Cellularisation in the endosperm of *Arabidopsis thaliana* is coupled to mitosis and shares multiple components with cytokinesis

Mikael Blom Sørensen[1,2], Ulrike Mayer[3], Wolfgang Lukowitz[4], Hélène Robert[1], Pierre Chambrier[1], Gerd Jürgens[3], Chris Somerville[4], Loic Lepiniec[5] and Frédéric Berger[1,*]

[1]Laboratoire de Reproduction et Développement des Plantes, UMR 5667, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, F-69364 Lyon, Cedex 07, France
[2]Plant Research Department, PRD-301, Risø National Laboratory, PO Box 49, DK-4000 Roskilde, Denmark
[3]ZMBP – Center of Plant Molecular Biology, Developmental Genetics, University of Tübingen, Auf der Morgenstelle 3, D-72076 Tübingen, Germany
[4]Carnegie Institution of Washington, Department of Plant Biology, 260 Panama Street, Stanford, California 94305, USA
[5]Laboratoire de Biologie des Semences, UMR INRA/INA-PG, Route de Saint-Cyr, 78026 Versailles Cedex, France
*Author for correspondence (e-mail: frederic.berger@ens-lyon.fr)

SUMMARY

Distinct forms of cytokinesis characterise specific phases of development in plants. In *Arabidopsis,* as in many other species, the endosperm that nurtures the embryo in the seed initially develops as a syncytium. This syncytial phase ends with simultaneous partitioning of the multinucleate cytoplasm into individual cells, a process referred to as cellularisation. Our in vivo observations show that, as in cytokinesis, cellularisation of the *Arabidopsis* endosperm is coupled to nuclear division. A genetic analysis reveals that most *Arabidopsis* mutations affecting cytokinesis in the embryo also impair endosperm cellularisation. These results imply that cellularisation and cytokinesis share multiple components of the same basic machinery. We further report the identification of mutations in a novel gene, *SPÄTZLE*, that specifically interfere with cellularisation of the endosperm, but not with cytokinesis in the embryo. The analysis of this mutant might identify a specific checkpoint for the onset of cellularisation.

Movies available on-line

Key words: *Arabidopsis thaliana*, Endosperm, Seed, Cytokinesis, *SPÄTZLE*

INTRODUCTION

Cytokinesis partitions the cytoplasm of a dividing cell and involves a precisely orchestrated interaction between the cytoskeleton and vesicle trafficking that leads to synthesis of new plasma membrane. In higher plants, a specialised cytoskeletal structure supports the de novo formation of a cell wall during cytokinesis. The components of this cellular assembly, the phragmoplast, include microtubules, actin microfilaments and Golgi-derived vesicles (Staehelin and Hepler, 1996; Heese et al., 1998; Sylvester, 2000). At the end of telophase, elements for membrane and cell wall synthesis are transported by the phragmoplast to the interzone where the cell plate forms a membrane-bound compartment enclosing the immature wall. Phragmoplast and cell plate expand laterally until the cell plate fuses with the parental plasma membrane and cell wall. At the onset of mitosis, the site of fusion is marked by a transient array of microtubule, the preprophase band (PPB).

The prominent role played by the microtubule cytoskeleton in this process is well supported by genetic evidence. Mutations in the *Arabidopsis PILZ* genes encoding the microtubule folding cofactor complex (Steinborn et al., 2002) severely disrupt microtubule organisation and result in a complete block of mitosis and cytokinesis both in embryo and endosperm (Mayer et al., 1999). Other mutations, such as *tonneau1* and *fass/tonneau2,* more specifically affect organisation of cortical microtubules: *fass/ton2* embryos, for example, lack both the cortical array of parallel microtubules and the PPB (Traas et al., 1995; McClinton and Sung, 1997). As a consequence, *fass/ton2* cells undergo unordered expansion in all dimensions and form oblique irregular cell walls very unlike the well-ordered cell files that are representative of *Arabidopsis* embryos (Torres-Ruiz and Jürgens, 1994; Traas et al., 1995).

Several mutations that affect cytokinesis in the *Arabidopsis* embryo have been isolated, including *knolle* and *keule* (Mayer et al., 1991). *KNOLLE* encodes a member of the syntaxin family, a class of membrane-bound receptors required for docking and fusion of vesicles at the target membrane (Lukowitz et al., 1996). The KNOLLE protein only accumulates at mitosis and localises to the plane of division, indicating that its specific role is to mediate membrane fusion at the cell plate (Lauber et al., 1997). The *keule* (Assaad et al., 1996) and *hinkel* (Strompen et al., 2002) mutants display cytokinesis defects during embryo development similar to

those in *knolle*. The *KEULE* gene encodes a member of the Sec1 family (Assaad et al., 2001). SEC1 proteins regulate vesicle trafficking and interact with syntaxins. *E. coli*-expressed KNOLLE binds KEULE from plant extracts in vitro, suggesting that the two proteins interact directly during cytokinesis. In support of this, the *knolle keule* double mutant displays a synthetic embryo-lethal phenotype, with cytokinesis completely prevented (Waizenegger et al., 2000). The *HINKEL* gene encodes a kinesin-like protein apparently involved in reorganisation of phragmoplast microtubules (Strompen et al., 2002). Additional mutations in genes non-allelic to *KNOLLE*, *KEULE* and *HINKEL* but with similar embryo-lethal phenotypes have recently been isolated. These mutants include *runkel* (Nacry et al., 2000), *open house* (allelic to *findling*; W. Lukowitz and C. Somerville, unpublished) (Nacry et al., 2000) and a novel allele of *pleiade*, first isolated as a root-specific cytokinesis mutant (Hauser and Bauer, 2000). The functions and products of the mutated genes are not known.

In flowering plants the pollen delivers two male gametes that fuse with the egg cell and the central cell present in the ovule. The ensuing fertilisation product of the central cell develops as the endosperm. In *Arabidopsis* the early endosperm development is characterised by a series of synchronised nuclear divisions that are not followed by cytokinesis (Mansfield and Briarty, 1990a). This results in the formation of a large multinucleate cell, a syncytium. The syncytial *Arabidopsis* endosperm contains three distinct domains (Brown et al., 1999). In this study we refer to these as the micropylar endosperm, surrounding the embryo at the anterior pole (MCE), the peripheral endosperm (PEN) and the chalazal endosperm (CZE) at the posterior pole, reflecting the arrangement along the anterior-posterior axis defined by the site of delivery of the sperm (anterior). While nuclei in the MCE share a common mass of cytoplasm, each nucleus in the PEN is associated with its individual cytoplasm surrounded by a dense array of microtubules in a structure termed a nucleocytoplasmic domain (NCD) (Brown et al., 1999). In MCE and PEN pseudo-synchronous mitoses take place at different paces (Boisnard-Lorig et al., 2001). At the globular stage of embryogenesis, the syncytial endosperm contains approximately 100 nuclei (Stage VIII). At this stage, the formation of cell walls that partition nuclei and cytoplasm into individual cells is initiated in the MCE. This process, termed cellularisation, was reported to proceed as a wave across the PEN towards the CZE (Mansfield and Briarty, 1990b; Brown et al., 1999). Cellularisation establishes one endosperm cell layer and precedes further cell division. In maize and in barley, complex microtubule rearrangements take place prior to endosperm cellularisation. During cellularisation so-called adventitious or cytoplasmic phragmoplasts are observed at the junctions of opposing ends of radial microtubules that encircle each nucleus (Brown et al., 1994). Similar adventitious phragmoplasts are seen during *Arabidopsis* endosperm cellularisation (Brown et al., 1999). Recently, detailed studies of endosperm cellularisation in *Arabidopsis* have confirmed that cell wall formation is mediated by specialised types of phragmoplasts and cell plates (Otegui and Staehelin, 2000b). The syncytial-type cell plates go through a series of maturation steps that are similar, but not identical, to those observed in conventional cytokinesis (Otegui and Staehelin, 2000a). While cytological descriptions of endosperm cellularisation thus indicate its unique features, evidence for the accumulation of both KNOLLE (Lauber et al., 1997) and the dynamin-like protein ADL1A (Otegui et al., 2001) in both somatic- and syncytial-type cell plates hints at shared components in the two processes.

