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Novel phenotypes and developmental arrest in early *embryo specific* mutants of maize

Thierry Heckel[1], Karine Werner[1], William F. Sheridan[2], Christian Dumas[1], Peter M. Rogowsky[1]

[1]RDP, UMR 9938 INRA-CNRS-ENSL, ENS-Lyon, 46 Allée d'Italie, F-69364 Lyon Cedex 07, France
[2]Department of Biology, UND, PO Box 9019, Grand Forks ND 58202, USA

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Abstract. *Embryo specific* (*emb*) mutants exhibit aberrant embryo development without deleterious effects on endosperm development. We have analyzed five *emb* mutants of maize, which, based on their developmental profiles can be divided into two groups: mutants arrested at early stages and mutants with novel phenotypes. The members of the first group resemble wild-type proembryos and never reach other developmental stages. In the second group the tube-shaped mutants *emb**-*8522* and *emb**-*8535* completely lack apical-basal differentiation, while in mutant *emb**-*8516* a second embryo-like structure arises from the suspensor. The five *emb* mutations analyzed are non-allelic and two of the mutations are very likely caused by insertion of the transposon *mutator*, opening the door for their molecular analysis.

Key words: Embryo development – *Embryo specific* mutants – Mutant (maize, *emb*) – *Mutator* tagging – *Zea mays* (mutants)

Introduction

During plant embryogenesis a single-celled zygote develops into a highly organized multicellular embryo with the basic components of the future plant: shoot apical meristem, root meristem, epidermis, vascular system and in some cases even leaf primordia. Numerous mutants with aberrations in this developmental program have been isolated in genetic model species such as *Arabidopsis thaliana* and *Zea mays* (for recent reviews, see Goldberg et al. 1993; Meinke 1995; Sheridan 1995; Kaplan and Cooke 1997; Laux and Jürgens 1997; Berleth 1998). In *Arabidopsis* the class of *embryo defective* (*emb*) mutants encompasses mutants that will not germinate, that show aberrant seedling structure and that are altered in embryo pigmentation. In maize the same abbreviation *emb* (*embryo specific*) has been restricted to mutants producing non-viable embryos in the presence of a normal endosperm. Attempts to saturate the *Arabidopsis* genome with embryonic mutants led to the mapping of 110 *emb* mutations and to the estimation that approximately 500 genes could potentially cause an *emb* phenotype (Franzman et al. 1995). Several mutations have been analyzed at a molecular level after cloning of the corresponding genes (Castle and Meinke 1994; Shevell et al. 1994; Lukowitz et al. 1996; Hardtke and Berleth 1998; Moussian et al. 1998). In several cases the genes code for putative regulatory proteins that potentially play key roles in embryogenesis, in others the molecular analysis suggests functions not readily explaining the mutant phenotype.

One fundamental question in embryogenesis concerns the process of pattern formation (Jürgens et al. 1994), which defines the spatial relationships of the different parts of the embryo. Contrary to animal systems, cell lineage does not seem to be important in embryonic pattern formation in plants, which is instead regulated by the exchange of positional information between neighboring cells that are often clonally unrelated (Mayer and Jürgens 1998). A comparison of pattern formation between the two major model species, the dicot *Arabidopsis* and the monocot *Zea mays*, is difficult for several reasons. Firstly, in contrast to *Arabidopsis*, cell divisions in young maize embryos follow no obvious geometric pattern and are variable between individuals. Secondly, in maize the embryo axis defined by the shoot apical meristem and the root meristem is oblique to the axis established by the embryo proper and the suspensor. Finally, in maize, dormancy occurs later in the development, leading to the formation of six leaf primordia in the embryo in contrast to *Arabidopsis* where there are no leaf primordia and the embryo is much less mature. Recently a new model for the evaluation of embryo mutants has been proposed that

Abbreviations: AIMS = amplification of insertion mutagenised sites; DAP = days after pollination; PCR = polymerase chain reaction

Correspondence to: P.M. Rogowsky;
E-mail: progowsk@ens-lyon.fr; Fax: 33 (4) 72 72 86 00

is not based on embryo shapes and is applicable to both species (Kaplan and Cooke 1997). In chronological order it proposes the following key processes: formation of the linear proembryo, differentiation into embryo proper and suspensor, initial histogenesis (formation of protoderm, cortical precursor, vascular precursor), meristem organization and initial organogenesis (cotelydon).

