 5 I&J). This is not consistent with this affect 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 ABA-induced (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 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); Supplemental File 1). Chromatin immunoprecipitation (ChIP) on endosperm-enriched fractions of mature *ice1-2 pICE1:ICE1-GFP* or wild type (Col) seeds was used to test for evidence of ICE1 association 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* (Supplemental Figure 4). However, the *ice1-2 pICE1:ICE1-GFP* line demonstrated enrichment over 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 2kb 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 *ABI3* transcript levels, so we therefore conclude that ICE1 represses *ABI3* transcription. Because the *AFL* transcription factors directly up-regulate ABA synthesis in Arabidopsis seeds (Gazzarrini et al., 2004), our data suggests that ICE1 promotes dormancy through modulation of *AFL* transcription factor levels in the endosperm.

## Discussion

The acquisition of seed dormancy has allowed plants to establish seed banks and correctly time their germination with seasonal cues. We demonstrate herein that loss of function of *ICE1* and/or *ZOU* result in seeds with increased primary dormancy and elevated accumulation of ABA (Figures 1, 2 & 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 are separable processes. 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 likely present in the endosperm (Figure 4). The SCBA data demonstrates that mature *ice1* endosperm is working 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 endosperms of seeds and bolsters the increasing body of evidence demonstrating the endosperm is the primary site of dormancy and germination control 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 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 embryonic cuticle biogenesis 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 are essential for omega-7 monounsaturated fatty acid biosynthesis via transcription of two Δ9 acyl-ACP desaturases *AAD2* and *AAD3* (Troncoso-Ponce et al., 2016) and inhibit *AFL* gene activity and thus delay 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 incubations of *ice1-2* or *zou-4* seeds do 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 'nondeep simple' (Baskin and Baskin, 2014). *Thalictrum mirabile* (Ranunculaceae) exhibit nondeep 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 a 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.

# Materials and methods

## 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 Soppe. *aba2-1* (MacGregor et al., 2008) was a kind gift from Jocelyn Malamy. Segregating

populations of *ice1-3* (SALK_003426, N503426, not previously characterized) and *zou-3* (WiscDsLox465F5, 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 in Supplemental Table 1. *zou-3* is in the Col background, despite what is stated elsewhere (Yang et al., 2008).

Plants were sown, grown, and harvested per the methods described in MacGregor et al. (2015). Great care was taken 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 on and stratified at 4°C for 2–4 d on MS agar plates (4.4 g l−1 Murashige and Skoog (MS) basal salt mixture, Melford Laboratories cat. no. M0221 with 0.9% agar Sigma Aldrich cat. no. A1296). Seedlings were grown in growth cabinets at 22°C for 10–14 d with 12 : 12 h light : dark cycles before being transplanted to 40 cell trays containing John Innes Seed Compost. Plants were grown under well-watered conditions at 22°C under standard long days using fluorescent white light at 80–100 μmol m−2 s−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.

## 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). 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) for the desired length and/or put directly into a 12 : 12 h white light (80–100 μmol m−2 s−1) : dark light regime at 22°C in a Panasonic MLR growth cabinet (Panasonic) for germination. Exogenous gibberellic acid (Gibberellin $A_3$ Sigma Aldrich G7645), 10 mM potassium nitrate, norflurazon (norflurazon PESTANAL®, Sigma Aldrich 34364) or the appropriate solvent controls were supplemented to the molten water agar in the concentrations indicated in the figures. Germination was scored as the emergence of the radicle using a Leica MZ6 stereomicroscope after seven days of exposure to warm light incubation unless otherwise indicated. For each data point, germination frequency (%) was calculated as the percentage of seeds germinating from a minimum of 20 seeds from five biological replicates, which were

defined as seeds from different mother plants. Data are shown as averages of the biological replicates ± standard error. If statistics are shown, Student's T-Tests were performed on arcsine-transformed data and the single asterisk indicates significance of $P<0.05$ and double $P<0.01$. The germination phenotypes of *ice1* and *zou* are robust. Each experiment was repeated multiple times with comparable results being produced from different repeats; for clarity data from one experimental replication are shown.

