ZHOUPI controls embryonic cuticle formation via a signalling pathway involving the subtilisin protease ABNORMAL LEAF-SHAPE1 and the receptor kinases GASSHO1 and GASSHO2

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SUMMARY
Seed production in angiosperms requires tight coordination of the development of the embryo and the endosperm. The endosperm-specific transcription factor ZHOUPI has previously been shown to play a key role in this process, by regulating both endosperm breakdown and the formation of the embryonic cuticle. To what extent these processes are functionally linked is, however, unclear. In order to address this issue we have concentrated on the subtilisin-like serine protease encoding gene ABNORMAL LEAF-SHAPE1. Expression of ABNORMAL LEAF-SHAPE1 is endosperm specific, and dramatically decreased in zhoupi mutants. We show that, although ABNORMAL LEAF-SHAPE1 is required for normal embryonic cuticle formation, it plays no role in regulating endosperm breakdown. Furthermore, we show that re-introducing ABNORMAL LEAF-SHAPE1 expression in the endosperm of zhoupi mutants partially rescues embryonic cuticle formation without rescuing their persistent endosperm phenotype. Thus, we conclude that ALE1 can normalize cuticle formation in the absence of endosperm breakdown, and that ZHOUPI thus controls two genetically separable developmental processes. Finally, our genetic study shows that ZHOUPI and ABNORMAL LEAF-SHAPE1 promotes formation of embryonic cuticle via a pathway involving embryonically expressed receptor kinases GASSHO1 and GASSHO2. We therefore provide a molecular framework of inter-tissue communication for embryo-specific cuticle formation during embryogenesis.

KEY WORDS: Cell death, cuticle, Embryo, Endosperm, Seed development, Signalling

INTRODUCTION
Seed production in angiosperms requires a tight coordination of the development of the two zygotic products of double fertilization: the embryo and the endosperm. In the model species Arabidopsis thaliana, the endosperm is a largely transitory structure, which has been shown to play an important role in driving the expansion of the maternally derived seed coat early in seed development (Garcia et al., 2003), but which then breaks down, permitting the developing embryo to fill the seed at later stages. At maturity, only a single-cell layer of endosperm tissue remains surrounding the dormant embryo (Bethke et al., 2007; Penfield et al., 2004). The importance of the coordination of endosperm breakdown with embryogeny is exemplified in basic helix-loop-helix transcription factor ZHOUPI loss-of-function mutants, in which endosperm breakdown does not occur, leading to the production of a persistent endosperm and an embryo of drastically reduced size (Kondou et al., 2008; Yang et al., 2008).

ZHOUPI is expressed exclusively in endosperm cells surrounding the developing embryo, and it is therefore likely that its role in driving endosperm breakdown is cell-autonomous (Yang et al., 2008). Interestingly however, the phenotype of zou mutants is not restricted to endosperm persistence and decrease in embryo size. A second embryonic phenotype, consisting of cuticle abnormalities and an extreme desiccation intolerance upon germination, has also been described (Kondou et al., 2008; Yang et al., 2008). Consistent with cuticular abnormalities, the embryos of zou mutants adhere tightly to surrounding endosperm tissues both during development and upon germination, when the remains of the persistent endosperm can be observed on the cotyledon surface (Yang et al., 2008). Despite these severe seed and seedling phenotypes, zou mutant seeds germinate and give rise to phenotypically normal homozygous plants, if seedlings are maintained in a humid environment during their early development.

The epidermal phenotype of zou mutants is intriguing, as cuticle biogenesis is widely understood to be an epidermal property and thus to be mediated by the epidermal cells of the developing embryo. Two possible, and not necessarily mutually exclusive, explanations could account for the cuticular defects in zou mutants. First, the close proximity of the abnormally persistent endosperm in zou mutants could make it difficult for the epidermal cuticle of the embryo to mature correctly, and lead to a boundary ‘blurring’ between the two structures. Alternatively, ZOU could regulate embryonic cuticle formation independently of endosperm breakdown, via a non-cell-autonomous pathway.

