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## ***fist*: an *Arabidopsis* mutant with altered cell division planes and radial pattern disruption during embryogenesis**

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**Abstract** A T-DNA-tagged, embryo-defective *Arabidopsis thaliana* mutant, *fist*, was identified and shown to exhibit defects in nuclear positioning and cell division orientation beginning at the four-cell stage of the embryo proper. Cell division orientation was randomised, with each embryo exhibiting a different pattern. Periclinal divisions did not occur after the eight-cell embryo proper stage and *fist* embryos lacked a histologically distinct protoderm layer. Terminal embryos resembled globular-stage embryos, but were a disorganised mass containing 30–100 cells. Some terminal embryos (5%) developed xylem-like elements in outer surface cells, indicating that the *fist* mutation affects radial pattern. A soybean  $\beta$ -conglycinin seed storage protein gene promoter, active in wild-type embryos from heart stage to maturity, was also active in terminal *fist* embryos despite their disorganised globular state. This indicated that some pathways of cellular differentiation in *fist* embryos proceed independently of both organised division plane orientation and normal morphogenesis. Endosperm morphogenesis in seeds

containing terminal *fist* embryos was arrested at one of three distinct developmental stages and appeared unlinked to *fist* embryo morphogenesis. The  $\beta$ -conglycinin seed storage protein gene promoter, normally active in cellularised wild-type endosperm, was inactive in *fist* endosperm, indicating abnormal development of *fist* endosperm at the biochemical level. These data indicate that the *fist* mutation, either directly or indirectly, results in defects in cell division orientation during the early stages of *Arabidopsis* embryo development. Other aspects of the *fist* phenotype, such as defects in endosperm development and radial pattern formation, may be related to abnormal cell division orientation or may occur as pleiotropic effects of the *fist* mutation.

**Key words** Cell division · *Arabidopsis thaliana* · Embryo mutants · Endosperm · T-DNA

### **Introduction**

Control of cell division orientation is thought to be a critical aspect of morphogenesis in plants. Plant cells are glued together by the middle lamella and, thus, are not able to move relative to one another during development. As a consequence, organ and body shape are generated by other cellular processes, including controlled cell division orientation (Francis and Halford 1995). This is especially evident during early embryogenesis in species such as *Capsella* and *Arabidopsis* (Johansen 1950; Mansfield and Briarty 1991) where cell division orientations are invariant. Control over cell division orientation may also be important in the initiation of new organs. For example, during leaf, petal, sepal and lateral root development, new organs are initiated by a change in cell division orientation (Esau 1977; Lyndon 1983).

The mechanisms controlling cell division orientation in plants are not understood. Mutant analysis suggests that control of cell division orientation is coordinated with that of other shape-generating processes, such as the rate of cell division and the rate and plane of cell expansion. For example, periclinal chimeras containing a

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mutation that slows growth of cells from one cell layer can be compensated for by accelerated growth of wild-type cells (Stewart et al. 1974). Similarly, a maize mutant (*tangled-1*) with altered cell division orientation has essentially normal leaf shape (Smith et al. 1996). These data suggest that if a defect in cell division rate, cell division orientation, cell expansion rate or cell expansion orientation occurs, it can be compensated for by a modulation in the activities of the other three unaffected processes and that cell division orientation is possibly controlled by general mechanisms controlling organ shape.

Division plane orientation in plants is correlated with changes in the microtubular cytoskeleton (Cyr 1994; Kropf 1994). In higher plants, the preprophase band (ppb) that appears early in the G<sub>2</sub>-phase of the cell cycle predicts the plane of cell division (Gunning and Wick 1985). Thus, the ppb appears to be a marker of the plane of cell division orientation (Wick 1991). Little is known about what influences the temporal and spatial positioning of the ppb or how it predicts the plane of cell division (Francis and Halford 1995).

One candidate for a controller of cell division orientation in *Arabidopsis* is TON. The *ton* mutant has been characterised with respect to microtubule arrays and is defective in ppb formation and in the alignment of interphase cortical microtubules, suggesting that TON is necessary for division plane alignment and cell expansion, respectively (Traas et al. 1995). Another candidate for controlling division plane orientation is the rice OSH-1 gene product. Ectopic expression of OSH-1 in tobacco results in alteration in cell division plane orientation specifically during leaf primordia initiation (Yutaka et al. 1996).

Several *Arabidopsis* mutants identified at the seedling stage as pattern formation mutants have been shown to exhibit altered cell division orientation during embryogenesis. These mutants include *gnom* (Mayer et al. 1993), *monopterous* (Berleth and Jürgens 1993), *fass* (Torres-Ruiz and Jürgens 1994), *knolle* (Lukowitz et al. 1996) and *ton* (Traas et al. 1995). The GNOM (EMB30 allele; Shevell et al. 1994) and KNOLLE (Lukowitz et al. 1996) genes have been cloned, and they encode proteins related to yeast secretory proteins and syntaxins, respectively. While the cellular function of GNOM remains unclear, KNOLLE appears to be important for cell plate formation during cytokinesis, and both genes suggest the importance of membrane trafficking in cell wall synthesis and plant development (Dupree 1996).

We have used T-DNA insertional mutagenesis of *Arabidopsis* as a system to identify molecules essential for embryo development (Meinke 1985; Feldmann 1991). We have identified mutants defective over a wide range of embryo stages (Goldberg et al. 1994; West et al. 1994; Yadegari et al. 1994). In this paper, we present the initial characterisation of *fist*, a T-DNA-tagged mutant that exhibits alterations in cell size, shape and nuclei position from the four-cell embryo proper stage. The terminal phenotype of *fist* is a disorganised cell mass approximating the size of a wild-type mid to late globular stage embryo. *fist* also exhibits defects in radial patterning and

endosperm development. Molecular characterisation of *fist* may provide information on processes controlling cell cleavage plane orientation and tissue differentiation events during radial axis formation in *Arabidopsis*.

