

Cell cycle changes during callus initiation from cultured maize embryos. An autoradiographic study

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SUMMARY

Cell cycle changes were examined in various scutellar regions of immature maize embryos during a total culture period of 72 h on a modified MS agar medium containing 1 mg l^{-1} 2,4-D and 6% sucrose. At intervals of 8 h embryos were sampled and transferred to the MS medium supplemented with $5 \mu\text{Ci ml}^{-1}$ [³H]thymidine and incubated for 8 h. The fraction of labelled nuclei was determined for each interval and a comparison was made between the scutellar regions. During the first day, identical changes in the fraction of labelled nuclei and the mitotic index were observed in all scutellar regions. After the first day, however, various regions showed different cell cycles. The areas from which callus originated were mitotically active, showing high numbers of labelled nuclei, up to 60%. Furthermore, a difference was observed between the adaxial and the abaxial regions concerning the sequence of cell cycle changes. It is concluded that, after the initiation of the *in-vitro* culture, a shock response and a growth response are manifested, as observed by changes in the cell cycle. These responses proved to be different for the various regions of the immature embryos.

Key-words: autoradiography, cell cycle, embryogenesis, *in-vitro* culture, *Zea mays* L.

INTRODUCTION

The majority of plant regeneration from cereal explants takes place through an embryogenic callus phase. From this callus, shoots (Green & Phillips 1975) and somatic embryos (Armstrong & Green 1985; Kamo *et al.* 1985; McCain *et al.* 1988) develop, which can grow further into complete plants. Embryogenic callus also plays an important role in the production of cell suspension and protoplast cultures with regeneration capacity (Green *et al.* 1983; Vasil & Vasil 1986; Kamo *et al.* 1987). Induction of this callus type is therefore of great importance, e.g. in plant regeneration, genetic manipulation and somaclonal variation.

Many articles deal with the efficiency of the culture of embryogenic callus from cereals by the use of different genotypes (Duncan *et al.* 1985; Tomes 1985) or immature explant sources (Rhodes *et al.* 1986; Suprasanna *et al.* 1986). Other papers report on the study of

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morphogenetic processes during callus initiation by histological techniques (Springer *et al.* 1979; Vasil & Vasil 1982; Wernicke *et al.* 1982; Vasil *et al.* 1985). However, only a few studies have been published on the ultrastructural level of callus initiation in cereals (Kott *et al.* 1985; Fransz & Schel 1987), while cell cycle data about this early period are completely lacking. Most reports on the cell cycle during callus formation deal with work on dicotyledons, the Jerusalem artichoke in particular (Yeomon *et al.* 1965, 1966, 1967, 1968).

By the use of an *in-vitro* culture system with immature embryos from the maize inbred line A188, embryogenic callus was obtained from more than 90% of the cultured embryos. This callus mainly developed from the scutellar regions of the embryos (Fransz & Schel 1987; Fransz 1988). The high frequency of callus formation gave the opportunity to examine the early period of culture, during which no macroscopical changes are yet visible. The main objective of this study was to correlate cell cycle events in maize with callus initiation, and to examine cellular changes in different scutellar subregions during culture.

MATERIALS AND METHODS

Culture. Immature embryos (approximately 1.5 mm) were excised 12 days after pollination from caryopses of maize (*Zea mays* L.), strain A188. They were cultured as described before (Fransz & Schel 1987). The solid medium was composed of the inorganic constituents of Murashige & Skoog (1962) and the organic compounds as described by Green & Phillips (1975). The medium further contained 6% sucrose and 1 mg l^{-1} 2,4-dichloro-phenoxy-acetic acid (2,4-D).

Labelling of the embryos with [^3H]thymidine. From the onset of culture ($t=0$), four embryos were transferred every 8 h to the same medium supplemented with $5 \mu\text{Ci ml}^{-1}$ [^3H]thymidine (specific activity 5.0 Ci mmol^{-1} , Amersham, UK). They were incubated on labelled medium for 8 h. From the start of the culture ($t=0$), and after each labelling period, the embryos were sampled and fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at room temperature. After several rinses in the same buffer for a period of 2 days, the specimens were dehydrated and embedded in 2-hydroxy ethyl-methacrylat (Technovit 7100, Kulzer, Wehrheim, FRG). Longitudinal sections (3 μm) were cut on a Reichert-Jung Supercut 2050.

