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The endosperm-specific *ZHOUPI* gene of *Arabidopsis thaliana* regulates endosperm breakdown and embryonic epidermal development

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During *Arabidopsis* seed development, the growing embryo invades and consumes the surrounding endosperm tissue. The signalling pathways that coordinate the separation of the embryo from the endosperm and the concomitant breakdown of the endosperm are poorly understood. We have identified a novel bHLH transcription factor, *ZHOUPI* (*ZOU*), which mediates these processes. *ZOU* is expressed exclusively in the endosperm of developing seeds. It is activated in the central cell immediately after fertilization and is initially expressed uniformly in endosperm, subsequently resolving to the embryo surrounding region (ESR). However, *zou* mutant embryos have defects in cuticle formation and in epidermal cell adhesion, suggesting that *ZOU* functions non-autonomously to regulate embryonic development. In addition, the endosperm of *zou* mutant seeds fails to separate from the embryo, restricting embryo expansion and resulting in the production of shrivelled collapsed seeds. *zou* seeds retain more endosperm than do wild-type seeds at maturity, suggesting that *ZOU* also controls endosperm breakdown. We identify several target genes whose expression in the ESR is regulated by *ZOU*. These include *ABNORMAL LEAF SHAPE1*, which encodes a subtilisin-like protease previously shown to have a similar role to *ZOU* in regulating endosperm adhesion and embryonic epidermal development. However, expression of several other ESR-specific genes is independent of *ZOU*. Therefore, *ZOU* is not a general regulator of endosperm patterning, but rather controls specific signalling pathways that coordinate embryo invasion and breakdown of surrounding endosperm tissues.

KEY WORDS: Endosperm, Embryo, Epidermis, *Arabidopsis*

INTRODUCTION

In angiosperm seeds, developing embryos are surrounded by the second zygotic product of double fertilization, the endosperm (Olsen, 2004). In many dicotyledonous plants, such as *Arabidopsis*, the endosperm is largely transient and is consumed during seed development. In *Arabidopsis*, a single specialized cell layer, which has been compared with the aleurone layer of monocotyledonous plants, is all that remains of the endosperm by seed maturity. Although the importance of the endosperm in embryo/seedling nutrition is uncontested, it may also provide developmental signals to the young embryo (Berger, 2003; Berger et al., 2006). In both monocotyledonous and dicotyledonous species, the region of the endosperm that directly surrounds the developing embryo (the embryo surrounding region or ESR) expresses a unique complement of genes, supporting the idea that it has a specialized function. Although several of these ESR-specific genes encode proteins with obvious metabolic roles, others encode small secreted peptides, some of which are related to the precursor of CLAVATA3 (CLV3), the likely ligand of the CLAVATA1 receptor-like kinase, supporting the supposition that the ESR may be involved in signalling (Opsahl-Ferstad et al., 1997; Sharma et al., 2003). However, no precise developmental role for any of these peptides has yet been demonstrated. Recent studies have also shown that the developing embryo can send developmental signals to the endosperm, as fertilization of the egg cell alone is sufficient to trigger proliferation of the central cell (Nowack et al., 2006).

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One potentially important role that the endosperm could play in embryogenesis is in defining the continuous cuticular layer at the boundary between the developing embryo and the endosperm. This structure is necessary to prevent organ fusion after germination, and it may also prevent fusion of the embryo surface with surrounding endosperm tissues during seed development, thus allowing normal embryo growth. In addition, the presence of a watertight cuticle surrounding the mature embryo is crucial in protecting it from desiccation upon germination (reviewed by Jeffree, 2006). Although the production of cuticle is generally considered to be a strictly epidermal property, a cuticularized cell wall has been reported to surround the unicellular zygote in *Citrus*, well before a defined protodermal cell layer is formed (Bruck and Walker, 1985). Interestingly, the expression of epidermal markers, such as the transcription factors *ATML1* and *PDF2*, which are required for epidermal specification in *Arabidopsis*, is also detected well before protoderm formation (Abe et al., 2003; Lu et al., 1996). Thus, the outer layer of embryonic cells display epidermal characteristics, including the presence of cuticularized tissue, from an extremely early stage in embryogenesis.

Several recent publications have supported a role for the endosperm in embryonic cuticle production and the specification of embryonic epidermal identity in *Arabidopsis*. A secreted subtilisin-like serine protease ABNORMAL LEAF SHAPE1 (ALE1), which is expressed predominantly in the ESR appears to be required for normal cuticle production in *Arabidopsis* embryos (Tanaka et al., 2001). Plants that lack *ALE1* produce embryos that adhere to the endosperm during development. Moreover, germinating seedlings are extremely sensitive to desiccation owing to abnormal cuticle production on their cotyledons. However, if *ale1* seedlings are grown in vitro in high humidity, and then transferred to soil, they survive and their adult leaves have a normal cuticle structure,

consistent with their cuticle defects being derived from abnormalities arising during embryogenesis. Mutations in either of two embryonically expressed genes encoding RLKs, *ACR4* or *ALE2*, leads to a significant enhancement of the relatively mild ale1 phenotype. In double mutants, epidermal specification (as judged by the expression of the epidermal markers such as *ATML1*) is also partially lost (Tanaka et al., 2007; Watanabe et al., 2004). Neither *acr4* nor *ale2* mutants show a marked seedling phenotype, although both have cotyledon cuticle abnormalities, and the synergistic interactions of these genes may indicate that ACR4 and ALE2 are required to perceive a signal processed by ALE1. Such a function for ALE1 would also be consistent with the known role of several other subtilisin proteases in processing ligand signals (Berger and Altmann, 2000; Cui et al., 1998; Julius et al., 1984; Rose et al., 1996). Two other embryonically expressed receptor-like kinase encoding genes, *GASSHO1* and *GASSHO2*, also act redundantly to allow normal embryonic cuticle formation (Tsuwamoto et al., 2008), although how these genes interact genetically with *ALE1*, *ACR4* and *ALE2* has yet to be determined.

In this paper, we show that the transcription factor encoded by the *ZHOUPI* (*ZOU*) (Chinese for 'shrivelled') gene of *Arabidopsis* is a key player in controlling ESR-expressed genes involved in the development of the embryo surface, including *ALE1*. The *ZOU* gene is widely conserved and occurs in plant groups that lack endosperm (gymnosperms) or seeds (Lycophyta). We propose that *ZOU* plays a pivotal role in the crosstalk between the embryo and surrounding nutritive tissues. In angiosperms, this is necessary to promote both cuticle formation and the separation of embryo from the surrounding tissues, whereas in more primitive plants it may permit invasive growth of embryos into nutritive tissue of the female gametophyte.

MATERIALS AND METHODS

Plant materials

The semi-dominant *zou-1D* allele arose in a conditional *curly leaf* (*clf*) mutant background (*clf-50 CLF::CLF-GR*, Ws ecotype) described previously (Schubert et al., 2006). The mutant was backcrossed several times to Ws ecotype to transfer the *zou-1D* mutation into a *CLF+* background and all experiments were performed using this line.

