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RESEARCH REPORT

# Endosperm breakdown in *Arabidopsis* requires heterodimers of the basic helix-loop-helix proteins ZHOUPI and INDUCER OF CBP EXPRESSION 1

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## ABSTRACT

In *Arabidopsis* seeds, embryo growth is coordinated with endosperm breakdown. Mutants in the endosperm-specific gene *ZHOUPI* (*ZOU*), which encodes a unique basic helix-loop-helix (bHLH) transcription factor, have an abnormal endosperm that persists throughout seed development, significantly impeding embryo growth. Here we show that loss of function of the bHLH-encoding gene *INDUCER OF CBP EXPRESSION 1* (*ICE1*) causes an identical endosperm persistence phenotype. We show that ZOU and ICE1 are co-expressed in the endosperm and interact in yeast via their bHLH domains. We show both genetically and in a heterologous plant system that, despite the fact that both ZOU and ICE1 can form homodimers in yeast, their role in endosperm breakdown requires their heterodimerization. Consistent with this conclusion, we confirm that ZOU and ICE1 regulate the expression of common target genes in the developing endosperm. Finally, we show that heterodimerization of ZOU and ICE1 is likely to be necessary for their binding to specific targets, rather than for their nuclear localization in the endosperm. By comparing our results with paradigms of bHLH function and evolution in animal systems we propose that the ZOU/ICE1 complex might have ancient origins, acquiring novel megagametophyte-specific functions in heterosporous land plants that were conserved in the angiosperm endosperm.

**KEY WORDS: Endosperm, Seed, Transcriptional regulation**

## INTRODUCTION

Angiosperm seed development necessitates coordinated growth of the two products of fertilization, i.e. the endosperm and the embryo, within the maternally derived seed coat. In wild-type *Arabidopsis*, the syncytial endosperm expands soon after fertilization and then cellularizes (Garcia et al., 2005; Ingram, 2010; Sørensen et al., 2002). The space occupied by the endosperm after its cellularization is subsequently appropriated by the developing embryo as it expands, so that by the end of seed development only a single layer of endosperm cells remains. The process of endosperm breakdown is regulated by a unique and highly evolutionarily conserved basic helix-loop-helix (bHLH) transcription factor, ZHOUPI (ZOU). In *zou* mutants, the endosperm persists throughout seed development, significantly impeding the growth of the embryo and giving rise to a collapsed seed phenotype upon desiccation (Xing et al., 2013; Yang et al., 2008).

In addition to regulating the breakdown of the endosperm, ZOU regulates the expression of the subtilisin serine protease ALE1 in the endosperm, which in turn acts with two embryo-expressed receptor kinases, GASSHO1 and GASSHO2 (GSO1 and GSO2), to regulate embryonic cuticle biogenesis. Mutants in *ALE1*, *GSO1* and *GSO2* have no measurable defect in endosperm breakdown but produce abnormal embryonic surfaces (Tanaka et al., 2001; Xing et al., 2013; Yang et al., 2008).

bHLH transcription factors are known to act combinatorially as homo- or heterodimers to regulate developmental processes in both animals and plants (Jones, 2004; Massari and Murre, 2000; Pires and Dolan, 2010). To understand more about ZOU function, we investigated whether this unique protein might act in combination with other bHLH transcription factors in order to carry out one or both of its roles in the developing seed.

## RESULTS AND DISCUSSION

To identify potential protein partners of full-length ZOU protein, a yeast two-hybrid screen was carried out using a split ubiquitin-based system in which the bait protein is presented as a membrane-anchored fusion (Möckli et al., 2007). Two independent cDNA clones encoding similar truncated versions of the bHLH protein INDUCER OF CBP EXPRESSION 1 (ICE1, also known as SCREAM; AT3g26744) were obtained in a screen of 1.2 million clones (Fig. 1A; see Materials and Methods). Both clones encoded the bHLH domain-containing C-terminal region of the ICE1 protein (ICE1-C). ICE1-C was confirmed as a true interactor of ZOU by retransformation with the ICE1-C-containing prey vector and either the ZOU-containing bait vector or an empty bait vector, with growth on selective media only being observed in the former case (Fig. 1B).

