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New embryo lethals in *Arabidopsis thaliana*: basic genetic and morphological study

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Abstract

Six different mutations with defects in immature seed development have been identified during screening of a T-DNA collection of *Arabidopsis thaliana*. The mutations were confirmed to be monogenic and recessive-lethal by genetic analysis. Mutant embryos were blocked in certain steps in the process necessary for embryo viability and development, and therefore they belong to the embryo-lethal class of mutants. The genetic and morphological studies of T-DNA mutations affecting embryo development are presented. The youngest embryos with a defect were observed at the globular stage in the VIII-64 mutation. Externally located cells, precursor of the protoderm, were characterised by abnormal cell division. VIII-41 mutation with a defect at the late globular stage was arrested at the globular-heart stage transition. VIII-111 mutation showed defect at heart stage of embryogenesis with atypical development of cotyledon primordia. The defect was associated with abnormal pattern of cell division constituting the precursor of the shoot apical meristem. In VIII-82 mutation defect in torpedo stage with asymmetric cotyledons was observed. Cotyledon stage of embryos and chlorophyll defect were observed in VIII-75 mutant. Abnormal suspensor consisting of two columns of cells was observed in 280-4-4 mutation. Newly identified embryo-lethals can serve as starting material for more detailed genetic and molecular studies.

Introduction

Embryogenesis in higher plants, that is, embryo development, begins with the fertilisation of the egg cell and zygote formation and ends with embryo germination resulting from terminal meristem activity. Plant embryos are morphologically simple but on molecular level complex (Howell, 1998). Developing embryo of most cotyledonous angiosperms proceeds through a series of shape changes, going from zygotic to preglobular, through globular, heart and torpedo to the mature cotyledon embryo. Many genes are expressed during this cascade process in a highly co-ordinated manner to ensure that the zygote develops into an organised multicellular structure. Genetic approaches have been particularly useful for identifying important genes involved in embryo development. Much of the work in this field has been done using *Arabidopsis thaliana* L. (Heynh.) as a model for dicotyledonous species. Genetic analysis of embryogenesis in plants has revealed a great number of genes responsible for the formation of viable seeds. Jürgens et al. (1991) estimated that about 4000 genes are required for normal embryo development.

Mutagenesis with classical and biological mutagens has revealed two types of embryo mutants. The first one affects patterning in embryo development and the other one is embryo-lethal mutants. Embryo-lethal (*emb*) mutations represent the most common heritable defect identified after mutagenesis in *Arabidopsis* that interferes with embryo development (Franzmann, Yoon & Meinke, 1995). Except for *Arabidopsis* (review McElver et al., 2001), embryo-lethal mutations have been described in maize (Clark & Sheridan, 1991) and petunia (Souer et al., 1996). In *Arabidopsis*, *emb* mutations

represent 3–5% of T-DNA mutagenised population (Despres, Delseny & Devic, 2001). Franzmann, Yoon and Meinke (1995) presented the chromosomal locations of 110 *emb* genes, 26 of them were tagged with T-DNA. Approximately 250 embryo-defective mutants of *Arabidopsis* have been isolated and characterised in the Meinke laboratory at Oklahoma State University (Meinke, 1994; http://mutant.lse.okstate.edu/embryopage/emb_list.html). Recently, 354 T-DNA tagged mutants defective in embryo development have been identified in a project aimed at saturation mutagenesis of *EMB* genes in *Arabidopsis* (McElver et al., 2001).

In *Arabidopsis* the number of *EMB* genes with essential and non-redundant functions is estimated at approximately 500 (Franzmann, Yoon & Meinke, 1995; Despres, Delseny & Devic, 2001). *emb* seeds are phenotypic uninformative as to the role of the corresponding *EMB* genes in embryogenesis and display only defective development. Embryo-lethal mutants are blocked in a particular step of the process necessary for embryo viability and development and are maintained as heterozygotes. Mutants have defects in important developmental regulators or in simple metabolic functions (Howell, 1998). There are two particular modes necessary for signalling in the embryo, long-range signals *via* auxin and cell wall components. Each plays an important role in co-ordination of gene expression (Souter & Lindsey, 2000). Despite the availability of a large number of mutants, very few *EMB* genes have been molecularly characterised and cloned so far (Shevell et al., 1994; Meinke, 1995; Tsugeki, Kochieva & Fedoroff, 1996; Zhang & Somerville, 1997; Grossniklaus et al., 1998; Patton et al., 1998; Uwer, Willmitzer & Altmann, 1998; Albert et al., 1999; Apuya et al., 2001).