The recessive, loss of function *zou2-4* alleles were obtained from the Nottingham *Arabidopsis* Stock Center. The *zou2* allele (Ws background) arose in the FLAG collection of T-DNA insertions (Samson et al., 2002) and corresponded to FLAG 400A08. The *zou-3* allele (Ws background) was obtained from the University of Wisconsin collection of T DNA insertion lines (Sussman et al., 2000) and corresponded to WiscDsLox465F5. The *zou-4* (Col-0 background) allele was obtained from the Gabi-Kat collection of T-DNA inserts (Rosso et al., 2003) and corresponded to GABI_584D09. The position of the T-DNA inserts was confirmed by PCR amplification and sequencing of genomic DNA flanking the inserts. The *ale2-1* and *ale1-1* mutations were provided by Hirokazu Tanaka (Tanaka et al., 2001; Tanaka et al., 2007). The *acr4-2* mutation has been described previously (Gifford et al., 2003). The *ARF12* and *ARF21* reporter lines were provided by Dolf Weijers. The reporter line N9185 was obtained from the Haseloff collection of enhancer trap lines (http://www.plantsci.cam.ac.uk/Haseloff/construction/GAL4Frame.html). The marker lines *pATML1::GFP-ATML1* and *pACR4::H2B-YFP* have been described previously (Gifford et al., 2003).

To propagate *zou* mutants, seeds were sterilized and germinated on sterile tissue culture medium comprising 0.5 MS salts (Duchefa), 0.3% sucrose, 1% agar. Seedlings were transferred to soil about 10 days after germination, when first leaves were easily visible.

Protein sequence alignments

Protein sequences similar to ZOU were retrieved using the BLASTP program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to search plant genome databases (http://www.plantgdb.org/). The *Selaginella moellendorffi* sequence was retrieved using the TBLASTN program to query the *Selaginella* DNA sequence database (http://selaginella.genomics.purdue.edu/cgi-bin/blast_tmpl_s.cgi). We used the software NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/) (Brunak et al., 1991; Hebsgaard et al., 1996) and comparisons with the ZOU sequence to predict intron/exon boundaries in the *Selaginella* sequence. The protein sequence alignments were generated using the programs T-Coffee and ClusalW2 available at http://www.ebi.ac.uk/t-coffee/.

Isolation of *ZOU* gene and transgene construction

Southern analysis showed that *zou-1D* plants carried a single pSKI074 T DNA insertion (data not shown). The T DNA contained a selectable marker (kanamycin resistance) that co-segregated with *zou-1D* (data not shown), suggesting that the insertion was the cause of the mutation. The sequences flanking the pSKI074 T-DNA insertion at *zou-1D* were isolated using the plasmid rescue technique described previously (Weigel et al., 2000). We confirmed the predicted intron-exon structure of *ZOU* by isolating a cDNA from silique tissue and determining its sequence, which matched that in databases (Swissprot Accession Number, NM 103864). Total RNA was extracted using Trizol Reagent (Invitrogen) and RNeasy columns (Qiagen) according to manufacturer's instructions. First-strand cDNA was prepared from 0.5 μg total RNA primed with oligo-dT primer using ImProm-II reverse transcription system (Promega) according to the manufacturer's instructions. The *ZOU* cDNA was amplified by PCR using primers ZOU-F 5′-GGCTCTAGATGACTAATGCTCAAG and ZOU-R 5′-CAGGTC-GACAACTCAAACCGAAGC. The resulting product was cloned in the plasmid vector pGEM-T (Promega) to generate clone pSY3.

To generate the *35S::ZOU* construct, the *ZOU* cDNA was excised from pSY3 by digestion with *Xba*I and *Sal*I, and subcloned into the binary vector pFP101 (generous gift from Francois Parcy) under control of CaMV 35S promoter to generate clone pSY5. To generate the *ZOU::ZOU-GFP* reporter, the Gateway modified primers ZOUGWF (5′-GGGGACAAGTT-TGTACAAAAAAGCAGGCTGCCTGACCATAACAACCTATATCTC) and ZOUWGR (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-TAGAGATGAAAAATATAACACCAGTTC) were used to amplify the *ZOU* genomic region from plant DNA (Col-0 ecotype) by PCR with the hi-fidelity *Phu* DNA polymerase (NEB). The PCR product was cloned into the Gateway entry vector pDONR207 by recombination with BP enzyme mix (Invitrogen) to create clone pSY13. The clone pSY13 was then recombined with the Gateway compatible binary vector pMDC111 (Curtis and Grossniklaus, 2003) to create clone pSY14 encoding an in-frame fusion of GFP to the C terminus of the ZOU protein. To create the *ZOU::H2B-YFP* reporter, a 1.6 kb region upstream of the *ZOU* ATG codon was amplified by PCR (as above) using primers ZOUSALIF (5′-GTCGACTGTGG-TGGCATAATACGA) and ZOUSALIR (5′-GTCGACTGCTCAT-TTTACCCTTTT). The resulting product was subcloned into the vector pSC-B (Stratagene), excised by digestion with *Sal*I and cloned into the binary vector pMD4 (Gifford et al., 2003) containing the *H2B-YFP* fusion.

Analysis of gene expression

RT-PCR analysis of gene expression was performed as described previously (Chanvivattana et al., 2004) using the following primers: *ZOU* ZOUD3F, 5′-GCTGACTATCTGTGGGAATG; ZOUD3R, 5′-AACTCGGAT-TTACCTGTGCT; *EiF4A* EiF4AF, 5′-TTCGCTCTTCTCTTTGCTCTC; and EiF4A-R, GAACTCATCTTGTCCCTCAAGTA. In situ hybridization analysis was as described previously (Chanvivattana et al., 2004), with the exception that plant tissue was wax embedded using an automated processor (Leica Tissue Processor TP1050) and embedding centre (Leica EG 1160). The *ZOU* probes were made from plasmid pSY3. To make the *ALE1* probe, a region of ALE1 was amplified from silique cDNA using primers ALE5-2 (5′-TGAAACTAATGACAACATACACTCCC) and ALE3-2 (5′-ACA-TATCACGATACTTCCAAAAACTGC). The resulting product was subcloned into pGEM-T vector (Promega). Plasmids were linearized by digestion with appropriate restriction enzyme, and RNA probes made using T7 or SP6 RNA polymerase as described previously (Chanvivattana et al., 2004).

For real-time PCR measurements, RNA was extracted from seedlings and siliques using Trizol Reagent (Invitrogen) and RNeasy columns (Qiagen) according to manufacturers instruction's. First-strand cDNA was prepared

from 0.5 μg total RNA primed with oligo-dT primer using ImProm-II reverse transcription system (Promega) according to manufacturer's instructions. PCR reactions were performed in triplicate and the products quantified using a Rotor gene RG-3000 real-time PCR machine and associated software (Corbett Research) to assay SYBR green fluorescence. PCR reactions were made using SYBR green Jump-Start mix (Sigma). *ZOU* was amplified using primers ZOUD3F and ZOUD3R (above). *ALE1* was amplified using primers ALE1F (5′-CTTCTCAGGCCAAGAAACTC) and ALE1R (5′-TTTGCCAGACTTGTTGAGGA). *AtSUC5* was amplified using primers AtSUC5-F (5′-ATCGAAGAAACTTTACGACCAAGG) and AtSUC5-R (5′-TTAACGCTAAGACTCCACTAACC). Results were normalized using the EiF4A gene primers described above.

