# The *globby1-1* (*glo1-1*) mutation disrupts nuclear and cell division in the developing maize seed causing alterations in endosperm cell fate and tissue differentiation

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*Accepted 30 June 2003*

Development 130, 5009-5017

doi:10.1242/dev.00692

## Summary

**Cereal endosperm tissues account for most of the world's calorific intake, yet the regulation of monocot seed development remains poorly understood. The maize endosperm originates with a series of free-nuclear divisions, followed by cellularisation and subsequent formation of a range of functional cellular domains. We describe the isolation and characterisation of a mutation that induces aberrant globular embryo and endosperm morphology, *globby1-1* (*glo1-1*). Our data indicate that *glo1-1* plays a role in nuclear division and cytokinesis in the developing seed. Pattern formation in the embryo is severely impaired with development arresting at premature stages, while in the endosperm, the effects of the *glo1-1* mutation are manifest at the free-nuclear or syncytial stage. During cellularisation, and at later stages of development, aberrant cell division and localised domains of cell proliferation are apparent in *glo1-1* endosperms. As a consequence, cell fate acquisition and subsequent differentiation of endosperm tissues are affected to varying degrees of severity. To date, it has been hypothesised that BETL cell fate is specified in the syncytium and that cell files subsequently develop in response to a gradient of signal(s) derived from the maternal pedicel region. Based on our findings, however, we propose that specification of BETL cells is an irreversible event that occurs within a narrow window of syncytial development, and that BETL cell identity is subsequently inherited in a lineage-dependent manner. Additionally, our data suggest that acquisition of aleurone cell fate does not solely rely upon signalling from the maternal surrounding tissue to the periphery of the endosperm, as previously thought, but that other factor(s) present within the endosperm are involved.**

Key words: *globby1-1*, Endosperm development, BETL, Aleurone, Cell fate, Cell proliferation, Cytokinesis, Maize

## Introduction

Control of cell division and expansion is crucial for developmental patterning and is likely to be mediated by factors operating at different organisational levels (Irish and Jenik, 2001). Although some mutations causing altered division rates or aberrant cytokinesis affect overall plant morphogenesis (Liu et al., 1997), others do not (Blilou et al., 2002; Cleary and Smith, 1998). However, the increasing number of mutations reported to generate cytokinetic defects that lead to alterations in cell fate (Assaad et al., 1996; Chen and McCormick, 1996; Kessler et al., 2002) further demonstrate the importance of the precise timing and proper execution of cell division in plant organogenesis.

The maize seed is composed of an embryo and a persistent endosperm, and is proving an effective model system for the study of basic mechanisms of plant development. The endosperm is a simple structure that originates as a triploid central cell, the result of the fusion of the diploid central cell nucleus with one of the two sperm cell nuclei (Kiesselbach, 1949). A succession of free-nuclear divisions results in the formation of the single-celled syncytium, which bears strong similarities to the *Drosophila* blastoderm. Cellularisation of the syncytium is initiated by the formation of radial microtubule systems (RMS), which emanate from the nuclear envelopes. Adventitious phragmoplasts are deposited at the points of intersection, forming open-ended tube-like alveolar structures with the open end facing the interior of the endosperm (Brown et al., 1994; Olsen et al., 1995). Coordinated mitotic divisions of nuclei within alveoli are followed by cytokinesis, thus giving rise to a single peripheral layer of cells and a new layer of open-ended alveoli (Olsen, 2001). Cellularisation proceeds in a centripetal manner, as this cycle is repeated, until the endosperm becomes fully cellular.

The molecular systems regulating early endosperm development, including the transition from syncytial to cellular stages, remain unclear. Studies in *Arabidopsis* have shown that the process of endosperm cellularisation shares multiple components with cytokinesis (Sørensen et al., 2002). However, the isolation of a small but significant number of mutations which specifically affect either cytokinesis only in the embryo (Assaad et al., 1996) or cellularisation of the endosperm (Liu

and Meinke, 1998; Sørensen et al., 2002), point to the existence of additional mechanisms operating in these two structures (Dickinson, 2003).

Unlike *Arabidopsis*, maize endosperm develops four structurally and functionally distinct tissues – aleurone, basal endosperm transfer layer (BETL), embryo-surrounding region (ESR) and starchy endosperm (SE) – which are believed to be specified during the free-nuclear to cellularisation stages of development (Becraft, 2001; Olsen, 2001). A significant number of mutants with defective aleurone development have thus far provided valuable insight into aleurone cell fate and differentiation (Becraft and Asuncion-Crabb, 2000; Becraft et al., 1996; Lid et al., 2002; Shen et al., 2003). By contrast, no mutants with structural defects in either the BETL or the ESR domains alone have been identified, and as a result, little is known of the mechanisms regulating BETL and ESR formation (Becker et al., 1999; Becraft, 2001).

We describe here a recessive lethal mutant characterised by a distinctive globular embryo and endosperm morphology, termed *globby1-1* (*glo1-1*). A key feature of the *glo1-1* phenotype is aberrant nuclear and cell proliferation in the early syncytial and cellular endosperm, respectively, which to our knowledge has not been previously reported for monocots. As a consequence of these early abnormalities, localised disruptions in cell organisation of the BETL arise, and some cells with aleurone characteristics form ectopically in the regions of SE and BETL. These findings are inconsistent with a previous model for aleurone development, where it was proposed that aleurone cell fate was acquired through signalling from the maternal tissue to the endosperm surface, mediated via the CRINKLY4 receptor kinase (Olsen, 1998). Instead, our data point to the presence of other unknown developmental cue(s) operating within the starchy endosperm that confer aleurone cell identity.

## Materials and methods

### Genetic stocks, mapping and growth conditions

The *glo1-1* and *glo1-2* mutants were isolated from active *Activator* (*Ac*) transposon containing lines maintained in a standard W22 inbred. B-A translocation stocks and the following genetic stocks located on chromosome 1L were used for allelism tests with heterozygous *glo1-1*/+ plants: *cp3*-N888A, *cp**-N918A, *cp**-N948A, *dek22*-1113A, *dek2*-1315A, *dek**MS6214, *gm**-N1303 and *ptd1*, and were provided by the Maize Stock Center (Urbana, IL, USA). The embryo and aleurone-specific pVP1::GUS seeds, and BETL-specific pBET1-GUS (Hueros et al., 1999) seeds were kindly provided by Drs. P. Perez (Biogemma, France) and R. Thompson (Max-Planck-Institut, Germany), respectively.