Genetic approaches to elucidate these processes in monocots have mainly focused on rice (Hong et al. 1995, 1996) and maize. In maize, two groups of mutants have been relevant to studies of embryogenesis. In the large class of *dek* (*defective kernel*) mutants (Neuffer and Sheridan 1980; Sheridan and Neuffer 1980) the development of both the embryo and the endosperm are significantly altered. Developmental profiles of some *dek* mutants have been established giving interesting insights into embryo morphogenesis (Clark and Sheridan 1986, 1988; Sheridan and Thorstenson 1986). In order to complete the phenotypic description by a molecular analysis, more recent mutant screens for *dek* mutants have focused on insertional rather than chemical mutagenesis (Scanlon et al. 1994, 1997). In the second group of *emb* mutants (Sheridan and Clark 1993) the development of the embryo is profoundly altered without disrupting growth of the endosperm. This group of mutants is potentially more interesting for the isolation of genes involved in pattern formation because the fact that the endosperm develops normally reduces the probability that housekeeping genes are disrupted.

In a transposon tagging mutagenesis, 51 *emb* mutants had been isolated by Clark and Sheridan (1991). We report here the detailed analysis of a subgroup of five mutants that were thought to have developmental blocks at very early stages of embryogenesis after a preliminary characterization at kernel maturity. We present evidence that the five mutations are not allelic, that the mutants never reach the initial histogenesis step (coleoptilar stage) and that at least two of the mutations are caused by insertion of a member of the *mutator* family.

Materials and methods

Plant material. Mutants *emb*-8516*, *emb*-8522*, *emb*-8535*, *emb*-8543* and *emb*-8547* (Clark and Sheridan 1991) were maintained as heterozygotes. They were grown in the field and systematically back-crossed over at least four generations to a genetic stock carrying the *R-scm2* allele (Clark and Sheridan 1991) responsible for anthocyanin coloration of the scutellum of the embryo. To ascertain the presence of the mutation the pollen donors were self-pollinated and scored for ears with a segregation ratio of 3:1 for wild type:*emb* phenotype kernels.

Mapping with TB-A translocation lines. The chromosome arm locations of *emb* mutants were determined with the following B-A translocation lines (Beckett 1978): TB-1Sb, TB-1La, TB-3La-2S, TB-1Sb-2L, TB-3Sb, TB-4Sa, TB-4Lf, TB-5Sc, TB-5La, TB-6Sa, TB-6Lb, TB-6Lc, TB-7Sc, TB-7Lb, TB-9S-4L, TB-9Sd, TB-10Sc and TB-10L (Maize Stock Center, Urbana, Ill., USA). The presence of the translocation chromosome in the pollen used for crosses to *emb* mutants was verified by parallel crosses to appropriate tester lines (Maize Stock Center). For each *emb* mutant, 100 normal-looking kernels were taken from a self-pollinated, segregating ear (back cross 4) and planted. Groups of five of the resulting plants were pollinated by one given translocation line. At maturity a minimum of 100 kernels was scored per ear. The presence of at least eight kernels with an *emb* phenotype was required to conclude that an arm-locating cross had been accomplished. To exclude an arm location the pollinations of all 5 plants per group had to be successful, the parallel test crosses had to confirm the presence of the translocation in the pollen and the ears had to show a maximum of one *emb* kernel per 100 analyzed.

Allelism tests. For each test, nine plants of a given *emb* mutation (statistically 1/3 +/+ and 2/3 +/*emb*) were double-pollinated (Sheridan and Clark 1987): half the ear was self-pollinated, the other half pollinated by pollen of a second *emb* mutant. In each case, three plants were pollinated by the same pollen donor, which was also self-pollinated. Only cases where both the self-pollinated half of the female and the self-pollinated ear of the male showed one quarter *emb*/*emb* kernels were informative (statistically 4/9).

For the double pollination (Sheridan and Clark 1987) the silks were cut 1 cm above the tip of the ear 24 h before pollination. Just prior to pollination a cardboard divider was inserted by a vertical cut in the middle of the regrown silks. One half was pollinated and the silks of the other half cut back to avoid contamination. One day later the second half was pollinated and the silks of the first half were cut. Two weeks after pollination the cardboard divider was replaced by a V-shaped incision in the tip of the ear retracing the same direction. The incision was deep enough (approx. 1 cm) to remain visible throughout harvest and drying. A minimum of 50 kernels was scored in the central zone of each half. A ratio of *emb* phenotype to wild-type kernels of $<2\%$ was taken as evidence for a wild-type zone, a ratio between 20% and 30% as evidence for a segregating zone.