In terms of cuticle formation, the angiosperm embryo is unique, as all other cuticle-bearing surfaces in the aerial part of the plant are juxtaposed either by the atmosphere, or by other epidermally derived cuticle-bearing surfaces on adjacent organs. The cuticularization of juxtaposed surfaces has been shown to be extremely important for the definition of organ boundaries, both during development and postgenitally. Mutants with compromised cuticles often show extensive organ fusions (Kurdyukov et al., 2006a; Kurdyukov et al., 2006b; Lolle et al., 1992; Pruitt et al., 2000; Wellesen et al., 2001; Yephremov et al., 1999). Several genetic lesions that affect cuticle biogenesis either exclusively, or predominantly, in the developing embryo have been described.
consistent with the hypothesis that the unique situation of the embryo requires the activity of specific developmental pathways for the production of a functional cuticle. These lesions include the simultaneous loss of two related and functionally redundant receptor-like kinase-encoding genes, GASSHO1 and GASSHO2 (GSO1 and GSO2) (Tsuwamoto et al., 2008), and mutations in a gene encoding a Subtilisin-like Serine protease, ABNORMAL LEAF SHAPE1 (ALE1) (Tanaka et al., 2001; Tanaka et al., 2004). Although GSO1 and GSO2 are expressed in the embryo, the expression of ALE1 is restricted to the endosperm surrounding the embryo (Tanaka et al., 2001). An extracellular localization for ALE1 protein has been predicted, leading to speculation that endosperm-derived information could be important for normal embryonic cuticle formation (Rautengarten et al., 2005).

The expression patterns of ZOU and ALE1 are strikingly similar, and we have previously shown that the transcription of ALE1 is strongly reduced in zou mutants and that ZOU is epistatic to ALE1, consistent with both genes acting in the same developmental pathway (Yang et al., 2008). The cuticle phenotype of ale1 mutants is reminiscent of, although milder than, that of zou mutants. However, despite the fact that the endosperm tissues have been reported to adhere to the surface of ale1 mutant embryos during their development (Tanaka et al., 2001), it has so far been unclear whether an endosperm breakdown defect is associated with loss of ALE1 function. Two scenarios can be envisaged: (1) ALE1 acts downstream of ZOU in a pathway regulating both endosperm breakdown and cuticle formation; (2) ZOU regulates two developmental processes - endosperm breakdown (via an ALE1 independent pathway) and embryo cuticle formation via a non-cell-autonomous pathway involving ALE1. In order to distinguish between these hypotheses, we have analysed whether ALE1 plays a role in regulating endosperm cell death, and investigated whether the cell death regulation and cuticle formation functions of ZOU can be separated during seed development. We have also studied the genetic relationship between the endosperm-specific genes ALE1 and ZOU, and the genes encoding the embryonically expressed receptors GSO1 and GSO2, to elucidate their potential roles in signalling between the endosperm and the embryo.

MATERIALS AND METHODS
Plant materials and growth conditions
Mutant lines used in this study have been published previously with the exception of ale1-3 (SAIL_736_D09/N862715) and ale1-4 (SAIL_279_C04/N812918). General plant culture conditions were long (16 hour) days at 21°C. For staged material for phenotypic and transcriptional analysis, seeds were spread uniformly on 15 cm plates containing 1 MS Basal Salts (Duchefa), 0.3% sucrose and 0.4% Phytagel (Sigma) (pH 5.8). Seeds were incubated at 4°C overnight before visualization under DIC optics using a Zeiss AX10.

Seed clearing
To visualize and stage developing seeds, siliques were opened with a sharp needle and samples were incubated at 4°C overnight before visualization under DIC optics using a Zeiss H11003. Applied and samples were incubated at 4°C overnight before visualization using a spectrophotometer.

Test for seedling desiccation tolerance
Batch of 100 seeds were counted, plated on MS-Agar (0.5% Sucrose), stratified at 4°C for 3 days, and allowed to germinate in the standard growth chamber conditions for 7 days. All resulting seedlings were transferred to small pots containing moist compost in mixed trays, and covered with a loosely fitted lid for 3 days to allow root establishment. Pots were rotated in a random block design in trays, ensuring an equal exposure to potential border effects for each genotype. Moreover, trays were rotated once daily in order to minimise any heterogeneity owing to shelf position. The lid was then completely removed. Trays were kept uniformly moist, and seedling survival was counted after a period of 3 weeks.

Resin embedding and sectioning
Mature seeds were removed from siliques and vacuum infiltrated at 4°C with 4% paraformaldehyde + 5% glutaraldehyde in PBS (pH 7) with 1% Triton-X-100 (Sigma), 0.1% TWEEN (United States Biological) and 1% DMSO. Samples were incubated at 4°C overnight with continuous shaking, rinsed thoroughly with ice-cold PBS and dehydrated through a cold ethanol series into dry ethanol. Ethanol was then substituted sequentially for LR-White Hard Grade resin over 4-5 days. Samples were incubated in several changes of 100% resin over at least 3 days before placing individual seeds into 50, snap-fit, gelatine capsules (Agar Scientific, G3740). Resin was polymerized for 24 hours at 60°C. Sections (1 μm) were cut using glass knives on an ultramicrotome, and dried onto glass slides where they were stained for 10 seconds at 70°C with 1% Toluidine Blue/1% borax before rinsing with distilled water, drying and mounting in Eukitt mounting medium (Fluka). Alternatively, fixed, dehydrated samples were embedded in Technovit 7100 resin following the manufacturer’s instructions (Heraeus Kulzer GmbH). Sections (1.5 μm) were cut and mounting a metal knife on a Zeiss Hm355 S microtome, floated onto glass slides, dried, oxidized for 10 minutes in 1% periodic acid, washed for 5 minutes under running tap water, stained for 5 minutes in Schiff’s reagent (Sigma-Aldrich), washed for 5 minutes, dried and mounted as above.