For genetic analysis and mapping, plants were grown in fields at Cornell University (Aurora, NY). Glasshouse grown plants were used for all other analyses and either grown at Oxford or Jealott's Hill, under the following regime: 16 hours day length (supplemented with metal halide lamps at 250 mmols, when required) at 22-28°C during the day, and at 16-20°C at night. Humidity levels were set at ~40-50% daytime and ~60-70% at night.

### Histology

Plants heterozygous for the *glo1-1* allele were self-pollinated to recover mutants, which segregated as a single recessive Mendelian trait (data not shown). For analysis of early endosperm developmental stages, kernels were harvested from the mid portion of the ear and the remaining kernels were left to mature to score the mutant phenotype. Fresh hand sections of mature kernels were obtained after imbibing seeds on wet tissue for 2 days, and cutting through the central longitudinal axis with a sharp blade. For phenotypic descriptions of developing embryos, we followed nomenclature according to Abbe and Stein (Abbe and Stein, 1954).

### Microscopy

For epifluorescence microscopy, kernels were trimmed along the mediolateral axis and fixed with FAA (5% formaldehyde, 5% acetic acid, 45% ethanol) for 15 minutes under a gentle vacuum. After infiltration, samples were left overnight at 4°C in fresh fixative, dehydrated through a graded ethanol series, cleared with Histoclear (National Diagnostics, Hull, UK) and wax embedded in Paraplast Plus (Sigma, St Louis, MO). Sections were cut at 8-10 μm, mounted on Superfrost Plus slides (BDH) and stained with DAPI (Ruzin, 1999) at a final concentration of 1-2 mg/ml in Vectashield (Vector laboratories, Peterborough, UK). Slides were examined with a Zeiss Axiophot microscope using a 50 W mercury lamp and the following filter set: 365 nm excitation, 395 nm dichroic and 420 nm long-pass emission.

For light microscopy and transmission electron microscopy (TEM), tissue was fixed overnight in 100 mM phosphate buffer (pH 7.5), with 4% paraformaldehyde and 1% gluteraldehyde at room temperature. The material was then washed in four 15 minutes changes of phosphate buffer and treated in 1% aqueous osmium tetraoxide buffer, followed by dehydration in a graded acetone series and embedding in epoxy resin. Thick sections were cut at 2 μm with a tungsten-coated glass knife, affixed to pre-treated glass slides, stained with Toluidine Blue O (Feder and O'Brien, 1968) and viewed under bright field optics using a Zeiss AxioPhot microscope. Ultra-thin sections were cut using a diamond knife at 80 nm, stained with uranyl acetate and lead citrate on a 2168 Ultrostainer (Carlsberg System), and collected on 2 mm copper grids coated with Butvar B98 support film. Grids were examined with a JEOL 2000EX TEM operating at 80 kV accelerating voltage and negatives were digitally imaged after conventional development.

### mRNA in situ hybridisation

In situ hybridisation was performed on developing kernels at 7 and 10 days after pollination (dap) according to Jackson (Jackson, 1991), with minor modifications. Briefly, kernels were trimmed along the medial-lateral axis and immediately fixed in ice-cold FAA, dehydrated in an ethanol series, and embedded in wax. Sections were cut at 10-12 μm and affixed onto pre-treated Superfrost Plus slides (BDH). Riboprobes were labelled using the DIG RNA labeling mix (Boehringer Mannheim, catalogue number 1175025) according to manufacturer's instructions, and slides were hybridised overnight at 50°C. Slides were viewed with a Zeiss AxioPhot microscope under DIC3-5 optics and images were digitally recorded.

### GUS marker gene analysis

Plants were genotyped for β-glucuronidase (GUS) transcriptional fusions via PCR using GUS-specific oligonucleotides (data not shown), and backcrossed to *glo1-1*/ + plants for three successive generations. Kernels were cut longitudinally and GUS was detected histochemically according to a method previously described (Jefferson et al., 1987), with slight modifications. After staining at 37°C overnight, the material was fixed in phosphate buffer and wax embedded, as described above. Sections were cut at 12-15 μm and viewed under Nomarski optics.

## Results

### Identification of the *glo1-1* mutant

The *glo1-1* mutant and a second allele, *glo1-2*, were both isolated from an active *Ac* transposon population and segregated as single recessive seed lethal traits. Although the degree of

phenotypic severity varies within individual segregating ears (Fig. 1A,B), both *glo1-1* and *glo1-2* mutant kernels are small in size and possess identical phenotypes (data not shown). For this reason, only the *glo1-1* allele has been used in the phenotypic characterisation described below. An *Ac* element linked to the *glo1-1* mutation was identified through Southern hybridisation and a region flanking the *glo1-1*-linked *Ac* element was cloned and mapped to position 1L, bin 1.07 between SSR loci umc1358 and umc1128 using the Maize Missouri IBM mapping population. Furthermore, the *glo1-1* mutation was uncovered by crosses to the B-A translocation stock TB-1La (Beckett, 1978), thus confirming its position to the long arm of chromosome 1 (Fig. 1C). Complementation analysis of *glo1-1* with other known seed mutants located on chromosome 1L demonstrated that *glo1-1* and *gm**-N1303 (Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980) were allelic (data not shown).

To ascertain whether the lethality of the *glo1-1* embryo was a consequence of abnormalities observed in the endosperm, we attempted to create mosaics with a hyperploid endosperm (*glo1-1*/ *glo1-1*/+ /+) and hypoploid (*glo1-1*/–) embryo (Fig. 1C,D). If the embryo lethality was due to the absence of the GLO1 product in the endosperm, then we reasoned that we should be able to rescue the mutant phenotype in the hyperploid endosperm and in the hemizygous embryos. As shown in Fig. 1D, many of these kernels lacked a distinct embryo, suggesting that *glo1* is necessary and expressed in cells of the developing embryo. Furthermore, progeny containing hypoploid (*glo1-1*/ *glo1-1*/ –) endosperms and hyperploid (*glo1-1*/+ /+) embryos (Fig. 1D) failed to germinate, indicating that expression of *glo1* in embryo tissues is necessary, but not sufficient, for proper kernel development.