We report in vivo observation of cellularisation in the PEN of *Arabidopsis* and show that it follows nuclear division, as in conventional cytokinesis. We evaluate the differences and similarities between conventional cytokinesis and endosperm cellularisation using a genetic approach. We analyse the effect on endosperm cellularisation of six *Arabidopsis* mutations that affect cytokinesis in the embryo. This analysis shows that most embryo cytokinesis-defective mutants display similar defects in endosperm cellularisation. In addition, we characterise mutant alleles of a novel gene, *SPÄTZLE*, in which endosperm cellularisation is defective but cytokinesis is normal in the embryo and in plants homozygous for the mutation.

MATERIALS AND METHODS

Plant strains

Ecotypes used as wild types were Landsberg *erecta* and WS. Alleles used in the L*er* ecotype were *fas/ton2* (Torres-Ruiz and Jürgens, 1994), *knolle-AP6-16* (Lukowitz et al., 1996), *keule-Ap77* (Lukowitz et al., 1996) and *runkel* U381. Mutant alleles of other genes required for cytokinesis in the embryo *runkel-7* and *hinkel* were generated in the L*er* ecotype at the Max Planck Institute in Tübingen while *OX10*, *pleiade-4* and *open house-1* were generated in the L*er* ecotype at the Carnegie Institution. The lines DRU42 and DQB12 were generated in the WS ecotype in Versailles (Bechtold et al., 1993). The KS117 GFP marker line has been described elsewhere (Sørensen et al., 2001).

Feulgen staining and confocal microscopy

Individual *Arabidopsis thaliana* siliques were opened with two shallow longitudinal cuts on either side of the false septum. Siliques were stained with Schiff's reagent (Sigma) and embedded in LR-white (Sigma) according to the method of Braselton et al. (Braselton et al., 1996). All mutant lines were initially propagated as heterozygotes and produced siliques that contained both wild-type seeds and seeds that displayed the mutant phenotype. Seeds that originated from individual siliques were isolated in each preparation in order to be able to compare the mutant and wild-type development at corresponding stages. Confocal microscopy was performed using a Zeiss LSM-510 microscope using the 488 nm excitation line of an argon laser and a long pass emission filter at 510 nm.

Time-lapse imaging of cellularisation

Seeds of an endosperm marker line from the enhancer trap collection of J. Haseloff were placed in a small moist chamber as described previously (Boisnard-Lorig et al., 2001). Imaging of the GFP fluorescence (488 nm excitation; 510-550 nm emission) was performed for 12 to 24 hours using a time-lapse automated recording of confocal sections every 10 minutes. We used a ×16 objective n.a. 0.7. with oil immersion. 1024×1024 pixel confocal sections were processed using Photoshop 5.0 (Adobe). Movies were produced with Metamorph.

Genetic mapping

The homozygous mutant line DRU42 in WS ecotype was crossed to wild-type Landsberg *erecta*. F$_2$ seeds from F$_1$ progeny heterozygous for the DRU42 mutation were used to generate the mapping population. The phenotype of individual F$_2$ plants was determined and the wild-type plants were used to investigate recombination events

with polymorphic markers (CAPS and SSLP). 49 plants were analysed for three markers per chromosome.

RESULTS

Cellularisation in the peripheral endosperm is coupled to mitosis

Previous analyses of *Arabidopsis* endosperm using microscopical observations of fixed tissues have shown that cellularisation begins at the MCE when the embryo has reached the late globular stage. This corresponds to the end of the stage VIII as defined by the number of mitotic rounds in the syncytial endosperm (Boisnard-Lorig et al., 2001). During the transition from the triangular to the early-heart stage of embryogenesis, cellularisation takes place in the PEN, apparently following a wave-like pattern initiated at the anterior pole (Mansfield and Briarty, 1990b). At the completion of cellularisation, anticlinal walls are established in the entire PEN. The endosperm then comprises approx. 200 nuclei (stage IX) (Scott et al., 1998). At late heart stage, a near-synchronous round of mitosis (developmental stage X) followed by what appears to be a conventional cytokinesis allows the formation of periclinal walls, defining an outer peripheral cell layer and an inner layer that undergo another round of cell division (stage XI, Fig. 4A) (Brown et al., 1999).

To perform a detailed analysis of the timing and dynamics of endosperm cellularisation we have taken advantage of an *Arabidopsis* enhancer trap line that expresses a variant of the green fluorescent protein, mGFP5 (Haseloff et al., 1997), uniformly in the developing endosperm (Boisnard-Lorig et al., 2001). mGFP5 is retained in the endoplasmic reticulum and during cytokinesis fluorescence from mGFP5 accumulates at the forming cell plate (Haseloff, 1999). This labelling permits in vivo observation, using confocal microscopy, of endosperm at the transition between the stages VIII and IX when PEN cellularisation takes place. At the end of stage VIII, cellularisation is complete at the micropylar pole around the embryo but the PEN remains syncytial (Fig. 1A, 0 minutes; MCE shown only in supplementary material). At this point the NCDs remain unchanged for an extended period, without any signs of cell wall formation or division. In the data presented here, this period is more than 3 hours (Fig. 1A,B). In multiple observations, each with a duration greater than 6 hours we did not observe any extension into the PEN of the cell walls present in the MCE. At the transition to stage IX, simultaneous nuclear divisions are initiated in the micropylar mitotic domain and propagate rapidly as a wave across the PEN (Fig. 1C; total duration approximately 40 minutes, the anterior pole is toward the top of the figure) as reported for earlier developmental stages (Boisnard-Lorig et al., 2001). In the already cellularised MCE, GFP fluorescence is detected in a small band between sister nuclei, about 15 minutes after karyokinesis (see movie: http://dev.biologists.org/supplemental/). This band expands centrifugally as in conventional cytokinesis and delineates the nascent cell wall between the two daughter cells. In the syncytial PEN a GFP signal is first detected between post mitotic nuclei that still form a distinguishable pair, 30-40 minutes after telophase (Fig. 1D). Over a period of 60 minutes after karyokinesis, cellularisation becomes apparent throughout the peripheral endosperm (Fig. 1E). It was often

