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In vitro development of maize immature embryos: a tool for embryogenesis analysis

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Abstract

During monocot embryo development, the zygote goes through a proembryo stage characterized by a radial symmetry and later becomes a true embryo with a bilateral symmetry. In order to determine culture conditions for immature embryonic stages, proembryos and embryos were isolated from controlled pollinated maize plants and developed *in vitro*. Precise culture conditions were determined for each type of explant: a monolayer system for embryos using NBM medium enriched with maltose (0.25 M) but without hormones, and a bilayer system for proembryo stages using N6 medium supplemented with maltose (0.35 M) and zeatin (3 mM). Morphological, cytological, and *in situ* hybridization analysis have shown that the resulting embryos (stages 1–2), developed *in vitro*, were similar to those formed *in vivo* and subsequently gave rise to fertile plants. This work demonstrates that successful embryo differentiation is dependent on specific parameters including the genotype, the nature of the carbon source, the type and concentration of hormones used and orientation of the embryos on the medium. The potential use of these results for embryo rescue and mutant analysis are discussed.

Key words: Maize, proembryo *in vitro* culture, plant regeneration, ISH-*Ltp2*.

Introduction

Embryonic development in plants starts after double fertilization and leads to the formation of an embryo and a nutritive tissue, the endosperm, which are both situated within the growing seed. Embryogenesis can be divided schematically into three phases. The first phase is characterized by pattern formation, morphogenesis and differentiation of the basic tissues (epidermis, ground and vascular tissues). The second phase is marked by the accumulation of storage products and during the last phase the seed dehydrates and enters dormancy. Recent studies have emphasized the importance of the first phase which seems to be crucial for further normal embryo development (Mayer *et al*., 1993; Goldberg *et al*., 1994; Jürgens, 1995; Breton *et al*., 1995). However, little information is available about the mechanisms which govern the establishment of the embryo structures and the differentiation of the endosperm tissue. Two main approaches are being applied to identify genes involved in these processes: isolation of genes using differential screening techniques and gene identification through mutant analysis. In maize, which is our plant model for embryo studies, (Sheridan and Clark, 1987; Dumas and Mogensen, 1993) no genes specifically expressed in the early embryo have been isolated. Conversely, three genes expressed in the early endosperm have been identified, *Bet 1* (Hueros *et al*., 1995), *End 1* (Doan *et al*., 1996) and *Esr* (Opsahl-Ferstad *et al*., 1997). In the same species numerous mutants have also been described, among which the *dek* (defective kernel) mutants affect only the endosperm or both endosperm and embryo, and *emb* (embryo-specific) mutants which affect only the embryo (Sheridan, 1988; Clark and Sheridan, 1991; Sheridan and Clark, 1993). The embryos of some *dek* and *emb* mutants are blocked in the early phase of development, at globular, transitional and coleoptilar stages (names of stages according to Randolph, 1936; Kiesselbach, 1949; Abbe and Stein, 1954). One way to understand the effect of these mutations and to study the genetic control of early embryogenesis is to restore the development of the arrested embryos through *in vitro* techniques by addition of a specific

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nutrient or growth factor (Sheridan and Neuffer, 1980; Devic *et al*., 1996). Such a strategy implies that appropriate media and culture conditions have first to be tested with wild-type embryos. Although the *in vitro* development of maize coleoptilar stage has been accomplished (Mòl *et al*., 1995) it has yet to be established for younger stages such as the proembryo and transitional stages. This paper describes here *in vitro* culture conditions for these stages. The explants were isolated manually from controlled fertilized ears and transferred on to a culture medium where they reached stages 1 and 2 (Fig. 1). In order to assess the conformity of these *in vitro* developed embryos relative to their counterparts *in planta*, morphological, cytological and *in situ* hybridization analyses were carried out. The *in vitro* developed embryos were similar to *in planta* ones and were able to germinate and develop into fertile plants.

Materials and methods

Source plants

Seeds of *Zea mays* L. lines A188 or F485 were supplied by INRA Plant Breeding station (Domaine de Crouelle, Clermont-Ferrand, France). Maize plants were grown under controlled conditions as described in Mòl *et al*. (1993). The first ears appearing on the plants (A188 or F485) were covered with paper bags before silk emergence and were pollinated with A188 pollen when the optimum length of silk receptivity (12–14 cm, 6–8 cm, respectively) was reached. Ears were removed from the plants at 6–7 d after pollination (DAP) for culture experiments and at 12–13 d for control analysis (morphology, cytology, biochemistry, *in situ* hybridization). The emerged silks and outer husks were removed and the ears were surface-sterilized with 70% ethanol.