Detection of new mutants in embryo development is required for subsequent genetic and molecular analysis. In our work, six mutants with defects in immature seed development have been identified during screening of T-DNA collection of *A. thaliana*. Here we present the genetic and morphological study of T-DNA mutants affecting embryo viability. The work is aimed at identification of genes acting during embryo development.

Materials and methods

Arabidopsis *lines and growth conditions*

All T-DNA lines used in this study were obtained from C. Koncz, MPI Kőln, Germany. *A. thaliana* plants of Columbia ecotype (Col) were transformed by *Agrobacterium tumefaciens* strain carrying the transformation vector named pTAc1 constructed by L. Szabados (personal communication) with the constitutively active 35S promoter of cauliflower mosaic virus (CaMV) and four copies of an enhancer element from the 35S CaMV at the right T-DNA border. In all mutants the *hygromycin phosphotransferase* (*hpt*) selectable gene was included in the T-DNA. Root transformed plants were marked T_0, hygromycin resistant plants selected in T_0 progeny – T_1, progeny of a T_1 selfed plant – T_2. Six embryo lethal mutations were identified (VIII-41, VIII-64, VIII-75, VIII-82, VIII-111, 280-4-4) during screening of T_1 and T_2 families.

Plants were grown to maturity in a controlled climate cultivation chamber, at 20–22°C and irradiation of 70 $\mu mol\, m^{-2}\, s^{-1}$, under 16 h light/8 h dark cycles.

Genetic analysis of mutants

Embryo-lethal mutations were determined in T_1 and T_2 generations of hygromycin resistant plants by the presence of siliques containing normal and defective embryos in seeds and were observed in a 3:1 ratio (Műller, 1963). Three middle contiguous siliques from each T_1 plant were scored for embryo-lethal mutations before the seed coat of normal seeds became brown (Gichner et al., 1994). The seeds from each positively determined heterozygous mutant plant were separately harvested and in T_2 progeny of each mutant plant a 3:1 ratio was verified for at least 10 heterozygous plants. A 1:2 ratio of standard homozygotes and heterozygotes was tested among the progeny of heterozygous plants in T_2 generation (30–60 plants). In immature siliques phenotypes of embryos in seeds were observed and scored under a Nikon SMZ-2T dissecting microscope.

Microscopic study

Embryos of mutant and wild-type seeds in different stages of development were dissected from immature siliques and a clearing treatment was used (Mayer et al., 1991). Immature seeds were transferred to Eppendorf tubes and fixed in 96% ethanol and acetic acid (3:1, v/v) for 2 h at room temperature. After washing three to four times in 96, 70, 30% ethanol and finally in distilled water, the immature seeds were cleared in chloral hydrate, water and glycerol (8:3:1, w/v/v) for 30 min to 1 h at room temperature and older stages of seeds were cleared at 60°C. For embryos at globular

stage of development or younger ones, the protocol described by Motamayor et al. (2000) was applied. The pistils were fixed in formaldehyde, propionic acid and 50% ethanol (5:5:90, v/v) for 3 h at room temperature. A mixture of 85% lactic acid and phenol (2:1, w/w) was used for clearing. A differential interference contrast (DIC) Olympus BX-60 microscope was used after clearing treatment. Photographs were taken using Olympus camera and Lucia 4.21 software. At least 60 mutant seeds were analysed for each mutation.