## Double mutant creation and confirmation

Double mutants between *ice1-2* and *dog1-2*, *aba2-1*, or *pICE1:ICE1-GFP* were obtained by using pollen from homozygous donors to fertilize emasculated homozygous *ice1* plants, allowing the F1 generation to self, and screening the F2 seeds for the *ice1* shrivelled seed phenotype. Putative *ice1* homozygotes were then sown on plate supplemented with 1% sucrose and transferred to soil once established for further growth. Homozygosity of both mutations were confirmed by PCR (primer details in Supplemental Table 1) in the case of *ice1-2*, *dog1-2* and *aba2-1*, or by the ubiquitous presence of GFP fluorescence in the stomata of two generations of seedlings for *pICE1:ICE1-GFP*. GFP fluorescence of 500 to 530nm was visualized using a standardized GFP protocol on a stereo-dissecting microscope.

## Confocal microscopy

Developing seeds of *pICE1:ICE1-GFP* in *ice1-2* were excised from the siliques, mounted in water between a microscope slide and coverslip and were visualised on a Leica SP8X confocal microscope using Argon ion laser at 488nm to excite both GFP and auto fluorescence; emission of GFP was collected at 500 to 530nm and the auto fluorescence 600 to 630nm. A 63x/1.2 water immersion objective lens was used. The Z series in Figures C-F were collected at 0.5 micron intervals. Images were processed using Image J (https://imagej.nih.gov/ij/) in which max projections were made and scale bars added. The composite image was made by the Leica LAS X software. The stage of development was verified by chloral hydrate clearing of seeds after microscopy.

## Seed coat bedding assays

Seed coat bedding assays were performed using freshly harvested seed 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 5 biological replicate batches of 100 mg 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 in conditions above directly into RNAlater (Sigma Aldrich cat. no. R0901), which was subsequently removed before the seeds were flash frozen in liquid nitrogen and stored at -80°C until required for analysis. 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) according to the manufacturer's protocol. First-strand cDNA was synthesized with 1 µg of total RNA in 20 µl reactions using Superscript III Reverse Transcriptase (Invitrogen cat. no. 18080-044) and Oligo(dT)12-18 (Sigma Aldrich cat. no. 18418-012) according to the manufacturer's instructions. 180 µl water was added before the qPCR step. Gene expression analysis was determined in a BioRad CFX CFX96 instrument using the primers indicated in Supplemental Table 1 and Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies cat. no. 600883) according to both manufacturer's protocols.

## Chromatin Immunoprecipitation

Freshly harvested seed from wild type and *ice1-2 pICE1:ICE1-GFP* or wild type Columbia plants were grown under standard long-day greenhouse conditions were surface-sterilized for 3 minutes in bleach and washed at least four times with sterile water. Sterile seeds were plated out onto filter paper in petri dishes containing 20 µM paclobutrazol (Sigma Aldrich cat. no. 46046). The petri dishes were sealed with micropore tape and incubated in 12 : 12 h white light (80–100 µmol m−2 s−1) : dark light regime at 22°C in a Sanyo MLR growth cabinet (Panasonic) for 24hrs. Glass microscope slides were used to squeeze seeds until the embryos were forced from the endosperm and seed coat, all of which were collected into a 50ml tube. An endosperm and seed coat enriched fraction was obtained by spinning these mechanically disrupted seeds at 4000 rpm for 10 minutes 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 minutes under vacuum. Fixed tissues were quenched with a final concentration of 125mM glycine under vacuum for five minutes 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 Supplemental Table 1.

### 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 *ABA2* (AT1G52340), *ABI3* (AT3G24650), *ABI4* (AT2G40220), *ABI5* (AT2G36270), AT2G23230, *CACS* (At5g46630), *CYP707A1* (AT4G19230), *CYP707A2* (AT2G29090), *DOG1* (AT5G45830), *ICE1* (AT3G26744), *MYB118* (AT3G27785), *NCED6* (AT3G24220), *NCED9* (AT1G78390), *ZOU* (AT1G49770). Primer sequences used are detailed in Supplemental Table 1. 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).

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## Author Contributions

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

## Short Supplemental Files Legends

Supplemental Figure 1: The dormancy phenotypes of *ice1* and *zou* are repeatable and robust.

Supplemental Figure 2: The increased dormancy of *ice1* or *zou* can be rescued by exogenous gibberellin ($GA_3$) in a concentration dependent manner or by after-ripening.

Supplemental Figure 3: ICE1-GFP is located in the nuclei of both stomata in true leaves and endosperm of developing seeds.

Supplemental Figure 4: Chromatin immunoprecipitation (ChIP) using endosperm-enriched fractions of *ice1-2 pICE1:ICE1-GFP* shows no evidence for enrichment at putative ICE1 binding sites in the promoters of CYP707A2, CYP707A1, NCED6, or NCED9.