difficult to observe more precisely the extension of the band of GFP fluorescence between individual nuclei because of growth of the seed that displaced the observed nuclei out of the focal plane. Initiation of PEN cellularisation was, however, repeatedly (*n*=13) observed immediately after the eighth wave of mitosis that crosses the endosperm from the anterior to the posterior pole. Cellularisation is completed without any further divisions occurring (Fig. 1F). Fortuitous observation of a developing seed fixed at a point during the advancement of the eighth mitotic round revealed further details of the first steps of cellularisation. Fig. 1G-J are periclinal views of dividing nuclei positioned along the posterior to anterior axis. The mitotic stage seen mostly frequently at the posterior (i.e. the
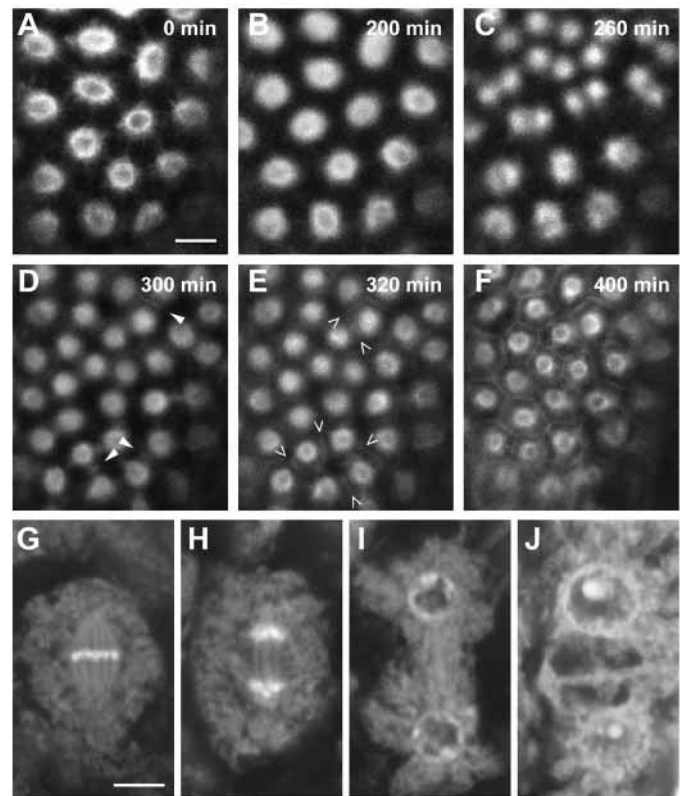
Fig. 1. Initiation of cellularisation is coupled to mitosis. (A-F) Time series visualising nuclear division and cellularisation in the PEN (see movie: http://dev.biologists.org/supplemental/). The in vivo progression of cellularisation was monitored by time-lapse confocal microscopy of living seeds from the GFP-expressing line KS22. GFP accumulates at sites of forming cell walls between sister nuclei (arrowheads). This series is part of an 8 hour recording of endosperm development. Frames were acquired every 10 minutes. Images in A and F mark the beginning and end of the series, respectively, and were recorded with a decreased acquisition rate to give better resolution. 200 minutes after the beginning of recording, a mitotic wave was initiated at the anterior pole (upper side of the section) and crossed the PEN (B,C). Immediately after mitosis cell plates are visible between sister nuclei (D, arrowhead) and slightly later between non-sister nuclei (E, arrowhead). Cellularisation appears to be complete 6 hours after the mitotic wave (F). (G-J) Detail of the formation of a cell plate between sister nuclei following the 8th mitotic cycle in the PEN observed in fixed material. (G) Metaphase, (H) anaphase, (I) telophase, and (J) the clear formation of a cell plate. Scale bar: 20 μm (A-F); 5 μm (G-J).

more recently produced) is metaphase (Fig. 1G). Toward the anterior pole anaphase (Fig. 1H) and telophase (Fig. 1I) are observed successively, immediately followed by formation of a structure resembling a cell plate between separating sister nuclei (Fig. 1J). It thus appears that cellularisation follows a series of events initiated in conjunction with a synchronised division, where a cell plate is first formed between sister nuclei, followed rapidly by assembly of cell walls between non-sister nuclei throughout the PEN, resulting in a layer of cells at the periphery of the central vacuole.

Cytokinesis genes are required for endosperm cellularisation

To address the question of whether cellularisation and conventional cytokinesis are under the same genetic and molecular control, we analysed endosperm development in several cytokinesis mutants. Visual screens of *Arabidopsis* embryos or seedlings with altered morphology have identified numerous genes required for cytokinesis. Mutants with very similar phenotypes include *KNOLLE* (*KN*) (Mayer et al., 1991), *KEULE* (*KEU*) (Mayer et al., 1991), *HINKEL* (*HIN*) (Strompen et al., 2002), *RUNKEL* (*RUK*) (Nacry et al., 2000), *OPEN HOUSE* (*OPN*) (W. L. and C. S., unpublished) and *PLEIADE* (*PLE*) (Hauser and Bauer, 2000).

The phenotype of *knolle* and *keule* mutant embryos is easily discernible from the wild type, which has ordered files of regularly shaped cells (Fig. 2A). Both mutants are characterised by the presence of cells and nuclei of irregular shapes and sizes in addition to giant multinucleate cells (Fig. 2C,E). Nevertheless, the endosperm development is affected differently by these two mutations. In all *keule* seed analysed (*n*>30) cellularisation was indistinguishable from that in wild type, beginning at the MCE (Fig. 2C) and subsequently forming a layer of regularly positioned hexagonal cells in the PEN (Fig. 2D). Following cellularisation *keule* endosperm cells begin anticlinal division, forming a multilayered endosperm identical to that observed in wild-type seeds with early torpedo stage embryos. In contrast, *knolle* endosperm was not cellularised in the vast majority of seeds observed (*n*>50), with both MCE (Fig. 2E) and PEN (Fig. 2F) remaining syncytial. In approximately 10% of the seeds some degree of cellularisation was apparent, most frequently limited to the MCE. However, one case of cellularised PEN with multiple cell layers, containing multinucleated cells and irregularly shaped nuclei, was also observed. This is reminiscent of the phenotype variation observed in *knolle* embryos, containing a mixture of apparently normal and defective cells, with the difference that, being a synchronised event within each endosperm domain (MCE or PEN), defective cellularisation will be evident throughout the entire domain. Subsequently, individual cells might be affected by aberrant cytokinesis when additional cell layers are formed.

Double mutant *knolle/keule* embryos display a remarkable additive phenotype, where cytokinesis is completely absent (Fig. 2G) (Waizenegger et al., 2000). In accordance with the conclusion that *KNOLLE* is required for cellularisation, the
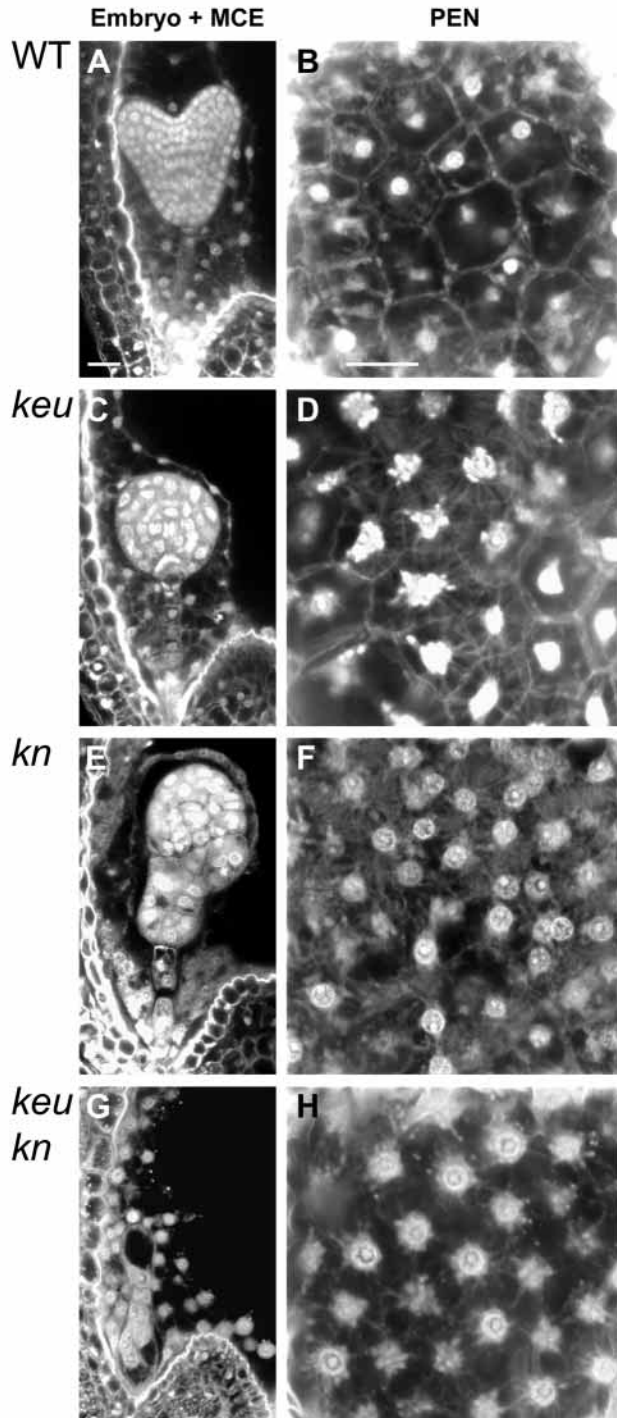
Fig. 2. Comparison of the effect of *knolle* and *keule* on cytokinesis and on the cellularisation in the endosperm. Seeds with embryos at late heart stage originate from heterozygous mutant plants. Homozygous mutant seeds are identified by their embryo phenotype and seeds with a wild-type phenotype from the same silique are used as controls. (A,B) Wild-type reference seed with late heart stage embryo and fully cellularised endosperm around the embryo (A) and in the PEN (B). (C,D) *keule* mutant seeds contain an embryo with multiple defect in cytokinesis, and multinucleate enlarged cells (C) whereas the endosperm cellularisation is not affected (D). (E,F) *knolle* produces seeds with embryos defective in cytokinesis (E) and non-cellularised endosperm (F). Embryos of the double mutant *knolle/keule* are characterised by a complete absence of cytokinesis and are reduced to multinucleated tubes (G). However the defect of cytokinesis in the endosperm is no more pronounced than in *knolle* (G,H). Scale bars represent 20 μm.