Further constructions encoding full-length ICE1 (ICE1-FL) or ICE1-C as bait or prey fusions were used to test interactions with full-length ZOU (ZOU-FL) or with a truncated version lacking the bHLH domain (ZOU-C). Results are summarized in Fig. 1B. The ICE1-FL protein showed only a weak interaction with ZOU-FL in yeast. Although the ZOU-FL construct interacted strongly with ICE1-C, ZOU-C showed no interaction, suggesting that, as expected for this protein family, the bHLH domain-containing regions of the two proteins mediate their interaction (Jones, 2004). Homodimerization of both ZOU-FL and ICE1-C was detected in yeast.

ZOU expression is strictly localized to the developing endosperm (Yang et al., 2008). *In situ* hybridizations were carried out in developing seeds and, consistent with *in silico* data (Le et al., 2010), expression of *ICE1* was detected in all three seed tissues, the

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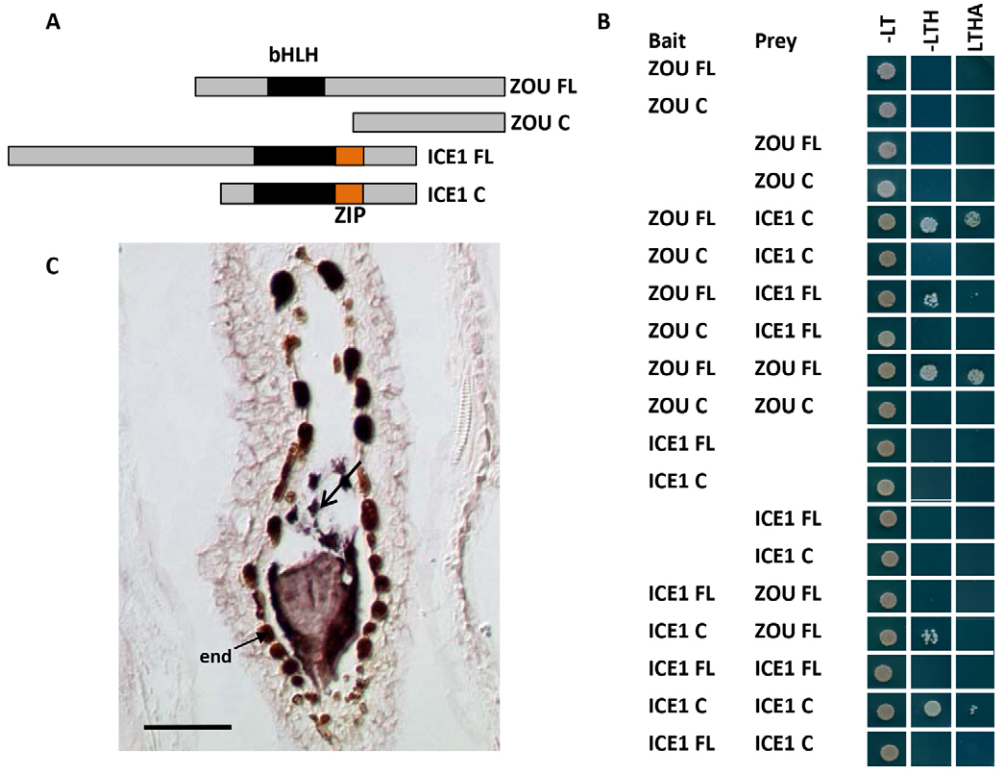


**Fig. 1. ZOU and ICE1 interact in yeast and are co-expressed during endosperm development.** (A) The proteins used in yeast two-hybrid experiments. (B) Summary of interactions tested in yeast. Empty boxes indicate empty vector controls. Growth on minimal non selective (–LT) and selective [–LTH (low stringency) and –LTHA (high stringency)] media is shown. (C) *In situ* hybridization shows strong expression of *ICE1* transcripts in the embryo-surrounding region (ESR) of the endosperm. Dark brown staining of the endothelium (end) is an artifact. True staining in the ESR is seen as a blue/black coloration (arrows). Weak signal is also present in the embryo and testa. Sense controls are presented in supplementary material Fig. S1. Scale bar: 100 µm.

endosperm, the embryo and the testa. Expression in the embryo-surrounding endosperm was particularly strong (Fig. 1C; supplementary material Fig. S1). *ZOU* and *ICE1* are therefore co-expressed in the endosperm during seed development.