Isolation and culture of pretransitional (6 DAP) and transitional (7 DAP) embryos

Ovaries were dissected and the fertilized embryo sacs containing well developed endosperm and embryos removed and transferred into drops of MS medium liquid (Murashige and Skoog, 1962) supplemented with 0.35 M sucrose for cell survival. The embryos were isolated with insect needles and immediately transferred on to the culture medium. For transitional embryos (7 DAP) (genotype A188 × A188) the basal medium was NBM (Mòl *et al*., 1993) supplemented with different concentrations of sucrose or maltose (0.18 M and 0.26 M) and solidified with gelrite (0.1%, w/v). Other assays were carried out using the same solid media overlaid with a thin layer of liquid medium. The pretransitional (6 DAP) proembryos (genotypes, A188 × A188 or F485 × A188) were first transferred on to a bilayer culture system with N6 (Chu *et al*., 1975) as the basal medium. The bottom layer was supplemented with 0.09 M sucrose and solidified with 0.8% (w/v) Sea Plaque Agarose (Tebu-France 50102). The upper layer was prepared with the same basal medium (N6) supplemented with 0.35 M sucrose or 0.30 M maltose and with or without cytokinins: BAP (benzylaminopurine, Sigma B 3408) 17 mM or zeatin (Sigma Z0876), 0.03, 0.3 or 3 mM. The medium was solidified with 0.8% (w/v) Sea Plaque Agarose. In this double layer system the young embryos were placed at the interface of the two layers. In all cases the sugared basal media and the hormones were filter-sterilized.

Development of in vitro*-cultured embryos (stages 1–2) into plantlets and mature plants*

When the 7 DAP transitional embryos reached stages 1–2 (it is difficult to distinguish stage 1 or stage 2 in culture), they were

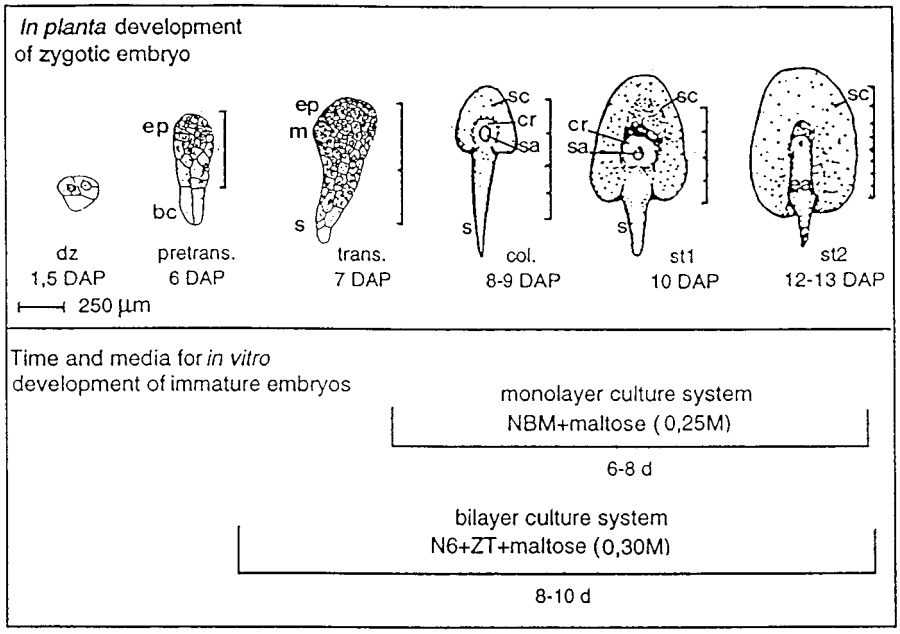

Fig. 1. Comparative development of maize embryos *in planta* (adapted from Randolph 1936; Kiesselbach, 1949; Abbe and Stein, 1954) and *in vitro*. Stages: dz, dividing zygote; pretrans, pretransitional proembryo; trans, transitional proembryo; col, coleoptilar stage; st1, stage one; st2, stage 2; DAP, days after pollination; d, days of *in vitro* culture; ZT, zeatin; bc, basal cells; ep, embryo proper; s, suspensor; m, meristem; sc, scutellum; sa, shoot apex; cr, coleoptilar ring; ea, embryo axis.