Supplemental File 1: putative ICE1 binding sites in the targets in Figure 5.

Supplemental Table 1: Primers used in MacGregor et al.

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

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# Figures Legends

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 wild type (Col-0, blue diamonds), *ice1-2* (red squares), *ice1-3* (green triangles), *zou-3* (purple X's), *zou-4* (cyan asterixis), and the *ice1-2*/zou-4 double mutant (orange circle) 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 to with stratification at 4°C for seven 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 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 Xs). **D.** The germination frequency for seeds of wild type (Col 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 three days at 4°C. **E.** The germination frequency of freshly harvested seeds matured at 16°C of 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 5 or more biological replicates of Col or *ice1-2* respectively with at least 20 seeds per batch, or 6 Col♀ ice1-2♂ or 8 ice1-2♀ Col♂ individual siliques with an average of 15 seeds per silique ± SE. For all, significant differences by Student's t- test on arcsine- transformed germination data where *, $P < 0.05$; **, $P < 0.01$.

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), *ice1-2* and *zou-3* 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) 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, blue diamonds) or *ice1-2* (red squares) from seeds shown in **B**. Data are averages of five or more biological replicate seed batches with at least 15 seeds per batch ± SE.

Figure 3: ABA biosynthesis and *DOG1* are required for the seed dormancy phenotype, but not the seed morphology phenotype, of *ice1*. **A.** The germination frequency for seeds of wild type (Col-0, blue diamonds), *ice1-2* (red squares), *aba2-1* (green triangles), and the *aba2-1/ice1-2* double mutant (purple Xs) matured at 22°C without or with stratification at 4°C for the given times. **B.** The germination frequency for seeds of wild type (Col-0, blue diamonds), *ice1-2* (red squares), *dog1-2* (green triangles), and the *dog1-1/ice1-2* double mutant (purple Xs) matured at 22°C without or with stratification at 4°C for the given times. For **A** and **B**, data are averages of five or more biological replicate seed batches with at least 50 seeds per batch ± SE. **C** and **D**. 50mm squares showing representative seeds from **A** and **B**.

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 Abscisic Acid

(ABA) from freshly harvested seed from four or more biological replicates of wild type (Col), *ice1-2*, or *zou-4* seeds matured at 22°C or wild type (Col) 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 (SCBA) using wild type (Col) or *ice1-2* embryos on water agar, Col endosperm, or *ice1-2* endosperm photographed every 24 hours for 72 hours. Intact seeds of each genotype sown on water agar are shown for reference.

Figure 5: *ice1* changes the expression of many genes including some in ABA biosynthesis, catabolism and/or signalling, however only *ABA INSENSITIVE 3* is significantly misregulated in developing *ice1* seeds. Wild type or *ice1* cDNA from developing seeds at globular, heart, torpedo or green cotyledon (Green Cot) stage were examined using qPCR for the expression of *ZHOUPI* (*ZOU*), *MYB118*, the embryo specific At2g23230, *ABSCISIC ACID INSENSITIVE4* (*ABI4*), *ABSCISIC ACID INSENSITIVE5* (*ABI5*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), the 9-cis-epoxycarotenoid dioxygenases *NCED6* and *NCED9*, and the Abscisic Acid 8'-hydroxylases *CYP707A1* and *CYP707A2* that were normalised against a clathrin adaptor complex subunit (CACS, At5g46630, (Nelson et al., 2009)). Similar data were found for normalisation against the control gene At4g12590 (Saez-Aguayo et al., 2017). Wild type (Col) is represented as black diamonds and *ice1-2* as grey squares with a hatched line. Data are averages of three biological replicate seed batches ± SE.

Figure 6: Chromatin immunoprecipitation (ChIP) using endosperm-enriched fractions of *ice1-2 pICE1:ICE1-GFP* (grey bars) shows enrichment at regions of the *ABI3* promoter that contain putative ICE1-binding sites. This enrichment is not seen in wild type (Col, black bars). Data represents the average ± SE of three biological replicates per locus. Primers in the 3'UTR of ACTIN2 (from Adams 2015) were used as a negative control and in the promoter of CBF3 as a positive control. The lower pannel represents the *ABI3* and *ABI5* promoters with the qPCR targets and putative ICE1 binding sites indicated.

Figure 7: Model summarising how ICE1 and ZOU repression of the *AFL* transcription factor *ABI3* 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), up-regulate 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 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 also is a transcriptional activator of *MYB118* (Barthole et al., 2014).