ICE1 has well characterized roles in regulating both tolerance to cold stress (Chinnusamy et al., 2003; Lee et al., 2005; Miura et al., 2007) and stomatal development, in which the class III bHLH protein ICE1 and its close homolog SCREAM2 (SCRM2, also known as ICE2) have been shown to interact with the class Ia bHLH proteins SPEECHLESS, MUTE and FAMA (Kanaoka et al., 2008). We obtained the previously characterized null *ICE1* allele *ice1-2* (Kanaoka et al., 2008) and observed that homozygous plants produced shrivelled seeds (Fig. 2A-C). This phenotype could be complemented by an ICE1:GFP protein fusion expressed under the *ICE1* promoter (supplementary material Fig. S2), confirming that this phenotype is caused by loss of function of *ICE1*. In resin-embedded silique samples, we observed that, like *zou-4* mutants, *ice1-2* mutant seeds have a persistent endosperm and much reduced embryo (Fig. 2E-G). To test for seedling cuticle defects, *ice1-2*, *zou-4* and Col-0 seedlings were treated with the hydrophilic dye Toluidine Blue. Both *ice1-2* and *zou-4* seedlings showed similar, high levels of Toluidine Blue uptake compared with Col-0 seedlings (Fig. 2I-L). Thus, *ICE1*, like *ZOU*, is involved both in endosperm degradation and seedling surface formation.

The expression of five previously identified targets of ZOU activity (Xing et al., 2013), including *ALE1*, was investigated in staged *zou-4*, *ice1-2* and Col-0 siliques containing embryos between the late globular and late heart stage of development. The transcription of all five genes was found to be lost in both the *zou-4* and *ice1-2* mutant background, showing that, consistent with their similar mutant phenotypes, ICE1 and ZOU regulate common targets (Fig. 3A; supplementary material Fig. S3). To test whether, like *ICE1*, *ZOU* and *ALE1* (Yang et al., 2008), the other potential targets were strongly expressed in the endosperm, *in situ* hybridizations were carried out. Strong endosperm expression was detected in each case (Fig. 3C-E; supplementary material Fig. S3) and was not detected in either *zou-4* or *ice1-2* mutant backgrounds (Fig. 3F-H and supplementary material Fig. S3 show results for *zou-4*), consistent with the regulation of expression of these genes by ZOU and ICE1 in the endosperm.

To investigate the genetic relationship between *ZOU* and *ICE1* during seed development, double mutants were generated between *zou-4* and *ice1-2*. Double-mutant plants were viable and their seed and seedling permeability phenotypes were indistinguishable from those of either single mutant (Fig. 2D,H,I). To test whether *ZOU* and *ICE1* act sequentially in a transcriptional cascade, their expression levels were compared in wild-type, *zou-4* and *ice1-2* mutant backgrounds. No significant misregulation of *ICE1* was observed in *zou-4* mutants, or vice versa (Fig. 3B). The independence of the phenotypes of *ice1-2* and *zou-4* is therefore likely to be due to a non-redundant functional role for the ICE1/ZOU heterodimer in seed development.

In addition to the recessive loss-of-function allele *ice1-2*, previous studies have described *ice1-1/Scrm-D*, which is caused by a point mutation giving rise to a single amino acid substitution in the ICE1 protein (Chinnusamy et al., 2003; Kanaoka et al., 2008; Miura and Ohta, 2010). During stomatal development, this mutation causes a strong semi-dominant gain-of-function phenotype opposite to that observed in *ice1-2 scrm2* loss-of-function mutants, so that *Scrm-D* plants produce stomata over most of their cotyledon and leaf surfaces (Kanaoka et al., 2008). Despite this phenotype, *Scrm-D* plants are fertile, although silique initiation is slow and erratic. Mature seeds of *Scrm-D* homozygous plants were indistinguishable from those of wild type (Fig. 4G). When homozygous *Scrm-D* plants were crossed reciprocally with *ice1-2*, the resulting seeds resembled those of wild type, consistent with the *Scrm-D* allele functionally compensating for the presence of the recessive *ice1-2* allele in the heterozygous state during seed development.