transferred on to a solid medium composed of MS salts (Murashige and Skoog, 1962), with sucrose 0.09 M, and Sea Plaque Agarose 0.8% (w/v) in the dark. As soon as the embryos germinated they were transferred into transparent boxes (10/5 cm, ht/diam) containing MS medium in diffused light. Three different sucrose concentrations were tested (0.01, 0.03 and 0.09 M). After 10 d, plantlets were well developed. They were acclimatized progressively in soil. Reciprocal crosses with seed-derived plants were carried out in order to check the fertility of regenerated plants.

The 6 DAP proembryos, were transferred on to a N6 medium supplemented with 0.09 M sucrose when they had reached stages 1–2. Other assays were carried out on MS/2 medium with only 0.03 M sucrose. Phytagel was added at the concentration of 0.3% (w/v). As described above, the plantlets were transferred into soil.

Cytology

In total 40 *in vivo* and *in vitro*-developed embryos were analysed cytologically by light microscopy following paraffin microtomy and staining with safranin and fast green according to Jensen (1962).

In situ *hybridization*

Maize caryopsis collected *in vivo* at 12–13 DAP with embryos at stages 1–2, and *in vitro*-cultured embryos which had reached the same stage (1–2), were fixed in 0.1 M phosphate buffer pH 7.4 with 4% paraformaldehyde at 4 °C for 48 h, rinsed twice in phosphate buffer for 15 min, dehydrated through an ethanol series and embedded in paraffin (paraplast + M, Prolabo, Lyon, France). Sections (10 mm thick) were dried on to slides at 45 °C overnight, dewaxed with Histoclear (Prolabo, Lyon, France) and rehydrated through an ethanol series. Slide pretreatment before hybridization was performed as described by Opsahl-Ferstad *et al.* (1997). 11-UTP-fluorescein-labelled probes were produced by *in vitro* transcription according to the manufacturers instructions (RNA colour kit for *in situ* hybridization, Amersham Les Ullis, France). Sense and antisense riboprobes were transcribed from an *Ltp2* cDNA clone (gift from P. Puigdomenech, SSIC, Barcelona, Spain). Hybridization was then performed as described by Opsahl-Ferstad *et al.* (1997). Sections were counterstained with 0.5% fast green and photographs were taken with a camera on a Zeiss inverted microscope (IM2).

Results

Proembryos develop normally in vitro *and generate fertile plants*

Figure 2 shows the general development of transitional (A) and pretransitional (B) embryos. Stage 1 and 2 embryos were obtained through *in vitro* culture of the two types of explants (C, D, E). Regenerated fertile plants (F) and seed set (G). A cytological section of a stage 2 embryo is presented in H. (For identification of embryo stages see Fig. 1.)

Transitional embryos (700 μm)

These are recognizable by their well-differentiated suspensor and embryo proper, within which the first meristem is initiated (Fig. 2A). In the culture conditions used in this study, transitional embryos are formed *in planta* after 7 DAP. Different concentrations of sugars in NBM medium were tested for stimulation of *in vitro* development (Table 1). The best results were obtained when the medium was supplemented with 0.25 M maltose (Fig. 2C). There was no difference between culturing with or without a liquid layer of medium, so for subsequent experiments the simplest culture conditions (i.e. NBM medium and 0.25 M maltose without addition of liquid medium), were chosen. To check the efficiency of the method, a significant number of proembryos (546) were isolated and cultured. It has been noticed that the orientation of the developing embryos was crucial for their subsequent differentiation. Since the transitional embryos had not acquired their bilateral symmetry it was impossible to distinguish the future dorsal (scutellum) and ventral (embryo axis) regions. Consequently, the development in culture was precisely monitored and as soon as the coleoptilar stage was formed, it was ensured that each embryo was situated so that its scutellum was against the medium. Table 2 shows two series of experiments with or without reorientation. The correct positioning of young embryos on the medium led to a clear increase in the percentage of stage 1–2 embryos obtained (66–88%).