### The *glo1-1* mutation disrupts early endosperm development

To determine when in endosperm development GLO1 function is required, serial sections of sibling kernels were compared from wild-type and self-pollinated *glo1-1*/+ heterozygous plants between 2 and 4 dap. We examined endosperms that ranged from syncytial, to cellularising to fully cellular stages of development. In wild-type syncytia, nuclei are suspended in a thin layer of cytoplasm surrounding the large central vacuole (Fig. 2A). Remarkably, subtle structural differences and anomalies in the distribution of nuclei were observed in ~25% of syncytia examined from segregating, but not wild-type, ears (Fig. 2B). We interpret these anomalies as indicating the lack of GLO1 in these syncytia. At a point when the first cell layer forms in wild-type syncytia (Fig. 2C), we noticed an unusual

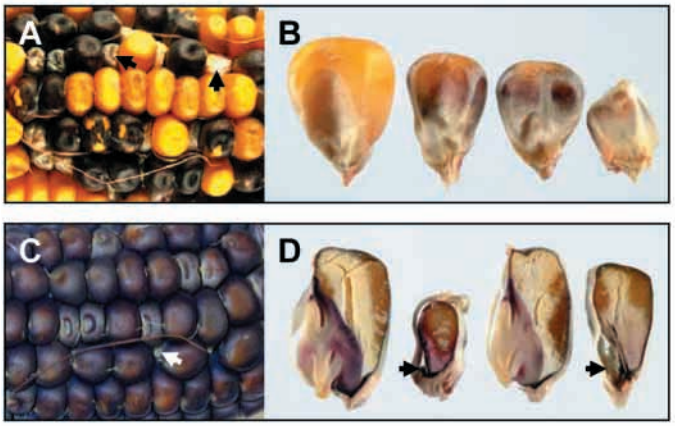


**Fig. 1.** The *glo1-1* kernel phenotype. (A) Ear segregating for *glo1-1* mutant kernels (arrow). (B) Germinal faces of a mature wild-type (far left) and three *glo1-1* kernels, showing a range of typical mutant phenotypes. (C) *glo1-1* uncovered by TB-1La (white arrow). (D) Longitudinal section through a wild-type kernel (far left), kernel with hypoploid (*glo1-1*/ *glo1-1*/–) endosperm and hyperploid (*glo1-1*/+/+) embryo (arrow, middle left), kernel with hypoploid (+/+/–) endosperm and hyperploid (+/+/+) embryo (middle right), and kernel with hyperploid (*glo1-1*/ *glo1-1*/+ /+) endosperm and hypoploid (*glo1-1*/–) embryo (arrow, far right).

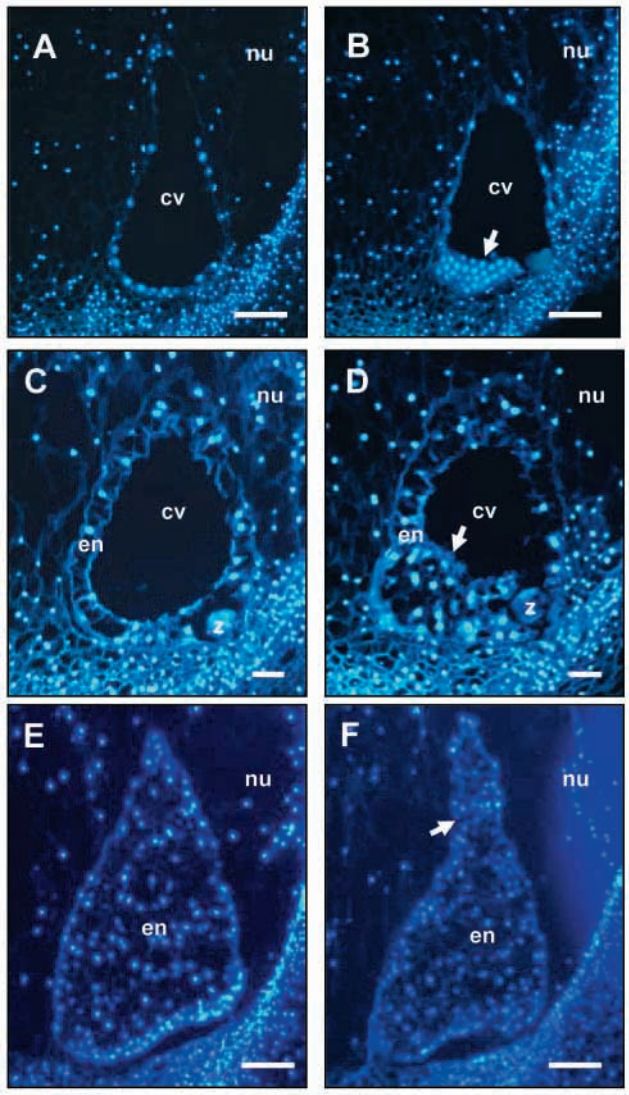


**Fig. 2.** Effects of the *glo1-1* mutation on early endosperm development. Developing endosperm in (A,C,E) wild-type and (B,D,F) *glo1-1* mutants. (A) Wild-type syncytium. (B) Abnormal accumulation of nuclei in *glo1-1* basal syncytium (arrow). (C) Wild-type cellularising endosperm with first cell layer formed. (D) *glo1-1* endosperm showing abnormal cellularisation in the basal region (arrow). Fully cellular (E) wild-type endosperm and (F) *glo1-1* endosperm with constrictions around the apical region (arrow) and abnormal basal endosperm morphology. en, endosperm; nu, nucellus; p, pericarp; z, zygote. Scale bars: 50 μm.

overproliferation of cells confined to basal regions of endosperms interpreted as *glo1-1* (Fig. 2D). By 4 dap, wild-type endosperms were fully cellular and had a rounded, conical shaped morphology (Fig. 2E), whereas putative *glo1-1* endosperms often showed signs of constrictions around the mid-apical region (Fig. 2F) that became greatly accentuated during later stages of development. Our current data thus indicate that the *glo1-1* mutation affects both nuclear divisions in the syncytium and endosperm cellularisation (summarised in Table 1), which dramatically alter subsequent development of the mutant endosperm.