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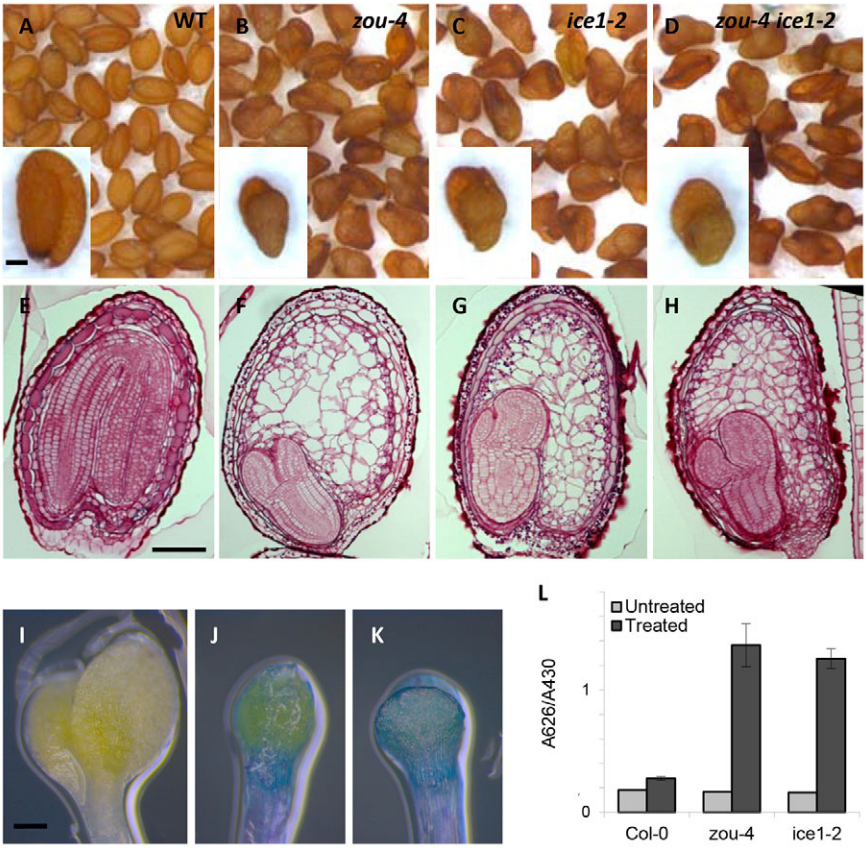


**Fig. 2. *ZOU* and *ICE1* show similar loss-of-function phenotypes and act in the same pathway with respect to endosperm breakdown.** (A-D) Dry seeds from Col-0, *zou-4*, *ice1-2* and *zou-4 ice1-2* plants. (E-H) Resin sections of mature non-desiccated seeds from Col-0, *zou-4*, *ice1-2* and *zou-4 ice1-2* plants, respectively, colored using the PAS reaction. (I,J) Cotyledons of etiolated 7-day-old seedlings of Col0 (I), *zou-4* (J) and *ice1-2* (K) stained with Toluidine Blue. (L) Quantification of Toluidine Blue (abs. 626 nm) uptake by 20 10-day-old light-grown seedlings, normalized to chlorophyll content (abs. 430 nm). Values indicate means ± s.d. of four biological replicates. Scale bars: 100 µm.