endosperm of *knolle*/*keule* seeds remained syncytial (Fig. 2G,H). In contrast to the embryo, no new endosperm phenotype was observed and the endosperm phenotypes of *knolle* and *keule*/*knolle* mutants appeared identical.

Endosperm cellularisation in plants mutant for *HINKEL*, which encodes a kinesin-related protein, and for *OPEN HOUSE* for which the gene product has not yet been identified,
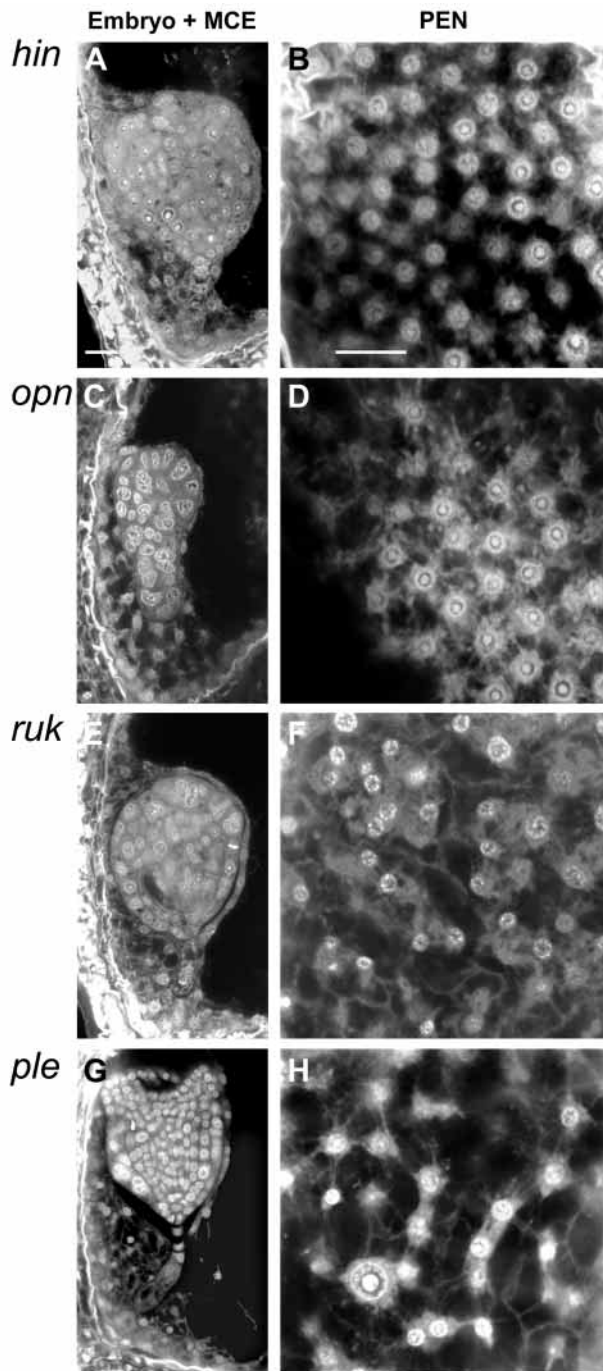
Fig. 3. Cytokinesis-related genes are required for endosperm cellularisation. (A,C,E,G) Both the embryo and the MCE, have defective cytokinesis in the mutants *hinkel* (A) *open house* (C) *runkel* (E) and *pleiade* (G). (B,D,F,H) Corresponding confocal sections of the PEN with obvious defects in cellularisation. Scale bars: 20 μm.

follows the pattern of *knolle* mutants. Although generally remaining syncytial (Fig. 3A-D), partial cellularisation, particularly in the MCE, was occasionally observed.

The endosperm phenotypes of *runkel* and *pleiade* are less severe than those of *knolle*, *hinkel* and *open house,* although cellularisation clearly requires these genes in order to proceed normally (Fig. 3E-H). Partial cellularisation of the MCE (Fig. 3E,G) was observed in almost all seeds analysed. Cellularisation in the PEN, albeit defective and displaying multinucleate cells (Fig. 3F), nuclei of different sizes (Fig. 3H) and incomplete cell walls (Fig. 3F,H) was likewise observed in most seeds. In *pleiade* the relatively mild cellularisation phenotype is accompanied by a correspondingly mild cytokinesis phenotype, with the embryo maintaining a correct morphology until cotyledon development (Fig. 3G). Additionally, the first isolated mutant alleles of this gene allow germination and plant development, with cytokinesis being affected only in the root (Hauser and Bauer, 2000).

A common feature of the cellularisation mutants described here is the high density of NCDs aligned along the peripheral cell wall in the PEN (Fig. 2F,H, Fig. 3B,D). This indicates that, in the absence of cellularisation, the syncytial PEN will continue divisions at least through nine mitoses.

Periclinal cell wall formation is defective in *fass/ton2*

The *fass/ton2* mutation affects embryo morphogenesis by interfering with cell wall orientation and cell shape, resulting in compressed embryos with seemingly randomly arranged cells and abnormal tissue organisation (Torres-Ruiz and Jürgens, 1994). Although endosperm cellularisation takes place in *fass/ton2* seeds, closer examination reveals differences between wild-type and *fass/ton2* endosperm. The number of cell layers in completely cellularised seeds are frequently reduced from four in the wild type (Fig. 4A) to two or three in *fass/ton2* (Fig. 4B). Observations at the cellularisation front, just after the first anticlinal division reveal that these differences are caused by the inability of the *fass/ton2* mutant to correctly form periclinal cell walls separating newly divided sister nuclei (Fig. 4D). The layer of small peripheral cells is not continuous in *fass/ton2* endosperm and cells of the inner layers of the PEN are longer than in the wild type possibly as a result of the absence of periclinal divisions (Fig. 4B,E).