Manipulation of DNA. For DNA gel blots, plant DNA was isolated from young leaf material (Dellaporta 1994), digested with restriction enzymes (Boehringer) and separated on 0.7% agarose gels. The DNA fragments were transferred in 0.4 N NaOH to Hybond N^+ nylon membranes and hybridized according to the instructions of the manufacturer (Amersham). The most-stringent washes were 0.1× saline sodium citrate buffer (SSC) and 0.1% SDS for 15 min at 65 °C. Radioactive DNA probes were obtained with the random-primed DNA labeling kit (Boehringer). The DNA fragments used were internal fragments of different *mutator* elements excluding the inverted repeats (kindly provided by V. Chandler): a 650-bp *Ava*I/*Bst*NI fragment (Mu1), a 320-bp *Sma*I/*Dde*I fragment (Mu2), a 1000-bp *Xba*I/*Hin*dIII fragment (Mu3), a 650-bp *Eco*RI/*Tth*111I fragment (Mu4), a 800-bp *Sal*I fragment (Mu5), a 360-bp *Eco*RI/*Ava*I fragment (Mu6), a 160-bp *Eco*RI/*Bst*XI fragment (Mu7), a 730-bp *Pst*I/*Sal*I fragment (Mu8) and a 1400-bp *Eco*RI/*Bam*HI fragment (MuDR).

For the amplification of insertion mutagenised sites (AIMS) technique (Frey et al. 1998) the DNA was cut with *Tru*9I (Promega), an isoschizomer of *Mse*I allowing the use of the described *Mse*I adapter and *Mse*I selective primer. The biotinylated Mu-specific primer (GAGAAGCCAACGCCA**W**CGCCTC**Y**AT TTCGTC) and the Mu nested primer (TC**Y**ATAATGGCAA TTATCTC) were degenerated in additional positions (bold letters). Reamplified AIMS products were cloned and sequenced using the pGEM-T Easy Vector System (Promega) and the PRISM ready reaction AmpliTaq FS kit (Perkin Elmer), respectively.

Co-segregation analysis. In the first instance the co-segregation analysis was performed on pools of DNA. Starting with 30 normal-looking kernels (statistically 1/3 +/+ and 2/3 +/*emb*) of a self-pollinated ear of backcross 4, for each *emb* mutant the genotype of the resulting plants was determined by scoring the ears obtained by self pollination. Heterozygous +/*emb* plants were identified by the presence of at least 20% *emb*/*emb* kernels, homozygous +/+ plants by the absence of such kernels. Equal amounts of DNA extracted previously were mixed to generate DNA pools of all +/

+ plants and all +/*emb* plants. Both DNA pools were digested with *Sst*I or with *Eco*RI and *Hin*dIII and probed successively with probes specific of the eight mutator elements. If a band was present in the mutant pool and absent in the wild-type pool, the DNA gel blot was repeated with individual DNA samples. Similarly, the AIMS technique (Frey et al. 1998) was in the first instance applied to DNA pools. Bands specific to the +/*emb* pool were re-amplified, cloned and sequenced. A primer turned towards the *mutator* element was designed in the AIMS product and the individuals were tested by standard polymerase chain reaction (PCR) using this primer and the Mu nested primer. With either one of the two techniques, co-segregation was established when all individual +/*emb* DNA samples contained the band, while it was absent in all +/+ DNA samples.

Preparation of cytological sections. Using the same plants as for the co-segregation analysis, for each *emb* mutant five plants were chosen at random. At 9 days after pollination (DAP) the upper third of the ear was harvested by a horizontal cut through the protecting husk leaves. The procedure was repeated at 16 DAP for the middle third, while the bottom third was left on the plant. The immature kernels were removed from the harvested part, fixed in *p*-formaldehyde and dehydrated as described previously (Opsahl-Ferstad et al. 1997). At maturity the remaining third of the ear was scored and +/*emb* plants identified by segregation of the respective *emb* mutation on self-pollinated ears. Only the fixed kernels of heterozygous plants were embedded in paraffin, sectioned and stained (Opsahl-Ferstad et al. 1997).

Results

Isolation and propagation of mutants. Out of a group of 51 *emb* mutants (Sheridan and Clark 1993), 5 *emb* mutants with developmental blocks at early stages of development were chosen for further analysis. According to a preliminary classification, at kernel maturity the development of mutants *emb**-*8516*, *emb**-*8522*, *emb**-*8535*, *emb**-*8543* and *emb**-*8547* was arrested before the coleoptilar stage. Since the mutations had been isolated in active *mutator* stocks it was important to create neutral genetic backgrounds prior to a detailed phenotypic or co-segregation analysis. Consequently, the five mutants were systematically back-crossed to the colored stock Rscm2 carrying the *R-scm2* allele responsible for anthocyanin coloration of the scutellum of the embryo as well as of the aleurone layer. All subsequent experiments were performed with plants back-crossed at least four times to the respective recurrent parent.