The *in vitro*-cultured embryos reaching stages 1–2 (Fig. 2D) were transferred to a new medium containing MS salts + 0.09 M sucrose, for germination. For subsequent growth, the germinated embryos were placed on MS medium, with or without 0.01 M sucrose. Using these media, in one experiment, 127 embryos (stages 1–2) gave 100% germination. These were separated into two equal groups: of those placed on MS medium 62% gave plantlets and of those grown on MS medium with 0.01 M sucrose, 73% generated plantlets. If the sucrose concentration was higher than 0.01 M, calli developed at the base of the plantlet.

Pretransitional proembryos

Embryos at this stage (400 μm) (Fig. 2B) were composed of an embryonic region with small cells and a basal extension with larger cells, the suspensor. Using the genotype A188 × A188, and media containing maltose or sucrose and several different hormone concentrations, no development was observed (data not shown). Therefore another genotype, F485, recommended by Bulant-Robert (1996), was used (Table 3). N6 medium with sucrose did not allow embryo development. In contrast, when maltose and BAP or zeatin were added to N6 medium, embryo differentiation occurred and led to the development of stage 1–2 embryos. The best results were obtained with zeatin, whereas BAP was less efficient. Plantlets were recovered as described above.

In all these experiments *in vitro* plantlets easily yielded plants in soil, although their development was dependent