To assess whether *Scrm-D* affects endosperm development, the structure of developing *Scrm-D* homozygous seeds was examined. Although the endosperm of heart stage *Scrm-D* homozygous seeds started to cellularize apparently normally (Fig. 4A,D), by the torpedo stage, when the majority of wild-type endosperm is cellularized, the endosperm of *Scrm-D* homozygous seeds is densely packed around the periphery of the embryo cavity and the cell walls are particularly difficult to distinguish [phenotype observed clearly in 13 of 17 *Scrm-D* seeds sectioned at this stage, and in no simultaneously processed wild-type seeds (*N*=15)] (Fig. 4B,E). Mature non-desiccated *Scrm-D* homozygous seeds showed a large gap between the cotyledons and the hypocotyl (17 out of 19 seeds sectioned), which was not apparent in wild-type seeds processed simultaneously (*N*=16) (Fig. 4C,F). The phenotype of *Scrm-D* homozygous seeds is suggestive of precocious breakdown of the endosperm cell structure, confirming that *Scrm-D* is a hypermorphic allele of *ICE1* with respect to endosperm breakdown. Interestingly, homozygosity for *Scrm-D* did not significantly alleviate the small embryo phenotype of the *zou-4* seed. In this background the endosperm was well cellularized and persistent at seed maturity, as in *zou-4* mutants (Fig. 4H,I). Thus, the precocious endosperm breakdown phenotype of *Scrm-D* homozygous mutants is dependent upon the presence of functional ZOU protein within the developing seed.

Our results suggest that heterodimerization of ZOU and ICE1 is strictly necessary for target gene activation. The gene *At3g08900* was selected as a likely direct target of ZOU/ICE1, since its expression in endosperm is more than 1000-fold downregulated in both *zou-4* and *ice1-2* mutant backgrounds (supplementary material Fig. S3). We tested the activation of the *At3g08900* promoter by ZOU, ICE1 or SCRM-D and by combinations of ZOU with ICE or SCRM-D in a *Physcomitrella patens* protoplast system (Thévenin et al., 2012), and confirmed that only combinations of ZOU with ICE or SCRM-D could activate promoter activity to levels above background (supplementary material Fig. S3).

To eliminate the possibility that the formation of the ZOU/ICE1 heterodimer is necessary for protein localization, constructs expressing functional ZOU and ICE1 tagged with GFP under their native promoters (Yang et al., 2008) (supplementary material Fig. S2) were crossed into the reciprocal mutant background. Despite complementing the *ice1-2* mutant seed phenotype and being clearly visible in stomatal guard cells (supplementary material Fig. S2), the ICE1-GFP fusion protein could not be clearly visualized in the developing endosperm in any background. However, nuclear ZOU-GFP protein was present in both wild-type and *ice-2* mutant seeds, confirming that ICE1 is not necessary for ZOU localization in endosperm nuclei (Fig. 4J,K).

The roles of SPEECHLESS, MUTE, FAMA, ICE1 and SCRM2 in the regulation of sequential developmental decisions in stomatal development show similarities with bHLH-regulated cascades in animals controlling developmental processes, including vertebrate myogenesis and *Drosophila* neurogenesis (Serna, 2009). In each case, tissue-specific factors preferentially form heterodimers with structurally distinct and more widely expressed bHLH proteins (Jones, 2004; Massari and Murre, 2000; Murre et al., 1989). Here we show that another highly tissue-specific bHLH protein, ZOU, also forms heterodimers with ICE1 to regulate endosperm breakdown. As has previously been observed in interactions between tissue-specific and ubiquitous bHLH proteins in animal systems, we show that although both ICE1 and ZOU can form homodimers in yeast, ICE1/ZOU heterodimers are responsible for the biological activity of these proteins in the endosperm (Jones, 2004; Massari and Murre, 2000; Murre et al., 1989).

