A mutational approach to the study of seed development in maize

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Abstract
The maize seed comprises two major compartments, the embryo and the endosperm, both originating from the double fertilization event. The embryogenetic process allows the formation of a well-differentiated embryonic axis, surrounded by a single massive cotyledon, the scutellum. The mature endosperm constitutes the bulk of the seed and comprises specific regions containing reserve proteins, complex carbohydrates, and oils. To gain more insight into molecular events that underlie seed development, three mono- genic mutants were characterized, referred to as emp (empty pericarp) on the basis of their extreme endosperm reduction, first recognizable at about 12 d after pollination. Their histological analysis reveals a partial development of the endosperm domains as well as loss of adhesion between pedicel tissues and the basal transfer layer. In the endosperm, programmed cell death (PCD) is delayed. The embryo appears retarded in its growth, but not impaired in its morphogenesis. The mutants can be rescued by culturing immature embryos, even though the seedlings appear retarded in their growth. The analysis of seeds with discordant embryo–endosperm phenotype (mutant embryo, normal endosperm and vice-versa), obtained using B–A translocations, suggests that emp expression in the embryo is necessary, but not sufficient, for proper seed development. In all three mutants the picture emerging is one of a general delay in processes related to growth, as a result of a mutation affecting endosperm development as a primary event.

Key words: Embryo, emp mutants, endosperm, maize, PCD, seed development.

Introduction
Cereal grains are the most important sources of foods. The endosperm, a simple tissue surrounding the embryo, stores proteins and carbohydrates during seed development, while the scutellum, an embryonic structure, that is generally considered as functionally equivalent to the cotyledons in dicotyledons (Kiesselbach, 1949; Van Lammeren, 1986; Vernoud et al., 2005), accumulates oils. The recent revival of interest in the study of the genetic control of seed development in cereals is probably due to the opportunity it offers to improve some important traits such as yield and quality. The maize seed consists of the embryo and endosperm, the two products of double fertilization, and of a seed coat, the pericarp, of maternal origin (Kiesselbach, 1949). The embryo follows a developmental pattern characterized by a sequence of phases, the first one where the embryo proper is formed consists of histodifferentiation, followed by the acquisition of polarity and bilateral symmetry and morphogenesis, a maturation phase where reserve food accumulates, dehydration, and quiescence (Kiesselbach, 1949; Vernoud et al., 2005). For the maize endosperm, four developmental phases are recognized: syncytium formation, cellularization, differentiation, and cell death (Olsen, 2001). The differentiated endosperm consists of four major cell types or domains: the starchy endosperm, representing the central bulk; the single-cell aleurone layer covering the periphery of the
endosperm; the embryo-surrounding region, consisting of the cells lining the region where the embryo develops; and the basal endosperm transfer cells involved in the transport of nutrients. Each cell type has distinct morphology and function and is characterized by specific gene expression (Lopes and Larkins, 1993; Olsen, 2001; Bommert and Werr, 2001). During maize kernel development, starchy endosperm cells undergo PCD, a process that is initiated at approximately 16 days after pollination (DAP), of which the pattern and timing of progression is well established and appears to be related to cell age (Buckner et al., 1998). A cell death process also occurs in the placento–chalazal (P–C) cell layers in maternal pedicel tissues, as recently described by Kladnik et al. (2004). Mutants affecting endosperm development represent an important tool for discovering the identities of the corresponding genes and elucidating their roles. Several mutants with defects in the endosperm have been described and are broadly referred to as dek (defective kernel) mutants (Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980).