Quantitative gene expression analysis
In order to obtain staged material, all plants were grown under near identical conditions. Every siliques on the main inflorescence spike of selected individuals of each genotype was dissected and cleared in order to ascertain the synchronicity of the population. Pools of siliques were then harvested from the remaining individuals in such a way that each sample contained one discrete developmental stage. A single biological replicate contains three or four siliques from one individual plant. Total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma). Total RNAs were digested with Turbo RNA-free DNase I (Ambion) according to the manufacturer’s instructions. RNA concentration and integrity were measured after DNase digestion with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies). RNA (1 μg) was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer’s protocol. PCR reactions were performed in an optical 96-well plate in the StepOne Plus Real Time PCR System (Applied Biosystems), using Platinum SYBR Green qPCR SuperMix-UDG in a final volume of 20 μl, according to the manufacturer’s instructions. The following standard thermal profile was used for all PCR reactions: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 20 seconds. Amplicon dissociation curves, i.e. melting curves, were recorded after cycle 40 by heating from 60°C to 95°C with a ramp speed of 1°C/minute. Data were analyzed using the StepOne Software v2.2.
RESULTS

The ZOU target gene ALE1 is involved in embryonic cuticle formation but not endosperm autolysis

The expression of ALE1 is dramatically downregulated in zou mutant backgrounds (Yang et al., 2008). As ALE1 is the only ZOU-regulated gene for which mutants share elements of the zou phenotype (namely cuticle defects in cotyledon tissues), we decided to further explore the function of ALE1 in seed development, and in particular whether it plays any role in regulating endosperm breakdown. The first published mutant alleles of ALE1, ale1-1 and ale1-2, were isolated in the Landsberg erecta (Ler) background (Tanaka et al., 2001). ale1-1 was subsequently introgressed into the Columbia-0 (Col0) background by successive backcrossing. In order to confirm that the phenotype of this line corresponds to that of ale1 loss of function in a pure Col-0 background, we also characterized two new T-DNA insertion alleles of ALE1, which we have named ale1-3 and ale1-4. ale1-3 contains a T-DNA insertion in the last intron of ALE1, whereas in ale1-4, a T-DNA is inserted just upstream of the start of transcription, where it would be expected to interfere with regulatory sequences (Fig. 1A). Q-RT-PCR analysis of ALE1 transcripts in cDNA from young siliques of wild-type plants showed strong expression of ALE1 from the globular stage onwards. Although ale1-1 siliques expressed transcripts at low levels (supplementary material Fig. S1), ale1-3 and ale1-4 siliques were found to express no detectable ALE1 transcripts at mid-seed development (supplementary material Figs S1, S2) and thus represent true null alleles of ALE1.

Consistent with transcriptional analysis, ale1-1, ale1-3 and ale1-4 homozygous seedlings showed patches of staining on cotyledons when treated with Toluidine Blue, suggesting that they show similar cuticle defects (Fig. 1D,E; supplementary material Fig. S3) (Tanaka et al., 2004). These patches tended to be larger in ale1-3 and ale1-4 than in ale1-1, and were generally smaller than those previously seen for ale1-1 in the Ler background. As previously...
reported, zou-4 seedlings showed a strong staining with Toluidine Blue, consistent with severely compromised cuticle function (Fig. 1C; supplementary material Fig. S3) (Yang et al., 2008). Wild-type seedlings showed no visible permeability to Toluidine Blue stain (Fig. 1B; supplementary material Fig. S3). In order to further investigate this phenotype, we developed a method of quantifying Toluidine Blue uptake by seedlings grown in vitro, and stained for a defined period of time (see Materials and methods). Briefly, Toluidine Blue enters cotyledons via the defective cuticle and binds strongly to cell walls within the organ (Tanaka et al., 2004). Washing to remove excess external Toluidine Blue has little effect on internalized stain. Internalized stain can, however, be solubilized in 80% ethanol and quantified spectroscopically (Terry et al., 2000). This method proved robust, and allowed us to measure Toluidine Blue uptake relative to cotyledon volume (approximated by Abs 430, an absorbance peak for Chlorophyll A, see supplementary material Fig. S10 for details). Although we could easily distinguish Col0, ale1 mutants and zou-4 using this method, we were not able to show a statistically significant difference in cuticle defects between ale1-1, ale1-3 and ale1-4 (Fig. 1F; supplementary material Fig. S3).