### The *glo1-1* mutation affects cellular organisation and cytokinesis in embryo and endosperm

The comparisons of embryos isolated from 8 dap wild-type and *glo1-1* kernels indicated that *glo1-1* mutants showed a greatly retarded growth, appearing as mid-transition phase embryos while their wild-type siblings had entered the early coleoptilar phase (data not shown). A comparative analysis of wild-type and *glo1-1* mid-transition stage embryos revealed profound differences in cellular anatomy (Fig. 3A,B). In wild-type, the mid-transition stage embryo is located within the ESR domain of the endosperm and consists of the apical embryo proper and basal suspensor (Fig. 3A). The embryo proper is characterised by small cytoplasmically dense cells surrounded by a single peripheral layer of protodermal cells, while cells of the suspensor are larger and vacuolated (Fig. 3A). By contrast, an irregular protoderm was often observed in *glo1-1* embryos, accompanied by an occasional misplacement of densely cytoplasmic cells located in the abnormal suspensor (Fig. 3B). At later stages, mutant embryos either degenerated, some completely, or persisted as a mass of undifferentiated cells, lacking any visible signs of a shoot apical meristem (SAM), while only 16% formed rudimentary primary roots upon germination (data not shown).

At 8 dap, mutant endosperms were reduced in size relative to their wild-type siblings and *glo1-1* endosperm tissues showed developmental abnormalities. In many *glo1-1* mutant kernels the BETL region, which is located at the endosperm-maternal tissue interface (Fig. 3C), was disrupted. The severity of this disruption varied from a small number of comparatively undifferentiated cells (Fig. 3D) to a total disorganisation of cells. Conventional microscopy of these maturing mutant kernels also revealed purple pigmentation in a significant number of cells resembling anthocyanin pigment seen in the aleurone (data not shown). In contrast to the uniform patterning of cells in the wild-type SE (Fig. 3E), striking abnormalities occurred in the *glo1-1* SE (Fig. 3F), including the clustering of small cells, to an apparent loss of cell adhesion and unusual cell wall thickenings. The monolayer of aleurone cells that regularly invests the larger starchy cells in wild-type endosperms (Fig. 3G) was also disrupted in *glo1-1*, principally on the abgerminal face of mutant endosperms (data not shown). Other abnormalities included areas containing multiple aleurone layers (Fig. 3H), irregular proliferation of aleurone at the endosperm apex (Fig. 3I), and regions devoid of aleurone altogether (Fig. 3J). To summarise, pattern formation is severely disrupted in *glo1-1* embryos, whereas endosperm patterning appears to be affected to a lesser extent as the ESR, BETL, SE and aleurone domains are more or less established.

Interestingly, cell wall projections or 'stubs' (Fig. 3F,I,J) and multinucleate cells were occasionally observed in both embryo (data not shown) and endosperm cells (Fig. 3D,I). Using TEM to investigate further these possible cytokinesis-associated defects, we examined wild-type and mutant kernels in the sub-aleurone region, as it is the most actively dividing area in the 8 dap endosperm (Olsen et al., 1999). In wild-type cells, the protoplast surface was lined by regular aggregations of ER (Fig. 4A), a feature absent in the mutant (Fig. 4B,E). Irregular deposits of cell wall material (Fig. 4B) and the appearance of striking cell wall 'stubs' in the mutant, comprising all elements of the normal cell wall (Fig. 4B,C), were also observed in addition to multinucleate cells (Fig. 4D). Furthermore, cell-cell adhesion appeared to be poor in *glo1-1*, with interfaces between cells frequently featuring gaps and lacunae (Fig. 4E,F). Interestingly, multivesicular bodies (MVBs) were commonly associated with irregular cell wall thickenings, where they appeared to secrete their contents through the plasma membrane (Fig. 4E). Strikingly, large intercellular lacunae that characterise the SE region in *glo1-1* endosperms were bordered by isodiametric cells with dense cytoplasms, which were microscopically indistinguishable from aleurone cells (Fig. 4F).

### Expression patterns of basal endosperm domains are altered in *glo1-1*

mRNA in situ hybridisation on wild-type and *glo1-1* endosperms was performed to assess the early expression patterns of genes normally restricted to the ESR and to the BETL. The ZmESR2 probe (P. Rogowsky, Lyon), was used to detect all three ESR-specific transcripts (ZmESR1, ZmESR2 and ZmESR3) (Opsahl-Ferstad et al., 1997). In 7 dap wild-type kernels, ZmESR transcripts were highly abundant in the ESR (Fig. 5A), whereas in 7 and 10 dap *glo1-1* endosperms, ZmESR transcripts were restricted to the basal extremity of the suspensor, in close contact with the pericarp (Fig. 5B,C).

Similarly, expression of an early BETL-specific gene, *ZmMEG1-1* (J.F.G.-M., unpublished) was dramatically altered in *glo1-1* endosperms. At 7 dap, ZmMEG1-1 transcripts were abundant throughout the wild-type BETL (Fig. 5D), while in

**Table 1. Summary of events in early endosperm morphology caused by the *glo1-1* mutation**

| Days after pollination | Developmental stage | Wild-type phenotypic description | *glo1-1* phenotypic description |
|---|---|---|---|
| 2 | Free-nuclear (syncytium) | Nuclei arranged equidistantly in peripheral syncytium | Accumulation of nuclei in basal syncytium |
| 3 | Cellularising endosperm | Inward periclinal cell division initiating centripetal cellularisation | Abnormal cellularisation in the basal region |
| 4 | Fully cellular endosperm | Plump, conical-shaped endosperm | Irregular endosperm morphology; constrictions often seen in apical region |

*glo1-1*, transcripts were present at lower levels and were often restricted to small fields within the transfer tissue (Fig. 5E). To investigate further anomalies in the *glo1-1* BETL at later stages of development (14 dap), plants carrying a *BET1*-specific promoter-GUS fusion (Hueros et al., 1999) were introgressed in the *glo1-1* background (Fig. 5F). In all circumstances, GUS staining was only ever detected in the BETL region, although expression of the pBET1-GUS reporter was frequently observed to be 'patchy' (Fig. 5G) in the basal region of *glo1-1* endosperms. Thus, it appears that the *glo1-1* mutation results in structural irregularities and altered patterns of gene expression in basal endosperm (ESR and BETL) domains.