## Materials and methods

### Plant material

The *fist* mutant was isolated from a collection of lines of *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (WS) mutagenised by T-DNA insertion (Feldmann and Marks 1987; Feldmann 1991; Yadegari et al. 1994). The original line number for this mutant was A2179, and it can be obtained from the *Arabidopsis* Biological Resource Center, Ohio State University, USA, as stock number CS2683. Plants were grown in a mixture of white sand, peat and perlite (2:2:1) either in growth chambers at 25°C with a 16/8 h light/dark cycle or in the greenhouse. The *fist* phenotype remained constant in either environment. As this recessive mutation is lethal, plants were maintained as heterozygotes which produced 25% mutant seeds upon self-fertilisation. Attempts to germinate *fist* seeds and to rescue *fist* embryos *in vitro* were unsuccessful. *Arabidopsis thaliana* lines containing a chimeric GUS fusion gene regulated by the  $\beta$ -conglycinin  $\alpha'$  subunit promoter (Hirai et al. 1994) were kindly provided by Satoshi Naito, Hokkaido University, Japan.

### DNA isolation and analysis

Genomic DNA was isolated from heterozygous *fist* plants by the method of Konieczny and Ausubel (1993). Ten micrograms of DNA was digested with either *Eco*RI, *Sal*I or *Hind*III and resolved on 0.8% agarose gels. After transfer to Zeta-Probe nylon membrane (Bio-Rad), DNA was probed with T-DNA left-border and right-border probes (Castle et al. 1993), using high-stringency hybridisation at 65°C and washes up to 65°C in 0.1 $\times$ SSC, 0.1%SDS.

### Light and confocal microscopy

For light microscopy, seeds were fixed in 60% ethanol, 10% glacial acetic acid, 4% formaldehyde, or on occasion whole siliques were fixed in 4% paraformaldehyde, 0.25% glutaraldehyde in 0.05M PIPES buffer (pH 7). Tissue was fixed for 3–4 h at room temperature or overnight at 4°C. After fixing, seeds were further dehydrated to 70% ethanol and siliques were dehydrated through a graded ethanol series from 10 to 70% ethanol. Ethanol was replaced with LR gold resin (London Resin, UK). Following resin polymerisation, sections were cut 2–3  $\mu$ m thick and stained with 0.5% toluidine blue, then viewed and photographed on a Zeiss axioplan microscope fitted with an automatic camera attachment. Tissue for clearing was prepared as described by Stelly et al. (1984) except that haemalum staining was omitted.

For confocal microscopy, siliques between 3 and 8 mm in length were longitudinally slit before fixing in 4% glutaraldehyde in 12.5 mM sodium cacodylate buffer (pH 6.9) for 2 h to overnight. After dehydration through a graded ethanol series of 10 to 100% ethanol, siliques were cleared for 20 min in a solution of 2:1 (vol:vol) benzyl benzoate and benzyl alcohol. Tissue was mounted in immersion oil and imaged using the rhodamine channel on a Bio-Rad confocal microscope. This procedure identifies various cell structures and, in particular, causes bright autofluorescence of the nucleolus.

### GUS fusion gene introduction and GUS histochemical assays

To generate mutant *fist* plants carrying the  $\beta$ -conglycinin  $\alpha'$  subunit promoter-GUS fusion gene, recipient *fist* flowers were emasculated and stigmas were pollinated with pollen from plants homozygous for the GUS fusion gene. Resultant F<sub>1</sub> plants heterozygous

for *fist* were selected, selfed, and the seed was collected. Heterozygous *fist* plants were identified in the F<sub>2</sub> population and plants homozygous for the GUS fusion gene were selected after self-crossing and scoring for siliques that contain 75% seeds as wild-type and that stain positive for GUS activity and 25% of seeds as lethals. Wild-type and mutant seeds were dissected from developing siliques, prefixed by vacuum infiltrating with 0.1% formaldehyde, 0.1% Triton X-100, 0.1%  $\beta$ -mercaptoethanol and 0.1 M sodium phosphate (pH 7), and stained at 37°C overnight for GUS activity (Jefferson 1987). DMSO (10%) was added to the GUS assay medium, which was vacuum infiltrated into the seeds. After staining, seeds were fixed in 3% formaldehyde, 2% acetic acid in 0.1 M sodium phosphate buffer (pH 7) for 1 h, dehydrated using a graded ethanol series of 25 to 100%, cleared in methyl salicylate and viewed by light microscopy as above.

## Results

### Isolation of the *fist* mutant

As part of our screen of T-DNA mutagenised *Arabidopsis* lines, we focused on proembryo mutants that were likely to lead to identification of genes involved in early embryogenesis events (for primary screen details, see Yadegari et al. 1994). Fourteen lines were identified that aborted development prior to or around the globular stage. We examined the terminal embryo phenotypes in these 14 lines by DIC microscopy of whole cleared seeds (Stelly et al. 1984) and by light microscopy of sectioned seeds. Four lines were identified that exhibited defects in cell wall orientation, cell size and cell shape. One of these lines (Materials and methods), hereafter designated *fist*, was chosen for further analysis because it exhibited the most severe defect in cellular organisation, its phenotype co-