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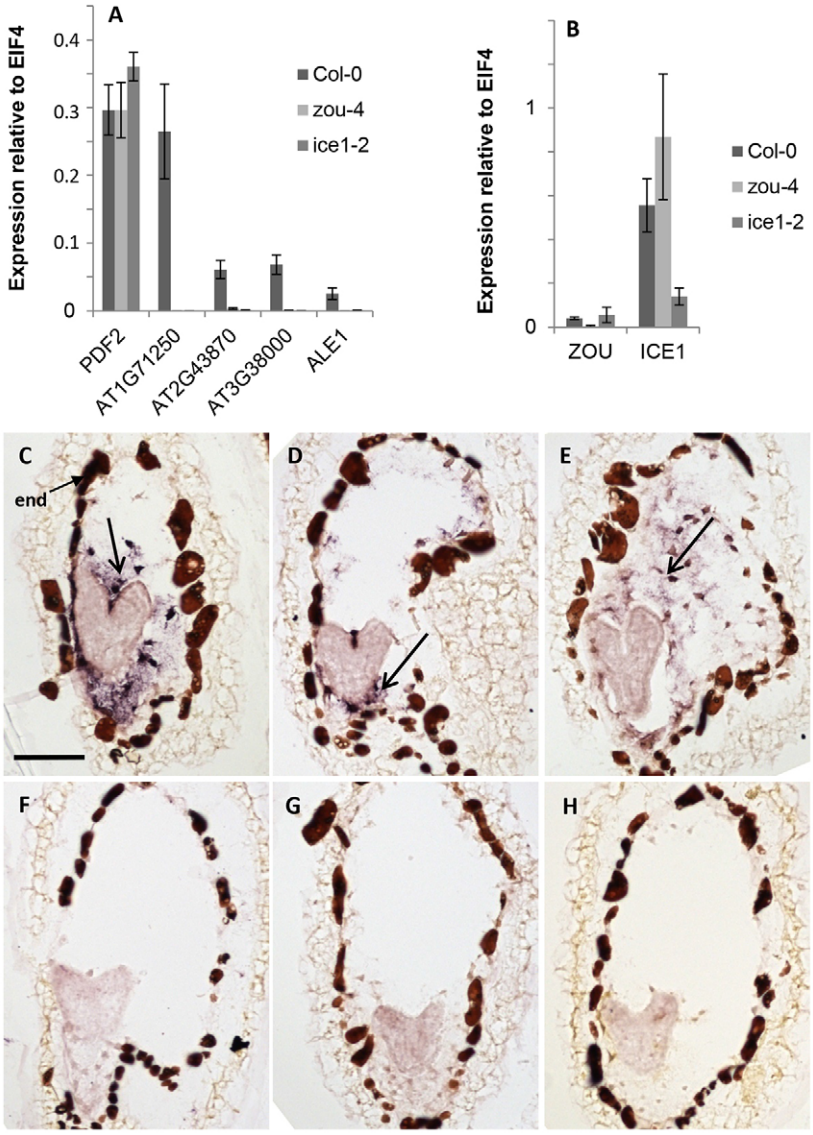


**Fig. 3. *ZOU* and *ICE1* share common ESR-expressed targets.** (A) Analysis of the expression of *PDF2* [unregulated control (Yang et al., 2008)] and the known ZOU-regulated genes *AT1G71250*, *AT2G43870*, *AT3G38000* and *ALE1* (Xing et al., 2013; Yang et al., 2008) in Col-0, *zou-4* and *ice1-2* backgrounds. (B) Expression of *ZOU* and *ICE1* in wild-type, *zou-4* and *ice1-2* backgrounds. qRT-PCR was carried out on staged silique material containing embryos at the mid-heart stage. *In situ* hybridization showing expression of (C,F) *AT1G71250*, (D,G) *AT2G43870* and (E,H) *AT3G38000* in the ESR of wild-type and *zou-4* seeds, respectively. Dark brown staining of the endothelium (end in C) is an artefact. True staining in the ESR is seen as a blue/black coloration (arrows in C-E). Values indicate means ± s.d. of three independent experiments. Scale bar: 100 µm.

Although ZOU possesses a unique C-terminal domain, recent phylogenetic analyses have placed it in bHLH class Ib1, relatively close to the class Ia SPEECHLESS, MUTE and FAMA proteins (Pires and Dolan, 2010). In contrast to ZOU, which is not clearly conserved in bryophytes (Yang et al., 2008), genes encoding both ICE1-like class IIIb and class Ia bHLH proteins can clearly be distinguished in the bryophyte *Physcomitrella*, which, like angiosperms, produces true stomata in the epidermal cell layer of the sporophyte (Vatén and Bergmann, 2012), leading to the suggestion that the class Ia/IIIb partnership might be ancient, possibly coinciding with the rise of the bryophytes and potentially contributing to the elaboration of true stomata (MacAlister and Bergmann, 2011; Vatén and Bergmann, 2012; Vatén and Bergmann, 2013).