Seed morphology was studied for all three alleles, and revealed a previously undescribed aspect of the ale1 phenotype: a significant rate of formation of visibly mis-shapen seeds. The frequency of misshapen seeds was higher in the ale1-4 background (74%) than in the ale1-1 background (47%). The abnormal seed phenotype is typically characterised by ‘lumpy’ seeds, which tend to be rounder than wild-type seeds, which are smoother and more elliptical (Fig. 2). In order to follow embryo development more closely, seeds were cleared and observed using DIC microscopy. Early embryo development in ale1-4 mutants and zou-4 was indistinguishable from that in the wild-type background (Fig. 3A,E,I). However, by the walking stick stage (Fig. 3D,H,L), mis-oriented embryo growth was observed at a frequency of 72% (n=150) in ale1-4 mutants (Fig. 3L) and 28% (n=170) in ale1-1 mutants. By this stage, embryo growth in zou-4 mutants (Fig. 3H) was severely retarded, and the persistent endosperm that characterizes zou mutants was evident. Although slight growth retardation was observed for adherent ale1-4 embryos, no significant endosperm persistence was noted in any of the ale1 alleles studied.

To further investigate endosperm persistence at seed maturity, mature green (non dessicated) seeds were fixed, resin embedded and sectioned. Wild-type seeds at this stage contain a single layer of specialized endosperm cells, which are maintained throughout, and necessary for, seed dormancy (Bethke et al., 2007) (Fig. 3M). In zou-4 mutants, a significant body of endosperm tissue persists at seed maturity (Fig. 3N; supplementary material Fig. S8), shrivelling upon desiccation to give zou seeds their distinctive appearance. Resin sections revealed that the specialized outer endosperm-cell monolayer seen in wild type is still differentiated normally in zou mutants, whereas the remaining persistent endosperm is composed of large, highly vacuolated cells with thin walls (Fig. 3N; supplementary material Fig. S8). In both ale1-1 and ale1-4 mutant backgrounds, only a single layer of endosperm cells was observed (Fig. 3O), similar to the situation in wild-type plants. Consistent with this observation, no seed shrivelling was apparent in any of the ale1 mutant backgrounds studied.

### The expression of a subset of genes misregulated in zou mutants, is also affected in ale1 mutants

In order to understand more about the molecular basis for the defects presented by zou and ale1 mutants, a transcriptomic analysis of siliques containing embryos at the late heart/early torpedo stage of development from zou-4, ale1-1 and Col0 plants was performed (see Materials and methods). Genes showing significant downregulation in zou mutant background fell into two classes: those whose expression was also significantly downregulated in ale1-4 (including the ALE1 gene), and those in...
whose expression levels in ale1 mutants were indistinguishable from those in wild type [including four of the six published ZOU targets previously identified by microarray analysis (Kondou et al., 2008)]. Confirmation of mis-regulation was carried out for a subset of genes predicted to be expressed in the endosperm and/or embryo based on in silico data (Le et al., 2010), using Q-PCR in developing siliques of zou-4, ale1-4 and Col0 plants grown under identical conditions. Data for three genes regulated specifically by ZOU, and two genes regulated both by ZOU and ALE1 are shown in supplementary material Fig. S4. These results support our phenotypic and genetic data showing that ZOU and ALE1 perform partially, but not totally, overlapping functions.

**ALE1 expressed under the SUC5 promoter rescues ale1 seed and cotyledon phenotypes**

Our results support a model in which ZOU regulates both cell breakdown in the endosperm and embryonic cuticle formation, whereas ALE1 is uniquely required for embryonic cuticle development. To investigate whether the two proposed functions of ZOU can be separated, a construct was generated that would permit the expression of ALE1 in a ZOU-independent fashion. Previous work from our laboratory had identified expression of the gene AtSUC5 (Baud et al., 2005), as being independent of ZOU activity (Yang et al., 2008). The published expression pattern of AtSUC5 is very similar to that of ZOU and ALE1, in that it is largely restricted to the endosperm surrounding the developing embryo during seed development (Baud et al., 2005). We therefore made a construct to express the ALE1 open reading frame under the control of the AtSUC5 promoter (pSUC5::ALE1), and thus render ALE1 expression independent of ZOU activity.