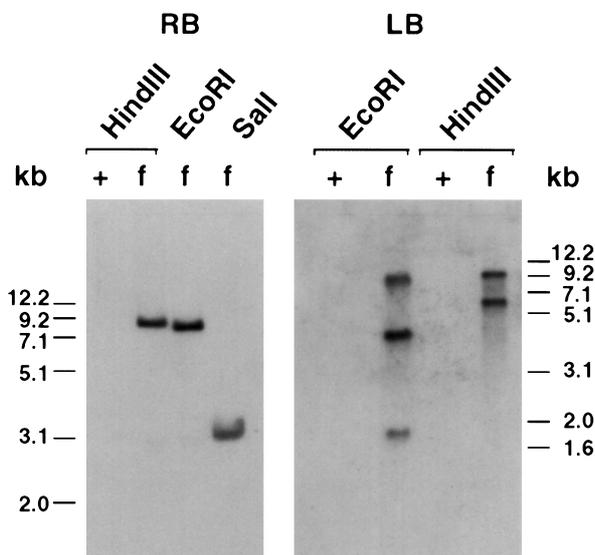
segregated with the T-DNA selectable marker gene (neomycin phosphotransferase) and the mutation was shown to be linked to a single T-DNA insertion following gel blot analysis of 50 segregants (Meinke 1985; Errampalli et al. 1991; segregation data not shown). Figure 1 illustrates gel blot analysis of *fist* DNA using T-DNA-specific probes. The presence of one T-DNA right border and two T-DNA left borders suggested a simple T-DNA insertion pattern. However, further sequence analysis (not shown) revealed a complex integration of additional *Agrobacterium* DNA adjacent to the T-DNA, which has impeded the characterisation of disrupted plant DNA.

Figure 2a–d shows that *Arabidopsis* embryo development proceeds in a series of developmental stages referred to as globular (Fig. 2a), heart (Fig. 2b), torpedo (Fig. 2c) and walking-stick (Fig. 2d) before the cotyledons fully recurve in the mature seed prior to desiccation (Mansfield and Briarty 1991; Bowman 1993; Jürgens and Mayer 1994). We examined the terminal phenotype of *fist* embryos in heterozygous *fist* siliques containing wild-type embryos at the walking-stick stage (Fig. 2d). Figure 2f–g shows that terminal *fist* embryos were similar in overall size, shape and cell number to mid to late globular wild-type embryos (Fig. 2e shows an early globular embryo) in that they contained between 30 and 100 cells in the embryo proper. However, in contrast to wild-type globular stage embryos, terminal *fist* embryos exhibited irregular cellular organisation and the cell walls of the embryos appeared to be in random positions. The *fist* embryo cells were large and vacuolate relative to wild-type embryo cells. Adhesions between cells appeared affected and this was independent of embryo cell number (compare Fig. 2e with Fig. 2f–j). At the basal end of the terminal *fist* embryo, a putative hypophysis region was present, but it was variable in the number of cells (Fig. 2g). The *fist* suspensor occasionally exhibited irregular, longitudinal cleavages in the cells proximal to the embryo proper in the most extreme examples of the terminal *fist* phenotype where there were also more cell divisions in the embryo proper (Fig. 2g). In most instances, however, *fist* suspensor morphology was indistinguishable from the wild-type morphology.

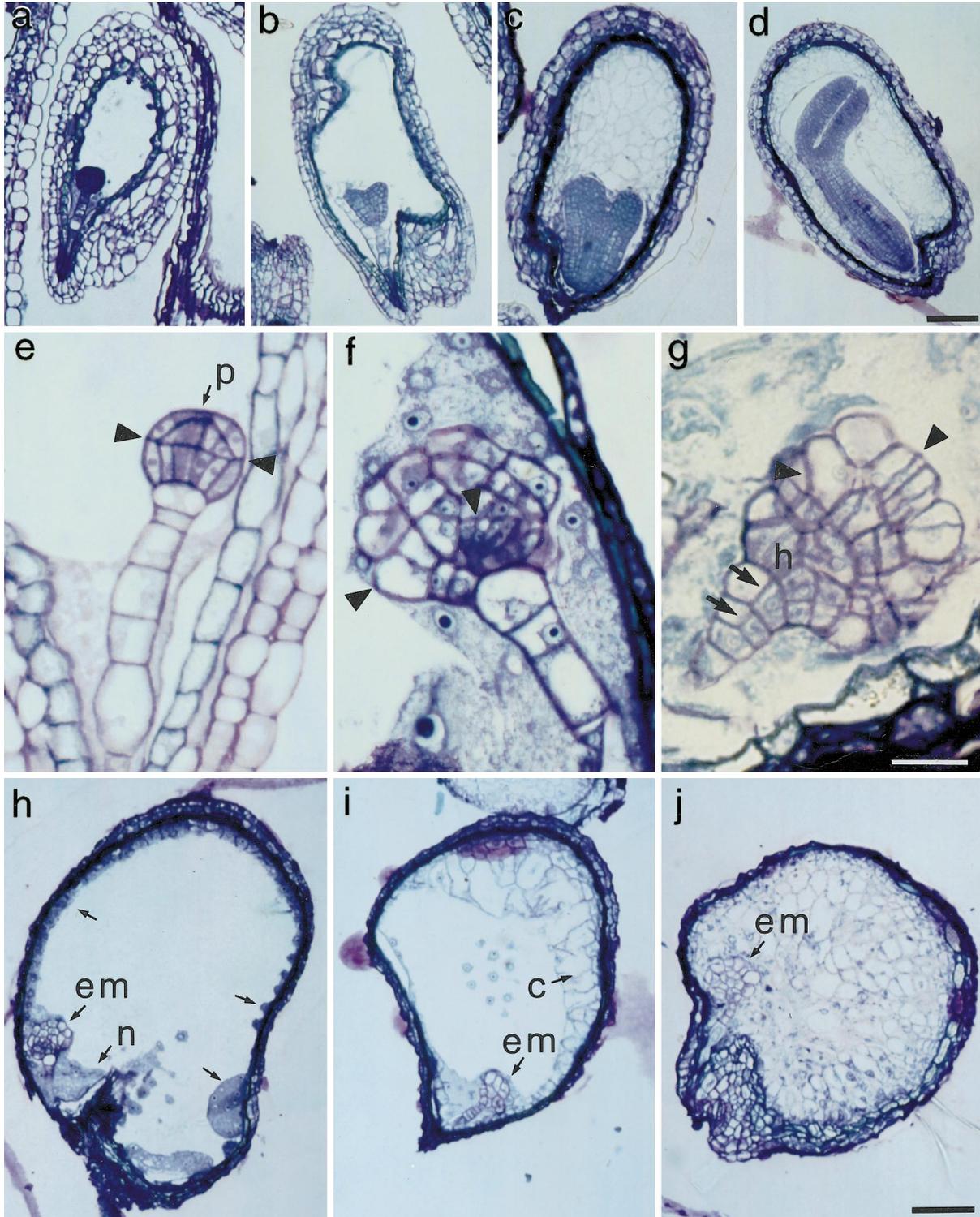
While some variation was seen in the cellular organisation of different *fist* embryos, there was, however, a consistent absence of a distinct protoderm, although no cell-type markers were used to verify this. In the wild-type, the protoderm layer is formed by a very early cell cleavage event preceding the globular stage of embryo development (Mansfield and Briarty 1991). The random organisation of cells in the embryo proper and the apparent lack of wild-type structural features such as a protoderm led us to classify *fist* as a potential embryo cell cleavage mutant.