In these mutants, both embryo and endosperm development is generally altered, but in most cases the developmental lesion has not been identified. The majority of these mutants grow into some form of a plant when germinated as mature seeds or cultured as immature embryos, while a minority lack this capacity. According to Sheridan and Neuffer (1981) they represent nutritional-type and developmental-type mutants, respectively. In addition, the analysis of non-concordant seeds with normal endosperm and mutant embryo or vice versa, obtained by crossing the mutants with pollen of the appropriate B–A translocations, suggests that the developmental fates of the endosperm and embryo are largely independent. In a few cases, however, the results indicate an interaction between the two compartments (Sheridan and Neuffer, 1986). A number of mutants have been isolated which block or alter aleurone differentiation over all or part of the endosperm (cr4, dek1, dil1, dil2, sal1, and Dap) (Becraft et al., 1996; Gavazzi et al., 1997; Becraft and Asuncion-Crabb, 2000; Lid et al., 2002, 2004, 2005; Shen et al., 2003) and their functions, at least for some of them, have been identified. A screening of a large collection of Mutator lines, segregating for maize dek mutants with various morphological defects, led to the observation that aleurone cells are always formed on the external surface of the endosperm. This suggests that the endosperm has an intrinsic developmental programme defining the surface cell layer as aleurone cells (Olsen, 2004). Aleurone cell fate specification was recently investigated by means of a novel maize endosperm in vitro culture method whereby the developing endosperm is completely removed from surrounding maternal tissues. Through this experimental approach, evidence was obtained that aleurone cell fate specification occurs exclusively in response to surface position and does not require specific, continued maternal signals input (Gruis et al., 2006).

In this paper, the isolation and characterization of three novel maize mutants are described, here referred to as empty pericarp (emp), with very reduced endosperm and loose pericarp (Fu et al., 2002; Fu and Scanlon, 2004), representing a subclass of the class of dek mutants (Scanlon et al., 1994). To date, the molecular basis of only one emp mutant (emp2) has been elucidated. emp2 encodes a protein with high similarity to HEAT SHOCK BINDING PROTEIN (HSP1), a negative regulator of the heat shock response (Fu et al., 2002). In the emp mutants described here, in spite of the profound alteration in the endosperm, a partial differentiation of the embryo is achieved. However, germination of the mature seeds is completely blocked. A characterization of these mutants is presented in terms of their genetics, their histology, and their in vitro germination. Data will also be presented on the effect of the mutation on the progression of cell death in the endosperm. This group of mutants is of interest for the analysis of both embryo and endosperm development and of the relationship between the differentiation of the endosperm and the progression of the cell death process.

Materials and methods

Isolation of mutants

The three emp mutants analysed here, emp*-7065, emp*-8075, and emp*-8077, were chosen out of a collection of mutants obtained by transpositional mutagenesis (Consonni et al., 1998). They were originally isolated in the F2 of selfed F1 plants derived by crossing a line maintained in our laboratory as inbred stock for at least four generations with a line carrying active Mu transposons (MuDR). They all behave as single gene recessive mutants in their original background and are propagated as heterozygotes, being lethal in the homozygous condition. Heterozygous +/emp plants, whose genotype was ascertained by selfing, were outcrossed as male parents to the W64A and A188 inbred lines to obtain vigorous F2 ears as source of material for the embryo rescue experiments. Mutant emp/emp seeds segregating upon selfing +/+plants are easily recognizable from normal sibs because of a drastic reduction in size and a pale translucent appearance.

Allelism test and chromosomal arm attribution of the emp mutants

The three mutants under test were crossed inter se in all pairwise combinations to assay their complementation pattern and progeny ears were analysed as previously described (Consonni et al., 2003). Their chromosomal arm location was established by crossing the progeny of outcrossed heterozygous +/emp plants with male parents including the entire set of B–A translocations covering about 90% of the genome. The source of these stocks is the Maize Stock Center, Urbana, Ill. USA. Ears (5–10) from these crosses were then scored for the presence of emp seeds with a frequency of 15–20% as evidence that the translocation is uncovering the mutant under test.

Embryo rescue

Immature F2 ears were left in a Na hypochloride:dH2O (1:1 v/v) solution for 30 min and then rinsed in sterile water. Mutant embryos
on a segregating ear are recognizable because of their reduced size. The small and normal-sized sibling embryos were excised from immature (18, 24, and 36 DAP, Days After Pollination) seeds and cultured on solidified MS medium. After 20 d of culture, germination percentage, as well as seedling elongation, were determined. The germination test on mature embryos was performed by culturing the entire seed since the removal of the embryo from the surrounding tissues is difficult and could impair its integrity.