Microscopy

The expression of GFP reporter gene constructs was monitored by fluorescence microscopy using an Olympus Fluoview confocal microscope. For DIC microscopy, seeds were cleared overnight in modified Hoyer's solution (chloral hydrate:water:glycerol in proportions 8 g:2 ml:1 ml) and examined using a Nikon Eclipse E600 microscope. For thin sections, seeds were submerged overnight in 30 mM PIPES (pH 7) solution containing 5% glutaraldehyde and 0.1% (w/v) Triton-X-100. Tissue was rinsed several times in 30 mM PIPES and dehydrated using an ethanol series. LR White hard grade resin (London Resin Company, Reading, UK) was infiltrated into tissue over a period of 5 days before embedding. Ultramicrotome-cut sections (1 μm) were stained with Toluidine Blue and visualized using the above Nikon microscope. For transmission electron microscopy (TEM), seeds were fixed and embedded as above, except that they were stained with 1% aqueous osmium tetroxide prior to embedding. Ultrathin sections were cut using a diamond knife, stained with 2% aqueous uranyl acetate, and viewed on a Philips CM 120 transmission electron microscope. Cryo-scanning electron microscopy studies were carried out using a Hitachi S-4700 scanning electron microscope (Hitachi High Technologies, Wokingham, UK).

Genetic analysis

For double mutant construction, *zou-4* mutants were crossed as females to *acr4-2*, *ale1-1* or *ale2-1* homozygotes (all alleles are in Colombia background, *ale2-1* mutants are female sterile). In all cases, *zou-4* homozygotes were pre-selected in the resulting F2 generation by only germinating the shrivelled (zou) seeds. To confirm that these plants were *zou-4* homozygotes, we also tested that their F3 progeny was 100% resistant to sulphadiazine antibiotic (the T-DNA allele at *zou-4* confers resistance). The *zou4 acr4-1* mutant was identified from F2 plants whose immature F3 seeds were round (phenotype maternally determined by *acr4-1* mothers). The *ale1-1 zou-4* double mutant was identified by a PCR-based genotyping assay using primers ALE1GenoF (5′-CGTGCTAGAATAGACGAAGG), ALE1GenoR (5′-CGTGGTGGAGATGGCAG) and ALE1Ds (5′-CCGTTTTGTATATCCCGTTTCCGT). The *ALE1+* allele produced a 388 bp fragment, whereas *ale1-1* harbours a *Ds* transposon insertion and produces a 300 bp fragment (Tanaka et al., 2001). Because *ale2* mutants are sterile, we first identified *zou-4/zou-4 ale2-1/+* F2 individuals using primers ALE2F (5′-AGGAACGCTTGATTGGGATG) and ALE2R (5′-GAAGTCAGCAGAGTCTGGTA) (the *ale2-1* mutation introduces a novel *Xho*I site within the amplified ALE2 fragment). These plants gave rise to F3 seeds that were germinated in sterile tissue culture and segregated about one-quarter of seedlings with severely defective cotyledons. We confirmed that these were *zou-4 ale2-1* double mutants by PCR-based genotyping.

RESULTS

ZOU encodes a conserved, basic helix-loop-helix (bHLH) class transcription factor

We identified the *ZOU* gene serendipitously, from a gain-of-function mutation, *zou-1D*, which we isolated by activation-tagging mutagenesis (Weigel et al., 2000) (see Materials and methods; see Fig. S1 in the supplementary material). The *zou-1D* mutation harboured a T-DNA insertion in the promoter of *At1g49770*, 119 nucleotides upstream of the ATG start codon (Fig. 1A). The insertion caused ectopic expression of *At1g49770* in leaves, but did not affect

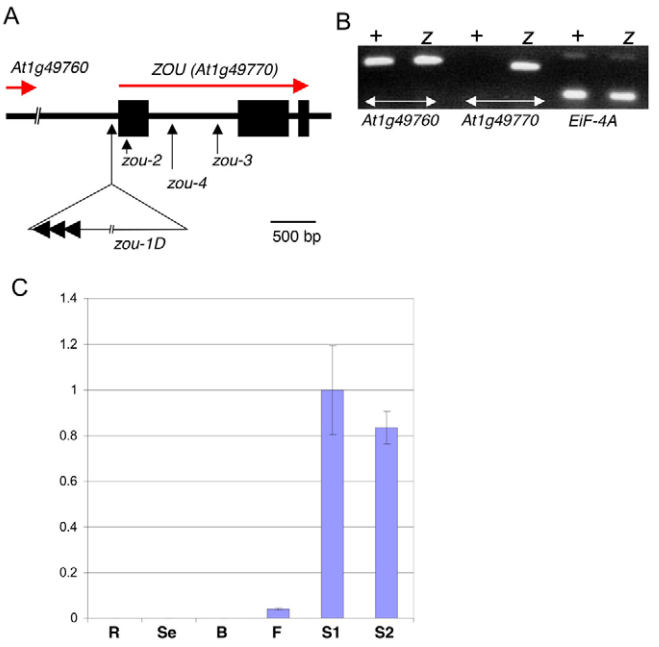

Fig. 1. Structure of *zou* mutant alleles and *ZOU* expression. (**A**) Structure of *ZOU* genomic region and position of T DNA insertions. Exons are indicated by black rectangles; the direction of transcription is indicated by red arrow. The pSKI074 T-DNA insertion in *zou1-D* is shown as a triangle with black arrowheads indicating the viral enhancer sequences. The origin of the *zou2-4* loss of function alleles is described in the Materials and methods. (**B**) RT-PCR analysis of gene expression in leaves of wild-type (+) and *zou-1D/–* (z) hemizygotes. Primer sets specific for three different genes were used, as indicated below the gel image. Eukaryotic translation initiation factor 4A (*EiF4A)* is a control for loading and RNA integrity. (**C**) Q-PCR analysis of *ZOU* expression in wild-type rosettes (R), seedlings (Se), unopened flower buds (B), opened flowers (F), siliques containing globular-heart stage embryos (S1) and siliques containing late heart and torpedo stage embryos (S2). Values are relative to expression in S1, which is designated as 1.0. No expression was detected in R, Se or F samples. Values are means of three replicates with standard error of mean.

the expression of *At1g49760*, the other gene neighbouring the insertion (Fig. 1B). The mis-expression of *At1g49770* caused leaf curling (see Fig. S1 in the supplementary material). However, the biological relevance of this phenotype is unclear as *ZOU* is not normally expressed in leaves and the leaf curling is not obviously related to the normal function of *ZOU*. In this study we therefore concentrate on the analysis of loss-of-function mutations.