### Early embryo divisions and tissue differentiation pattern are altered in the *fist* mutant

To determine what causes terminal *fist* embryos to exhibit irregular cellular organisation, we examined the early events of embryo development in this mutant. Early em-



**Fig. 1** Molecular characterisation of the T-DNA insertion in *fist*. Genomic DNA was isolated from mutant *fist* plants (*f*) and wild-type segregants (+) and digested with the restriction enzymes indicated. Gel blots were probed with radiolabelled DNA fragments spanning the right border (RB) and left border (LB) of the T-DNA used for mutagenesis, as described by Castle et al. (1993).



**Fig. 2** Histological analysis of *Arabidopsis thaliana* wild-type and *fist* mutant seeds. **a–d** Sections of wild-type seeds showing developmental stages of the embryo and endosperm. At the mid globular stage of embryo development (**a**) the embryo and suspensor are submerged in free-nuclear endosperm tissue. **b** Seed containing early heart stage embryo. Cellularising endosperm surrounds the embryo, and endosperm nuclei can be seen around the embryo sac wall. **c** At the early torpedo embryo stage, the endosperm is undergoing cellularisation. **d** Seed containing a walking-stick embryo and cellularised endosperm. **e** Section through a wild-type early globular embryo. The protoderm (*p*) is indicated, and the boundary between upper and lower parts of the embryo is marked by *arrow-*

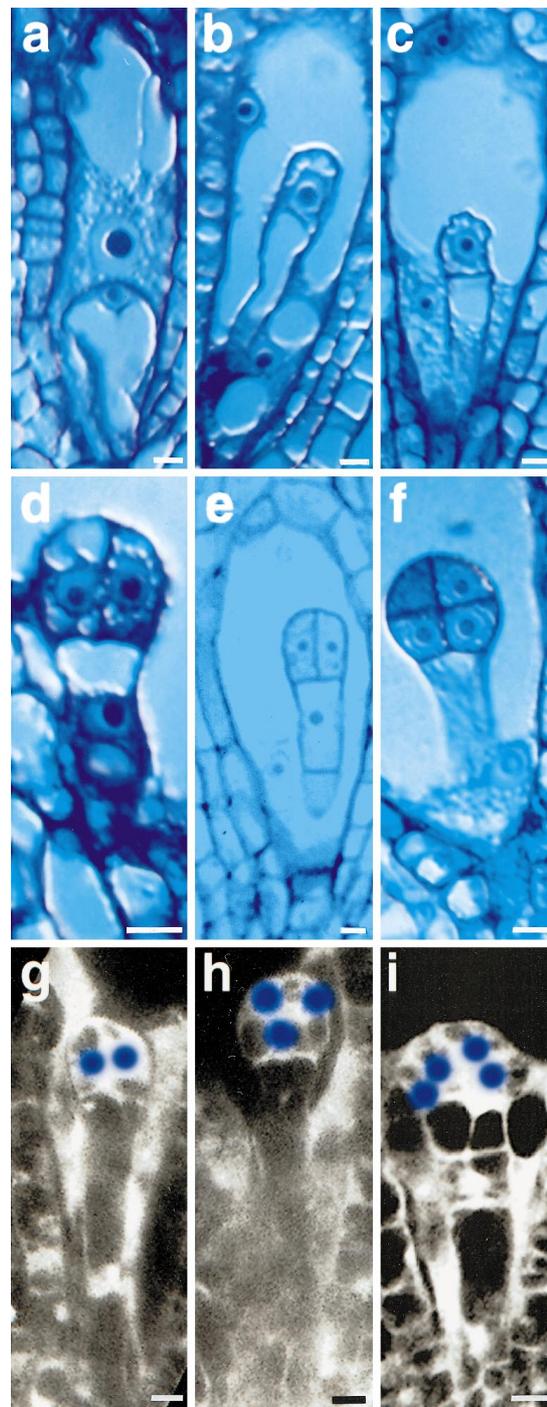
*heads*. **f, g** High magnification views of *fist* mutant embryos, illustrating abnormal cell positions and misshapen cells (*arrowheads*), and the lack of an outer protoderm layer. Longitudinal divisions are seen occasionally in suspensor cells proximal to the embryo proper as shown in (**g**) (*arrows*), and a putative hypophysis region (*h*) is indicated in (**g**) (*arrows*). **h–j** Sections through mutant seeds taken from siliques at the wild-type walking-stick embryo stage. The *fist* mutant embryo (*em*) has a disorganised globular structure in each case. *fist* endosperm phenotypes seen are: (**h**) type 1, free-nuclear, indicated by (*n*) and *arrows*; (**i**) type 2, partially cellularised (*c*) around the periphery of the embryo sac; and (**j**) type 3, completely cellularised. *Bars* 50  $\mu$ m for **a–d, h–j**; 20  $\mu$ m for **e–g**