To confirm the ability of this construct to recapitulate wild-type ALE1 expression and function, homozygous transformants in a Col0 background were crossed into the ale1-1 mutant background and F2 plants homozygous for both ale1-1 and the pSUC5::ALE1 transgene were selected for further analysis. The phenotypes of resulting plants were assessed both for cotyledon cuticle defects and for seed morphology, and showed a complete complementation of both phenotypes (supplementary material Fig. S5).

**Expression of ALE1 under the pSUC5 promoter complements zou cuticle defects, but does not restore endosperm autolysis**

The pSUC5::ALE1 construct was introduced into a zou-4 mutant background. Transformed lines were screened for ALE1 expression...
level by Q-PCR. Two lines with increased expression of ALE1 in a zou-4 background (line 8 and line 26) and one in a wild-type background were selected for further analysis. The expression of ALE1 in these lines was compared in detail with its expression in zou-4 mutants and in wild-type plants by collecting staged tissue samples corresponding to five developmental stages: inflorescence tips containing unopened flowers (flower), siliques containing preglobular stage embryos, siliques containing seeds with mid globular-stage embryos, siliques containing seeds with mid heart-stage embryos and siliques containing seeds with early torpedo stage embryos (see supplementary material Fig. S6 for staging). In wild-type tissues, the expression of endogenous ALE1 initiated at the globular stage and became increasingly strong at subsequent developmental stages (Fig. 4). Expression of ALE1 in zou-4 mutants was similar to wild type at the globular stage, but was then not maintained and fell back to very low levels at older stages. Expression of ALE1 in zou-4 plants transformed with pSUC5::ALE1 was considerably stronger than in either zou-4 or wild-type tissues at the globular stage, and then fell back to levels higher than those in zou-4 mutants, but lower than those in wild type at later stages. Wild-type plants containing pSUC5::ALE1 showed an ALE1 expression pattern that was effectively additive between wild type and transgene expression (Fig. 4). A basal level of ALE1 expression was also observed in inflorescence samples from all plants transformed with pSUC5::ALE1, suggesting that, as previously shown, pSUC5-driven expression is not entirely seed specific (Baud et al., 2005). It therefore appears that the pattern of expression of SUC5 during seed development is spatially similar, but temporally not identical to that of ALE1.

In order to ascertain the phenotypic effects of restoring ALE1 expression in a zou background, the zou-4 lines transformed with pSUC5::ALE1 were compared with zou-4 and wild type at the level of seed and seedling morphology, Toluidine Blue staining and seedling desiccation tolerance. At the level of seed morphology, both lines were indistinguishable from zou-4, in that they produced small, dark wrinkled seeds with an obvious ‘pouch’ of persistent endosperm surrounding a small embryo (Fig. 5E,F). Clearing of developing seeds from both transgenic lines and zou-4 revealed no visible difference in the amount of persistent endosperm observed at any developmental stage, although the ‘cavity’ surrounding the embryo was more often apparent in the transformed lines than in zou-4 (Fig. 5C,D). No phenotypic effects of overexpressing ALE1 in a wild-type background were observed. From these data, we conclude that no significant diminution in endosperm persistence is caused by the transcription of ALE1 in zou-4 mutants. This conclusion was confirmed by resin sectioning of seeds from the zou-4 line, showing the strongest level of ALE1 expression under the AtSUC5 promoter (line 8). The endosperm persistence in this line is indistinguishable from that in an untransformed zou-4 mutant (supplementary material Fig. S7).

When seeds were imbibed and dissected, it was considerably easier to separate the embryo and endosperm tissues of the zou-4

![Fig. 4. Expression of ALE1 relative to that of EIF4 in Col0, zou-4, zou-4[pSUC5::ALE1]-8, zou-4[pSUC5::ALE1]-26 and Col0[pSUC5::ALE1]-10 backgrounds. Staging of embryos is shown in supplementary material Fig. S6. Results are from biological triplicates, each of which was analysed in technical triplicate. Error bars represent s.d. between biological triplicates.](image)