Endosperm-specific cytokinesis-defective mutants define a novel gene, *SPÄTZLE*

In an attempt to identify mutants that specifically affect endosperm development but not embryonic development, we visually screened a subset of 150 lines from the Versailles collection of T-DNA insertion lines (20,000 lines pre-screened by L. L. for abnormal seed shape). Two lines, DRU42 and DQB12, were recovered that showed phenotypes with aberrant endosperm development but no apparent defect in the embryo. Complementation analyses revealed the two mutations to be allelic. Upon selfing, 24.7% (s.e.m.=0.7; *n*=500) of the seed from heterozygous plants displayed the mutant endosperm phenotype, indicating that the mutations are recessive and segregate in a mendelian fashion. In none of the lines was the mutant endosperm phenotype linked with the kanamycin resistance gene of the T-DNA, leading to the conclusion that the mutated gene is not tagged by a complete T-DNA. Mapping with PCR based markers indicated that the *SPÄTZLE* gene is
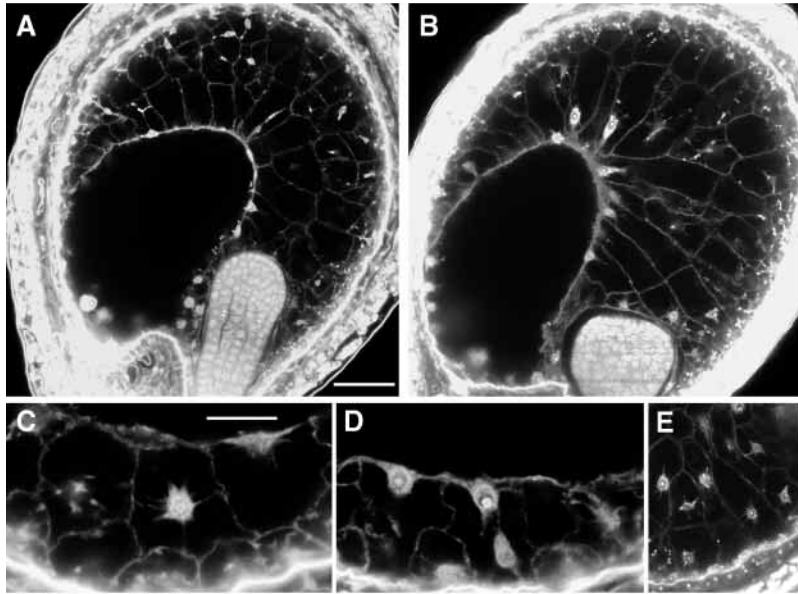
Fig. 4. *FASS/TON2* is required for correct periclinal divisions in the peripheral endosperm. Confocal sections of seeds from heterozygous *fass/ton2* parent plants. (A) Wild-type reference seed with torpedo stage embryo and cellularised endosperm. (B) *fass/ton2* mutant seed from same silique as A. (C,D) Details of first periclinal division in the PEN of wild type (C) and *fass/ton2* (D). (E) Absence of thin outer cell layer in cellularised PEN in *fass/ton2*. Scale bars: 100 μm (A,B) and 25 μm (C,D,E).

located on chromosome 1 approximately 5 cM south of the SSLP marker ACC2.

As with the other mutants that we analysed, *spätzle* displayed a variable range of phenotypes. At late-heart stage of embryogenesis, when endosperm cellularisation is complete in wild type (Fig. 2A), no sign of cell wall formation could be detected in the most frequent mutant phenotype, either in the MCE or in the PEN (Fig. 5). Until endosperm stage VIII, *spätzle* endosperm develops normally and the PEN contains regularly organised NCDs (Fig. 5C), as in wild type just prior to cellularisation. The PEN undergoes at least one additional mitotic division resulting in a syncytium with increased density of NCDs (Fig. 5D), similar to that of *knolle*, *hinkel* and *open house* as described above. Subsequently, the number of NCDs in the PEN continuously reduces while the

size of individual nuclei increases. After stage VIII, we observed various degree of fusion of NCDs (Fig. 5E). Division of nuclei in these binucleate NCDs apparently leads to lack of nuclear separation upon mitosis (Fig. 5E) and ultimately to fusion of nuclei (Fig. 5F). This process can continue until the PEN contains a few giant NCDs, each with one or more giant multinucleolated nuclei (Fig. 5G). This pattern of endosperm development distinguishes *spätzle* from the other cytokinesis mutants where enlarged nuclei were always limited in frequency and accompanied by partial cellularisation of the PEN (e.g. Fig. 3H). At all stages analysed, in seeds displaying the *spätzle* endosperm phenotype embryos did not show any visible defects associated with cell division, such as incomplete cell walls, enlarged nuclei or multinucleate cells, which are typical of all the other cytokinesis mutants
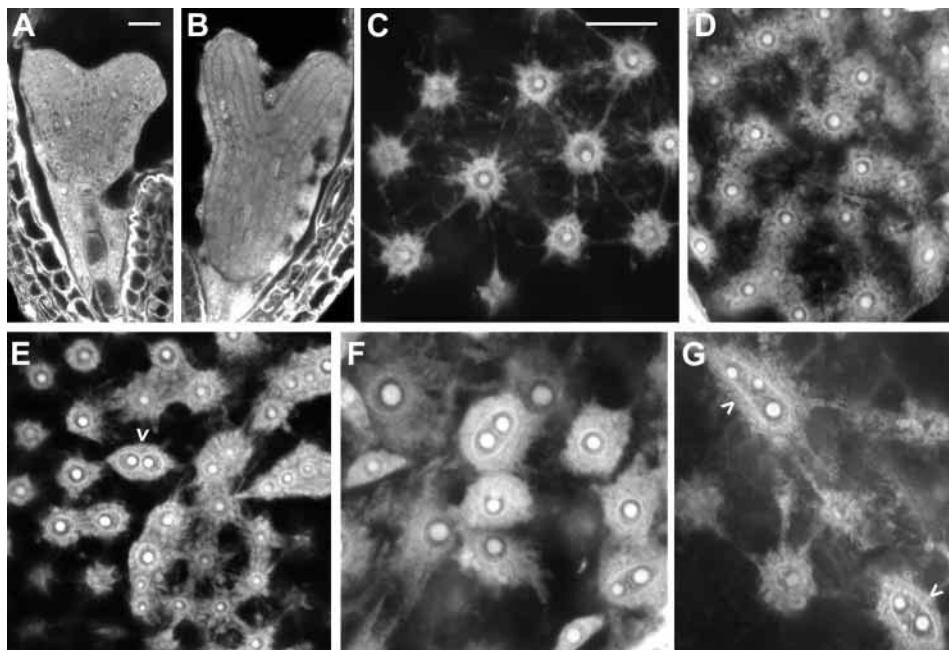
Fig. 5. The *SPÄTZLE* gene is required for endosperm cellularisation, but not for cytokinesis in the embryo. (A,B) The *spätzle* embryo does not show any morphogenetic defect (A, heart stage, B, torpedo stage) although it is surrounded by non cellularised MCE. (C) The PEN does not undergo cellularisation during the heart stage. However division of nuclei is maintained but is not always followed by proper separation of NCDs (D,E) and in some cases nuclei remain attached by incompletely separated nuclear envelopes (E, arrowhead). Hence multinucleated NCDs form and nuclei fuse (F) leading to large nuclei that display multiple nucleoli (G, arrowheads). Scale bars represent 20 μm.

described here (Figs 2 and 3). Moreover, morphogenesis and pattern formation in *spätzle* embryos is normal (protoderm, vascular tissue, root and shoot embryonic meristems) (Fig. 5A). After torpedo stage, most *spätzle* embryos show retardation in overall growth (Fig. 5B), perhaps resulting from the inability of the defective endosperm to nurture the embryo. Mutant seeds, which can be recognised by their slightly shrunken appearance and slightly smaller size at maturity, are viable, can germinate on soil and give rise to homozygous *spätzle* plants. In those plants we could detect neither morphological defects nor growth retardation. Fertility was not altered, and homozygous *spätzle* plants produced 100% seeds with non-cellularised endosperm. Similar observations were made for both *spätzle* alleles, DRU42 and DQB12. These observations strongly support the conclusion that the *SPÄTZLE* gene product is required specifically for cellularisation in the endosperm but not for embryogenesis.