*ZOU* expression in *Arabidopsis* is strictly limited to the endosperm, which in angiosperms is thought to be the sexualized homolog of the megagametophyte in lower land plants. Neofunctionalization of ZOU might have arisen after restriction of its expression to the astomatous gametophyte generation in a lycophyte ancestor, permitting rapid sequence divergence of ZOU relative to its more widely expressed and promiscuous class IIIb partner (ancestral ICE1). Neofunctionalization after changes in expression patterns has been evoked to explain the evolution of tissue-specific bHLH proteins involved in neural development in animal lineages (Simionato et al., 2008; Vervoort and Ledent, 2001). Detailed analysis of the expression patterns of class IIIb, class Ia and, where present, class Ib genes in lower plant species should serve to test this hypothesis in future studies.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Mutant *Arabidopsis thaliana* lines used in this study have been published previously. All plant lines were plated on Murashige and Skoog (MS) media (with or without selection), stratified for 3 days at 4°C, and germinated in a Lemnagen growth cabinet under long days (16 hours light) at 21°C for 7-10 days before transferring to soil in identical growth room conditions.

### Genotyping

Plant DNA was extracted using a rapid CTAB isolation technique as described (Stewart and Via, 1993). *zou-4* genotyping was carried out as previously described (Tanaka et al., 2001; Xing et al., 2013; Yang et al., 2008). Genotyping for *ice1-2* was performed using primers *ICE T-DNA F* and *ICE T-DNA R* for the wild-type allele and *ICE T-DNA F* with a SALK left border (LB) primer for the mutant allele (supplementary material Table S1).

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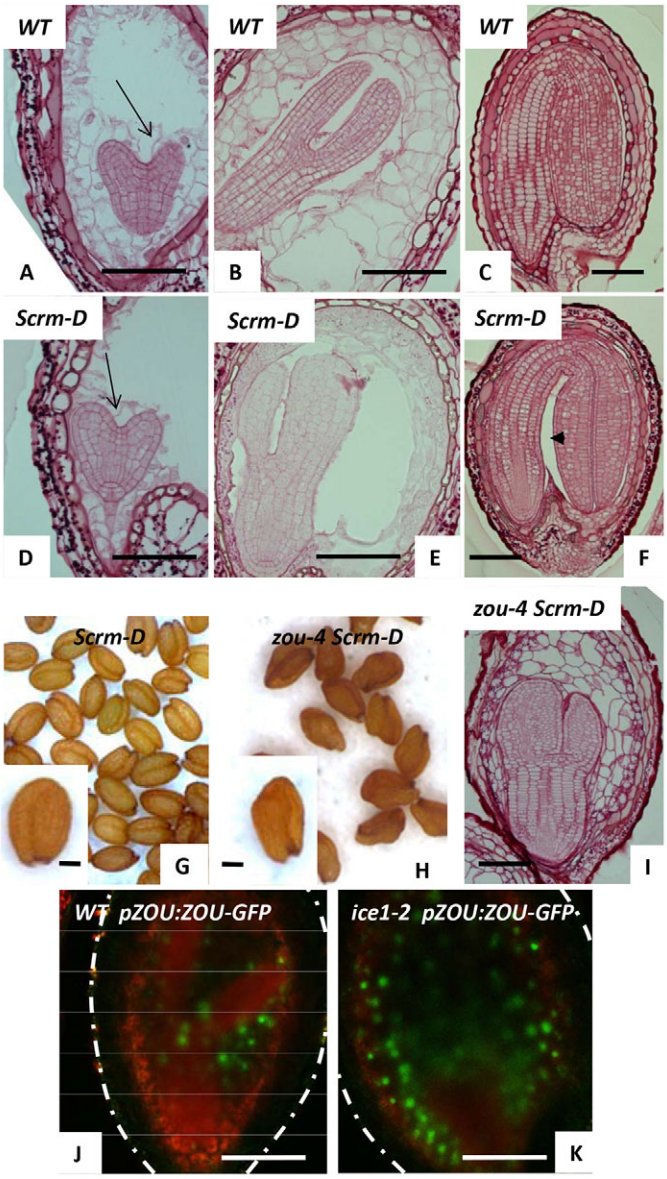