Mapping of emb mutations to chromosome arms. Since the five selected *emb* mutants had similar phenotypes, it was important to determine the number of genes affected. Two of the mutations had been mapped to chromosome arms during the original characterization (Sheridan and Clark 1993). In new experiments we used seeds of self-pollinated ears rather than crosses to increase the frequency of informative +/emb heterozygotes over +/+ wild-type plants. All five *emb* mutants were crossed by the "basic set" of B-A translocation lines. However, not all crosses were informative because in some cases the parallel cross to tester lines was not successful.

Our results allowed the mapping of *emb**-*8543* to chromosome arm 10S and of *emb**-*8547* to chromosome arm 6L (Table 1). In the second case the position was in conflict with the original position on 4L (Sheridan and Clark 1993). This could be due to the presence of two *emb* mutations in the original material or to a secondary insertion of a *mutator* element during one of the back crosses. No chromosome arm location was achieved with *emb**-*8516* or *emb**-*8535*. This could be explained by the experimental limitations cited above or by a location proximal to the chromosome breakpoints of the translocation lines. However, for both mutations a location to the parts of 9S and 10S covered by the respective translocation lines could be excluded due to the absence of an *emb* phenotype and co-comitant positive results of the crosses to tester lines. Taken together, the results showed that at least three different loci were affected in the five *emb* mutants.

Allelism tests. Allelism tests were performed to gain further insight into the number of different genes mutated in the five *emb* mutants. Putting together the results of crosses in both directions, informative results were obtained for 6 of the 10 combinations possible (Table 1). In all cases the two mutations in question were non-allelic. Some tests were not carried out due to differences in flowering time. Others were not conclusive because none of the crosses involved two heterozygous

Table 1. Characteristics of *emb* mutants

Mutant	Chromosome arm location	Allelism tests	Tagging by mutator	Phenotype
*emb**-*8516*	not located (not 9S, not 10S, no data 6L)	not *emb**-*8535*, not *emb**-*8547*	yes	second structure
*emb**-*8522*	9S	not *emb**-*8535*, not *emb**-*8543*	yes	uniform tube
*emb**-*8535*	not located (not 9S, not 10S, no data 6L)	not *emb**-*8516*, not *emb**-*8522*, not *emb**-*8543*, not *emb**-*8547*	not found	uniform tube
*emb**-*8543*	10S	not *emb**-*8522*, not *emb**-*8535*	not found	block
*emb**-*8547*	6L	not *emb**-*8516*, not *emb**-*8535*	not found	block

+/*emb* plants. The results allowed allelism between the two mutations not mapped to chromosome arms and the other three mutations to be excluded. Therefore five different genes were affected in the five mutants.

Developmental profile of emb mutants. The original description and classification of the five *emb* mutants was based on light microscopy of the mutant embryos at maturity. To gain further insight into the developmental aberrations, cytological sections of early developmental stages were analyzed. Five sister plants (statistically 1/3 +/+ and 2/3 +/*emb*) were self-pollinated, and at 9 DAP and 16 DAP, 20 kernels were chosen at random and fixed. The genotype of the plants was determined at maturity and all kernels of mutant ears were embedded in paraffin and sectioned. This technique allowed observations of embryo size and shape (Fig. 1A–L) and, at higher magnifications, of cell size and type (Fig. 1M–R). The phenotypes observed were homogenous between kernels of the same ear as well as between sister plants. Wild-type controls showed that under our growth conditions the formation of the linear proembryo, the differentiation into embryo proper and suspensor, the formation of the protoderm and the formation of the coleoptile primordium had been accomplished at 9 DAP (Fig. 1A,M). In addition, the shoot apical meristem, the root meristem, several leaf primordia and the vascular system were clearly distinguishable at 16 DAP (Fig. 1G).

In mutant *emb*-8516* two embryo-like structures were found, both of which were retarded in their development (Fig. 1B,H,N). Observation of adjacent sections suggested that the separation of the two structures was already present at 9 DAP. The separation was clearly visible at 16 DAP when both structures showed an apical-basal differentiation into a roundish upper part and a tubular lower part reminiscent of the division into embryo proper and suspensor. However, with the exception of the partial formation of a protoderm in the upper structure (Fig. 1N), all the other attributes seen in wild-type controls at the same stages (Fig. 1A,G,M) were missing.

Mutants *emb*-8522* (Fig. 1C,I,O) and *emb*-8535* (Fig. 1D,J,P) formed a tubular structure of seemingly undifferentiated cells. While at 9 DAP a handful of smaller cells was occasionally observed at the apical end, at 16 DAP no apparent size difference was noticed between apical and basal cells (Fig. 1O,P). Between 9 DAP and 16 DAP the structures increased in size without showing any of the developmental characteristics seen in wild-type controls.