The *ZOU* gene is predicted to encode a transcription factor of the basic helix-loop-helix (bHLH) class, designated bHLH95 in previous analyses of the *Arabidopsis* genome (Heim et al., 2003; Toledo-Ortiz et al., 2003). Genome database searches revealed that the ZOU protein is widely conserved in plants (see Fig. S2 in the supplementary material): homologues were found both in other dicotyledonous species, including grape (*Vitis vinifera*) and barrel medic (*Medicago truncatula*) and also in more distantly related angiosperms (e.g. rice, a monocot); furthermore, ZOU homologues were also found outside flowering plants in the gymnosperms [Sitka spruce (*Picea sitchensis*)] and club mosses (*Selaginella moellendorffii*). Protein sequence alignments showed that two regions of the ZOU protein were well conserved: the bHLH domain (residues 66-124) and a region of unknown function near the C terminus of the protein (residues 215-301, see alignment in Fig. S2 in the supplementary material). It is

Table 1. Genetic analysis of recessive *zou* mutations

	Shrivelled seed	Normal seed	χ^2 test
zou-2/ZOU$^+$ self	115	375	0.61, *P*>10%
zou-3/ZOU$^+$ self	123	386	0.19, *P*>10%
zou-4/ZOU$^+$ self	141	391	0.64, *P*>10%
zou-2/zou-2 self	100	0	
zou-2/zou-2♀ × *ZOU*$^+$/*ZOU*$^+$♂	4	131	
zou-2/zou-2♂ × *ZOU*$^+$/*ZOU*$^+$♀	0	151	
zou-2/zou-2 ZOU::ZOU-GFP/– self	204	507	5.2, *P*>1%

The shrivelled seed phenotype was fully penetrant in progeny of selfed *zou2/zou2* parents. When the *ZOU::ZOU-GFP* transgene was introduced into the *zou2* background, the seed shrivelling was complemented in 15 out of 32 primary transformants (plants which had more than 50% normal seeds). Analysis of the progeny of one T1 complementing plant hemizygous for a single locus transgene insert shows slightly fewer normal seeds than the 75% expected if the transgene complements in dominant fashion (χ^2 test for 3:1 distribution is statistically significant at 5% level but not at 1% level). This difference is probably because the expression of the transgene was unstable; for example, in siliques produced late in development, few seeds showed transgene expression.

likely that these proteins are ZOU orthologues as each is more similar to ZOU than to any other *Arabidopsis* protein. Using these criteria, no clear ZOU orthologues were identified in the bryophyte *Physcomitrella patens* or in the green alga *Chlamydomonas reinhardtii.* The *ZOU* gene is therefore ancient and is likely to have arisen early in vascular plant evolution. In addition, the *Arabidopsis* ZOU protein is more similar to homologues in other plant species than to any of the other *Arabidopsis* bHLH proteins [estimated to be between 132 and 146 in number (Heim et al., 2003; Toledo-Ortiz et al., 2003)], suggesting that it is a unique gene in *Arabidopsis*.

Loss-of-function mutations show that *ZOU* normally functions during seed development

A previous study of *Arabidopsis* bHLH genes suggested that *ZOU* (there termed bHLH95) expression was specific to flowers and/or siliques (Heim et al., 2003). Quantitative RT-PCR (Fig. 1C) confirmed and extended this: *ZOU* was not expressed in whole seedlings, rosette leaves or unopened flower buds, but was detected at a low level in opened flowers and more strongly in siliques, suggesting that it normally functions in siliques or seed development. To determine the loss-of-function phenotype, we obtained three independent alleles (*zou-2*, *zou-3* and *zou-4*) from collections of T-DNA insertion lines (Fig. 1A). All three alleles conferred very similar phenotypes, with *zou-2* being most severe, consistent with the location of the T-DNA insertion in the first exon. Plants heterozygous for *zou* were normal; however, when self-pollinated about one quarter of their seeds (see Table 1) were mis-shapen and shrivelled at maturity (Fig. 2A,B). In *zou-2* homozygotes, all seeds were shrivelled, but when they were crossed either as male or female parent to wild type, the resulting seeds were normal (Table 1). Together, these observations indicated that *zou-2-4* are recessive loss-of-function mutations with normal transmission through male and female gametophytes and that *ZOU* acts zygotically (rather than maternally) to control seed development. The seed shrivelling phenotype co-segregated with antibiotic resistance conferred by the T DNA insertion at *zou-2* (data not shown) and could be complemented by transformation with a genomic *ZOU* clone (Fig. 2C; Table 1), confirming that it was caused by loss of *ZOU* function. We renamed the *At1g4977/bHLH95* gene *ZHOUPI* (Chinese for 'shrivelled') in reference to the seed phenotype.

zou embryos show abnormal adhesion to endosperm and have cuticle defects

Mature seeds of *zou* mutants contained smaller embryos than wild type (Fig. 2D), suggesting that the shrivelling of *zou* seeds occurs because the embryo fails to fill the seed. However, *zou* seeds were viable (see below), indicating that *zou* embryos do not undergo premature developmental arrest. To determine when *zou* mutations first affect embryo development, developing seeds were cleared and examined using differential interference contrast (DIC) microscopy. Up until the early heart stage of development, *zou* embryos appeared normal and the surrounding endosperm became cellularized around early heart stage, as in wild type (Fig. 2E,F). Subsequently, *zou* embryos failed to elongate and were shorter and wider than wild-type torpedo and later stage embryos. Whereas in wild type the cotyledons elongate and bend over, so that a large embryo fills the seed, in *zou* embryos the cotyledons remained short and stumpy, and were usually more widely separated (Fig. 2G,H). The failure of *zou* embryos to elongate may be due to their abnormal adhesion to the surrounding endosperm, which physically impedes growth. In wild-type seeds, the endosperm in the region surrounding the embryo (ESR) separates from the embryo from the heart stage of embryogenesis onwards; at the torpedo stage of development, little or no endosperm remains between the cotyledons (Fig. 2G). By contrast, *zou* embryos continue to adhere to endosperm throughout embryogenesis and considerable amounts of endosperm are present between cotyledons (Fig. 2H). To characterize the adhesion of endosperm to embryos more clearly, we examined thin sections of resin embedded seeds using light microscopy. This confirmed that in *zou* seeds the endosperm remains stuck to the embryo over its entire surface (Fig. 2I,J). Similar, but less severe, defects in *ale1* mutants (Tanaka et al., 2001) have been attributed to impaired cuticle formation during embryogenesis. To examine surface development in *zou* embryos, seeds were embedded in resin, stained with osmium tetroxide and ultra-thin sections examined using transmission electron microscopy (TEM). Wild-type torpedo stage embryos were covered by a cell wall with a continuous electron dense cuticularized layer (Jeffree, 2006) (CL). An overlying translucent cuticle proper (Jeffree, 2006) (CP) was also present, although visualization of this at high resolution in seed tissue is challenging. We did not observe any obvious discontinuities in the CL of *zou* mutant embryos at the same developmental stage, but no CP was apparent, and the cuticularized cell wall was extensively fused to endosperm cell wall (Fig. 2K,L).

The separation of embryo from endosperm during angiosperm embryogenesis correlates with breakdown of the endosperm cells surrounding the developing embryo (Briggs, 1993). As this separation does not occur efficiently in *zou* mutants, we examined whether the endosperm is more persistent in *zou* seeds than in wild type. After imbibition of mature wild-type seeds, the embryo could easily be separated from the single layer of residual endosperm cells by extrusion. By contrast, in *zou* mutant seeds, the embryo and endosperm adhered strongly. Furthermore, the chalazal pole of the endosperm, which was not invaded by the embryo, formed a sac-like structure. When the embryo was cut away, a considerable quantity of abnormal paste-like tissue was extruded from the endosperm cavity (see Fig. S3 in the supplementary material).