Patterning of the endosperm is not dependent on cellularisation

We have previously shown that the *FIS* (Fertilisation Independent Seed) genes are required for correct anterior-posterior polarisation in the *Arabidopsis* endosperm and that, additionally, endosperm cellularisation is impaired in a *fis* mutant background (Sørensen et al., 2001). To investigate whether a general connection exists between cellularisation and patterning, we analysed development of the CZE in two representative cytokinesis mutants. As in wild type (Fig. 6A), *knolle* posterior pole endosperm is characterised by the presence of a chalazal cyst and one or more nodules, both containing multiple nuclei in a common cytoplasm (Fig. 6C). Likewise, *spätzle* CZE morphology appears normal (Fig. 6E),

but the cytological distinction between chalazal nodules and giant NCDs in the PEN is not as clear as in the wild type or as in *knolle*. In the two mutants *knolle* and *spätzle* we introgressed the marker line (KS117) with an exclusive GFP expression at the posterior pole of the endosperm (Fig. 6B). The expression of the marker is restricted to the CZE in all seeds of both *knolle* (Fig. 6D) and *spätzle* (Fig. 6F) siliques. This confirms the correct patterning of the endosperm in the absence of cellularisation in cytokinesis-defective mutants as well as in *spätzle*. Cellularisation therefore does not appear to be necessary for the expression of endosperm polarity.

DISCUSSION

Genetic analysis suggests that endosperm cellularisation and somatic cytokinesis share components

In *Drosophila* the embryo develops initially as a syncytium and cellularises according to mechanisms distinct from those observed during cytokinesis. The analysis of cellularisation-defective mutants (Schweisguth et al., 1991; Schejter and Wieschaus, 1993; Miller and Kiehart, 1995) has shown that despite an apparent mechanistic difference at the cellular level, many components are shared between cellularisation and cytokinesis. We have undertaken a similar genetic approach in *Arabidopsis*. Six mutants that affect cytokinesis in the embryo were analysed and, with the exception of *keule*, found to be defective in endosperm cellularisation. Endosperm cellularisation does not take place or is incomplete while nuclear division is not directly affected. However, at later stages after partial cellularisation, larger nuclei are present in
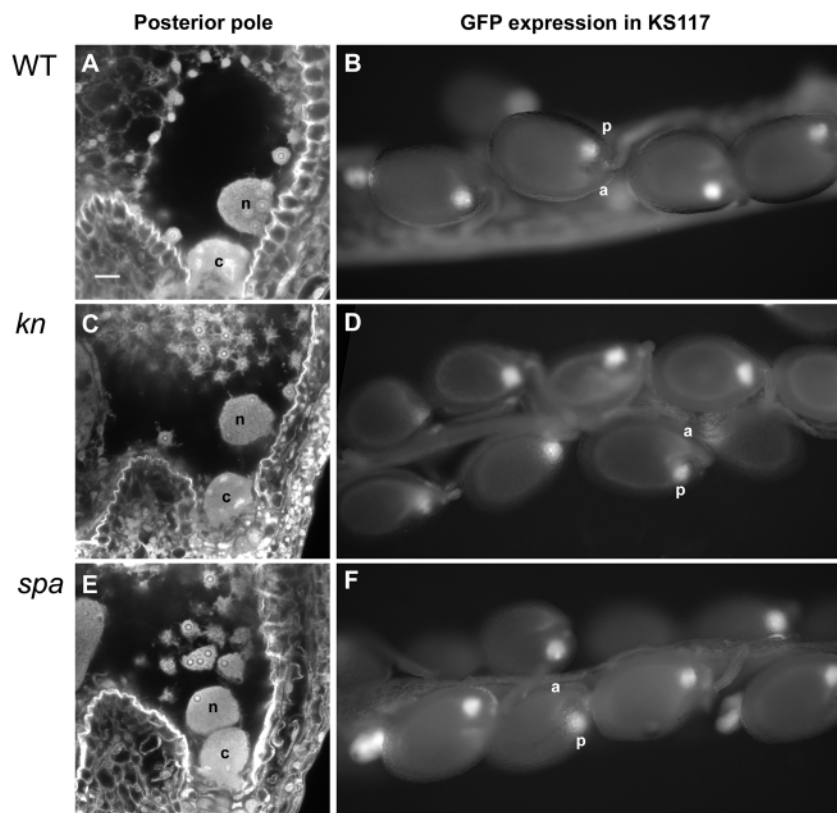
Fig. 6. Anteroposterior polarity in cellularisation-defective mutants. (A,C,E) Cross sections of the posterior most part of the endosperm. In the wild-type the posterior-most part of the endosperm does not cellularise. (A) This part remains syncytial and contains two types of multinucleated large NCDs, the nodules (n) and the cyst (c). (C,E) In both *knolle* and *spätzle* backgrounds nodules and cysts are observed at the posterior pole similar to that in the wild type (early torpedo stage). In *spätzle* endosperm (E), the coalescence of NCDs is observed in the non-cellularised PEN and its distinction from the CZE is less clear than in the wild type or in *knolle*. (B,D,F) GFP expression in the enhancer trap line KS117. (B) After the globular stage, the posterior pole remains the only site where the GFP marker is expressed (Sørensen et al., 2001). (D,F) However, the expression of the marker KS117 is confined to the posterior pole as in the wild type in both *knolle*/+ (D) and *spätzle/spätzle* (F) backgrounds. Scale bar: 20 μm.

some larger cells, presumably due to coalescence of mitotic spindles.

Among the genes investigated, *KNOLLE*, *KEULE* and *HINKEL* have been cloned and found to encode proteins potentially involved in vesicle trafficking. *HINKEL* encodes a novel kinesin that is localised to the cell plate during cytokinesis in a pattern complementary to that of the KNOLLE protein (Strompen et al., 2002). KNOLLE is localised to forming cell plates in cellularising endosperm (Lauber et al., 1997), in agreement with the absence of cellularisation observed in *knolle*. Direct interaction between the KNOLLE syntaxin and the KEULE sec1-related protein has been demonstrated and it is therefore surprising that *keule* mutant endosperm has a wild-type appearance. It is not known whether KEULE is present in the endosperm. In contrast to the embryo, the endosperm of the *knolle keule* double mutant does not show an additive phenotype to that observed in *knolle*. It is thus likely that KEULE either is not expressed during endosperm cellularisation or that a functionally redundant gene exists. It has been suggested that each Sec1 protein interacts with several syntaxins (Sanderfoot et al., 2000). Our observation that KNOLLE but not KEULE is required for endosperm cellularisation suggests that the KNOLLE syntaxin is likely to interact with other Sec1 proteins than KEULE in the endosperm.

Absence of endosperm cellularisation has also been reported in mutants affected in structural elements such as microtubules. Mutations in the *PILZ* group of genes (Mayer et al., 1999) that includes *ttn1* and *ttn5* (McElver et al., 2000; Tzafrir et al., 2002) affect the complex of tubulin-folding cofactors and cause disruption to the microtubule cytoskeleton (Steinborn et al., 2002). The absence of microtubules thus explains the lack of cytokinesis in the embryo and of cellularisation in the endosperm of *pilz* mutants. Other *titan* mutants (Liu and Meinke, 1998), including *titan-7* and *-8* encoding chromosome scaffold proteins (Liu et al., 2002) show a non-cellularised endosperm with giant nuclei likely to be the indirect consequence of defective chromosome separation.