Histological analyses

For light microscopy, immature wild-type and mutant seeds were removed from the cobs of segregating ears at successive times from 10 DAP to 29 DAP. Sections (8 μm) were stained with safranin-fast green or with DAPI (4, 6-diamidino-2-phenylidone, 1 mM in PBS). Photomicrographs were taken with Kodak Ektachrome 320T film (safranin-fast green) or with Kodak Ektachrome 64T film (DAPI).

DNA extraction and fragmentation analysis

Genomic DNA was extracted from developing wild-type and mutant kernels at 25, 28, 35, and 38 DAP according to a method previously described (Young et al., 1997). Briefly, 20 kernels for each sample were ground in liquid N₂ with a mortar and pestle to obtain a fine powder. 8 ml of extraction buffer (100 mM TRIS-HCl, pH 9.0, 20 mM EDTA, 200 mM NaCl, 1% (w/v) sarcosyl, and 10 μl ml⁻¹ β-mercaptoethanol) were then added, followed by 8 ml of phenol:chloroform (1:1, v/v) and samples were further ground. Samples were then centrifuged and the total nucleic acid was precipitated from the supernatant. The pellet was then dissolved with 800 μl of TE and treated with RNase (DNase-free). For DNA fragmentation analysis, 20 μg of each sample were resolved on 2% agarose gels containing 1× TBE (90 mM TRIS-borate and 0.1 mM EDTA), stained with ethidium bromide and photographed on a UV light box.

In situ detection of DNA fragmentation

To detect DNA fragmentation, the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-fluorescein nick end labelling (TUNEL) procedure was applied, as previously described by Giuliani et al. (2002). A negative control was included by omitting TdT from the reaction mixture. As a positive control, permeabilized sections were incubated with DNase before processing. Bright yellow spots overlapping nuclei (TUNEL positive) indicate the occurrence of DNA fragmentation. On the other hand, nuclei showing only a background green fluorescence are TUNEL negative and are therefore not undergoing PCD.

Results

The genetics of the emp phenotype

The emp mutants analysed here, belong to the class of dek mutants with the most severe reduction in endosperm development and they arise with a frequency of about one out of ten dek, as determined in a sample of dek mutants following chemical mutagenesis (R Pílu, unpublished results). They can be detected on a selfed +/emp ear as early as 10–12 DAP, on the basis of their reduced size and a pale, translucent appearance. Their endosperm has a soft and fluid consistency, whereas their embryo appears smaller than that of wild-type sibs and retarded in its morphogenetic development. Mature mutant seeds appear flattened and smaller than the wild-type sibs since they lack a well-developed endosperm (Figs 1a, b, 4d; see Supplementary Fig. 1d and Supplementary Fig. 2d at JXB online). Hence the ‘emp’ (empty pericarp) symbol applied to similar mutants in the literature. As to the embryo, the analysis of dissected mature seeds under low magnification microscopy indicates that the embryo proper is no longer recognizable. In its place, one can observe a scutellum-like structure in emp*-7065 mutant embryos (Fig. 4d) and a less organized structure surrounded by a cavity in emp*-8075 (see Supplementary Fig. 1d at JXB online) and in emp*-8077 (see Supplementary Fig. 2d at JXB online).

The origin of the three mutants can be traced back to a cross between a line carrying active Mutator elements (MuDR) and the same genetic stock. Their inter se crosses established that they define three separate genes, whereas their crosses with the B–A stocks allowed their attribution to the following chromosomal arms: 1L for emp*-7065, 3S for emp*-8075, and 9S for emp*-8077.

emp mutations affect endosperm and embryo development: For each mutant the histological analysis was performed at two developmental periods: 10–14 DAP and 25–29 DAP, when mutant and wild-type kernels are distinguishable. A sample of seeds were excised from different ears, fixed, and analysed at a histological level. The genotype of each ear was confirmed by looking at its segregation at maturity.