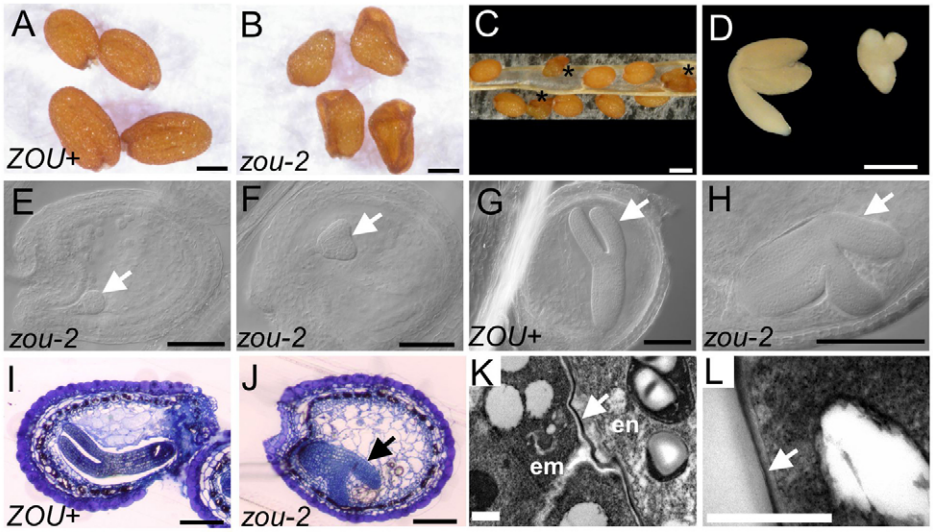

Fig. 2. Phenotype of *zou* mutant seeds. (**A**) Seeds from wild-type plant. (**B**) Shrivelled seeds from self-pollinated *zou-2*/+ plant (the bulk of seeds were wild-type and non-shrivelled). (**C**) Dissected silique of self pollinated *zou-2*/*zou-2 ZOU::ZOU-GFP*/– primary transformant. The majority of seeds have a wild-type phenotype, owing to complementation by the transgene. Some seeds (asterisk) do not inherit the transgene, owing to segregation, and therefore have *zou* mutant phenotype. (**D**) Embryos dissected from mature seeds of wild-type (left) and *zou-2* mutants (right). (**E-H**) Cleared seeds viewed using DIC microscopy. Arrows indicate embryos which are at globular (E), heart (F), late torpedo (G,H) stages. (**I**,**J**) Light microscope images of 1 μM sections of resin-embedded seeds. Arrow in J indicates the mis-shapen *zou* embryo, which has adhered to the surrounding cellularized endosperm. (**K**,**L**) TEM images of ultra thin sections of osmium stained seeds with torpedo stage embryos. The cuticularized layer (CL) is indicated by arrows. In *zou-2* (K), unlike *ZOU*+ (L), endosperm (en) is appressed to the cuticle of the embryo (em). Scale bars: 200 μm in A-H; 100 μm in I,J; 500 nm in K,L.

ZOU is needed for normal epidermal development in seedlings

The shrivelled *zou* seeds were viable and germinated to produce seedlings that survived when grown in conditions of high humidity, but desiccated and died when grown in low humidity. This phenotype is commonly found in mutants with defects in cuticle or epidermis that impair water retention. We therefore stained whole seedlings with Toluidine Blue, as this has been shown to provide a rapid test for cuticle and/or epidermal defects (Tanaka et al., 2004). Whereas wild-type plants were unstained, in *zou* mutants the hypocotyl and the cotyledons, but not the leaves, stained strongly (Fig. 3A,B). This suggested that the defects in *zou* mutants were restricted to organs that initiate during embryogenesis (i.e. hypocotyls and cotyledons, but not leaves), consistent with the seed-specific expression of *ZOU* (Fig. 1C; Fig. 4). Furthermore, when *zou* mutants were grown in tissue culture and transferred to soil after the first leaves had initiated, the mutants recovered and gave rise to normal plants, again suggesting that *ZOU* did not affect post-embryonic organ development.

Epidermal defects in *zou* seedlings were examined using scanning electron microscopy (cryo-SEM). The leaves of *zou* mutants were normal (Fig. 3C). The epidermal surfaces of *zou* cotyledons had normal pavement and stomatal cell types (Fig. 3D,E); however, large tears were frequently visible in the epidermis through which the underlying mesophyll cells protruded (Fig. 3C,F). As the protoderm appeared continuous in *zou* embryos (Fig. 2F,H,J), it is likely that the tears developed after germination, when cotyledon expansion occurred, owing to epidermal defects. Consistent with this, in less severely affected *zou* mutants, small holes were visible between cells, as if cell adhesion were compromised (Fig. 3G). To analyse post-germination cotyledon surface structure, TEM analysis was carried out on expanded wild-type and *zou* cotyledons 7 days post germination. Wild-type cotyledons were covered in a continuous cell wall with a pronounced CL and a clearly demarcated overlying CP. The CP was demarcated by a fine outer electron-dense line, possibly indicating an outer proteinaceous layer (Fig. 3J). In *zou* cotyledons, variability in thickness and discontinuities in the CP were evident, and the outer electron dense layer overlying the CP was more pronounced (Fig. 3K). This was not simply a consequence of tears, as abnormalities were visible in regions where the epidermal sheet was intact. In addition, remnants of endosperm were visible adhering to *zou*, but not to wild-type, cotyledons (Fig. 3L).

Genetic interactions of *ZOU*

We combined *zou* mutations with other mutations implicated in epidermal development, including *acr4*, *ale1* and *ale2* (see Materials and methods). The *zou-4* mutation was epistatic to *ale1-1* in double mutants, suggesting that *ZOU* and *ALE1* might act in the same pathway (data not shown). The *zou-4 acr4-2* seedlings had more severely defective cotyledons than either single mutant, suggesting that *ZOU* and *ACR4* may be involved in a common developmental process (Fig. 3H). The *zou-4 ale2-1* seedlings also had more severely defective cotyledons than either single mutant (Fig. 3I), consistent with previous observations that *ale1* mutations enhance *ale2* (Tanaka et al., 2007).

ZOU is expressed in the ESR of endosperm

To determine where *ZOU* was expressed, we localized *ZOU* mRNA by in situ hybridization to sections of developing seeds. We detected strong expression in the endosperm in the ESR in seeds containing embryos up to the torpedo stage, after which expression declined (Fig. 4A-C). No signal was detected when sense control probes were hybridized, confirming that the signal with antisense probes was specific for *ZOU* (Fig. 4D). Strikingly, we did not detect expression either in the embryo itself or in any of the maternal tissues in the seeds or siliques. The effects of *ZOU* on the embryonic epidermis are therefore non cell autonomous. To further characterize *ZOU* expression, we made two reporter gene constructs: a gene fusion in which the entire *ZOU* genomic region (i.e. upstream promoter and intragenic sequences) was fused in frame to GFP (*ZOU::ZOU-GFP*);