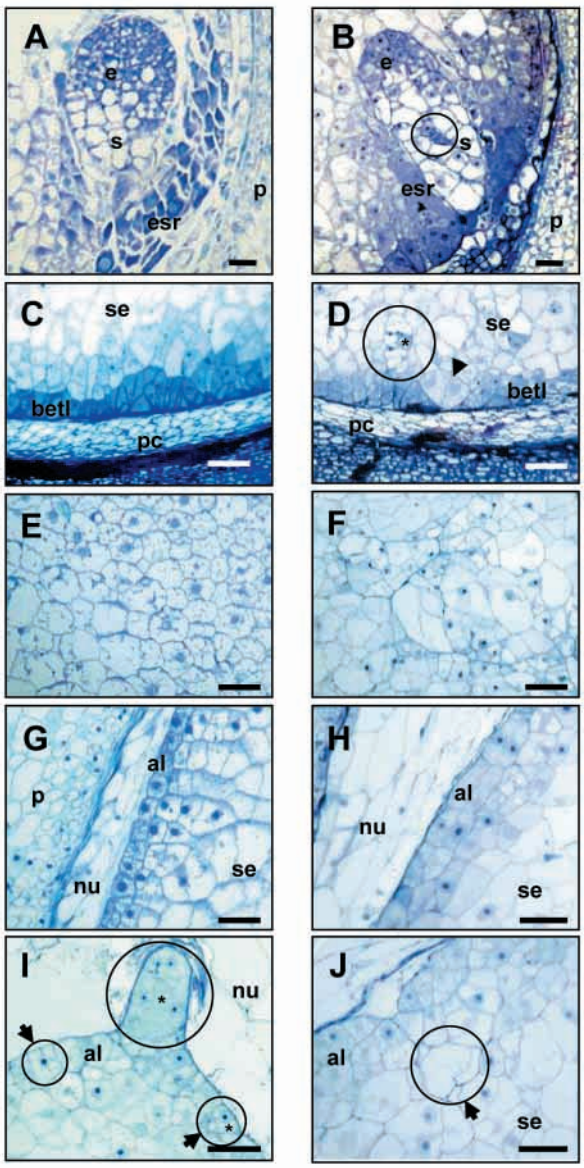


**Fig. 3.** Effects of *glo1-1* on embryo and endosperm morphology. Transition-stage embryo in (A) wild-type and (B) *glo1-1*, showing small, dense cytoplasmic cells (circle) located in the defective suspensor (s). Endosperms (8 dap) in (C,E,G) wild-type and (F,H-J) *glo1-1*. (C) Wild-type basal endosperm transfer layer (BETL; betl in figure). (D) Two large cells (designated with arrowhead) interrupt the arrangement of the *glo1-1* BETL. (E) Wild-type starchy endosperm (SE; se in figure). (F) Disorganised SE in *glo1-1* often containing clusters of small cells and enlarged cells. (G) Wild-type aleurone (al). (H,J) Aleurone development in *glo1-1*: (H) two aleurone layers, (I) abnormal proliferation of aleurone in the *glo1-1* apical endosperm and (J) lack of a regular aleurone layer. Additionally, cell wall stubs (arrows) and multinucleate cells (asterisks) were observed in *glo1-1* embryos (data not shown) and endosperms (D,F,I,J). Circles highlight areas of interest. e, embryo-proper; esr, embryo surrounding region; pc, placento-chalazal region. Scale bars: 50 μm in A-D,G,H,J; 100 μm in E,F,I.

### The identity and positioning of aleurone cells is irregular in *glo1-1*

To ascertain the positioning of aleurone cells in *glo1-1* endosperms, we introgressed pVP1-GUS plants (J.F.G.-M., unpublished data) in the *glo1-1* background, thus enabling GUS detection of aleurone cells. We examined 10 dap wild-type kernels and found GUS staining patterns confined to aleurone cells (Fig. 6A) However, in *glo1-1* endosperms, non-uniform expression in the aleurone was occasionally observed (data not shown) as was the unexpected presence of GUS in few cells of the central SE region (Fig. 6B). More frequently, GUS staining was observed in the BETL of severe *glo1-1* endosperms (Fig. 6C). An additional unique feature of the *glo1-1* phenotype was the appearance of discrete cysts encapsulated by extremely thick cell walls proximally located to peripheral regions of the endosperm. By using the pVP1-GUS reporter, we found that the layer of cells investing these ectopic structures also showed GUS staining (Fig. 6D), while cells of the interior of these structures contained well-defined starch grains and did not stain for GUS (Fig. 6E). Thus, our current data indicate that cells with many features of the aleurone are ectopically formed throughout the *glo1-1* endosperm.

## Discussion

A large number of maize seed mutants have been isolated (Neuffer and Sheridan, 1980; Scanlon et al., 1994; Sheridan and Neuffer, 1980), although only few have been comprehensively characterised to date (Becraft et al., 2002; Consonni et al., 2003; Fu et al., 2002; Lid et al., 2002; Shen et al., 2003). We describe here the phenotypic characterisation of the *glo1-1* mutation, which arose in an active transposon population. The distinctive mutant phenotype not only points to the nature of the gene affected, but when considered in the perspective of current knowledge, also provides further insight into the factors governing endosperm cell fate and tissue differentiation.