The phenotypes of the two mutants *emb*-8543* (Fig. 1E,K,Q) and *emb*-8547* (Fig. 1F,L,R) were similar. Proembryo-like structures with small apical and large basal cells were found at 9 DAP and 16 DAP. The formation of an embryo-proper-like bulb at the top of the embryo was observed in some individual cases but could not be generalized for any of the two mutations.

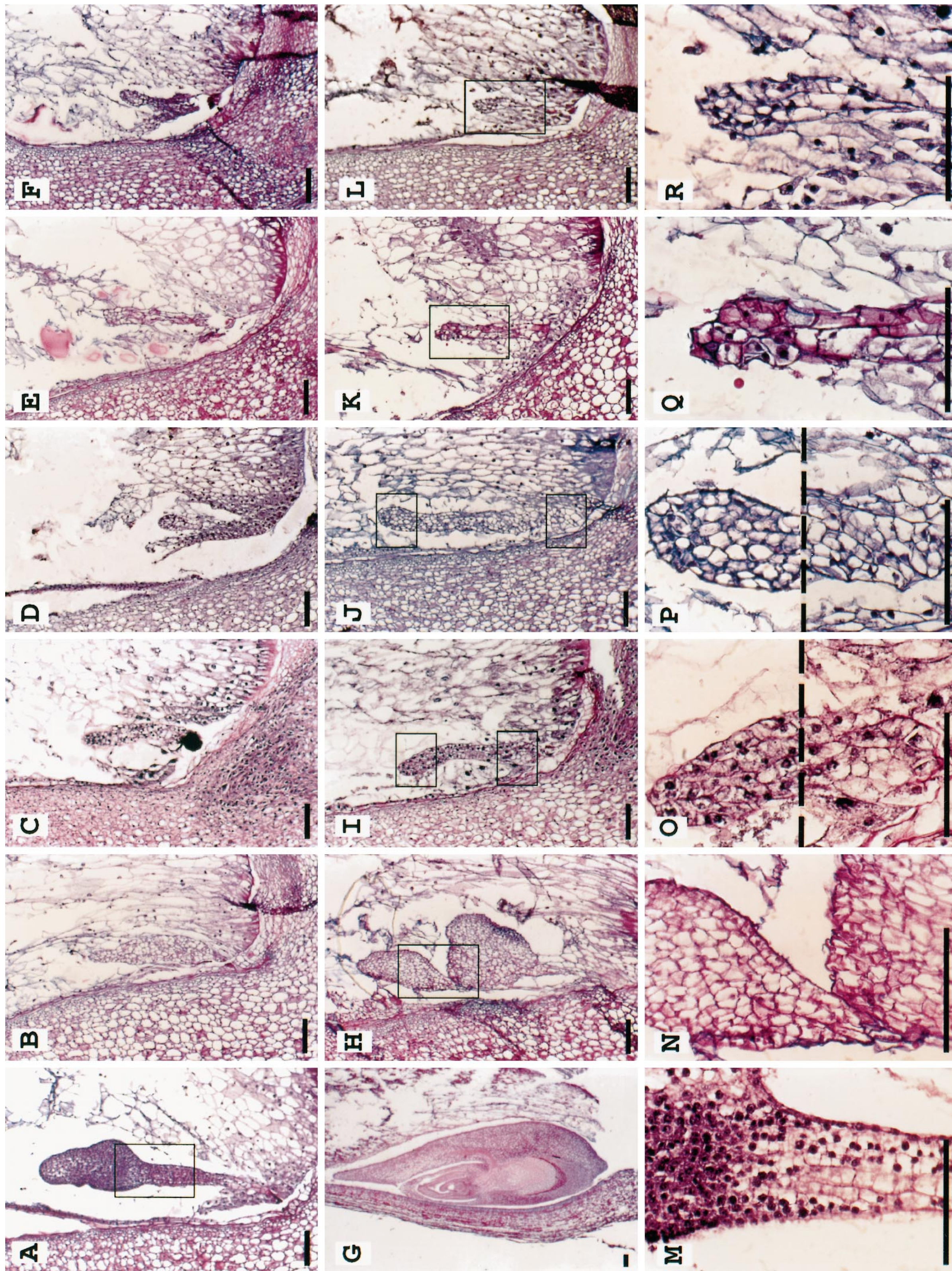
Co-segregation analysis. In maize the cloning of mutant alleles and their subsequent molecular analysis requires insertional rather than point mutations. Since the five *emb* mutants had been isolated from active Robertson's *mutator* maize stocks, they were likely to be caused by the insertion of one of the eight transposons that constitute the *mutator* family (Chandler and Hardeman 1992). To establish a genetic link between the *emb* phenotype and particular *mutator* bands in DNA gel blots, a co-segregation analysis was undertaken. It was carried out using at least back cross 4 to stock Rscm2 and population sizes ranging from 14 to 26 individuals.