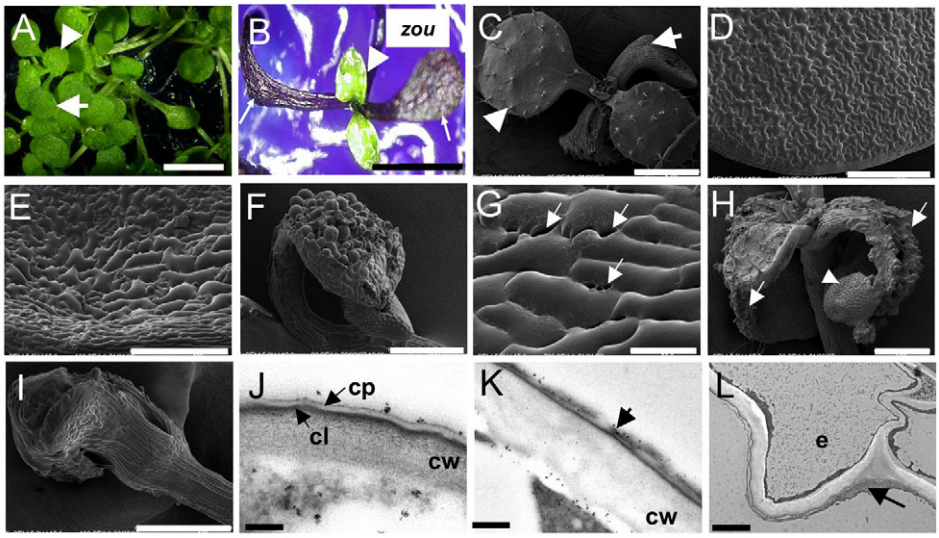

Fig. 3. Epidermal phenotypes of zou mutant seedlings. (**A**,**B**) Seedlings stained with Toluidine Blue. Arrowheads indicate leaves, arrows indicate cotyledons. Unlike wild type (A), in *zou-4* mutant (B) cotyledons and hypocotyls are stained strongly. (C-I) Cryo-SEM images of seedling epidermis. (**C**) *zou-4* mutant seedling. Leaves (arrowhead) are normal, whereas cotyledons (arrow) have large tears in the epidermis. (**D**) Epidermis of wild-type cotyledon, showing jigsaw-shaped pavement cells and stomata. (**E**) Epidermis of *zou-4* cotyledons. Normal cell types are present, although stomatal spacing is abnormal. (**F**) *zou-2* mutant cotyledon showing large tear, with protruding mesophyll cells. (**G**) *zou-4* mutant cotyledon showing gaps between pavement cells (arrows). The rough cell surfaces are due to adhering endosperm. (**H**) *zou-4 acr4-2* double mutant, showing severely defective cotyledons (arrows). Adhering seed coat is indicated by arrowhead. (**I**) *ale2-1 zou-4* double mutant cotyledons are minute, mis-shapen and partially fused. (**J**) TEM image of wild-type adaxial cotyledon surface showing cell wall (cw) cuticularized layer (cl) and cuticle proper (cp). (**K**) TEM image of *zou-2* adaxial cotyledon surface showing thickness variability and discontinuity (arrow) of the cp. (**L**) *zou-2* cotyledon surface showing adhering endosperm tissue (arrow) on surface of embryo (e). Scale bars: 1 cm in A,B; 1 mm in C; 300 μm in D; 400 μm in E; 500 μm in F; 50 μm in G; 500 μm in H; 400 μm in I; 100 nm in J,K; 2 μm in L.

and a fusion of the *ZOU* upstream promoter sequences to a HISTONE 2B-YFP fusion protein (Boisnard-Lorig et al., 2001) (*ZOU::H2B-YFP*). Both constructs showed similar expression patterns. The *ZOU::ZOU-GFP* construct complemented *zou-2* mutants (Fig. 2C and Table 1), confirming that the ZOU-GFP protein fusion retained *ZOU*+ activity and that the construct was expressed correctly. The ZOU-GFP fusion protein was nuclear-localized, consistent with the predicted function of ZOU as a transcription factor, and was expressed in the ESR but not in the embryo (Fig. 4E). The non cell-autonomous effects of ZOU on the embryonic epidermis are therefore not due to the ZOU protein moving from endosperm to embryo. The ZOU-GFP fusion protein expressed from *ZOU::ZOU-GFP* was below the limits of detection by confocal microscopy until the early heart stage, possibly owing to post-transcriptional regulation. For this reason, we characterized the early expression of *ZOU* using the *ZOU::H2B-YFP* construct. *ZOU* was not expressed in the ovule prior to fertilization (Fig. 4F) but was strongly expressed in the central cell after fertilization (Fig. 4G). This is consistent with Q-PCR data which showed that *ZOU* expression was undetectable in unopened flower buds, but was detectable in opened flowers shortly after the point of fertilization (Fig. 1C). It was expressed uniformly throughout the endosperm during the early stages of its development when it comprised two to eight nuclei (Fig. 4H,I), but then became expressed more strongly at the micropylar pole during the 12-16 nuclei stage (Fig. 4J) and the 24-28 nuclei stages (Fig. 4K); it was largely restricted to the ESR by the 44-48 cell stage [Fig. 4L, stages as defined previously (Boisnard-Lorig et al., 2001)].

ZOU regulates *ALE1* in seeds

ZOU showed a very similar expression pattern to *ALE1*. The two genes have similar mutant phenotypes (Tanaka et al., 2001) and *zou* mutants are epistatic to *ale1* mutants, suggesting that they might act in a common pathway. To test whether *ZOU* regulates *ALE1*, consistent with the likely role of ZOU as a transcription factor, we localized *ALE1* expression in young siliques of wild-type and *zou-2* homozygotes. *ALE1* was strongly expressed in the ESR of the endosperm but not in the embryos of wild-type seeds (Fig. 5A,B). In *zou-2* mutants, *ALE1* expression was barely detectable at globular and heart stages (Fig. 5C,D), indicating that *ZOU* regulates *ALE1* expression. To test whether *ZOU* had a general role in regulating ESR identity, or rather was specific for genes such as *ALE1* (which have a role in embryo development), we analyzed the expression of *AtSUC5*, a sucrose transporter that is also expressed specifically in the ESR (Baud et al., 2005), using quantitative RT-PCR. Unlike *ALE1*, which showed an approximately fivefold decrease in expression, there was no significant change in *AtSUC5* expression in *zou* mutants (see Fig. S4 in the supplementary material). Likewise, the expression of reporters for the ESR-specific *AUXIN RESPONSE FACTOR 12* (*ARF12*) and *ARF21* genes (D. Weijers, personal communication) was unaffected in *zou* mutant seeds (data not shown), confirming that *ZOU* does not regulate all ESR-specific genes. However, the enhancer trap line N9185, which is expressed in wild-type ESR (Ingouff et al., 2005) was not expressed in *zou-4* (Fig. 5E,F), suggesting that the gene responsible for the N9185 pattern may also be a target of *ZOU* regulation. The enhancer trap T-DNA in N9185 is located on chromosome 4 (data not shown), and is therefore not associated with *ALE1* (*At1g62340*).

Because *zou* mutant seedlings had epidermal defects, we also introduced reporters for *ACR4* and *ATML1*, which show epidermal-specific expression. Both reporters were expressed normally in *zou-4* mutant embryos (Fig. 5G-J), consistent with the fact that protoderm specification appears largely normal in cleared and sectioned *zou* seeds.