Figure 2 presents the developmental profile of wild-type (Fig. 2a–c) and of emp*-7065 (Fig. 2d–f), emp*-8075 (Fig. 2g–i), and emp*-8077 (Fig. 2j–l) mutant seeds. At 10–14 DAP, in the three mutants the size of the starchy endosperm appears reduced to a variable extent and

![Fig. 1. (a, b) Phenotype of emp mutants. (a) Ear segregating for emp*-7065. Mutant kernels are indicated by arrows. (b) Mature mutant seeds viewed from the germinal side.](https://example.com/fig1.png)
embryo development is delayed (Fig. 2d, g, j) if compared to that of the wild type (Fig. 2a). Embryos appear at the transition–coleoptilar stage, since the embryo proper is separated from the suspensor and the development of the shoot axis is just beginning (Fig. 2d, g, j). At later stages (25–29 DAP), the mutants show a progressive increase in endosperm collapse (Fig. 2f, i, l), but an almost normal embryo development with clearly differentiated leaf primordia in \emph{emp*-8077} mutants (Fig. 2i), embryo at the transition-coleoptilar stage. (l) (25 DAP) Endosperm drastically reduced, aleurone present, embryo at L3-4 stage. (g-i) Mutant \emph{emp*-8075} (g, h) (10 DAP) reduced endosperm and BETL not fully developed (enlarged in h), embryo at the transition stage. (i) (29 DAP) Endosperm drastically reduced, aleurone present, embryo at L3 stage. (j-l) Mutant \emph{emp*-8077} (j, k) (13 DAP) endosperm reduced and incomplete differentiation of BETL (enlarged in k), embryo at the transition-coleoptilar stage. (l) (25 DAP) Endosperm drastically reduced, aleurone present, embryo at the coleoptilar stage. \emph{al} aleurone; BETL, basal endosperm transfer layer; \emph{em}, embryo; \emph{en}, endosperm; ESR, embryo surrounding region; \emph{lp}, leaf primordia; \emph{p}, pericarp; \emph{rp}, root primordium; \emph{s}, suspensor; \emph{sc}, scutellum. Bars=200 \mu m.

Discontinuity between pedicel and basal endosperm

Particular attention was devoted to the anatomy of the region between the basal endosperm and the placenta–chalazal (P–C) layers of the pedicel, the maternal diploid tissue at the base of the seed. With this aim wild-type (Fig. 3a) and mutant (Fig. 3b–d) longitudinal sections from 14 DAP seeds were stained with DAPI. During normal development a gradual loss of nuclei starting at 6 DAP creates the placenta–chalazal layer, consisting of two distinctive subdomains, a nucellar P–C layer and an integumental P–C layer (Fig. 3a). In the corresponding mutant regions (Fig. 3b–d), even though the enucleation process occurs correctly, one can see a withdrawal of the
pedicel from the endosperm, creating a gap between the two regions. P–C cells of the mutants are distinguishable from their wild-type counterpart. The wild-type nucellar P–C layer (Fig. 3a) consists of elongated and compact cells perpendicular to the direction of the solute transport, whereas in mutants (Fig. 3b–d) it consists of irregular, looser, and larger cells. The integumental P–C layer of the mutants is apparently formed by a lower number of cells.

**Mutant emp seedlings, obtained by culturing immature embryos, are not impaired in their morphogenesis**

The failure of emp mutants to germinate at maturity (Table 1), is in accordance with a lack of morphogenesis in the mature mutant embryo. In contrast, the histological picture of earlier developmental stages (at least in emp*-7065 and emp*-8075) (Fig. 2f, i) shows the presence of leaf and root primordia, even though their formation appears retarded in comparison to their pattern in wild-type seeds. To investigate the mutant germinability further, their embryo rescue was attempted at different developmental stages. Embryo cultures were initiated starting from 18 DAP when the mutants are recognizable from wild-type sibs on the basis of their reduced size. Attempts to rescue them at an earlier age, when they are first detectable, were unsuccessful. Mutant embryos of successive ages (18, 24, and 36 DAP) germinated. After 20 d of culture, a small seedling, consisting of a short shoot (0.2–0.9 cm) reaching the coleoptile stage and a primary root (0.2–1.7 cm length), was obtained. Normal seedlings of the same age had developed 3–4 leaves and a primary root (0.2–1.7 cm length), was obtained. Normal seedlings, obtained by culturing immature embryos, are not impaired in their morphogenesis (Table 1). The results of Table 1 show that mutant germination is clearly related to the age of the embryo for up to 24 DAP embryos. At 36 DAP, this trend of increased germination with the embryo age is maintained for one mutant (emp*-7065), whereas the two others show a decrease in their germination rate, that is as expected since the mature seed does not contain a properly organized embryo.