bryogenesis in wild-type *Arabidopsis* is characterised by a series of invariant cell cleavages (Mansfield and Briarty 1991), as illustrated in the bright-field micrographs shown in Figure 3a–f. Following fertilisation (Fig. 3a), the elongated zygote (Fig. 3b) undergoes an asymmetrical transverse division to produce a small apical cell and a larger basal cell, defining the embryo and suspensor domains, respectively (Fig. 3c). Two longitudinally oriented cleavages of the embryo proper cell generate a symmetrical four-cell embryo (Fig. 3d–e). At the four-cell stage, the four nuclei lie along the equatorial plane of the embryo and are positioned centrally within their respective cells (Fig. 3e). A transverse cleavage of each cell then produces a symmetric eight-cell globular embryo where the four cells in the top and bottom tiers will contribute to the apical and basal halves of the embryo, respectively (Fig. 3f). The eight nuclei are also organised into two superimposable tiers of four nuclei. Thus, the early stages of embryo development in wild-type *Arabidopsis* are characterised by a series of regular cleavages and also by precise nuclear positioning (Mansfield and Briarty 1991; Webb and Gunning 1991; Bowman 1993; Jürgens and Mayer 1994). We analysed embryos within siliques of heterozygous *fst* plants using confocal laser scanning microscopy (CLSM). The fixation method employed causes nucleoli to autofluoresce with a higher intensity than other cellular structures (Christensen et al. 1997). We used nucleolus position as a guide for nuclear position which, in turn, served as a marker for disruptions in the cleavage plane orientation process in embryos. CLSM enabled rapid determination of nuclei number and their relative positions, allowing processing of a larger number of samples than is normally possible by serial sectioning.

Siliques from heterozygous *fst* plants were assigned a stage based on the developmental stage of the morphologically normal (wild-type) embryos within that silique. Table 1 summarises these stages. Embryo development within a silique is not perfectly synchronous, and, as a consequence, siliques generally contained wild-type embryos at two developmental stages (Table 1). For each silique stage analysed, at least 100 embryos were observed.

Figure 3g–i shows CLSM images of *fst* embryos. In stage 1/2, 2/4 and 4/8 siliques, all embryos observed appeared morphologically normal, although at stage 4/8 some embryos contained fewer than four cells. By contrast, in stage 8/16 and 16/32 siliques, approximately 25% of the embryos observed exhibited abnormal morphology. Within stage 8/16 siliques, abnormal embryos were at the

**Fig. 3** Early cell cleavage patterns and nuclear position in the development of wild-type and *fst* mutant embryos. **a–f** Histological sections showing development of wild-type embryos. **a** Mature embryo sac. **b** Zygote with a large vacuole towards the micropyle and nucleus positioned towards the chalazal end of the cell. **c** Single-cell embryo proper after the first asymmetric zygotic cleavage. **d** Two-cell embryo proper. **e** Four-cell embryo proper. Two cells are visible in this section and the remaining two cells and their nuclei lie in the same positions in adjacent sections (not shown). **f** Eight-cell embryo proper. Four cells are visible and the nucleus occupies a central position in each cell.



**g–i** Optical sections of embryos obtained by confocal laser scanning microscopy. The fluorescence results from the fixing procedure, and the nucleoli have been artificially highlighted in blue. **g** Two-cell embryo proper from a stage 1/2 silique (Table 1). The two cells arose from a symmetrical longitudinal division of a single-cell embryo and deviation from the wild-type was not observed in any embryos up to this stage. **h** Four-cell *fst* embryo from a stage 8/16 silique. Three nuclei can be seen in the one optical section and are irregularly located compared with the nuclei in the wild-type four-cell embryo (**e**), which all lie close to the equatorial plane of the embryo proper. **i** Eight-cell *fst* embryo from a stage 16/32 silique. The four nuclei which can be seen in this section are at various spatial locations and not matched by the remaining four nuclei observed in different planes in other optical sections. Bars 10  $\mu$ m

**Table 1** Silique stages and types of embryos found in wild-type siliques examined by confocal microscopy

Stage	Embryo morphologies found
1/2	One-cell proembryo+two-cell embryo
2/4	Two-cell embryo+four-cell embryo
4/8	Four-cell embryo+eight-cell embryo
8/16	Eight-cell embryo+16-cell embryo
16/32	16-cell embryo+32-cell embryo

four-cell stage (Fig. 3h), and within stage 16/32 siliques, abnormal embryos were at the four-cell (Fig. 3h) and eight-cell (Fig. 3i) stages. These data indicate that developmental abnormalities are first detectable with CLSM at the four-cell stage and that one aspect of the *fist* defect is a developmental delay in embryo development.

In *fist* embryos at the four-cell stage, the four nuclei were not aligned along the equatorial plane of the embryo and were not positioned centrally within their respective cells (Fig. 3h) as they are in wild-type (Fig. 3e). Rather, nuclei were randomly positioned among the observed embryos (Fig. 3h) and, in all cases, were in configurations that did not relate to transverse divisions, which would normally occur in the transition between the four- and eight-cell stages (Webb and Gunning 1991). In *fist* embryos containing eight nuclei, two regular tiers of four nuclei were not observed and the nuclei were randomly positioned amongst the observed embryos. The cells of these abnormal eight-cell embryos were more vacuolate than their wild-type counterparts and often appeared bloated (Fig. 3i). The disorganised, random positions of nuclei correlated with altered cell wall position. This feature was continued in subsequent cell division cycles until the embryos arrested with a variable cell number and a single nucleus in each cell (Fig. 2f–g).