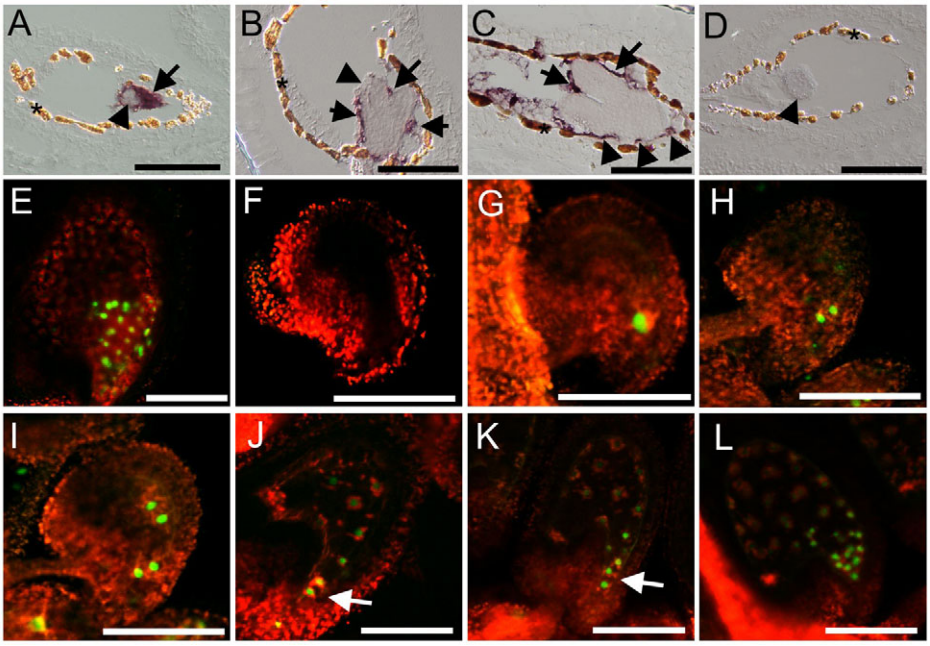

Fig. 4. *ZOU* expression pattern during seed development. (**A-D**) Images of in situ hybridizations of digoxigenin-labelled *ZOU* antisense (A-C) and sense (D) RNA probes to sections of siliques, viewed using DIC microscopy. Signal appears purple; the dark-brown staining of the endothelium (asterisk) does not represent signal. Embryos are indicated with arrowheads, the surrounding endosperm with arrows. (A) Seed with early globular stage embryo. (B) Seed with late heart stage embryo. (C) Seed with torpedo stage embryo. (D) Control hybridization with ZOU sense probe. (**E-L**) Confocal microscopy images in which autofluorescence appears red and GFP fluorescence green. (E) Seed of transgenic plant carrying *ZOU::ZOU-GFP* reporter gene fusion. The reporter is expressed in nuclei of the embryo surrounding region (ESR) that surrounds the late heart stage embryo. (F-L) Developing seeds of transgenic plant carrying *ZOU::H2B-YFP* reporter gene. (F) Unfertilized ovule shows no transgene expression. (G) Seed shortly after fertilization showing strong expression in central cell. (H) Seed with endosperm at the two-nuclei stage, with uniform expression in endosperm. (I) Seed with endosperm at the four-nuclei stage, with uniform expression in endosperm. (J) Seed with endosperm at 12- to 16-nuclei stage; expression is becoming stronger at micropylar pole of endosperm (arrow). (K) Seed with endosperm at 24- to 28-nuclei stage. (L) Seed with endosperm at 44- to 48-cell stage, expression is now largely confined to ESR. Scale bars: 100 μm.

ZOU is not sufficient to activate *ALE1*

The fact that *ZOU* is needed for *ALE1* expression in seeds raised the possibility that the curled leaves of *zou-1D* mutants could be a result of *ALE1* being activated by *ZOU* ectopically in leaves. To test this, we analysed *ALE1* and *ZOU* expression in seedlings by quantitative RT-PCR. Consistent with our RT-PCR results (Fig. 1B), *ZOU* expression was not detectable in wild-type seedlings, whereas in *zou-1D/+* seedlings it was readily detectable at about six times the level in wild-type siliques. No *ALE1* expression was detectable in wild-type or *zou-1D* seedlings (see Fig. S5 in the supplementary material). Therefore, *ZOU* expression is not sufficient to activate *ALE1* outside of the seed and the effects of *ZOU* mis-expression may be unrelated to the normal function of *ZOU* in seeds.

DISCUSSION

In this paper, we identify ZOU as a transcription factor whose expression is entirely restricted to the developing *Arabidopsis* endosperm during seed formation, but whose function mediates the normal development of neighbouring embryonic tissues. The potential non cell-autonomous role of the angiosperm endosperm as a source of developmental signals for the young embryo has long been a source of speculation. The observation that in vitro embryogenesis from cell cultures is often accompanied by the expression of endosperm-specific genes in non-embryogenic cells, led to the idea that the 'nurse-cell'-like capacities of these cells could be analogous to the developmentally important roles of the endosperm during zygotic embryo development (Magnard et al., 2000; Massonneau et al., 2005; van Hengel et al., 1998; Wiweger et al., 2003). However, the first concrete evidence that the endosperm might play a key role in *Arabidopsis* embryo development, distinct from its role in embryo nutrition, came only recently, with the characterization of the ESR gene *ALE1* (Tanaka et al., 2001). We show that *ZOU* regulates *ALE1* expression in endosperm, consistent with the similar expression patterns of the two genes. However, the zou phenotype is not simply a consequence of a loss of *ALE1* activity because *ale1* mutants are much less severe than *zou* mutants. In our growth conditions, *ale1* mutant seeds did not shrivel, they produced normal sized embryos and the epidermal morphology of their seedlings was only mildly affected. It is likely that *ZOU* regulates many targets additional to *ALE1*, as is supported by the loss of the N9185 reporter gene expression in *zou* mutants. Previous analyses of the *Arabidopsis* bHLH family indicated that all residues crucial for DNA-binding activity and dimerization are conserved in the ZOU bHLH and that it is likely to bind the G box sequence CACGTG (Heim et al., 2003; Toledo-Ortiz et al., 2003). Because mis-expression of *ZOU* in leaves is insufficient to activate *ALE1*, ZOU probably requires additional factors for activity. One possibility is that it acts as a heterodimer with another bHLH member, as is the case for several plant bHLH proteins (Heim et al., 2003; Toledo-Ortiz et al., 2003). Because ZOU is expressed in rather few cells in seeds (i.e. the ESR of endosperm), it is technically difficult to demonstrate direct binding in vivo using techniques such as chromatin immunoprecipitation.