Hitherto, comparisons of cellularisation with conventional cytokinesis has been based on different cytological analyses of wild-type tissues, and although the similarities of the two processes has been acknowledged (Olsen, 2001) most reports have emphasised their differences (Otegui and Staehelin, 2000a; Nacry et al., 2000; Verma, 2001). Our genetic analysis of the *knolle*, *hinkel*, *open house*, *runkel* and *pleiade* mutations directly involved in cytokinesis provides conclusive evidence that somatic cytokinesis and endosperm cellularisation share many components.

FASS is not required for endosperm cellularisation

The PPB is a temporary assemblage of microtubules that marks the future site of connection of the cell plate to the cell wall in plant cytokinesis and results in an F-actin-free zone. PPBs have not been observed during endosperm cellularisation but only during the subsequent developmental phase of the barley aleurone layer (Brown et al., 1994). In *Arabidopsis*, both cellularisation and subsequent periclinal divisions take place without a discernible PPB (Brown et al., 1999). The PPB is lacking in *fass/ton2* mutants and interestingly, *fass/ton2* cellularisation occurs normally in MCE and PEN but subsequent cytokinesis is impaired in the endosperm. This confirms that a PPB is not required for cellularisation, but, in contrast to earlier observations, it appears to indicate that the PPB is necessary for the first periclinal divisions to proceed correctly. Alternatively, the *FASS* gene product might be involved in an unknown process or structure directing periclinal divisions in *Arabidopsis* endosperm.

Endosperm cellularisation is coupled to mitosis as conventional cytokinesis

In previous observations on fixed seeds cellularisation appeared to progress as a wave from the MCE towards the CZE (Mansfield and Briarty, 1990b). Cellularisation was considered to be uncoupled from mitosis and no distinction was seen in the appearance of cell walls between sister or non-sister nuclei (Brown et al., 1999). In order to perform real time in vivo observations of this process we used the GFP variant mGFP5 that is targeted to the endoplasmic reticulum and accumulates around the forming cell plate in root meristem cells (Haseloff, 1999). Accumulation of RE on the side of cell plates in endosperm has been reported (Otegui et al., 2001) and this likely accounts for the labelling with mGFP5 that we observed. This property allowed us to visualise the time course of cellularisation in living seeds. Completely cellularised MCE was observed at the end of the globular stage of embryogenesis. At this stage, the endosperm contains approx. 100 nuclei (stage VIII). The wave of mitosis leading to stage IX is initiated in the MCE and propagates within 1 hour through the PEN, as observed already during syncytial divisions (Boisnard-Lorig et al., 2001). In vivo observations enabled us to establish that cellularisation in the PEN is taking place in conjunction with the 8th mitotic cycle. The cell walls first appear between sister nuclei, rapidly followed by walls completely surrounding individual NCDs, thereby defining cells. Although the connection between mitosis and cellularisation has not previously been observed in *Arabidopsis*, probably due to the short time scale (one hour) of these events, coincidence of cell plate initiation with nuclear division has been remarked on in *Ranunculus sceleratus* (Xu and van Lammeren, 1993). Indeed we observed typical intermediate structures with nascent cell walls forming between sister nuclei only in three fixed seeds amongst several thousands. Furthermore, our observations relate to the initial appearance of walls, not to the rate at which these might expand anticlinally to form the characteristic open-ended alveoli (Brown et al., 1999).

In *Arabidopsis* endosperm the link between cellularisation and mitosis reported in this study furthers the parallels between endosperm development and *Drosophila* embryogenesis. In *Drosophila* a series of rapid succession of pseudo-synchronous waves of mitosis cross the embryo until cycle 10. Then a marked and gradual increase in the length of the cell cycle culminates at cycle 14 in the cellularisation of the blastoderm (Edgar et al., 1986). In *Drosophila*, the signal that initiates blastoderm cellularisation relies on a nucleo-cytoplasmic threshold (Edgar et al., 1986). This might be the case as well in the *Arabidopsis* endosperm.

The *spätzle* mutant phenotype points to the existence of a cellularisation-specific checkpoint

We have isolated two alleles of a new mutant, *spätzle*, that are characterised by the absence of cellularisation in the endosperm but do not show cytokinesis defects in the embryo.

Mature seeds will germinate and the homozygous *spätzle* plant is indistinguishable from wild type. One possible explanation for the absence of defects in embryos and homozygous vegetative tissues is that *SPÄTZLE* encodes a gene involved in mechanisms common to cellularisation and cytokinesis, but functional redundancy prevents the appearance of an embryo phenotype, a situation that could mirror our interpretation of defects observed in *keule* embryos but not in the *keule* endosperm (see discussion above). Alternatively, *spätzle* mutants might be affected in a process specific to cellularisation.

Much like *spätzle*, the *fis* mutants produce endosperm that does not cellularise although the embryo does not display any defect in cytokinesis, is viable in *fis1/medea* and in *fis2* mutants (Sørensen et al., 2001) and can produce plants of wild-type appearance. The *FIS* genes, control multiple aspects of seed development including initiation of endosperm divisions (Ohad et al., 1999; Luo et al., 1999) and correct definition of the CZE (Sørensen et al., 2001). In contrast, endosperm polarity is clearly not affected in *spätzle* endosperm and the *SPÄTZLE* and *FIS* genes are probably not in the same genetic pathway.

Like *spätzle*, *titan3* does not affect embryo development, with the mutant phenotype being restricted to the endosperm, where giant nuclei and NCDs are formed (Liu and Meinke, 1998). However, *ttn3* endosperm is capable of cellularisation that takes place at the same time as in the wild-type seeds. The defective cellularisation in *spätzle* endosperm is probably not a consequence of nuclei enlargement. The gene product of *SPÄTZLE* is indeed required for and limited to the process of cellularisation, possibly being a structural protein specifically expressed in the endosperm. As an alternative hypothesis we propose that the primary defect in *spätzle* is related to the nucleo-cytoplasmic ratio or to its interpretation. Cloning the gene mutated in *spätzle* might allow distinction between these hypotheses.

REFERENCES

Assaad, F. F., Huet, Y., Mayer, U. and Jürgens, G. (2001). The cytokinesis gene *KEULE* encodes a Sec1 protein that binds the syntaxin KNOLLE. *J. Cell Biol.* **152**, 531-543.

Assaad, F. F., Mayer, U., Wanner, G. and Jürgens, G. (1996). The *KEULE* gene is involved in cytokinesis in *Arabidopsis*. *Mol. Gen. Genet.* **253**, 267-277.

Bechtold, N., Ellis, J. and Pelletier, G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci. Paris* **316**, 1194-1199.

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, W., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. and Berger, F. (2001). Dynamic analyses of the expression of the HISTONE::YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains. *Plant Cell* **13**, 495-509.

Braselton, J. P., Wilkinson, M. J. and Clulow, S. A. (1996). Feulgen staining of intact plant tissues for confocal microscopy. *Biotech. Histochem.* **71**, 84-87.

Brown, R. C., Lemmon, B. E. and Olsen, O. A. (1994). Endosperm development in barley – microtubule involvement in the morphogenetic pathway. *Plant Cell* **6**, 1241-1252.

Brown, R. C., Lemmon, B. E., Nguyen, H. and Olsen, O. A. (1999). Development of endosperm in *Arabidopsis thaliana*. *Sex. Plant Reprod.* **12**, 32-42.

Edgar, B. A., Kiehle, C. P. and Schuebiger, G. (1986). Cell cycle control by the nucleo-cytoplasmic ratio in early Drosophila development. *Cell* **44**, 365-372.

Haseloff, J. (1999). GFP variants for multispectral imaging of living cells. *Methods Cell Biol.* **58**, 139-151.