![Fig. 5. Reintroducing ALE1 expression into a zou-4 background partially rescues endosperm adhesion defects but does not rescue seed shrivelling. (A-D) Cleared embryos in seeds from Col0 (A), ale1-4 (B), zou-4 (C) and zou-4[pSUC5::ALE1]-8 (D) taken at the early torpedo stage in development. The growth defect in zou-4 mutants is evident at this stage. No obvious difference in embryo development is seen between zou-4 and zou-4[pSUC5::ALE1]-8, but separation between the embryo and endosperm (arrows) is observed in zou-4[pSUC5::ALE1]-8. (E,F) Seeds from zou-4 (E) and zou-4[pSUC5::ALE1]-8 (F) plants look identical. Arrows in E and F indicate the pouch of persistent endosperm present in zou seeds. Scale bars: 50 µm.](image)
lines transformed with pSUC5::ALE1, than of zou-4 mutant seed. It therefore appeared likely that expressing ALE1 might modify the cuticle defects observed in zou-4 mutants, and thus diminish adhesion between the embryo and endosperm. Consistent with this, visible differences in Toluidine Blue staining were discernible between seedlings of zou-4 and the pSUC5::ALE1 transformed zou-4 lines, suggesting that the cuticles of the latter were more intact than those of zou-4 mutants (Fig. 6A-C). These results were confirmed by spectrophotometry, which showed a significant diminution in cuticle permeability associated with the presence of the pSUC5::ALE1 transgene (Fig. 6D). Because cuticle defects have been shown to affect desiccation tolerance, and zou-4 mutant seedlings are extremely desiccation intolerant, we devised a quantitative test for resistance to desiccation, based on the ability of 7-day-old seedlings transplanted from agar plates to survive in moderately humid conditions on soil. Conditions were selected in which 95-100% of Col0 (desiccation tolerant) and ale1-1 seedlings, consistently survived transplantation, whereas ~50% of zou-4 seedlings died within 3 weeks of transplantation (see Materials and methods). Using these conditions, and growing plants under homogeneous conditions (mixed trays), we compared the desiccation tolerance of zou-4 mutants with zou mutants carrying the pSUC5::ALE1 transgene. Experiments involved the transplantation of the germinated plantlets from batches of 100 seeds, and were carried out in triplicate. They showed a significant increase in desiccation tolerance, particularly in line 8, consistent with a partial complementation of the zou-4 cuticle phenotype (Fig. 6E).

**ZOU, ALE1, GSO1 and GSO2 act in the same pathway to specify embryonic cuticle formation**

Double gso1-1/gso2-1 mutants show a severe defect in embryonic cuticle development that leads to extreme permeability of cotyledon surfaces to Toluidine Blue (Tsuwamoto et al., 2008). In addition, embryos adhere to endosperm during their development, and this causes them to bend abnormally as they elongate, giving rise to a misshapen-seed phenotype similar to, but more severe than, that seen in ale1 mutants (Fig. 7A-C). Despite their severe seed phenotypes, and consistent with the fact that the expression of GSO1 and GSO2 appears to be largely embryo specific during seed development, no persistent endosperm was observed in mature gso1-1/gso2-1 mutant seeds (Fig. 7C; supplementary material Fig. S8). We did, however, note that adult gso1-1/gso2-1 double mutants showed very subtle vegetative phenotypes, including slightly paler leaf colour, slightly retarded flowering and increased branching compared with wild-type plants, consistent with roles during post-germinative growth (data not shown).

In order to test genetically the relationship between GSO1-1/GSO2-1 function and the ZOU/ALE1 pathway, multiple mutant combinations were generated between gso1, gso2 and zou-4, and ale1-4. Cuticle permeability for single and multiple mutants was measured by quantification of Toluidine Blue uptake as described previously (Fig. 7G). As before, with Col0 seedlings, ale1-4 mutant seedlings show slight, but significant increases in cuticle permeability, whereas zou-4 mutant seedlings showed a strong increase in cuticle permeability. Consistent with previous
and Zou phenotype shown by In this paper, we aim to clarify a major issue regarding the mutant deposition.

either death defect present in the additional effects of the content) (supplementary material Fig. S10), and may also reflects partly attributable to their small size (and thus their low chlorophyll development.

Thus, GSO1 and GSO2 show epistasis with respect to embryonic cuticle formation, and probably act in the same developmental pathway. gso1-1/gso2-1/zou-4 triple mutants seedlings were small (similar to zou-4 mutant seedlings) (Fig. 7G; supplementary material Fig. S9). The apparent increase in cuticle permeability of gso1-1/gso2-1/zou-4 triple mutants compared with either zou-4 single mutants or gso1-1/gso2-1 double mutants, is partly attributable to their small size (and thus their low chlorophyll content) (supplementary material Fig. S10), and may also reflects the additional effects of the ALE1/GSO1/GSO2-independent cell-death defect present in the zou mutants, upon embryo cuticle deposition.