Periclinal division planes that occur at the eight-cell stage in wild-type embryos and give rise to a protoderm (Fig. 2e) in wild-type 16-cell embryos were never observed in *fist* embryos. As a consequence, *fist* embryos lacked a histologically definable protoderm layer. In the wild-type embryo, further divisions establish a radial axis of three layers in concentric arrangement that will differentiate into the three tissue layers of the embryo: the outer protoderm, middle ground meristem and central procambium (Goldberg et al. 1994). In approximately 5% of terminal *fist* embryos observed, cells in the outer layer exhibited features of differentiating xylem elements. Figure 4a–d shows bright-field micrographs of serial sections of a *fist* seed isolated from a silique in which wild-type embryos were at the curled-cotyledon stage (Fig. 2d). In the embryo shown in Fig. 4, a cluster of four cells at the surface of the embryo proper, immediately adjacent to the suspensor, showed spiral secondary cell wall deposits. This cellular feature is normally exhibited by differentiating xylem elements post-germination and is not observed in protoderm or differentiating epidermal cells. In late globular, wild-type *Arabidopsis* embryos, the procambium primordium that gives rise to procambium and ultimately xylem tissue consists of

eight narrow cells in the interior of the embryo lying directly above root primordium progenitor cells (Bowman 1993). Thus, the outer layer of the *fist* embryo is not a typical protoderm, but has some features of internally located cells. In addition, the xylem-like differentiation was premature, indicating that *fist* embryos have a heterochronic defect.

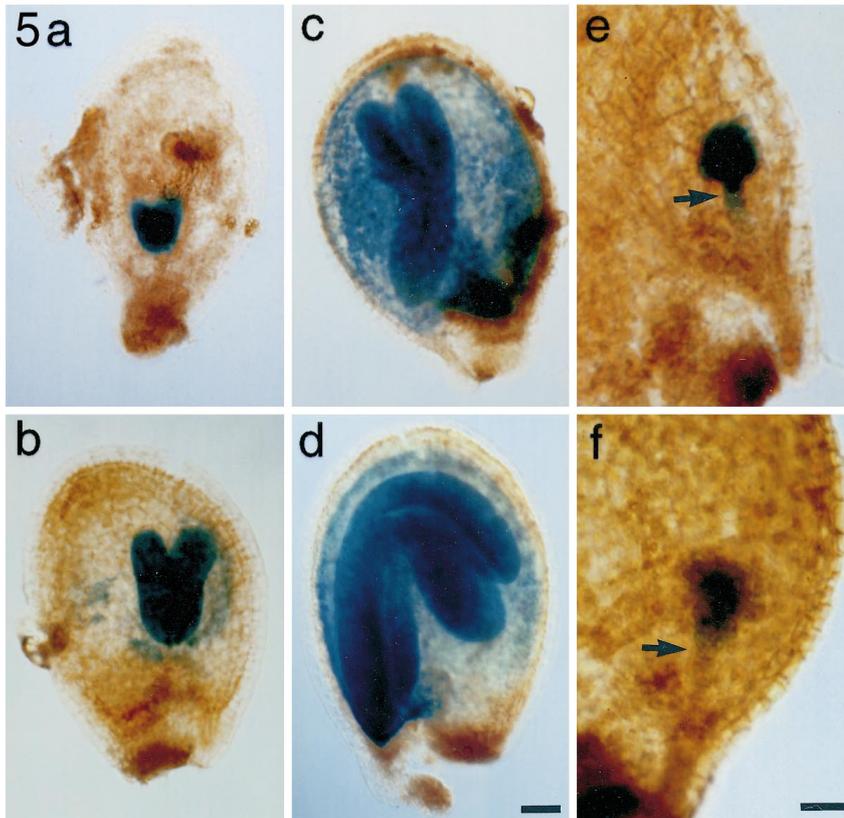
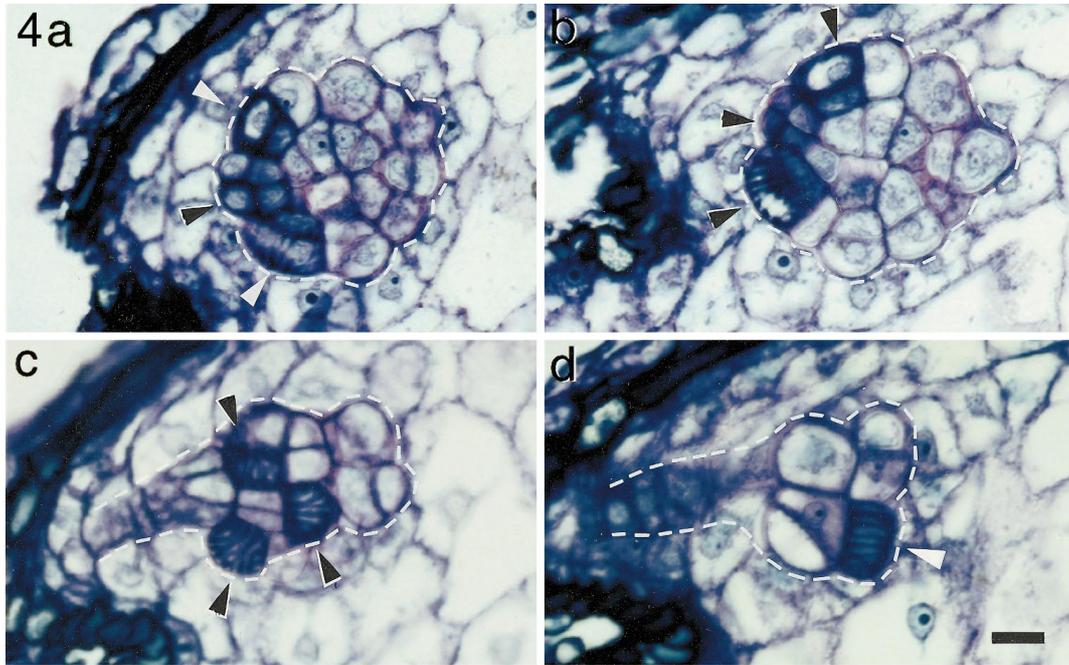
Taken together, these observations suggest that alterations in cleavage plane orientation processes occur early in *fist* embryogenesis, between the two-cell to four-cell embryo proper stages, and lead to alterations in cell wall orientation and position and eventually the terminal *fist* phenotype. The early periclinal divisions are not observed, and the positional cues establishing the correct location of the protoderm and procambium, two of the three tissues that comprise radial pattern, are disrupted in terminal *fist* mutants.