Results

Genetic analysis of mutants

After T-DNA mutagenesis, embryo-lethal mutations were screened by embryonic test (Müller, 1963). Six embryo-lethal mutations were determined by the presence of siliques containing wild-type and defective embryos in seeds in a 3:1 ratio ($N > 500$, Table 1). Standard homozygotes and heterozygotes in a 1:2 ratio were observed among the progeny of heterozygous plants in consequence of recessive homozygous lethality ($N > 50$). Thus, the mutations were confirmed to be monogenic and recessive-lethal as determined by χ^2 test for segregation ratios ($P > 0.05$). The only exception was VIII-64 mutation where more mutant seeds in siliques appeared than expected in monogenic segregation ratio.

Microscopic study

The immature seeds in wild-type Columbia ecotype were green (Figure 1), unlike the mutant immature seeds that remained white, and eventually shrivelled and turned brown (Figures 2(A)–7(A)). The phenotypes of mutant embryos were described after whole-mount clearing of immature seed tissues (Figures 2(B)–7(B)) and compared with wild-type ones (Figures 2(C)–7(C)).

Table 1. Segregation analyses of single tested embryo-lethal mutations

Mutation	Wild-type:mutant[a]	χ^2(3:1)
VIII-64	1647:717	35.80*
VIII-41	2839:951	0.01
280-4-4	437:150	0.10
VIII-111	1831:628	0.39
VIII-82	2371:823	0.25
VIII-75	1219:443	2.43

[a] No. of seeds in heterozygous plants.
* $P < 0.05$.

The youngest embryos with a defect were observed at the globular stage in VIII-64 mutation. The layer of externally located cells, precursor of the protoderm, was characterised by abnormal cell division (Figure 2(B)). As a consequence of this fact the cells showed various size and shape. Endosperm appeared to be hydrolysed early in this stage.

In VIII-41 mutation, defect was observed at the late globular stage, when the radial symmetry of the embryo was transformed into a bilateral symmetry by cotyledon primordia establishment. The development of mutant embryos was arrested at the globular-heart stage transition (Figure 3(B)).

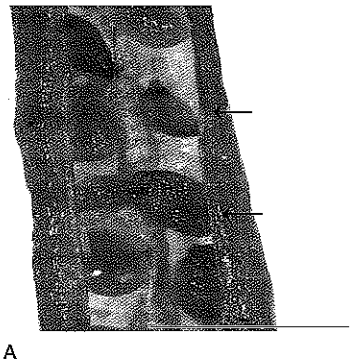
Abnormal suspensor consisting of two columns of cells was observed in 280-4-4 mutant (Figure 4(B)) in the early globular stage of embryogenesis. The main suspensor function is to provide nutrition and growth regulators to the embryo. This defect resulted in globular embryo abortion, probably as a consequence of malnutrition.

VIII-111 mutation showed defect with atypical development of cotyledon primordia at heart stage of embryogenesis (Figure 5(B)). Owing to the enlargement between cotyledon primordia, it could be suggested that the defect was associated with abnormal pattern of cell division constituting the precursor of the shoot apical meristem (SAM). This defect also resulted in heart embryo abortion.

In VIII-82 mutation, defects were observed in heart and torpedo stages with disorder in cotyledon establishment and development. Later during embryogenesis one cotyledon became smaller than the other (Figure 6(B)). The hypocotyl part of late torpedo embryo was abnormal, due to cell enlargement or atypical cell divisions. The embryo was arrested in the late torpedo stage.

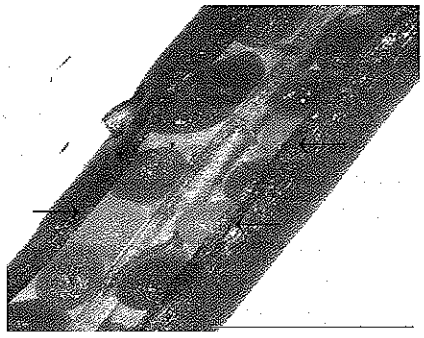
Cotyledon stage of embryos and chlorophyll defect were observed in VIII-75 mutation. In comparison with wild-type embryo, this mutation was characterised by normal embryo size and embryo development. However, from heart embryos up to maturity, alteration in pigmentation was observed and the embryos remained white (Figure 7(B)). Greening started at the heart stage of embryogenesis in wild-type embryos.