The genotype (+/+ or +/*emb*) of all individuals of the population was determined, then DNA pools of all +/+ and of all +/*emb* plants of each population were analyzed by DNA gel blot using two combinations of restriction enzymes and eight different *mutator* probes. In six cases a clearly distinguishable candidate band, which was present in the +/*emb* pool and absent in the +/+ pool was found. After repetition of the DNA gel blot with individual DNA preparations co-segregation was confirmed in two of the six cases. A 2-kb *Eco*RI/*Hin*dIII band obtained with the MuDR probe co-segregated in a population of 21 individuals with the phenotype of mutant *emb*-8516* and a 5-kb *Eco*RI/*Hin*dIII band obtained with the MuDR probe co-segregated in a population of 22 individuals with the phenotype of mutant *emb*-8522* (Fig. 2).

Since the DNA gel blots gave a low resolution of potentially interesting bands, we used in parallel the recently developed AIMS technique (Frey et al. 1998) to increase the resolution of the often numerous bands obtained with *mutator*. The technique was applied to the same +/+ and +/*emb* DNA pools used for the DNA gel-blot analysis. Distinct profiles were observed for every mutant as well as several candidate bands present in +/*emb* pools and absent in +/+ pools (Fig. 3A). Rather than testing the individuals with the labor-intensive AIMS technique, a conventional PCR approach was chosen. A total of four candidate AIMS bands was re-amplified, cloned and sequenced. A primer was designed in the flanking sequence and used in conjunction with the "Mu nested" primer situated in the inverted repeats of *mutator*. The PCR products were separated on agarose gels and hybridized to the corresponding cloned AIMS products. Perfect co-segregation was only found with mutant *emb*-8516*. In a population of 21 individuals a 89-bp band was present in all +/*emb* plants and absent in all wild-type plants (Fig. 3B). It is likely that this PCR product detected the same *mutator* element observed earlier as a 2-kb *Eco*RI/*Hin*dIII band in DNA gel blots.

To confirm the co-segregations found for mutants *emb*-8516* and *emb*-8522*, larger segregating popula-

Fig. 1A–R. Developmental profile of five *emb* mutants. Longitudinal sections of maize caryopses were stained using the periodic acid-Schiff procedure, counterstained with haematoxylin and photographed. Sister caryopses with wild-type (**A,G,M**) or mutant (**B–F,H–L,N–R**) phenotypes of mutants *emb*-8516* (**B,H,N**), *emb*-8522* (**C,I,O**), *emb*-8535* (**D,J,P**), *emb*-8543* (**E,K,Q**) and *emb*-8547* (**F,L,R**) were analyzed at 9 DAP (**A–F,M**) and 16 DAP (**G–L,N–R**). Bars = 100 μm. The parts enlarged in panels M–R are indicated by *black rectangles* in the respective panels above



tions of subsequent generations were analysed by PCR or DNA gel blot, respectively. By pooling all the results obtained for a particular mutant, perfect co-segregation was observed on 101 progeny of mutant *emb**-*8516* and on 79 progeny of mutant *emb**-*8522*.

Discussion

We present the genetic, phenotypic and molecular characterization of five mutants in early maize embryo development. These mutants represent five different