The ESR of the angiosperm endosperm is distinct structurally, and also in terms of gene expression, from the rest of the endosperm (Berger, 2003; Olsen, 2004). In both cereals and *Arabidopsis*, where the first few divisions of the central cell give rise to a free nuclear

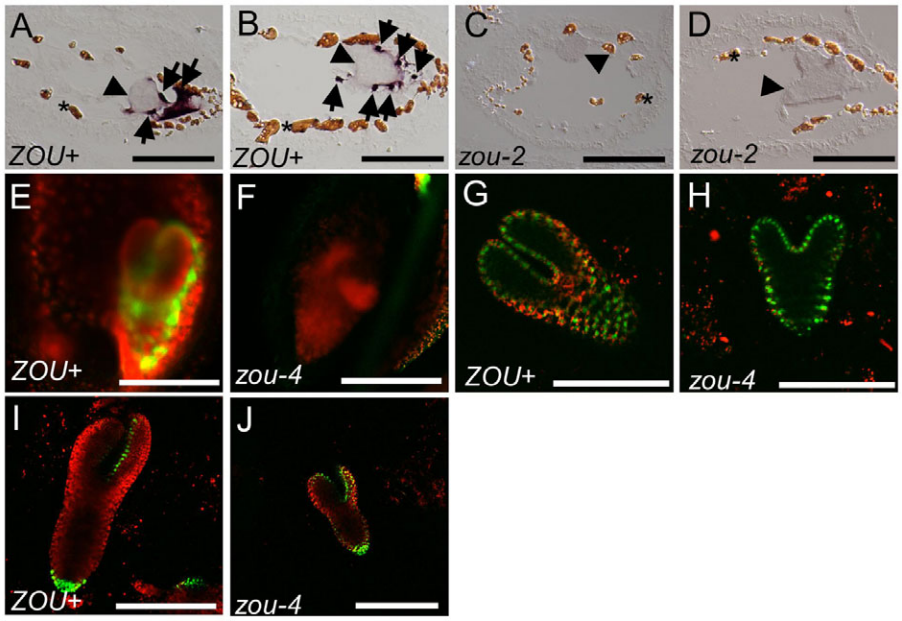

Fig. 5. Expression of diverse genes involved in endosperm and embryo development in zou mutant seeds. (**A-D**) Images of in situ hybridization of digoxigenin-labelled *ALE1* antisense RNA probes to sections of siliques, viewed using DIC microscopy. Embryo is indicated by arrowhead, endosperm by arrows. Little or no *ALE1* expression was detected in *zou-2* mutant seeds (C,D), whereas wild type showed intense signal in ESR (A,B). (**E**,**F**) Confocal images of reporter line 9185 in wild-type (E) and *zou-4* (F) seeds. (**G**,**H**) Images of reporter line *ATML1::GFP-ATML1* in wild-type (G) and *zou-4* (H) seeds. (**I**,**J**) Images of reporter line *ACR4::H2B-YFP* in wild-type (I) and *zou-4* (J) seeds. Scale bar: 100 μm.

syncitium, cellularization is initiated in the ESR. The ESR endosperm is also densely cytoplasmic and is closely associated with the developing embryo at early stages. This association is probably key for provision of nutrients to the young embryo, a supposition that is supported by the ESR-specific expression of the sucrose transporter AtSUC5, which is required for normal embryo growth in *Arabidopsis* (Baud et al., 2005). ZOU is the first transcription factor to be identified with an ESR-specific expression pattern, raising the possibility that it specifies the identity of the ESR region of the endosperm. We feel that this is unlikely for three reasons: first, the early morphology and development of *zou* endosperm, including the ESR, is indistinguishable from that of wild type; second, the expression of some ESR genes, such as *AtSUC5*, *ARF12* and *ARF21*, is not affected in *zou* mutants; finally, the expression of *ZOU* does not become wholly restricted to the ESR until the early heart stage. Therefore, rather than specifying the identity of the ESR region, it seems more likely that ZOU has more specific roles in endosperm and or embryo development.

The two roles of ZOU during seed formation

Although *zou* mutants form a defective epidermis, it is unlikely that *ZOU* is absolutely required for epidermal specification, as the expression of epidermal specific marker genes such as *ATML1* and *ACR4* appears normal in *zou* embryos. However, we have shown that the translucent cuticle-proper, which usually prevents cotyledon dehydration, is discontinuous in *zou* mutants. These discontinuities are not simply a result of the tears and holes that frequently develop in the epidermal surfaces of *zou* mutants, as they can be found in cuticle that overlies intact epidermal sheets. Thus, one function of *ZOU* is to allow formation of a normal cuticular membrane. It is possible that the cuticular defects are an indirect consequence of the persistent abnormal juxtaposition of living endosperm with the embryo. However, a very similar endosperm adhesion phenotype results when two embryo-specific receptor-like kinases, encoded by the *GSO1* and *GSO2* genes, are disrupted, indicating that endosperm adhesion is also controlled by the embryo and may be a result of cuticle defects (Tsuwamoto et al., 2008). We therefore suggest that *ZOU* regulates signalling between the endosperm ESR and the embryo that promotes normal cuticle deposition and is probably mediated by receptor-like kinases such as GSO1 and GSO2 in the embryo. Such signalling is probably two-way: *ZOU* expression becomes gradually restricted to the ESR as endosperm development progresses. The maintenance of *ZOU* expression in the endosperm cells directly in contact with the embryo may therefore be the result of signalling between the embryonic epidermis and the endosperm. Whether cuticle deposition is entirely mediated by the embryonic epidermal cells, or involves secreted compounds originating from the endosperm, is, as yet, unclear.

In *Arabidopsis* and many other dicotyledonous plants, the cellularized endosperm progressively degenerates as it is invaded by the expanding embryo, so that only a single layer – the aleurone – remains at seed maturity. By contrast, in mature *zou* seeds, much more endosperm persists, including non-aleurone tissues. Therefore, a likely second role of *ZOU* is to promote the expression of genes involved in degradation of endosperm cells around the expanding embryo. The *ZOU* target *ALE1* encodes a subtilisin-like serine protease that has been proposed to process ligands involved in epidermal specification/function (Watanabe et al., 2004). However, it is also possible that *ALE1* acts instead in the separation of the developing endosperm from the embryonic epidermis; for example, by promoting endosperm autolysis.

ZOU is therefore the first transcription factor to be identified that mediates two poorly understood processes in seed development – separation of the embryo from the endosperm and breakdown of the endosperm. Determining the targets of ZOU; for example, by transcriptional profiling of mutant seeds, should help identify the key factors that mediate these processes.

The ancestral role of *ZOU*

It is striking that *ZOU* is so widely conserved in plants. Within angiosperms, it is likely that the role of *ZOU* in the ESR is conserved as the rice *ZOU* homologue also shows seed-specific expression (Li et al., 2006). However, *ZOU* is also found both in seed plants that lack an endosperm (e.g. *Picea sitchensis*, a gymnosperm) and also in more basal vascular plant groups, such as the club mosses, which lack seeds altogether. A common feature of all these groups of land plants is that they have a specialized epidermis with cuticle, and that their embryos enlarge by invading and digesting surrounding maternal nutritive tissues (see Fig. S6 in the supplementary material). In gymnosperms, the female gametophyte proliferates to form a nutrient-rich tissue prior

to fertilization, and the growth of the embryo post-fertilization is invasive (see Fig. S6B in the supplementary material). Although club mosses such as *Selaginella* lack a seed, the embryo is similarly thrust into a nutrient-rich megagametophyte by a suspensor (see Fig. S6C in the supplementary material). It is therefore possible that the ancestral role of *ZOU* is to promote the separation of the embryo from surrounding gametophytic tissues, and the breakdown of these tissues, similar to its role in angiosperm endosperm. This would also be consistent with the current consensus that the endosperm is evolutionarily homologous to the gymnosperm female gametophyte [see Baroux et al. (Baroux et al., 2002) for recent discussion]. To test this further, it will be necessary to determine whether *ZOU* is also expressed in the ESR of the female gametophyte in these groups.

Note added in proof

While this article was under review, Kondou et al. (Kondou et al., 2008) published on RETARDED GROWTH OF EMBRYO1. The genes *ZHOUPI* and *RETARDED GROWTH OF EMBRYO1* both correspond to *At1g49770*.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/21/3501/DC1

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