#### A $\beta$ -conglycinin-GUS gene is expressed in *fist* embryos in the absence of normal morphogenesis

To determine whether *fist* embryo cells can activate transcription from the promoter of a seed storage protein gene as a marker of differentiation (Yadegari et al. 1994), we crossed a  $\beta$ -conglycinin-GUS fusion gene from a transformed *Arabidopsis* line into the *fist* mutant background and examined the expression patterns of the introduced gene in wild-type and *fist* seeds. Figure 5a–d shows the developmental expression pattern of the  $\beta$ -conglycinin-GUS gene in wild-type seeds. In wild-type embryos, expression of  $\beta$ -conglycinin-GUS was first detected at the early heart stage (Fig. 5a) and persisted through the torpedo (Fig. 5b, c) and curled-cotyledon (Fig. 5d) stages. Figure 5a–d shows that  $\beta$ -conglycinin-GUS expression was undetectable in the suspensor of wild-type embryos through to the torpedo stage (Fig. 5a–c), after which the suspensor disappears (Fig. 5d).

We analysed  $\beta$ -conglycinin-GUS expression in terminal *fist* embryos. To do this, we stained *fist* seeds dissected from heterozygous *fist* siliques containing wild-type embryos at the curled-cotyledon stage. These data are shown in Fig. 5e, f. Both wild-type embryos at the curled-cotyledon stage and terminal, globular-shaped *fist* embryos strongly expressed the  $\beta$ -conglycinin-GUS gene. The terminal *fist* expression pattern contrasts with that of wild-type embryos at the globular stage in which  $\beta$ -conglycinin-GUS gene expression was not detectable (data not shown). In contrast to wild-type embryos at any stage, some terminal *fist* embryos also expressed the  $\beta$ -conglycinin-GUS gene in the suspensor, specifically in the portion proximal to the embryo proper (Fig. 5e, f).

These data show that cell differentiation proceeds in *fist* embryos in the absence of normal morphogenesis and in the absence of normal appearance of embryo cell division planes. However, the spatial restriction of  $\beta$ -conglycinin-GUS gene expression to the embryo proper is relaxed in morphogenetically altered *fist* embryos with ectopic expression of the marker gene occurring in portions of the suspensor.



**Fig. 4a–d** Ectopic differentiation of xylem structures in the *fist* mutant embryo. Serial sections of a *fist* embryo stained with toluidine blue. The micropylar end of the embryo faces towards the left in each section. Embryo and suspensor cells in the plane of focus have been marked by *white lines* to distinguish them from surrounding cellular endosperm. Cells on the surface of the embryo containing xylem-like secondary wall thickenings are indicated by *arrowheads*. *Bar* 10  $\mu$ m

**Fig. 5a–f** Histochemical detection of GUS expression directed by the  $\beta$ -conglycinin promoter in wild-type and *fist* mutant seeds. **a–d** Wild-type seeds, showing expression of GUS in the early heart (**a**), early torpedo (**b**), late torpedo (**c**) and curled-cotyledon (**d**) stage embryos. GUS expression can be seen in endosperm surrounding the early torpedo stage embryo (**b**) and appears throughout the endosperm in older seeds (**c–d**). GUS activity was not detected in either embryo or endosperm of seeds containing embryos younger than heart stage (not shown). **e, f** GUS expression in *fist* seeds taken from siliques in which wild-type seeds contained curled-cotyledon embryos. Note the staining of suspensor cells (*arrows*) and the lack of endosperm staining. *Bars* 40  $\mu$ m for **a–d**, 30  $\mu$ m for **e–f**

## The *fist* mutation affects endosperm development

We analysed endosperm development in *fist* seeds to determine whether the *fist* mutation affected this structure. To do this, we sectioned seeds from heterozygous *fist* siliques containing wild-type embryos at the walking-stick stage. These data are shown in Fig. 2h–j. While all wild-type, walking-stick embryos were completely surrounded by cellularised endosperm (Fig. 2d), we observed three different types of *fist* endosperm pattern; they were present in approximately equal proportions. Type 1 *fist* endosperm pattern, shown in Fig. 2h, resembled that of wild-type seeds containing embryos at around the late globular stage (Fig. 2a; Mansfield and Briarty 1990a). Type 1 was free-nuclear endosperm, located predominantly in the micropylar and chalazal regions, but also surrounding the embryo and suspensor (Fig. 2h). Type 2 *fist* endosperm pattern (Fig. 2i) was similar to that observed in wild-type seeds containing heart stage embryos (Fig. 2b; Mansfield and Briarty 1990b). In type 2, partial endosperm cellularisation was observed in the embryo sac periphery from the micropylar region surrounding the embryo and extended towards the chalazal area of the embryo sac. Type 3 *fist* endosperm pattern was similar to that normally observed in wild-type seeds containing torpedo-stage embryos (Fig. 2c; Mansfield and Briarty 1990a, b) and was completely cellularised (Fig. 2j). The three *fist* endosperm patterns did not correlate obviously with a particular *fist* embryo size or disorganised cellular pattern. These data suggest that the *fist* mutation can result in the arrest of endosperm development at any of three distinct stages of endosperm cellularisation, or the mutation may simply cause variation in the rate of endosperm development. In either case, *fist* endosperm development is not tightly linked to *fist* embryo morphogenesis, as is the case in wild-type.