**Analysis of seeds with discordant embryo–endosperm phenotype**

The cross of the appropriate TB-A male parents to +/emp females should yield normal seeds, as well as two classes of non-concordant embryo-endosperm seeds, i.e. hypoploid emp/– embryo and hyperploid emp/emp/+ endosperm (class 1) and the reciprocal combination (class 2). Assuming 100% nondisjunction of the B–A chromosome and 2/3 of preferential segregation, class 1 and 2 should appear with a frequency of 8.3% and 16.6%, respectively, whereas wild-type seeds should amount to 75% (Beckett, 1978). The latter appear with a frequency close to the expected one and show normal development and morphogenesis (Fig. 4a; see Supplementary Fig. 1a and Supplementary Fig. 2a at JXB online). About 200 seeds obtained by crossing +/emp females to heterozygous B–A translocations uncovering the mutant were dissected and scored under low magnification. The observed values for the two classes are 9.0% and 22.7% for emp*-7065, 3.4% and 20.2% for emp*-8075, 8.8% and 20.8% for emp*-8077. Class 1 seeds (emp/– embryo and emp/emp/+ endosperm) are recovered with a frequency that is close to the expected one for emp*-7065 and emp*-8077, but significantly lower for emp*-8075. These seeds have a normal endosperm and a defective embryo with a globular shape and lacking shoot and root primordia, although increased in size when compared with the homozygous mutants (Fig. 4d; see Supplementary Fig. 1d and Supplementary Fig. 2d at JXB online).

Seeds of the second class (emp/emp/– endosperm and emp/+ embryo) are recovered with higher frequency over the expected one in all three mutants. This excess could be due to the presence of defective seeds frequently observed in crosses involving B–A translocations. This

### Table 1. Effect of embryo age on germination (%) of emp*-7065, emp*-8075, and emp*-8077 mutants, and wild-type sibs

<table>
<thead>
<tr>
<th>Embryo genotype</th>
<th>Germination (%) of embryos of increasing age (DAP)</th>
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<tbody>
<tr>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Wild-type</td>
<td>100</td>
</tr>
<tr>
<td>emp*-7065</td>
<td>27.5</td>
</tr>
<tr>
<td>emp*-8075</td>
<td>65.2</td>
</tr>
<tr>
<td>emp*-8077</td>
<td>83.0</td>
</tr>
</tbody>
</table>
class is not homogeneous, and exhibits various phenotypes that can be grouped in three categories each of them present in about the same frequency: lack of embryo, small embryos with partial or suppressed morphogenesis, and small embryos with root and shoot primordia. None of these seeds germinate. A representative embryo of the third category is shown in Fig. 4c; and in Supplementary Fig. 1c and Supplementary Fig. 2c at *JXB* online.

Although root and shoot primordia are clearly visible, the embryo size is reduced when compared with the wild-type one (Fig. 4a; see Supplementary Fig. 1a and Supplementary Fig. 2a at *JXB* online).

The discrepancies observed in the two classes may be due to differences in the rate of non-disjunction or preferential fertilization of the different B–A translocation stocks employed, as well as to differences in their genetic background.

**Mutant endosperm cells die later than wild-type cells**

The progression of programmed cell death (PCD) during *Emp* and *emp* whole kernels development was established on the basis of DNA fragmentation analysis and TUNEL (TdT-mediated dUTP-fluorescein nick end labelling) assay.