Autoradiography. Selected sections were mounted on ethanol-cleaned slides, dipped in Ilford K2 photographic emulsion diluted with aqua dest. (1:2), dried overnight at room temperature in open boxes and stored in closed boxes at 4°C. After a 2-week exposure period, the autoradiographic slides were developed in Kodak Microdol X for 4 min, rinsed in aqua dest. for 30 sec, fixed in Ilford Ilfospeed 200 fixer for 8 min, and rinsed in aqua dest. for 5 min. Sections were stained with 1% toluidine blue in 1% boric acid.

Measurements. The autoradiographic sections were examined with a light microscope at a magnification of $\times 200$. Drawings were made using a drawing-prism from median sections of the scutella. In these drawings the scutellum was subdivided into several regions as shown in Fig. 1. Two embryos were examined every 8 h. The nuclei in the scutellar parenchyma were used for measurement. All labelled and unlabelled nuclei,

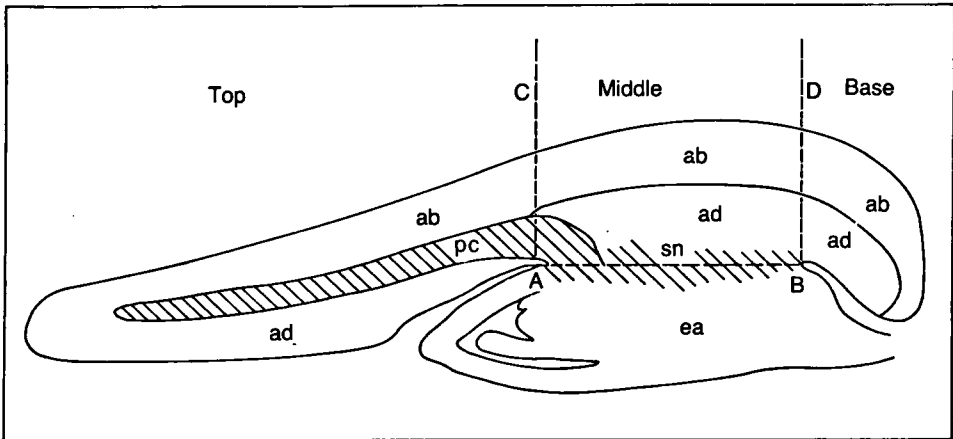


Fig. 1. Schematic representation of the several subdivisions of an immature embryo of *Zea mays* L. The points A and B are the attachment points of the coleoptile, respectively coleorhiza, to the scutellum. Lines AC and BD are drawn perpendicular to AB and divide the scutellum into a top, a middle and a basal region. The procambial strand (pc) divides the top region into an abaxial (ab) and adaxial (ad) part. For the middle region these parts are defined by a line at equal distances from the scutellar node (sn) and the scutellar epidermis. The most basal part shows no distinct discrimination between an abaxial and adaxial zone; this part was considered to be abaxial because of its similarity to labelling index as well as histological appearance. ea = embryo axis.

interphases as well as mitoses, were scored. Only nuclei that were distinctly covered by silver grains were counted as labelled nuclei.

RESULTS

Figure 2 illustrates the fractions of nuclei that incorporated [3 H]thymidine during an 8-h incubation in several regions of the scutellum. These fractions of labelled nuclei (FLN) represent the percentage of nuclei that were in the S-phase during 8 h. During the first 32 h of culture, similar changes were observed in the FLN for all subregions. The FLN was low during the initial 8 h. Thereafter, it increased by up to 60%. In general, the adaxial regions showed a lower FLN than the abaxial parts. After 32 h in culture, differences were found between the scutellar subregions. In the top regions and the adaxial base the FLN declined to values around 25%, while in the abaxial base and especially the middle region the FLN fluctuated with maximum values in the periods 40–56 h (adaxial middle), 48–56 h (abaxial middle and base) and 64–72 h (abaxial base).

On occasions, the adaxial subregions showed a change in FLN 8 h before the abaxial subregions. At 24 h in culture the adaxial FLN declined in the top and the middle, while the abaxial FLN decreased 8 h later. A similar situation was noted for the base at 32 h and 40 h in culture. An increase in FLN in the adaxial middle was observed in the period 40–48 h, which was 8 h sooner than the abaxial subregions. These results indicate the presence of a gradient in cell cycle activity from the adaxial region near the embryo axis towards the abaxial regions in the subepidermis of the scutellum.