### *glo1-1* is required for embryo and endosperm development

Using a range of microscopic techniques, in situ hybridisation and reporter gene assays, we have shown that early cellularisation events in the *glo1-1* endosperm are abnormal, and, as a result, lead to alterations in cell fate and subsequent differentiation of endosperm tissues. Examination of mutant embryos revealed severe cellular disorganisation with few recognisable tissues formed. Taken together, these findings suggest that the GLO1 product is essential for the regulation of cellular morphogenesis in maize embryo and endosperm tissues.

It is possible that the variation observed in endosperm and

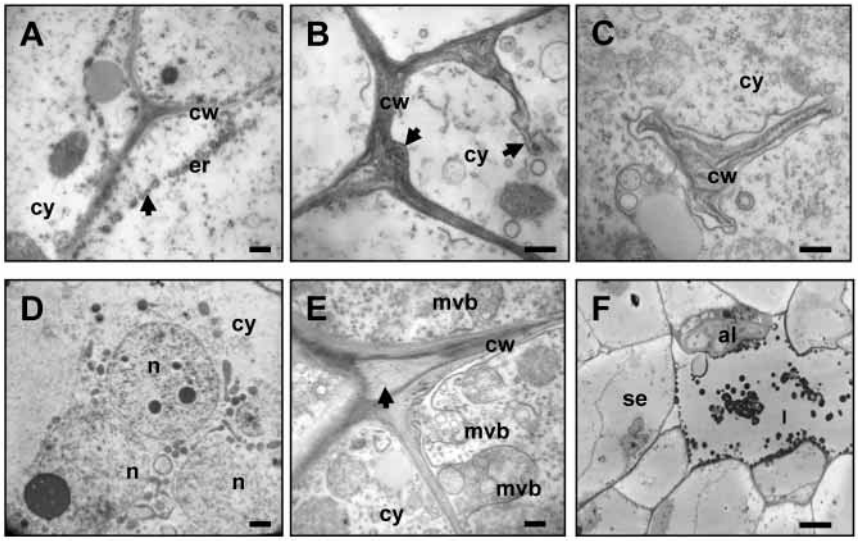


**Fig. 4.** TEM of *glo1-1* endosperms. (A) Endoplasmic reticulum (er) is regularly associated with cell walls (cw) in the wild-type. (B-F) *glo1-1* endosperms. (B) Cell walls are often thickened and incomplete in *glo1-1*. (C) Cell wall stub located in central cytoplasm (cy). (D) Cell with three nuclei (n). (E) Poor cell-cell adhesion (arrow) and multivesicular bodies (mvb). (F) Disrupted starchy endosperm (se) with an aleurone-like cell (al) adjacent to the lacuna (l). Scale bars: 200 nm in A-C,E; 1 μm in D; 10 μm in F.

embryo phenotypes among homozygous *glo1-1* mutants results from varying gene dosage. Through the use of a B-A translocation affecting the long arm of chromosome 1, additional copies of the *glo1-1* wild-type allele were introduced in either the embryo or endosperm independently, but this failed to rescue the mutant phenotype, suggesting that variations observed in *glo1-1* mutant phenotypes are not attributable to differences in gene dosage. These studies also demonstrated that the *glo1-1* mutation affects development of the embryo and endosperm independently. Moreover, we showed that wild-type (hyperploid) embryos were unable to develop in the presence of defective (hypoploid) endosperms, suggesting that under the *glo1-1* mutant condition, development of the embryo is strongly influenced by the endosperm.

Interestingly, we found *glo1-1* to be allelic to the EMS-induced mutant *gm**-N1303 (data not shown), originally described as a *germless* mutant by Neuffer and Sheridan (Neuffer and Sheridan, 1980). The variation in seed phenotype observed between the two alleles (data not shown) may be attributable to differences in genetic background (e.g. Gethi et al., 2002; Vollbrecht et al., 2000): *gm**-N1303 was described in a vigorous hybrid background, whereas the *glo1-1* allele is in a standard inbred line.

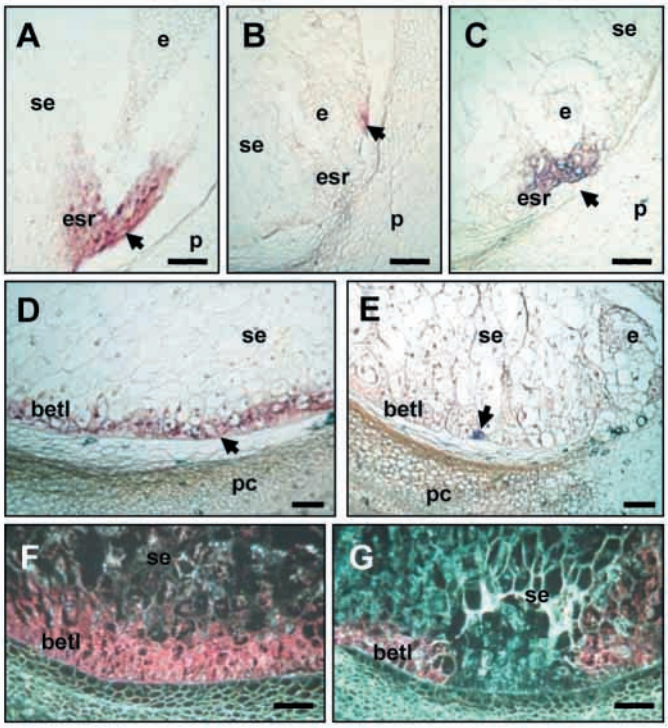


**Fig. 5.** Effects of *glo1-1* on basal endosperm-specific gene expression. mRNA in situ analysis using (A-C) *ZmESR* and (D,E) a BETL-specific marker (J.F.G.-M., unpublished). (A-C) *ZmESR* expression (shown in purple, arrows) in (A) 7 dap wild-type endosperm, and in *glo1-1* endosperms at (B) 7 dap and (C) 10 dap. BETL-specific gene expression in 10 dap (D) wild-type BETL and (E) *glo1-1* basal endosperm, where expression is confined to a single cell (arrow). pBET1-GUS expression (pink) in (F) wild-type and (G) *glo1-1* endosperms at 14 dap. Scale bars: 100 μm.