**DISCUSSION**

In this paper, we aim to clarify a major issue regarding the mutant phenotype shown by zou mutants: whether cuticular defects observed in zou mutants are an indirect consequence of the abnormal maintenance of intact endosperm cells in the region surrounding the embryo, or whether they indicate the loss of a specific and autolysis-independent pathway via which the endosperm regulates embryonic cuticle formation. We have focused on the gene ALE1, which we previously shown to be strongly downregulated in zou mutants. Previous analyses of ale1 mutants had shown abnormalities in embryonic cuticle formation, and adhesion to endosperm during seed development. However, they did not address directly whether ALE1 mutants showed any defects in cell death pathways. It was therefore unclear whether ZOU acted through ALE1 (and potentially other functionally redundant proteins) to regulate both cell death and cuticle formation, or whether ALE1 was involved uniquely in embryonic cuticle formation, making the two functions of ZOU potentially separable.

By identifying new null alleles of ALE1 and analysing these at the phenotypic level, we have shown that loss of ALE1 function affects embryonic cuticle formation, and that this causes embryonic adhesion to other seed tissues and seed malformation. However, ale1 mutants show no apparent defects in endosperm autolysis. We subsequently analysed the expression of several genes that we identified as being downregulated in zou mutant seeds, in ale1 mutants. Only a subset of these genes is significantly downregulated in ale1 mutants, further supporting our argument that the function of ALE1 only partially overlaps with that of ZOU.

The ultimate proof that the two functions of ZOU are separable is to rescue one without affecting the other. This was achieved by re-introducing ALE1 function into a zou mutant. We had previously results (Yang et al., 2008), zou-4 showed complete epistasis with ale1-4 using this technique and gso1-1/gso2-1 double mutants had extremely permeable cuticles (Tsuwamoto et al., 2008). Triple gso1-1/gso2-1/zou-4 and gso1-1/gso2-1/ale1-4 mutants were both viable. In the case of gso1-1/gso2-1/ale1-4 mutants, both plant phenotypes, and seed and seedling phenotypes were indistinguishable from those of gso1-1/gso2-1 triple mutant (Fig. 7C,D; supplementary material Fig. S9). In addition, cuticle permeability in the gso1-1/gso2-1/ale1-4 triple mutant was indistinguishable from that in the gso1-1/gso2-1 double mutant (Fig. 7G). In the case of gso1-1/gso2-1/zou-4, adult plants were indistinguishable from gso1-1/gso2-1 double mutants and seed phenotypes were indistinguishable from those of zou-4 single mutants (Fig. 7E,F).

Thus, GSO1 and GSO2 show epistasis with respect to embryonic cuticle formation, and probably act in the same developmental pathway. gso1-1/gso2-1/zou-4 triple mutants seedlings were small (similar to zou-4 mutant seedlings) (Fig. 7G; supplementary material Fig. S9). The apparent increase in cuticle permeability of gso1-1/gso2-1/zou-4 triple mutants compared with either zou-4 single mutants or gso1-1/gso2-1 double mutants, is partly attributable to their small size (and thus their low chlorophyll content) (supplementary material Fig. S10), and may also reflects the additional effects of the ALE1/GSO1/GSO2-independent cell-death defect present in the zou mutants, upon embryo cuticle deposition.

**Fig. 7. GSO1 and GSO2 act in the same embryonic cuticle development pathway as ALE1 and ZOU.** (A-F) Seed phenotypes of Col0, ale1-4, gso1-1/gso2-1, gso1-1/gso2-1/ale1-4, zou-4 and gso1-1/gso2-1/zou-4 homozygous mutant plants, respectively. No differences in seed phenotypes are visible between gso1-1/gso2-1 and gso1-1/gso2-1/ale1-4, or between zou-4 and gso1-1/gso2-1/zou-4 seeds. Arrows in E and F indicate the pouch of persistent endosperm present in zou seeds. (G) Toluidine Blue uptake in seedlings from these lines was quantified spectrophotometrically, showing no significant difference in Toluidine Blue uptake between gso1-1/gso2-1 and gso1-1/gso2-1/ale1-4. The difference between gso1-1/gso2-1 and gso1-1/gso2-1/zou-4 is largely explained by the small size of these seedlings (see supplementary material Fig. S10). Error bars represent s.d. between three biological replicates each containing 20 seedlings. Corresponding seedling phenotypes are shown in supplementary material Fig. S9.
shown that the expression of AtSUC5, the spatial distribution of which closely resembles that of both ZOU and ALE1, is not affected in zou mutants, providing a powerful means of specifically reintroducing ALE1 expression into zou mutants. In zou mutant plants transformed with pSUC5::ALE1, cuticle defects characterized by zou mutants are significantly alleviated, whereas endosperm autolysis remains defective to the same extent as in zou mutants.