Figure 3 shows the fractions of observed mitoses, expressed as the mitotic index (MI); labelled and unlabelled mitoses are indicated. At the onset of culture the scutellum tissue was mitotically active in all regions, with a mean MI of 0.05. At 8 h in culture the MI was completely reduced. A recovery in mitotic activity was observed after 16 and 24 h; the

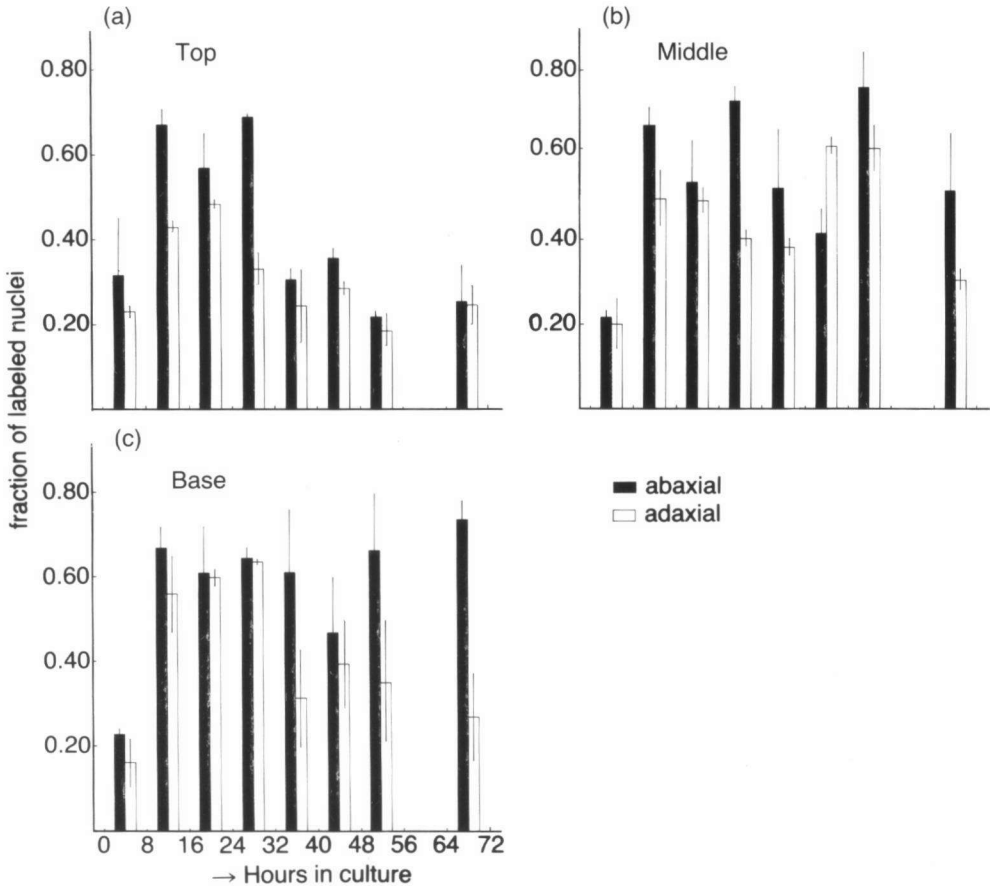


Fig. 2. Comparison of the fraction of labeled nuclei in the top, the middle and the basal region of scutella from cultured *Zea mays* L. embryos. The position of the bars corresponds to the 8-h labelling period preceding the fixation. The left bar (■) represents the abaxial region, while the right bar (□) represents the adaxial region. Each fraction is based on two measurements; standard errors are indicated by the vertical bars.

middle and basal regions showed only labelled mitoses, whereas in the top unlabelled mitotic figures were also detected. After 24 h in culture, the MI in the top region and the adaxial base declined and the few mitotic figures were usually observed close to the middle region. At this stage the top cells were elongated and their cytoplasmic density was low [Fig. 4(a)]. In the middle region and abaxial base the MI was constant or showed some fluctuations. Here, a maximum in cell division activity was noticed at 56 h.

In general, all scutellum regions showed labelled as well as unlabelled mitoses after an 8-h labelling period (Fig. 3). When embryos were labelled for only 4 h instead of 8 h, no labelled mitoses were observed in the parenchyma cells of the scutellum. Only in the procambium and the embryo axis were labelled mitoses observed [Fig. 4(b)].