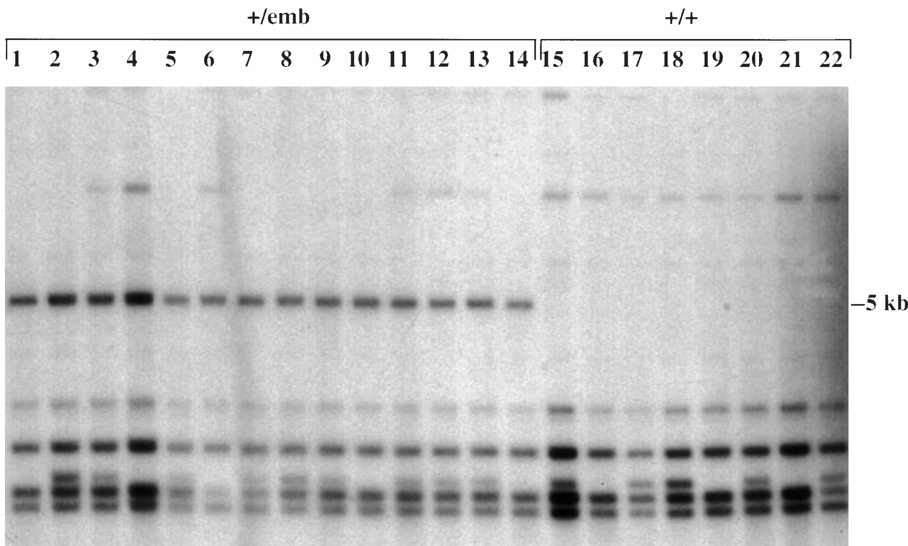


Fig. 2. Co-segregation analysis of mutant *emb**-*8522* by DNA gel blot. Genomic DNA of heterozygous +/*emb**-*8522* plants (*lanes 1–14*) and wild-type +/+ sister plants (*lanes 15–22*) was digested with *Eco*RI and *Hin*dIII, blotted and hybridized with a radioactively labeled 1.4-kb fragment specific to MuDR. The size of the band co-segregating with the *emb**-*8522* mutation is indicated

genetic loci and cause several clearly distinguishable phenotypes. We show that cloning of the mutated genes should be possible in at least two cases where genetic linkage has been established between the *emb* phenotype and DNA gel bands corresponding to specific *mutator* insertions.

Genetic analysis. Five different genes are affected in the five *emb* mutants under investigation. This result, based on chromosome-arm-location experiments and pairwise allelism tests further substantiates the current view that many different genes are involved in the early steps of plant embryogenesis. The fact that an *emb* phenotype can be caused by mutations in many different genes makes it difficult to screen for additional mutant alleles of the existing loci and thus to clarify gene function. However, after cloning of the affected gene the reverse genetics approach makes it possible to circumvent this potential problem.

Molecular characterization. We have shown that at least two of the five *emb* mutations (*emb**-*8516* and *emb**-*8522*) are likely linked to transposon insertions as expected, since stocks with active copies of the transposon *mutator* were used for the mutagenesis. Therefore, the cloning of the affected loci should be possible in the near future. While it is clear that the co-segregation data obtained so far need to be confirmed in larger populations and/or subsequent generations, the present numbers are very encouraging with respect to a molecular characterization. Molecular data will complement the phenotypic descriptions above, allowing a better understanding of the function and mode of action of the two genes in question.

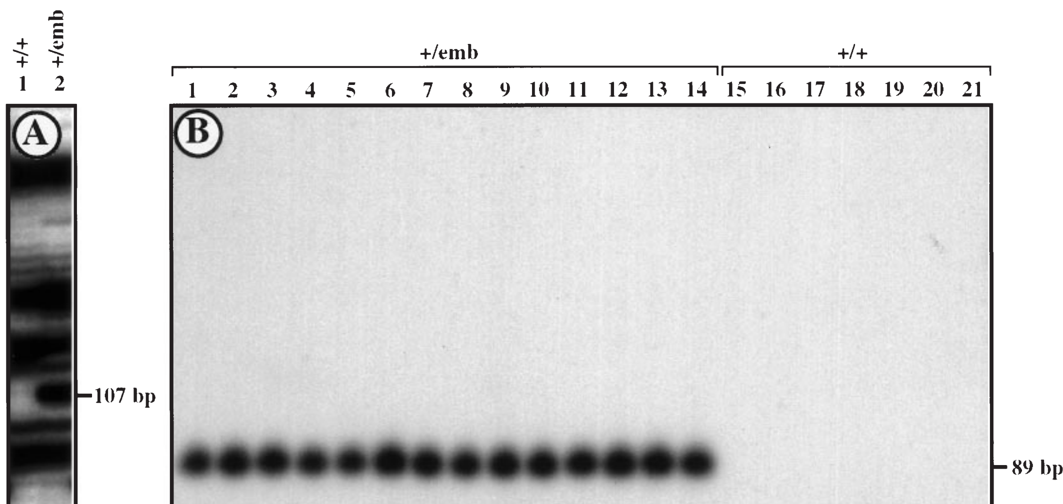


Fig. 3A,B. Co-segregation analysis of mutant *emb**-*8516* using the AIMS technique. **A** Banding pattern of the AIMS reaction. The products of the AIMS reactions performed on DNA pools of mutant *emb**-*8516* (*lanes 1, 2*) were separated on a 6% acrylamide gel. **B** DNA gel blot of PCR on individual plants. Genomic DNA of heterozygous +/*emb**-*8516* plants (*lanes 1–14*) and of wild-type sister plants (*lanes 15–21*) was amplified with primers "8516C" (located in the cloned AIMS product) and "Mu nested" (located in the inverted repeat). The PCR products were separated on a 2.5% agarose gel, blotted to Nylon membranes, hybridized with the cloned AIMS product and autoradiographed

It is not clear whether the remaining three mutations are caused by transposon insertion. Using both the classical DNA gel blot technique and the recently developed AIMS technique (Frey et al. 1998), no evidence for co-segregation has been found. This does not exclude the possibility of *mutator* insertion causing these mutations, because experimental limitations of both techniques make it difficult to visualize and score gel bands corresponding to every single *mutator* insertion. Other explanations include biological phenomena such as the imprecise excision or the silencing of *mutator* elements.