Figure 1.



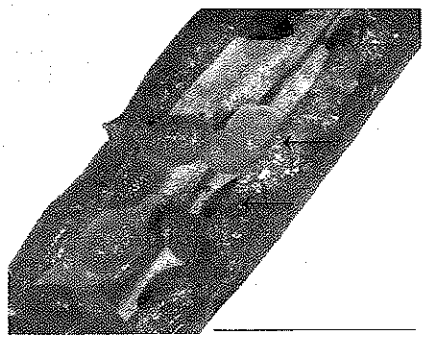
A

Figure 2.



A

Figure 4.



A

Figure 6.

Figure 1. Immature silique containing seeds from wild-type plant of *A. thaliana* ecotype Columbia. Bar = 1 mm.

Figure 2. VIII-64 mutation. Immature silique containing seeds from mutant plants (A) embryo of mutant plants (B) and wild-type embryo of Columbia plant (C) Bar = 1 mm (A), 100 μm (B, C).

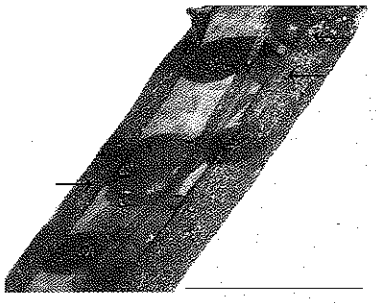
Figure 3. VIII-41 mutation. Immature silique containing seeds from mutant plants (A) embryo of mutant plants (B) and wild-type embryo of Columbia plant (C) Bar = 1 mm (A), 100 μm (B, C).

Figure 4. 280-4-4 mutation. Immature silique containing seeds from mutant plants (A) embryo of mutant plants (B) and wild-type embryo of Columbia plant (C) Bar = 1 mm (A), 100 μm (B, C).

Figure 5. VIII-111 mutation. Immature silique containing seeds from mutant plants (A) embryo of mutant plants (B) and wild-type embryo of Columbia plant (C) Bar = 1 mm (A), 100 μm (B, C).

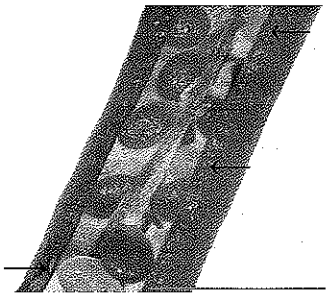
Figure 6. VIII-82 mutation. Immature silique containing seeds from mutant plants (A) embryo of mutant plants (B) and wild-type embryo of Columbia plant (C) Bar = 1 mm (A), 100 μm (B, C).

Figure 7. VIII-75 mutation. Immature silique containing seeds from mutant plants (A) embryo of mutant plants (B) and wild-type embryo of Columbia plant (C) Bar = 1 mm (A), 100 μm (B, C).



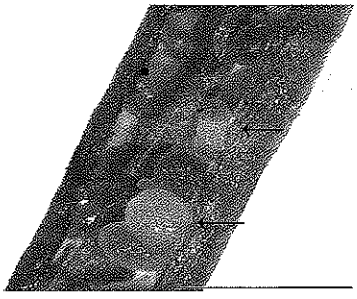
A

Figure 3.



A

Figure 5.



A

Figure 7.