## The *glo1-1* mutation predominantly affects nuclear division

Early endosperm development in many angiosperms is characterised by nuclear migration and suppression of phragmoplast formation (Brown et al., 1999; Brown et al., 1994; Olsen, 2001), requiring tight coordination between the cell cycle and cytoskeletal organisation. Phenotypic analysis of the *glo1-1* mutant suggests that *glo1-1* may affect nuclear division, perhaps through a deregulation, or partial loss of control of the cell cycle. Aberrant cellular development is often localised to small patches within *glo1-1* endosperms and may occur at different developmental stages, leading to the observed range of *glo1-1* mutant phenotypes. Thus, early proliferation of nuclei in the syncytium would induce a severe kernel phenotype, whereas random proliferation in the cellular endosperm would result in minimal disruption to tissue organisation. Cell wall aberrations and other defects typically associated with cytokinesis (see Söllner et al., 2002) were apparent in *glo1-1* kernels. These aberrations have been reported in several seed mutants, many of which have led to early developmental arrest of embryos (Consonni et al., 2003; Söllner et al., 2002). This link between defective cell division

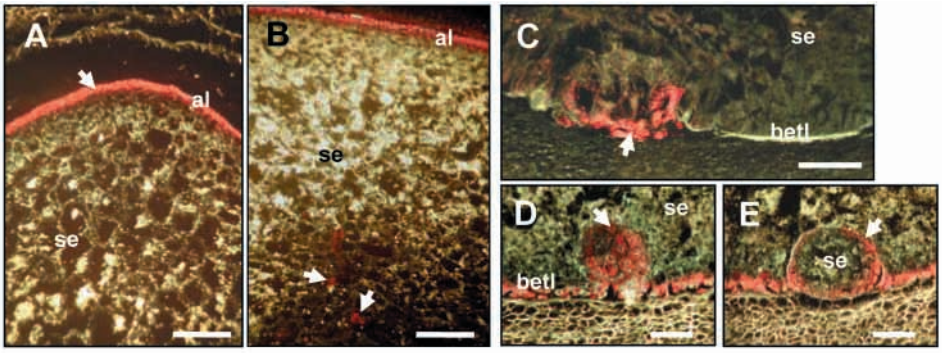


**Fig. 6.** Aleurone cell abnormalities in *glo1-1*. pVP1-GUS analysis in 10 dap wild-type (A) and *glo1-1* endosperms (B-E). GUS expression (pink) highlighting the (A) wild-type aleurone, and (B) *glo1-1* aleurone and some cells in the SE (arrows). GUS staining also in *glo1-1* basal transfer region (C-E) and in peripheral cells of ectopic cysts (D,E). Scale bars: 100 μm.

and suppression of embryo morphogenesis may account for the lack of pattern formation in *glo1-1* embryos.

The GLO1 gene product therefore seems necessary for both nuclear division and cytokinesis in the developing seed. Evidence from *Arabidopsis* is accumulating that the processes of mitosis and cytokinesis are intimately interrelated (Dickinson, 2003; Mayer et al., 1999; Sørensen et al., 2002), and it remains possible that GLO1 is involved only in an aspect of nuclear division which, when disrupted, causes secondary or downstream effects on cytokinesis. This is also true for *TITAN3*, which encodes an SMC2 condensin (Liu et al., 2002). Mutant endosperms lacking the functional TITAN3 protein develop giant nuclei in the syncytial cytoplasm, which consequently results in defective cellularisation (Liu and Meinke, 1998). Clearly, the role of GLO1 remains to be determined and efforts are now under way to clone the *glo1* gene using a closely linked *Ac* element in regional mutagenesis (Brutnell, 2002; Singh et al., 2003).

## Basal structures are predetermined in the syncytial endosperm

Disruptions to the basal endosperm region are witnessed from an early stage in *glo1-1* mutants, i.e. during syncytial development, when the ESR and BETL are thought to be specified (Becraft, 2001; Olsen, 2001).

Our data showed reduced levels of *ZmESR* gene expression in *glo1-1* kernels, often restricted to small regions of the ESR. This may be a consequence of either aberrant development of other endosperm tissues, impacting indirectly on the ESR, or defective development of the embryo caused by the *glo1-1* mutation. It has been proposed that the *ZmESR* gene products are involved in cross-talk between embryo and endosperm (Opsahl-Ferstad et al., 1997), as *ZmESR* expression is absent in spontaneously occurring embryo-less mutants (Opsahl-Ferstad et al., 1997). Our observations are consistent with the interpretation that correct establishment of the ESR relies upon successful development of the embryo – which is of course highly defective in *glo1-1* kernels.

In *glo1-1* endosperms, the BETL tissue was often disrupted to various degrees, and reduced levels of two BETL-specific transcripts were recorded – defects that are likely to result from abnormal syncytial development caused by the *glo1-1* mutation. As BETL identity is held to be acquired via maternally derived diffusible signal(s) or morphogen gradient(s) present in the syncytium (Becker et al., 1999), it follows that the irregularities in the syncytium induced by the *glo1-1* mutation may affect the ability of specific basal nuclei to perceive or act upon these positional cues. Our data therefore suggest that BETL specification is a fixed process, which occurs only during a short period in syncytial development. Nuclei that fail to perceive this information (either through the action of *glo1-1* or otherwise) are thus unable to differentiate as BETL cell types. Therefore, if the acquisition of BETL cell fate were both irreversible and to occur within a narrow developmental window, then 'BETL identity' must be passed on in a lineage-dependent fashion (see Fig. 7 for proposed model), which, although is rare for plants (Scheres, 2001), is common in animal systems such as *Drosophila* – where positional cues in the blastoderm lead to lineage-dependent differentiation (Lawrence and Struhl, 1996). In support of this hypothesis, we found that the BETL was frequently patchy in *glo1-1* endosperms. Additionally, it has been reported that following interploidy crosses, endosperms (with a paternal excess genomic contribution) often contain tiny interspersed clusters of BETL cells (Gutierrez-Marcos et al., 2003). As BETL cells are known to undergo a finite number of divisions