Haseloff, J., Siemering, K. R., Prasher, D. C. and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. USA* **94**, 2122-2127.

Hauser, M. T. and Bauer, E. (2000). Histochemical analysis of root meristem activity in *Arabidopsis thaliana* using a cyclin: GUS (beta-glucuronidase) marker line. *Plant Soil* **226**, 1-10.

Heese, M., Mayer, U. and Jürgens, G. (1998). Cytokinesis in flowering plants: cellular process and developmental integration. *Curr. Opin. Plant Biol.* **1**, 486-491.

Lauber, M. H., Waizenegger, I., Steinmann, T., Schwarz, H., Mayer, U., Hwang, I., Lukowitz, W. and Jürgens, G. (1997). The *Arabidopsis* KNOLLE protein is a cytokinesis-specific syntaxin. *J. Cell Biol.* **139**, 1485-1493.

Liu, C. M., McElver, J., Tzafrir, I., Joosen, R., Wittich, P., Patton, D., van Lammeren, A. A. and Meinke, D. (2002). Condensin and cohesin knockouts in *Arabidopsis* exhibit a titan seed phenotype. *Plant J.* **29**, 405-415.

Liu, C. M. and Meinke, D. W. (1998). The titan mutants of *Arabidopsis* are disrupted in mitosis and cell cycle control during seed development. *Plant J.* **16**, 21-31.

Lukowitz, W., Mayer, U. and Jürgens, G. (1996). Cytokinesis in the *Arabidopsis* embryo involves the syntaxin-related *KNOLLE* gene product. *Cell* **84**, 61-71.

Luo, M., Bilodeau, P., Koltunow, A., Dennis, E. S., Peacock, W. J. and Chaudhury, A. M. (1999). Genes controlling fertilization-independent seed development in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. USA* **96**, 296-301.

Mansfield, S. G. and Briarty, L. G. (1990a). Development of the free-nuclear endosperm in *Arabidopsis thaliana* (L.). *Arabidopsis Info. Serv.* **27**, 53-64.

Mansfield, S. G. and Briarty, L. G. (1990b). Endosperm cellularization in *Arabidopsis thaliana* (L.). *Arabidopsis Info. Serv.* **27**, 65-72.

Mayer, U., Herzog, U., Berger, F., Inze, D. and Jürgens, G. (1999). Mutations in the *pilz* group genes disrupt the microtubule cytoskeleton and uncouple cell cycle progression from cell division in *Arabidopsis* embryo and endosperm. *Eur. J. Cell Biol.* **78**, 100-108.

Mayer, U., Ruiz, R. A. T., Berleth, T., Misera, S. and Jürgens, G. (1991). Mutations affecting body organization in the *Arabidopsis* embryo. *Nature* **353**, 402-407.

McClinton, R. S. and Sung, Z. R. (1997). Organization of cortical microtubules at the plasma membrane in *Arabidopsis*. *Planta* **201**, 252-260.

McElver, J., Patton, D., Rumbaugh, M., Liu, C. M., Yang, L. J. and Meinke, D. (2000). The TITAN5 gene of arabidopsis encodes a protein related to the ADP ribosylation factor family of GTP binding proteins. *Plant Cell* **12**, 1379-1392.

Miller, K. G. and Kiehart, D. P. (1995). Fly division. *J. Cell Biol.* **131**, 1-5.

Nacry, P., Mayer, U. and Jürgens, G. (2000). Genetic dissection of cytokinesis. *Plant Mol. Biol.* **43**, 719-733.

Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J. J., Goldberg, R. B. and Fischer, R. L. (1999). Mutations in FIE, a WD polycomb group gene, allow endosperm development without fertilization. *Plant Cell* **11**, 407-416.

Olsen, O. A. (2001). Endosperm development: cellularization and cell fate specification. *Annu Rev. Plant Physiol. Plant Mol. Biol.* **52**, 233-267.

Otegui, M. S. and Staehelin, L. A. (2000a). Cytokinesis in flowering plants: more than one way to divide a cell. *Curr. Opin. Plant Biol.* **3**, 493-502.

Otegui, M. S. and Staehelin, L. A. (2000b). Syncytial-type cell plates: A novel kind of cell plate involved in endosperm cellularization of *Arabidopsis*. *Plant Cell* **12**, 933-947.

Otegui, M. S., Mastronarde, D. N., Kang, B. H., Bednarek, S. Y. and Staehelin, L. A. (2001). Three-dimensional analysis of syncytial-type cell plates during endosperm cellularization visualized by high resolution electron tomography. *Plant Cell* **13**, 2033-2051.

Sanderfoot, A. A., Assaad, F. F. and Raikhel, N. V. (2000). The *Arabidopsis* genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. *Plant Physiol.* **124**, 1558-1569.

Schejter, E. D. and Wieschaus, E. (1993). Functional elements of the cytoskeleton in the early Drosophila embryo. *Annu Rev. Cell Biol.* **9**, 67-99.

Schweisguth, F., Vincent, A. and Lepesant, J. A. (1991). Genetic analysis of cellularisation of the Drosophila embryo. *Biol. Cell* **72**, 15-23.

Scott, R. J., Spielman, M., Bailey, J. and Dickinson, H. G. (1998). Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development* **125**, 3329-3341.

Sørensen, M. B., Chaudhury, A. M., Robert, H., Bancharel, E. and Berger, F. (2001). Polycomb group genes control pattern formation in plant seed. *Curr. Biol.* **11**, 277-281.

Staehelin, L. A. and Hepler, P. K. (1996). Cytokinesis in higher plants. *Cell*, **84**, 821-824.

Steinborn, K., Maulbetsch, C., Priester, B., Trautmann, S., Pacher, T., Geiges, B., Küttner, F., Lepiniec, L., Stierhof, Y., Schwarz, H., Jürgens, G. and Mayer, U. (2002). The *Arabidopsis PILZ* group genes encode tubulin-folding cofactor orthologs required for cell division but not cell growth. *Gen. Dev.* **16**, 959-971.

Strompen, G., El Kasmi, F., Richter, S., Lukowitz, W., Assaad, F. F., Jurgens, G. and Mayer, U. (2002). The *Arabidopsis HINKEL* gene encodes a kinesin-related protein involved in cytokinesis and is expressed in a cell cycle-dependent manner. *Curr. Biol.* **12**, 153-158.

Sylvester, A. W. (2000). Division decisions and the spatial regulation of cytokinesis. *Curr. Opin. Plant Biol.* **3**, 58-66.

Torres-Ruiz, R. A. and Jürgens, G. (1994). Mutations in the *FASS* gene uncouple pattern-formation and morphogenesis in *Arabidopsis* development. *Development* **120**, 2967-2978.

Traas, J., Bellini, C., Nacry, P., Kronenberger, J., Bouchez, D. and Caboche, M. (1995). Normal differentiation patterns in plants lacking microtubular preprophase bands. *Nature* **375**, 676-677.

Tzafrir, I., McElver, J. A., Liu, C. M., Yang, L. J., Wu, J. Q., Martinez, A., Patton, D. A. and Meinke, D. W. (2002). Diversity of TITAN functions in Arabidopsis seed development. *Plant Physiol.* **128**, 38-51.

Verma, D. P. (2001). Cytokinesis and building of the cell plate in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 751-784.

Waizenegger, I., Lukowitz, W., Assaad, F., Schwarz, H., Jürgens, G. and Mayer, U. (2000). The *Arabidopsis KNOLLE* and *KEULE* genes interact to promote vesicle fusion during cytokinesis. *Curr. Biol.* **10**, 1371-1374.

Xu, H. and van Lammeren, A. (1993). Microtubular configurations during the cellularization of coenocytic endosperm in Ranunculus sceleratus L. *Sex. Plant Reprod.* **6**, 127-132.