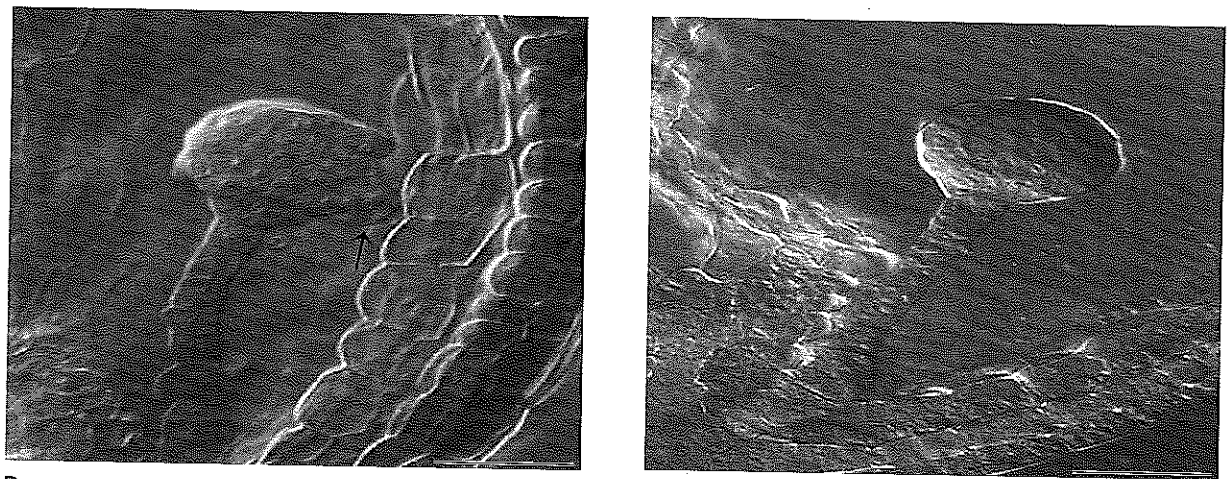


Figure 2. (*continued*).

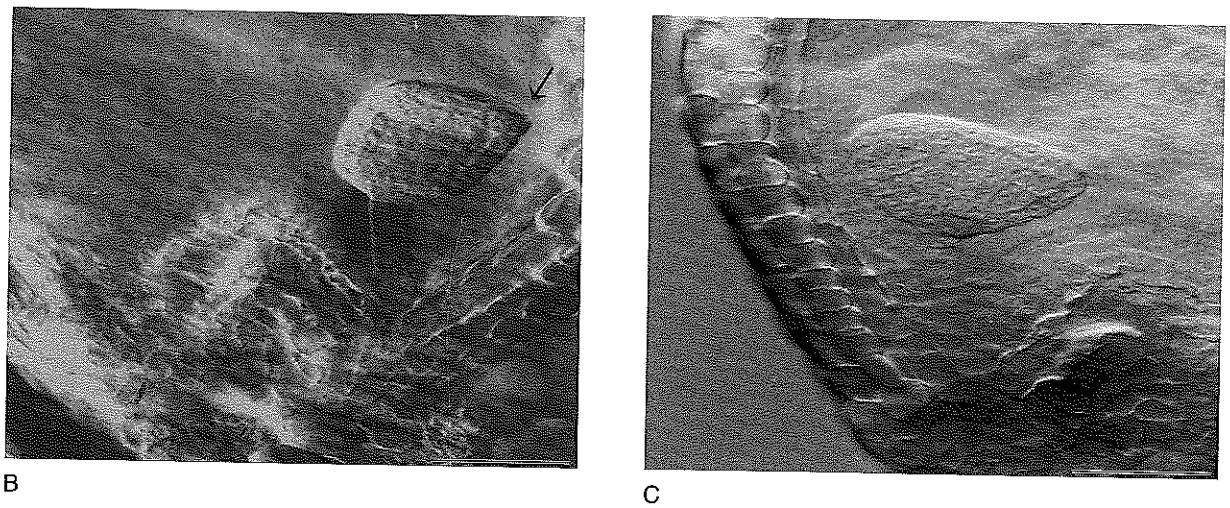


Figure 3. (*continued*).