To determine whether *fist* endosperm can enter a seed storage protein differentiation pathway, we analysed  $\beta$ -conglycinin-GUS expression during *fist* endosperm development. Figure 5b–d shows  $\beta$ -conglycinin-GUS gene expression during wild-type endosperm development. In wild-type,  $\beta$ -conglycinin-GUS expression was first detected soon after endosperm cellularisation in a region of the endosperm immediately surrounding the embryo at the early torpedo stage (Fig. 5b).  $\beta$ -Conglycinin-GUS expression then spread throughout the cellular endosperm (Fig. 5c) and continued at high levels in seeds containing embryos at the curled-cotyledon stage (Fig. 5d). At later stages, when the cotyledons had fully recurved and the endosperm was almost completely absorbed, endosperm expression diminished (data not shown).

We analysed  $\beta$ -conglycinin-GUS expression in the endosperm of seeds containing terminal *fist* embryos. Such seeds were extracted from siliques containing wild-type embryos at the curled-cotyledon stage. While we could detect strong  $\beta$ -conglycinin-GUS expression in terminal *fist* embryos, we did not detect  $\beta$ -conglycinin-

GUS expression in the endosperm of 50 *fist* seeds. As described above, at least one-third of the seeds containing terminal *fist* embryos developed a cellular endosperm. Thus, in contrast to wild-type seeds, that strongly express  $\beta$ -conglycinin-GUS in cellular endosperm,  $\beta$ -conglycinin-GUS expression is not detectable in cellular endosperm of *fist* seeds. Taken together, these data indicate that the *fist* mutation disrupts endosperm cellularisation and may affect differentiation.

## Discussion

The *fist* mutation is associated with altered cell division plane orientation in *Arabidopsis* embryos

In this study we analysed the effects of the *fist* mutation on seed development in *Arabidopsis* and observed that cleavage plane orientation is disrupted in the embryo, resulting in a globular arrested embryo containing irregular numbers of cells of varying size and shape. Although there is variability in the *fist* phenotype, the earliest visible effect of the *fist* mutation on embryo development was an altered positioning of cell nuclei at the four-cell embryo proper stage. It is not clear from this study whether the altered orientation of cell division planes is a primary or secondary effect of the *fist* mutation. Other features of the mutation include a delay in *fist* embryo development in relation to wild-type embryos and the lack of a regular protoderm layer in the embryo.

Of the published *Arabidopsis* mutants with alterations in cleavage plane orientation, *fist* shares some similarities with *knolle*, a radial pattern mutant that is defective in cell plate formation during cytokinesis (Lukowitz et al. 1996). However, unlike *knolle*, serial sectioning and examination of *fist* embryos by light microscopy showed that cell wall formation appeared complete and multiple nuclei were not observed in *fist* cells nor did nuclei appear significantly enlarged or variable in size amongst cells of the embryo (compare Fig. 2e with f and g). Crosses between *knolle* and *fist* heterozygotes have resulted in only wild-type progeny (W. Lukowitz, unpublished observations). This observation, together with the differences in the embryonic features between *knolle* and *fist* embryos, suggests that the two mutations represent different loci.

The *fist* mutation results in altered radial pattern formation in the embryo

Although cell division is affected early in *fist* embryogenesis, the embryos continue dividing, yet they do not form a normal globular embryo and cannot undergo transition to the heart stage. Radial pattern is affected in *fist* embryos because some develop spiral secondary wall thickenings akin to xylem elements in cells on the outer surface. Differentiation of xylem-like elements in the absence of embryo morphogenesis has previously been ob-

served in the *Arabidopsis* suspensor mutant *sus2-1*, but this occurred in the correct positional location in the interior of the embryo (Schwartz et al. 1994). The finding that xylem elements developed in cells at the outer surface of the *fist* embryo is significant and suggests that one effect of the *fist* mutation is to disrupt the positional differentiation of cell types that form the radial axis. Correct specification of the radial axis may need to occur before a normal root-shoot axis forms. However, given that *fist* is deficient in cleavage plane orientation, cotyledon and root territories may be specified, but it may not be possible to form these structures physically because appropriately oriented cleavages cannot be initiated.

#### Embryo and endosperm development is uncoupled in *fist* mutants

Cell differentiation of the *fist* embryo continued with respect to the activity of a seed storage protein gene promoter in the absence of embryo morphogenesis, and this is possibly due to the influence of an internal developmental "clock", as suggested for *raspberry* embryos (Yadegari et al. 1994). By contrast, the *fist* endosperm was unable to express the  $\beta$ -conglycinin-GUS fusion gene. Therefore, embryo and endosperm development have been uncoupled in this mutation. Whether the *fist* mutation directly affects endosperm development by influencing endosperm division pattern or interfering with some other metabolic process, or primarily affects the embryo, and embryo-endosperm interactions are perturbed as a result, remains to be determined. It is also possible that a certain stage of embryo development, not reached by *fist* embryos, may be required for some endosperm gene expression programs to be activated. Alternatively, the *fist* embryo may produce a signal that the *fist* endosperm is unable to perceive correctly.

#### FIST function during cell division in *Arabidopsis* embryos

The position and orientation of the first zygotic division and also the first division of the embryo proper appeared normal in *fist* mutant embryos. This observation implies that different cell division orientation controls may be in place at different stages of early embryogenesis in *Arabidopsis*, as has been shown to be the case in the early *Caenorhabditis elegans* embryo (Goldstein 1995). Alternatively, it is possible that additional maternal factors in the egg may aid in guiding the first few divisions of the embryo, and as these maternal factors are diluted out in the first few divisions, the mutant phenotype is manifested. The identity of the *FIST* gene remains to be determined. In the interim, cytological investigation of the microtubular cytoskeleton should provide information about the cell processes perturbed in this mutation.

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