**Fig. 4. *Scrm-D* mutants show a precocious endosperm breakdown phenotype that is hypostatic to the persistent endosperm phenotype of *zou-4*.** (A) Wild-type and (D) *Scrm-D* homozygous seeds at the heart stage. Nascent cells walls in the ESR are indicated with arrows. (B) Wild-type and (E) *Scrm-D* homozygous seeds at the torpedo stage. Endosperm cell walls are difficult to distinguish in the *Scrm-D* mutant. Wild-type (C) and *Scrm-D* homozygous mutant (F) mature (non-desiccated) seeds. The characteristic gap between the hypocotyl and cotyledons in *Scrm-D* seeds is indicated by an arrowhead. (G,H) Seeds of *Scrm-D* and *zou-4 Scrm-D* homozygous mutants. (I) Resin section of a mature, non-desiccated seed from a *zou-4 Scrm-D* homozygous mutant showing reduced embryo growth and cellularized, persistent endosperm. (J,K) Confocal microscopy images showing nuclear localization of ZOU-GFP (green) in the endosperm of wild-type and *ice1-2* mutant seeds. Outer limits of the testa are delineated. Scale bars: 100 µm.

### Toluidine Blue staining

Procedures for the quantification of Toluidine Blue uptake were as described (Xing et al., 2013). Etiolated seedlings were stratified for 3 days at 4°C, exposed to 4 hours of light in a Lemnagen growth cabinet at 21°C, and then plates were covered with two layers of metal foil before allowing seedlings to germinate and grow for 5 days. Coloration was carried out as previously described (Xing et al., 2013).

### Resin embedding

Resin embedding and Periodic Acid Schiff (PAS) staining procedures were carried out exactly as described previously (Xing et al., 2013).

### *In situ* hybridization

Methods were as previously described (Yang et al., 2008). Antisense probes were transcribed from linearized pCR BluntII-TOPO vectors (Invitrogen) containing full-length ORFs amplified from silique cDNA with *ICE 5′ YEAST* and *ICE 3′ YEAST BAIT*, *1g71250cdsFor* and *1g71250cdsRev*, *2g43870cdsFor* and *2g43870cdsRev*, or *At3g38000cdsFor* and *At3g38000cdsRev* (supplementary material Table S1).

### Quantitative gene expression analysis

Protocols for staging of plant material and qRT-PCR analysis, including qRT-PCR primers for all genes except *ICE1*, have been described previously (Xing et al., 2013; Yang et al., 2008). Levels of *ICE1* were detected using *ICE1-Q-L* and *ICE1-Q-R* (supplementary material Table S1).

### Yeast two-hybrid screen

Yeast two-hybrid experiments were carried out using the Dual Hunter split ubiquitin-based system from Dualsystems Biotech (Schlieren, Switzerland) (Möckli et al., 2007). All yeast protocols were performed exactly as described in the manufacturer's instructions. Single bait and prey constructs were made by cloning PCR-amplified cDNA fragments flanked by in-frame *Sfi*I sites into pCR BluntII-TOPO vector (Invitrogen), and then transferring *Sfi*I-excised inserts into the appropriate yeast vector. Primers used for specific constructs are listed in supplementary material Table S1. A normalized cDNA library was generated commercially by Dualsystems Biotech from RNA isolated from a pool of developing siliques containing embryos ranging from the zygote to the mid-torpedo stage of development. RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma). Library screening was carried out under stringent conditions on media lacking both adenine and histidine.

### *Physcomitrella* protoplast assay

Moss culture, protoplast preparation, vector pBS TPp-A and -B recombination, protoplast transformation and flow cytometry measurement were carried out as described (Thévenin et al., 2012) (see supplementary material Fig. S3).

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#### Competing interests

The authors declare no competing financial interests.

#### Author contributions

All authors performed experiments. G.D., A.C., P.W., B.D. and G.I. analyzed data. G.D., A.C., S.M. and G.I. prepared the figures. G.I. wrote the manuscript.

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#### Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.103531/-/DC1

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