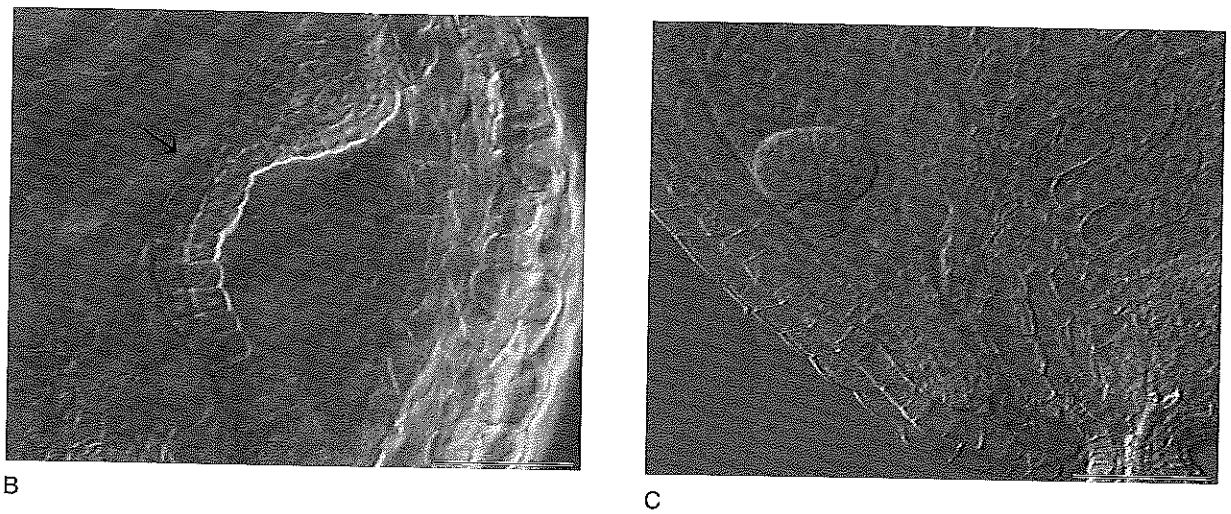
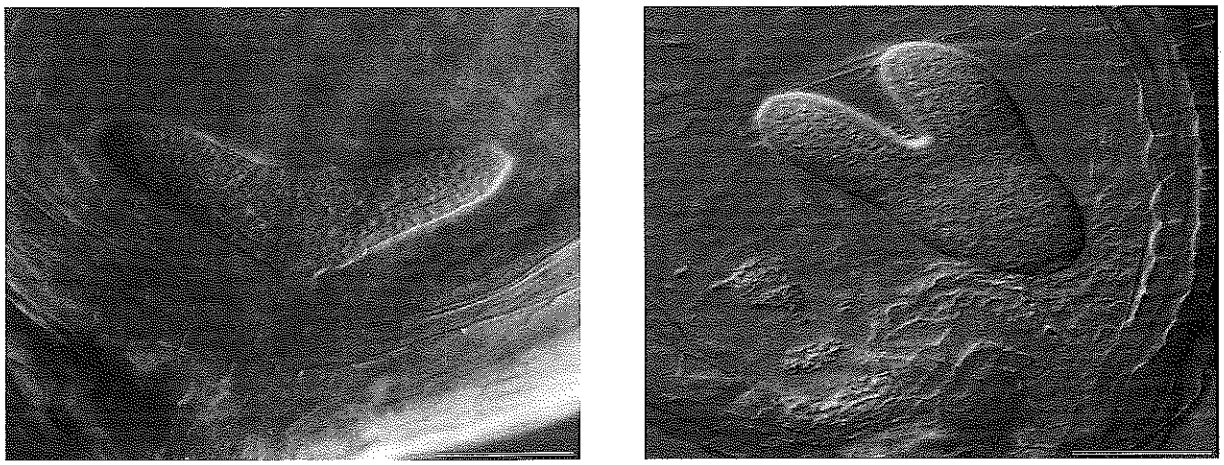
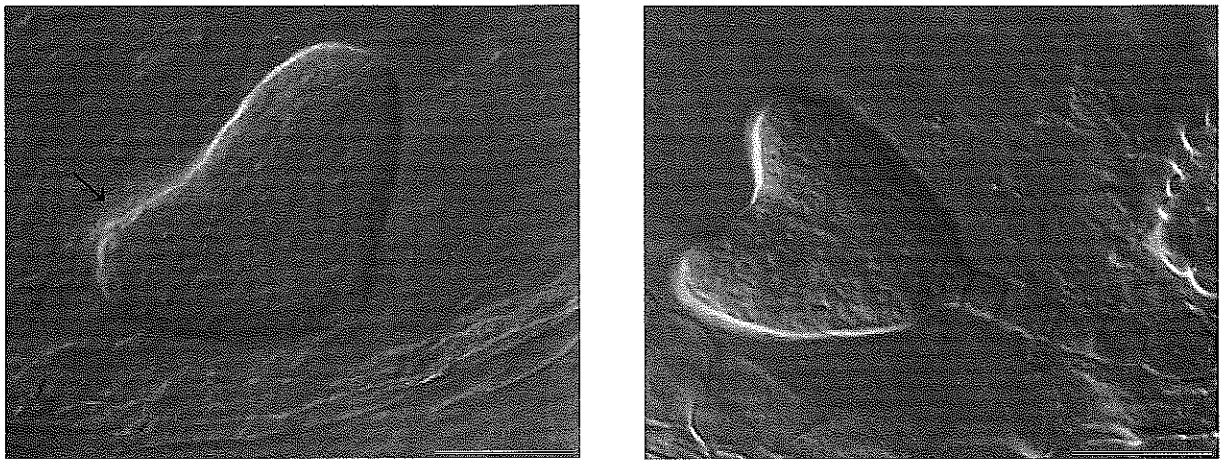