Although we can significantly alleviate cuticle defects in the zou mutant by reintroducing ALE1 expression under pSUC5, we could not fully rescue this aspect of the zou phenotype. One potential reason for this is that the temporal expression pattern of pSUC5 is not identical to that of ALE1, leading to lower ALE1 expression at later developmental stages in zou plants expressing pSUC5::ALE1 than in wild-type plants. However, pSUC5::ALE1 fully complements the ale1 mutant phenotype, suggesting that this is not a satisfactory explanation. The cuticular phenotype of null ale1 mutants is weaker than that of zou mutants, and it is therefore possible that ALE1 shows a partial functional redundancy with additional targets of ZOU that regulate embryonic cuticle formation. This possibility is currently under investigation. However, we cannot exclude a third factor, which is that persistence of the endosperm has some effect on cuticle formation. For example, persistent endosperm could physically impede the secretion of a normal cuticle even in the presence of ALE1.

Although our results show that ALE1 can normalize cuticle formation in the absence of endosperm autolysis, the issue still remains of how endogenous expression of ALE1 is regulated. It is not yet clear whether ALE1 is a direct target of ZOU, or whether its expression is regulated by other ZOU targets. We cannot formally exclude, for example, the possibility that components of the endosperm autolytic pathway are necessary for normal ALE1 expression. However, the fact that ALE1 expression is first detected well before the onset of autolysis makes this scenario unlikely.

ALE1 belongs to a family of secreted proteases that are widely implicated in cell signalling, and in particular in the processing of peptide ligands (Liu et al., 2009; Rautengarten et al., 2005). The fact that ALE1 acts to regulate cuticle biosynthesis independently of endosperm autolysis strengthens the argument for the presence of a non-autonomous signalling pathway by which endosperm-specific components (such as ALE1) regulate embryonic cuticle biosynthesis. We therefore asked whether any of the four receptor kinases previously implicated in the formation of embryonic epidermis might act in the same pathway as ALE1. Genes encoding two of these kinases, ABNORMAL LEAF SHAPE2 (ALE2) and ARABIDOPSIS CRINKLY4 (ACR4), show synergistic genetic interactions with ALE1 (Tanaka et al., 2007). Double mutants of either gene with ale1 show embryo lethality owing to dramatic increases in epidermal and cuticle defects compared with single mutants (Tanaka et al., 2007; Watanabe et al., 2004). Moreover, ale2 mutants are epistatic toacr4 mutants, suggesting that ACR4 and ALE2 act together in a pathway parallel to that containing ALE1 (Tanaka et al., 2007). We therefore tested whether two other redundantly acting receptor kinases, GSO1 and GSO2, might act in the ALE1 pathway. The complete epistasis we observed between gso1-1/gso2-1 double mutants and ale1 contrasts sharply with the synergistic interaction observed between ALE1 and ACR4 or ALE2, and provides convincing evidence that GSO1 and GSO2 act in the same pathway as ALE1, reinforcing previous observations suggesting that two major pathways are involved in regulation of epidermal development during Arabidopsis embryogenesis (Tanaka et al., 2007). Because GSO1 and GSO2 are strongly expressed in embryo tissues, whereas both ALE1 and ZOU show endosperm specific expression, it is tempting to speculate that the GSO receptor-kinases act redundantly to perceive a peptide ligand processed in the extra-embryonic space by ALE1 that is necessary for normal cuticle deposition on the embryonic surface. Although the identity and developmental origin of the putative ligand remain to be determined, these results represent a key indication that direct ligand-mediated signalling between the two zygotically derived seed components is necessary for normal embryonic development.

ZOU is an ancient and highly conserved transcription factor, with clear orthologues in monocotyledonous plants and even in gymnosperms and Selaginella. We have previously proposed that ZOU could have had an ancestral role in gymnosperms and clubmosses, permitting invasive embryo growth into maternally derived nutritive tissues. Subtilisin-like serine proteases are found in eudicot, monocot, gymnosperm, clubmoss and moss genomes. Interestingly, however, ALE1 does not have clearly defined orthologues outside the angiosperms. It is therefore possible that the function of ALE1 in regulating embryonic cuticle development was acquired during the radiation of the angiosperms. The concomitant and rapid development of the endosperm and embryo after fertilization of the angiosperm ovule may impose unique problems for both organisms, especially in terms of defining boundaries. We propose that ALE1 may have been recruited in some angiosperms to counter such problems.

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Supplementary material

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