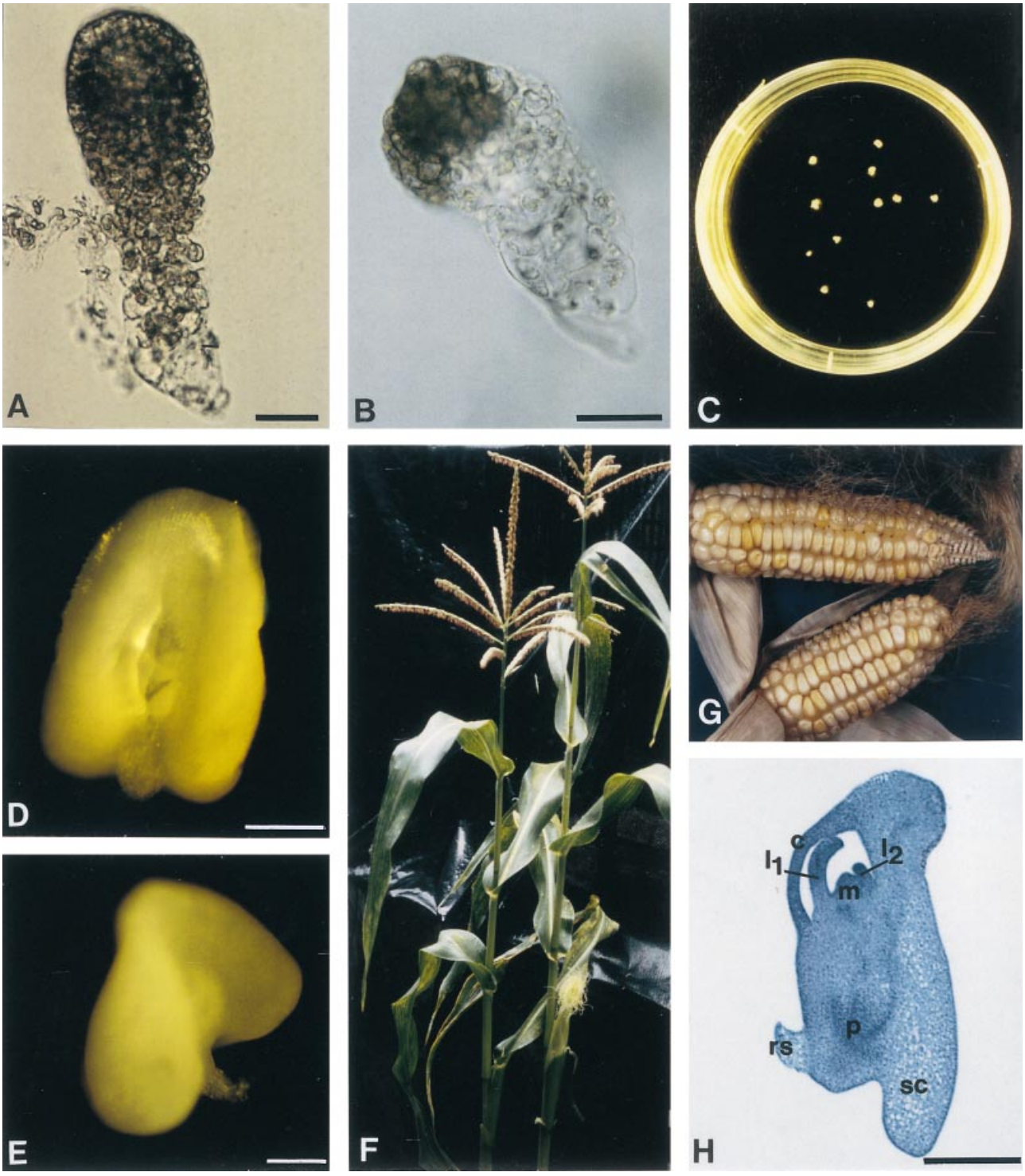

Fig. 2. *In vitro* development of immature embryos. (A) Transitional embryo (7 DAP); bar, 100 μm; (B) pretransitional proembryo (6 DAP); bar, 100 μm; (C) Petri dish (5 cm) showing stage 1–2 embryos developed from 10 transitional proembryos; (D) normal stage 2 embryo; bar, 0.45 mm; (E) stage 2 embryo with an enlarged scutellum; bar, 0.45 mm; (F) fertile plants regenerated from immature embryos; (G) seed set after crossing a regenerated plant with A188 pollen; (H) a longitudinal section of stage 2 embryo developed *in vitro*; bar, 0.45 mm. Normal shoot and root meristems are visible: c, coleoptile; l 1,2, leaf 1,2 primordia; am, apical meristem; sc, scutellum; p, procambium; rs, rest of suspensor.

Table 1. *Culture of maize (A188 × A188) transitional embryos*

Medium	Sugar (M)	Number of explants	Number of stage 1–2
NBM	Sucrose (0.18)	30	0
	Sucrose (0.26)	35	1
NBM	Maltose (0.17)	22	8
	Maltose (0.25)	20	14
NBM	Sucrose (0.18 + liq.)	20	0
	Sucrose (0.26 + liq.)	20	0
NBM	Maltose (0.17 + liq.)	20	7
	Maltose (0.25 + liq.)	20	15

on skilful manipulation and aftercare of cultures. However, it was confirmed that for both categories of explants (transitional and pretransitional proembryos) fertile plants and seed set were obtained (Fig. 2F, G).

In general, the length of culture required to develop stage 1–2 embryos from isolated proembryos was longer (1–2 d) than *in planta* (Fig. 1) and about 2 months were needed to produce a mature plant. It must be pointed out that seasonal conditions are very important for the correct development of the young proembryos. From May to September, their frequency of development was considerably increased.

The in vitro*-developed embryos are similar to those formed* in planta

Figure 2H shows the normal morphology of embryos developed in *in vitro* conditions. Their size is generally smaller than *in vivo* embryos by about one-third. Even in the most successful experiments, during the first phase of differentiation (i.e. from explant to stages 1–2) a number of proembryos did not develop or developed with a large scutellum (Fig. 2E). However, those with a large scutellum gave rise to plantlets. The normal embryos were morphologically similar to *in planta* embryonic stages in maize (Randolph, 1936; Kiesselbach, 1949; Abbe and Stein, 1954; Fig. 1). Analysis of cytological sections has confirmed these results and has shown that the meristems and leaf primordia formed *in vitro* closely resembled those occurring *in vivo*.

As another confirmation, *in situ* hybridization (ISH) with the *Ltp2* gene was used. In normal embryo development *Ltp2* is a good marker for protoderm which is the first embryonic tissue to be formed (Sossountzov *et al.*, 1991). ISH was performed on transverse or longitudinal sections of caryopses obtained *in planta* and of embryos (stage 1–2) developed *in vitro*. The *Ltp2* gene was expressed all around the scutellum and coleoptile in both types of embryos (Fig. 3B, C). No signal was detected using the sense probe (Fig. 3A).