Figure 4. (*continued*).



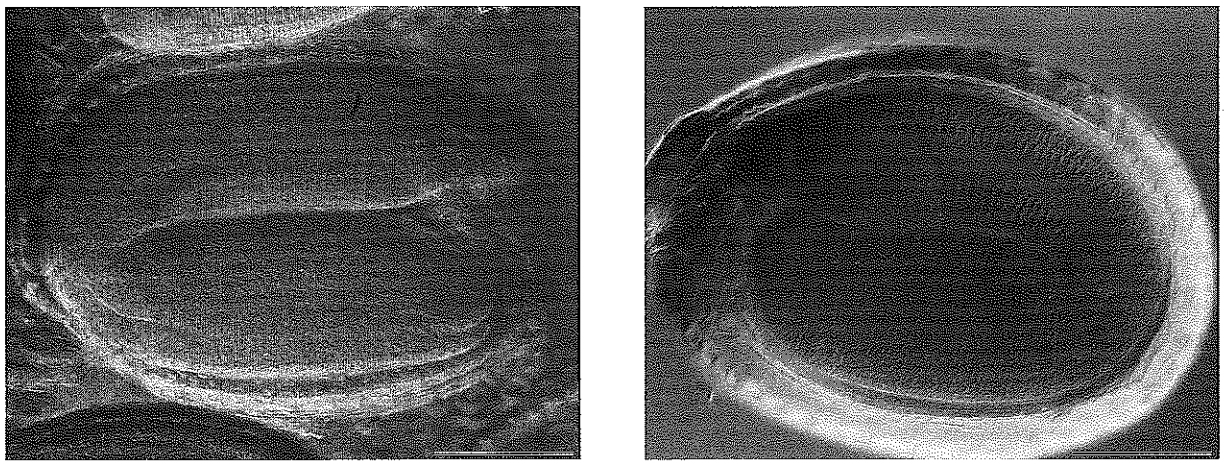
B C

Figure 5. (continued).



B C

Figure 6. (continued).



B C

Figure 7. (continued).

Discussion

Understanding of the regulation of plant embryogenesis at molecular level is still insufficient but it is in progress (McElver et al., 2001). Loss-of-function mutations have been carried out so far in embryogenesis. Mutation of a functionally redundant gene is not likely to lead to an easily recognisable phenotype because two or more genes can provide the same function. In addition, many genes function at multiple stages of development. Mutations in these genes may lead to early lethality or may be highly pleiotropic, which can mask the role of a gene in a specific pathway (Springer, 2000).