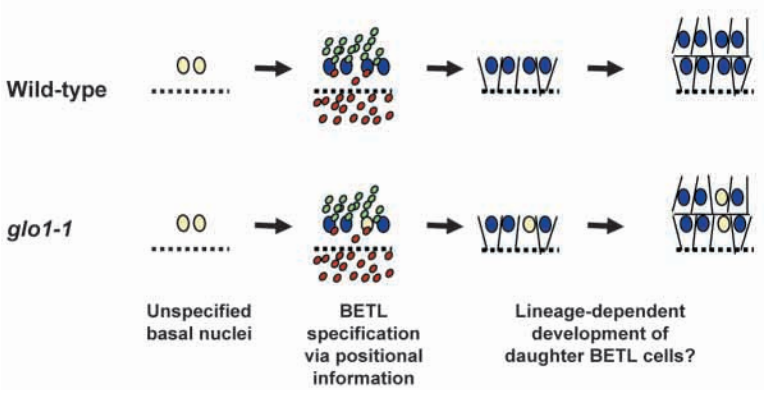


**Fig. 7.** Proposed model for BETL specification in wild-type and *glo1-1* syncytial endosperms. BETL specification of basal nuclei most probably occurs via positional information by unknown cue(s) either ascending from the maternal tissue (red circles), or already present in the syncytium (green circles), or derived from both. Given the abnormal arrangement of *glo1-1* basal nuclei, perhaps not all of them would perceive the positional information to assume BETL identity (blue), hence some nuclei would remain undifferentiated (yellow). Furthermore, we speculate that BETL specification is a determined event (see Discussion); hence, subsequent daughter cells would develop in a lineage-dependent fashion. Broken line indicates endosperm-maternal tissue interface.

(Slocombe et al., 1999), these clusters may have originated from a single nucleus that had initially perceived the developmental cues to assume BETL cell fate. The fact that cells occupying basal positioning in the *glo1-1* endosperm are not able to adopt BETL cell identity, together with evidence that BETL cells are observed in apical regions of endosperms following interploidy crosses (Gutierrez-Marcos et al., 2003), would imply that basal positioning is not strictly necessary for BETL cell fate. This contradicts previous models for BETL cell development, where, according to Becker et al. (Becker et al., 1999), induction of transfer cells is thought to occur either via a signalling mechanism or through mRNA transport present in the basal endosperm region.

### Aleurone cell formation is influenced by internal developmental cues

The plane of cell division is strictly controlled in aleurone cells by preprophase bands (PPB), defining the anticlinal plane of division, thus permitting lateral expansion of the aleurone layer (reviewed by Olsen, 2001). Data presented here revealed perturbations in the organisation of aleurone cells, which were mainly confined to the abgerminal face of *glo1-1* kernels – a pattern prevalent in many other mutants affecting early kernel development (Becraft and Asuncion-Crabb, 2000). These defects included the absence of aleurone cells and/or the presence of additional aleurone cell layers in regions of the *glo1-1* endosperm. Similarly, the *extra cell layers1* (*xcl1*) mutation causes aberrant periclinal divisions in the maize kernel, which results in the formation of extra aleurone layers (Kessler et al., 2002).

Little information exists of how aleurone fate is determined; however, the simplest model holds that aleurone cell fate is acquired through a steep ligand gradient away from the maternal cell wall (Olsen et al., 1998), which is perceived at the endosperm surface by the CRINKLY4 receptor kinase (Becraft et al., 1996). Based on microscopic observations and GUS marker gene expression analysis, our data unexpectedly revealed that aleurone-like cells have the ability to form from within the *glo1-1* endosperm. Thus, aleurone formation cannot exclusively rely upon maternally derived signals. Instead, we suggest that, at least in *glo1-1* endosperms, aleurone identity might be acquired internally, perhaps as a result of signals from within the SE itself. Certainly, studies have demonstrated that the aleurone and SE cells can develop from common progenitors and that neither cell type fates are terminally determined, as aleurone formation is dependent on the constant input of positional cues (Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002; Lid et al., 2002). This developmental plasticity is further supported by our observations: anthocyanin-like pigment (data not shown) and patchy reporter-GUS expression patterns observed in *glo1-1* basal endosperms suggest that the undifferentiated cells often observed within the BETL during early development are capable of assuming aleurone identity at later stages.

*glo1-1* endosperms thus contain two classes of aleurone-like cells, the first are peripherally located and bounded on their 'outward face' by a thickened wall, as in wild-type kernels (data not shown). The second class of cells, despite their location within the SE, are also bounded on one or more faces by thickened cell walls. Assuming differences in permeability between these thickened walls and others of the endosperm, it is possible that short-range cues that trigger aleurone formation may accumulate in these cells. This interpretation is also supported by emerging evidence that cell fate in plants is specified by short-range cell-cell interactions, mediated through signal transduction cascades, as occurs in *Drosophila* and *C. elegans* (Irish and Jenik, 2001; Marx, 1996).

We are indebted to Ian Hempshall and co-workers at Syngenta (Jealott's Hill, UK) for invaluable support and plant growth facilities. Cledwyn Merriman (Wellcome Trust Institute for Human Research, UK) and Judith Sheldon (Oxford Brookes University, UK) are kindly acknowledged for their help with resin sectioning and electron microscopy, as well as John Baker for image processing. We thank the Maize Genetics Cooperation Stock Center (Urbana, IL, USA) for providing the valuable seed stocks and the Maize Mapping Project Team (University of Missouri Columbia, MO, USA) for mapping information. We also thank Dr P. Becraft (Iowa State University, USA) for supplying the promoter-VP1-GUS construct and Dr P. Perez (Biogemma, France) for generation of transgenic plants. The pBET1-GUS seeds were a kind gift from Dr R. Thompson (Max-Planck-Institut für Züchtungsforschung, Germany) and the ZmESR2 plasmid was generously provided by Dr P. Rogowsky (RCAP ENS, Lyon, France). This work was funded by a BBSRC-CASE award and Syngenta.

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