**DNA fragmentation**

Internucleosomal DNA fragmentation was examined in both wild-type and mutant tissues at progressive stages of development (Fig. 5a–c). At the first stage analysed (25 DAP), most DNA from wild-type tissues appears to be extensively digested into low molecular weight fragments, whereas only a slight DNA fragmentation is observed in the three *emp* genotypes. In the mutants, the internucleosomal cleavage become more evident at 32 DAP and proceeds until 38 DAP, where an increased DNA degradation is clearly visible. For all genotypes, the comparison between wild-type and mutant patterns of DNA cleavage leads to the same results. Thus DNA fragmentation occurs earlier in non-mutant tissues, in comparison to *emp* endosperm, a conclusion confirmed by the detection of the DNA ladder in *Emp* wild-type endosperm analysed at 15 DAP (Fig. 5d).

**TUNEL procedure: in situ**

Incorporation of labelled nucleotides into DNA strand breaks has been assayed in wild-type and mutant *emp*-7065 longitudinal sections of 18 DAP seeds. TUNEL positive nuclei were detected in wild-type central endosperm as shown in Fig. 6b, whereas in the *emp*-7065 mutant endosperm only a few fluorescent nuclei are visible (Fig. 6c). Figure 6a shows the corresponding wild-type nuclei stained with DAPI. The mutant seed is presented at a lower magnification, in order to show the whole endosperm. The results obtained with these different approaches suggest that endosperm cell death is delayed in *emp* compared with wild-type seeds.

**Discussion**

In spite of the many mutants impairing seed development so far isolated in maize, very few have been thoroughly analysed in terms of tissue and domain organization. Here, three mutants impaired in both embryo and endosperm development are described.

**In the emp mutants both endosperm and embryo development are impaired**

The three recessive mutants here described (emp*-7065, emp*-8075, and emp*-8077) determine similar phenotypes and identify three different genes. In all three mutants the embryos appear retarded and partially

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**Fig. 4.** (a–d) Longitudinal sections of mature seeds obtained by crossing appropriate TB–A male parents to +/emp*-7065 females and of a *emp*-7065/emp*-7065 seed. (a) Wild-type seeds. (b) Hypoploid (*emp/–*) embryo and hyperploid (*emp/emp/+*) endosperm. (c) Hypoploid (*emp/emp–*) endosperm and hyperploid (*emp/+*) embryo. (d) An *emp*-7065/emp*-7065 seed. Bars=1.3 mm.
developed. However, the most evident lesion in all mutants is a drastic reduction in the endosperm size, causing a collapsed defective-kernel phenotype. Hence their classification as 'empty pericarp' (emp) mutants.

The small size of the emp endosperm may be the consequence of a low mitotic index, accounting for a reduction in cell number, and/or of defects in cell expansion and endoreduplication, causing a decrease in cell size. The histological and cytological observation of the endosperm suggests that the smaller mutant size can be due to a reduction in the total cell number, relative to wild-type siblings of the same age. As to the endosperm-specific domains of the mutants, the emp mutations described here clearly affect the basal transfer layer that appears impaired in its development. An elegant analysis of the early events in the syncytial and cellular endosperm of the globby-1 (Costa et al., 2003) mutant of maize, exhibiting an aberrant globular embryo and endosperm morphology, suggests that the specification of BETL cells is an irreversible event occurring during a short period of the syncytial development. Cells in this domain facilitate nutrient import into the maize kernel (Thompson et al., 2001).

Our study indicates that a functionally emp gene is required for the correct development of both seed compartments. This finding is in agreement with the previous study of the emp2 gene that clearly shows its involvement in the progression of both endosperm and embryo development (Fu et al., 2002).

Effect of the mutation on the seed connection to surrounding maternal tissues and on the two seed compartments

A correct development of the seed depends on the maternal tissues of the pedicel, since a close contact between the placento–chalazal cells of the pedicel and the basal endosperm is necessary for the transport of nutrients (Schel et al., 1984). Kladnik et al. (2004) reported that the establishment of a tissue connecting the developing seed with the mother plant is determined by a progressive loss of nuclei and demonstrated the occurrence of two different PCD processes leading to different structural and functional P–C layers. By analogy with conclusions on the miniature1 mutant (Kladnik et al., 2004), it may be supposed that emp alterations in maternal cells, such as an abnormal morphology of the nucellar P–C layer and a reduced number of integumental P–C layers, are controlled by the filial tissues. Metabolic changes in emp BETL may affect the cellular morphology of the pedicel tissues leading to a detachment between maternal tissue and mutant endosperm. Alternatively, the discontinuity might be the primary effect of the mutation, whereas lack of differentiation of the basal endosperm transfer cells may result from poor solute transport.