Phenotype of embryos in VIII-64 mutation with changes visible in precursor of protoderm is like the mutation in a gene involved in cytokinesis where embryo-arrested mutants are known as *raspberry* (Yadegari et al., 1994). Development of these mutants is blocked in the globular stage of embryo with visible protuberances in the outer cell layers giving the embryo a raspberry appearance. Similar to our mutation, *abp*1 *Arabidopsis* mutants deviate from normal development after the dermatogen stage (Chen et al., 2001) and are unable to make the transition to a bilaterally symmetrical structure. In addition and in contrast to our mutation, cell division is aberrant not only in embryo proper but even in the apical part of suspensor. A putative auxin receptor that mediates auxin-induced cell elongation and cell division proved to be encoded by *ABP*1 gene. Furthermore, *ACR*4 gene involved in epidermal differentiation encoding an *Arabidopsis* homologue of the maize putative receptor kinase *CRINCLY*4 (*CR*4) has been isolated (Tanaka et al., 2002).

According to Yadegari et al. (1994), mutants arrested in globular-heart transition of embryo development, for example, the VIII-41 mutant embryos, are thought to be defective in auxin metabolism or polar transport during early embryogenesis. Auxin has been proposed as a key signal molecule in providing positional information within the apical region of the embryo, particularly during the transition period from globular to heart stage (Souter & Lindsey, 2000).

The third mutation with embryo lethality in early embryogenesis is 280-4-4. Abnormal cell division in suspensor is apparently the reason for lethality. Besides the physical support for the developing embryo, the main role of the suspensor is to carry out embryo feeding (Yeung & Meinke, 1993). The suspensor cells would transport nutrients from the mother plant to the embryo as well as be themselves metabolic active for protein, auxin and gibberellin synthesis (Brady & Walthall, 1985). Any abnormality in suspensor formation might influence embryo viability. For example, *axr6* and *asf1* mutants also show inappropriate cell division in the suspensor (Hobbie et al., 2000) and as a consequence mutant embryos fail. Three classes of suspensor mutants in *Arabidopsis* have been described in detail (see review by Vernon et al., 2001). Our suspensor mutation seems to be different from these three classes. Suspensor defects arise primarily and embryo defects are the consequence of it. Neither secondary embryos nor polyembryony nor late embryo mutants and belated suspensor degeneration have been observed.

The common feature of the three above-mentioned mutations is alteration in early embryogenesis and embryo abortion in early stages of development. It seems likely that the failure in signalling or receptors for auxins has occurred. The nature of the signalling molecules produced either by the embryo-proper or the suspensor remains totally unknown, although the plant hormone auxin is a candidate (Souter & Lindsey, 2000). Furthermore, since morphogenesis of embryos depends almost entirely on cell division and cell expansion, cytokinesis genes also could be involved.

As for VIII-111 mutation, the embryonic defect was localised in the apical region of the heart embryo. This indicates loss-of-function of a gene required for the formation of a functional shoot meristem and for the separation of the cotyledon primordia.

VIII-75 mutant embryos achieved cotyledon stage of development and were like the other mutants with a chlorophyll defect, classified as *albino* mutants, but seeds did not germinate. Owing to normal embryo development, lack of pigmentation probably resulted from a direct effect on an *EMB* gene in chloroplast biogenesis or its function. In the majority of *emb* mutations, white or yellowish seeds and embryos result from a secondary effect of the mutations (Zhongsen & Thomas, 1998). Similar defects in chloroplast development from proplastid progenitors were previously described in *schlepperless* mutant and in addition, had highly reduced cotyledons (Apuya et al., 2001). These authors highlighted the importance of the chaperonin-60α protein for chloroplast development and subsequently for the proper development of the plant embryos and seedlings.

The identification and basic analysis of new mutants is the prerequisite for the more detailed study

of genetic determination of plant embryo development. Apparently, there are more mutants with the same or very similar embryo morphology and some of them can be allelic. Complementation tests should be performed between mutants mapped on the similar regions of the genetic map (if it is known). Once embryo mutants have been characterised, the next step is to clone the genes responsible and to elucidate their specific roles. None of the mutant phenotypes identified by us cosegregated with T-DNA; therefore genetic mapping using DNA markers could be used for further gene identification. Possible ways of gene identification are positional or map-based cloning methods (Tanksley, Ganal & Martin, 1995). Positional cloning requires high-resolution maps and involves merging of genetic and physical maps. For this reason and for complementation tests, genetic mapping of the mutant genes identified by us has been performed using DNA markers (Řepková, in preparation).

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