Discussion

Culture conditions for normal development of immature embryos in vitro

The experiments reported in this paper, emphasize the importance of several factors in the successful differentiation of immature embryos to stage 1–2 embryos.

The culture system must be adapted to the developmental stage of the proembryo. The transitional embryo resists dehydration within the culture dish. In contrast, the bilayer system, already established in previous work (Mòl *et al.*, 1993), seems to protect the younger, water-rich, proembryo (pretransitional) during culture, and so promotes its survival. The quality of nutrient supply is of great importance. In our cultures it has been shown that the nature of the sugar is crucial for the embryo development. Transitional embryos and pretransitional proembryos were able to develop only when maltose was used in the media instead of sucrose. Sucrose is converted to glucose and fructose. Fructose is considered to be less efficient as a carbon source (Vasil, 1984), thus perhaps the embryonic cells use glucose before and more rapidly than fructose. Because maltose is split into two glucose molecules, this homodisaccharide may be more efficient for the nutrition of the embryo. This conclusion is

Table 2. *Culture of maize (A188 × A188) transitional embryos*

Orientation	Medium	Sugar (0.25 M)	Number of explants	Number of stage 1–2	Number of plantlets
−	NBM	Maltose	346	228 (66%)	210 (61%)
+	NBM	Maltose	200	176 (88%)	168 (84%)

Table 3. *Culture of maize (F485 × A188) pretransitional proembryos*

Medium	Sugar (M)	Hormones (mM)	Number of explants	Number of stage1–2	Number of plantlets
N6	Sucrose (0.35)	17 BAP	51	0	0 (0%)
N6		0 BAP	73	28	19 (26%)
N6	Maltose (0.30)	0.03 ZT	52	34	30 (58%)
N6		0.3	69	49	35 (51%)
N6		3	54	21	6 (11%)