Mutant phenotypes. Two groups of mutants could be distinguished: mutants resembling wild-type embryos arrested at early developmental stages and mutants exhibiting features never seen during normal development. Systematic back crosses to standard lines over at least four generations allowed us to perform our experiments on vigorous material where the contribution of secondary mutations unlinked to the *emb* phenotype was probably greatly reduced. The development of wild-type maize embryos has been the subject of several detailed descriptions at the light-microscope (Randolph 1936; Abbe and Stein 1954) and electron-microscope levels (van Lammeren 1986). The mutant embryos of *emb**-*8543* and *emb**-*8547* found at 9 DAP strongly resemble younger wild-type embryos (3–4 DAP). They show an apical-basal differentiation with smaller apical and larger basal cells. The phenotype among sister kernels is variable with the transition between the upper and lower part being more or less marked, in some cases giving the impression of a true differentiation into suspensor and embryo proper. Between 9 DAP and 16 DAP no further development occurs, although the structures sometimes increase slightly in size. The phenotype of mutant *emb**-*8543* is thus consistent with that previously observed in mature kernels (Clark and Sheridan 1991; Sheridan and Clark 1993). This is not the case for mutant *emb**-*8547* (Clark and Sheridan 1991) whose suspensor thickening found at kernel maturity was not corroborated by our analysis of earlier stages. The explanation for this discrepancy may lie in the different chromosome arm locations obtained in the two studies, which suggest that the phenotypes described in the original mutant and its back cross 4 (this study) are caused by two different mutations. As in all the other mutants the parallel development of the endosperm is cytologically normal, suggesting that the mutations either do not affect simple housekeeping genes or that if housekeeping genes are affected, they are present in more than one copy and are differentially expressed in the embryo and in the endosperm. The two genes seem to be necessary for the progression of embryo development beyond the proembryo stage and could be involved in initial histogenesis and/or meristem formation.

The second group of *emb* mutants is characterized by aberrant development never seen during normal wild-type embryogenesis. For example the tubular structures seen in mutants *emb**-*8522* and *emb**-*8535* are different from proembryos because there is no difference in cell size between the upper and the lower end of the embryo. This becomes more obvious at 16 DAP when, in addition, the cells become slightly larger than wild-type suspensor cells. Since this phenotype can only be seen in cytological sections, it is understandable that it was confused with a simple arrest during the earlier microscopic description at kernel maturity (Clark and Sheridan 1991). Similarly to some *Arabidopsis* mutants in apical-basal pattern formation (Mayer et al. 1991) mutants *emb**-*8522* and *emb**-*8535* do not show any differentiation into embryo proper and suspensor. The tubular rather than spherical or irregular shape of the mutant embryo is very intriguing. It is an indication of preferential overall growth in one direction and implies the presence of some "up" or "down" information in the absence of a cytological differentiation. The mutant phenotypes corroborate the present working hypothesis that apical-basal differentiation, which may be laid down as early as in the zygote, precedes the differentiation into suspensor and embryo proper. They also show that neither the suspensor nor the embryo proper represent a "default" program because the tubular structures cytologically resemble neither one. The genes are likely involved in the very early steps necessary to translate the up/down information into embryo proper and/or suspensor morphogenesis.

In mutant *emb**-*8516* a second, roughly spherical structure develops from the suspensor, which at first sight can be interpreted either as a second embryo or a simple thickening of the suspensor. Somewhat similar phenotypes have been described in three groups of *Arabidopsis* mutants: *twn* (Vernon and Meinke 1994), *sus* (Schwartz et al. 1994) and *raspberry* (Yadegari et al. 1994). However, the developmental defects of the two structures, in which the irregularly shaped embryo proper shows only partial histogenesis and no meristem formation at 16 DAP, are much more severe than in *twn*, where the two embryos are viable. A comparison with the large and heterogenous group of *sus* and *raspberry* mutants is more difficult due to the differences in embryo development between *Arabidopsis* and maize and a final conclusion concerning the relation between *sus* and *raspberry* on one hand and *emb**-*8516* on the other will only be reached after the cloning of the corresponding genes in both species. The interesting phenotype with two embryo-like structures suggests that the gene might directly or indirectly interfere with suspensor identity.

The present study shows that the phenotypes of *emb* mutants in maize are varied and that they complement the knowledge gained by the analysis of *Arabidopsis* mutants. Where possible, a molecular study will be undertaken to furnish additional pieces to the jigsaw puzzle of plant embryogenesis.

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