Mutant embryos emp*-7065 and emp*-8075, even though retarded in their development, still show
differentiation of root and leaf primordia, whereas \textit{emp}*-8077 appears blocked at the coleoptilar stage. At maturity, in all three mutants, the microscopic analyses indicate that the embryo proper is not detectable, as if a ‘regression’ of the embryonic structures occurs during the last developmental stages. In accordance with this observation, embryo rescue experiments indicate that mutant germination is positively correlated to the age of the cultured embryo, up to 24 DAP, in \textit{emp}*-8075 and \textit{emp}*-8077 and up to 36 DAP in \textit{emp}*-7065, whereas total lack of germination is observed at maturity in all three mutants.

One can speculate that, if the arrest in endosperm formation observed in the three mutants is due to a block in the transfer of nutrients from the maternal tissues, the delay and the subsequent arrest in the embryogenetic process may be a consequence of the reduced or retarded passage of nutrients or other metabolites from the endosperm to the embryo.

The results obtained by analysing the progeny of crosses of the mutants with stocks carrying the appropriate B–A translocation indicate that hypoploid mutant endosperms (\textit{emp}/--) grown in the presence of hyperploid non-mutant endosperm (\textit{emp}/\textit{emp}/+/+) exhibit a partial repair in terms of growth, but not of morphogenesis. On the other hand, the analysis of the reciprocal class of seeds with hyperploid embryo (\textit{emp}/+/+ embryo) and hypoploid mutant endosperm (\textit{emp}/\textit{emp}/-- endosperm) show variability in embryo phenotypes ranging from the absence of an embryo to the presence of small embryos with partial morphogenesis.

Altogether these results indicate that the \textit{emp} genes act independently in controlling the morphogenetic pattern of the two seed compartments. However, although a functional \textit{emp} gene is required for embryo development, a normal endosperm can exert a positive influence on embryo’s growth.

\textbf{Timing of endosperm programmed cell death in relation to endoreduplication}

The death of the starchy endosperm cells is an active physiological process which occurs during seed development (Buckner et al., 1998). PCD initiates in the central region and expands towards the periphery (Young et al., 1997; Young and Gallie, 2000), following the same pattern as the endoreduplication process (Schweizer et al., 1995). A coincidence may therefore exist between the timing and progression of endoreduplication and that of cell death. The question arising is whether the two processes are coupled. Our data indicate that mutant cells show a marked delay in the progression of PCD when compared with their wild-type counterparts. These data are in contrast with the observation of Young et al. (1997) on \textit{shrunken2} genotypes, in which premature cell death takes place before the timing of reserve deposition. The difference may be ascribed to the different genetic defect in \textit{sh2}, involving starch synthesis, and to the elevated levels of ethylene found in \textit{sh2} kernels. In conclusion, the findings presented here indicate that an intact interface between maternal and filial tissues as well as the interaction between the two seed compartments are required for a proper seed development. The isolation of the genes corresponding to these mutants and the assignment of their functions should help to unravel the molecular basis of these observations.

\textbf{Supplementary data}

Supplementary data in the form of two figures can be found at JXB online. Fig. 1. Longitudinal sections of mature seeds obtained by crossing appropriate TB-A male parents to +/\textit{emp}-8075 females and of an \textit{emp}*/-8075/\textit{emp}-8075 seed. Fig. 2. Longitudinal sections of mature seeds obtained by crossing appropriate TB-A male parents to +/\textit{emp}*/-8077 females and of an \textit{emp}*/-8077/\textit{emp}-8077 seed.

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\textbf{References}


Kisselbach TA. 1949. The structure and reproduction of corn. Lincoln, NE: University of Nebraska Press.