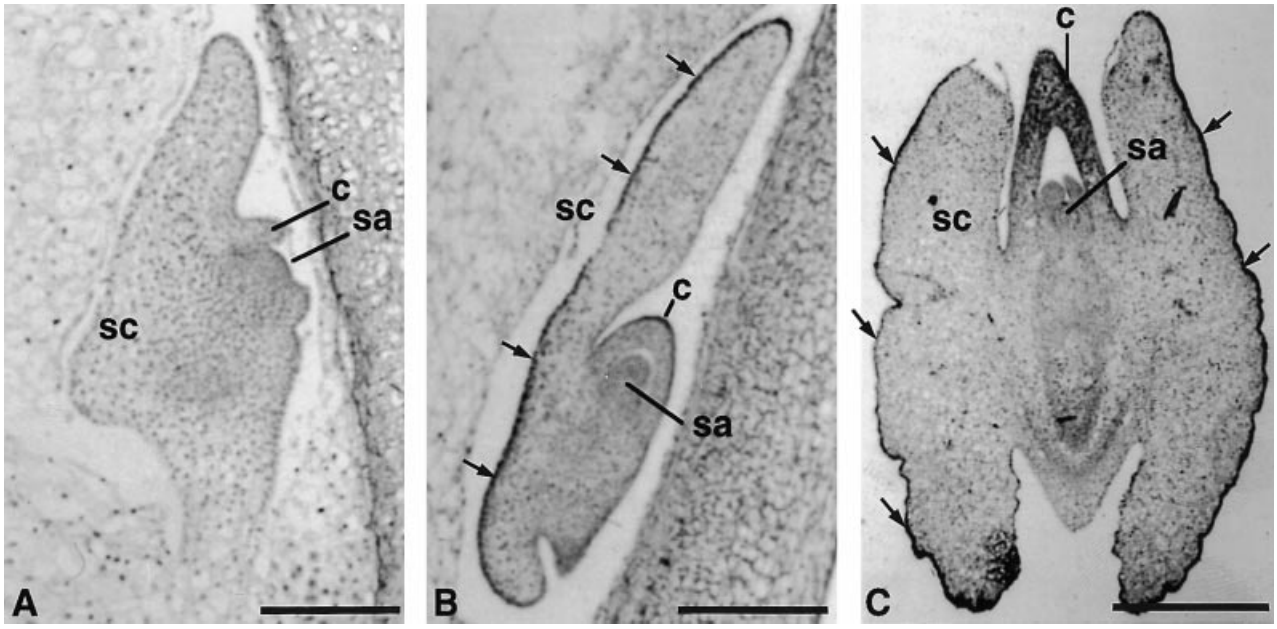

Fig. 3. *In situ* hybridization of maize embryos with *Lpt2* RNA probes. (A) Sagittal section of a caryopsis hybridized with an *Lpt2* sense probe. No staining is visible. (B) Sagittal section of a caryopsis hybridized with an *Lpt2* antisense probe: staining is visible around the scutellum (sc, arrows) and the coleoptile (c, arrow); (C) bilateral section of an *in vitro*-developed embryo. Hybridization with an *Lpt2* antisense probe. Staining is visible around the scutellum (sc, arrows) and the coleoptile (c, arrow); sa, shoot apex; bar, 0.45 mm.

supported by other authors (Stirn *et al*., 1995) who have demonstrated the stimulating role of maltose in androgenetic development of cereals.

Besides specific sugar requirements, exogenous hormones may be supplied. In the experiments described in this work, only pretransitional proembryos needed the addition of hormones. This suggests that the mother plant has already delivered sufficient growth factors to transitional embryos (7 DAP) to support development when they are removed from the plant, whereas younger embryos (6 DAP) need a hormonal complement to acquire competence for further differentiation. In fact, no precise knowledge is available concerning hormone concentration, balance and action during zygotic development. Only changes in 2,4-D (2,4-dichlorophenoxy acetic acid) and IAA (indole acetic acid) have been evaluated in different stages of somatic embryos (Michalczuk *et al*., 1992, and literature cited). According to these results and as also mentioned by Monnier (1976), the hormone requirement is variable according to the developmental stage. In these experiments, it has also been shown that zeatin is more efficient than BAP for embryo rescue. This result corresponds to previous work (Mòl *et al*., 1995) which demonstrated the positive effect of zeatin compared to BAP on the development of fertilized maize embryo sacs.

Correct embryo orientation on the medium is essential for their normal growth. Within the caryopsis in natural conditions, the scutellum is in close contact with the source of nutrients: the endosperm. The direct contact of the scutellum with the substratum may lead to an optimal efflux of nutrients from the medium to the embryo.

The last important factor is the effect of genotype on embryo development. Maize genotype A188 is well known for its suitability for *in vitro* culture (Armstrong and Green, 1985). However, in the first assays with pretransitional proembryos this genotype seemed to be recalcitrant. A recent report has drawn attention to highly vigorous maize hybrids that might be more suitable for *in vitro* culture (Bulant-Robert, 1996). Using one of these lines as female (F485 crossed by A188 pollen) has allowed the successful development of pretransitional proembryos.

Similarity between in vitro *and* in vivo *zygotic embryos is demonstrated by morphological and histological analysis and confirmed by* in situ *hybridization*

Cytological and anatomical analysis of stages 1 or 2 *in vitro* embryos showed that they were similar to *in planta* embryos demonstrated elsewhere (Randolph, 1936; Kiesselbach, 1949; Abbe and Stein, 1954). Stirn *et al*. (1995) described morphologically 'normal' somatic embryos but they were unable to germinate. Consequently, supplementary tests are necessary to compare development of embryos obtained through different conditions of culture. The expression of the *Ltp2* gene is similar in both embryos within caryopses at around stage 1, and in corresponding *in vitro* developed embryos at the same stages. This confirms the similarity between *in vitro* and *in vivo* zygotic embryos.

In conclusion, complete plant regeneration from maize embryos younger than coleoptilar stage was obtained through *in vitro* culture. This procedure yielded fertile plants within 2 months. It has also been shown that proembryos which reached stages 1–2 are analogous to corresponding zygotic embryos formed *in planta*.

This successful *in vitro* culture of very young maize embryos is promising for the elucidation of physiological and molecular mechanisms involved in early embryogenesis. Our experiments emphasize the different nutritional requirement of the two developmental stages analysed and help to understand physiological aspects of embryonic development. In addition, the *in vitro* conditions that have been determined now allow the rescue of mutant embryos at very early stages; this is an important tool in the identification of the biochemical pathways or genes affected by the mutation. As mentioned by Vasil (1991), 'It is hoped that synergism between *in vitro* technology and molecular biology will permit a better understanding of plant embryogenesis.'

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