After 56 h of culture, a region of meristematic cells, characterized by small cytoplasm-rich cells, was observed in the middle and abaxial basal scutellum [Fig. 4(c) (i)]. Cell divisions were found randomly in the subepidermal middle and basal region and most

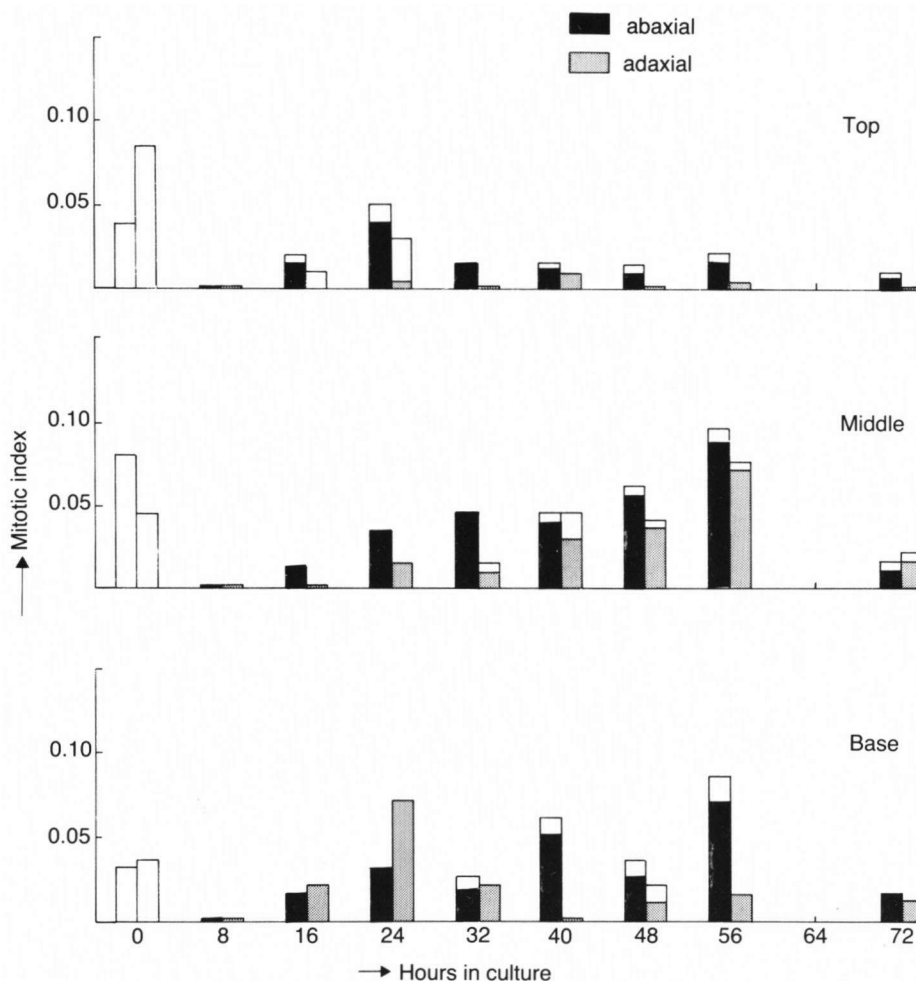


Fig. 3. Comparison of the mitotic indices in the top, the middle and the base, at various times after the onset of the culture of immature embryos from *Zea mays* L. (■), represents the abaxial region, while (▨) represents the adaxial region. The shaded areas mark the labelled fraction.

division planes were periclinal [Fig. 4(c) (ii)]. After 3 days, a clear protuberance could be observed in the scutellar base of some embryos [Fig. 4(d)]. These protuberances are known to develop into compact callus and scutellar bodies and, after removal of 2,4-D, into somatic embryos (Vasil *et al.* 1985; Frasz & Schel 1987; Frasz 1988).

The cells of the embryo axis showed no alterations of the cell cycle during the first 8 h in culture. Labelled nuclei, as well as cell divisions, were observed, which indicates that the culture response of these cells with respect to the cell cycle differed from the parenchyma cells of the scutellum. The procambium, which was recognized by the presence of small elongated cells, also showed continuous DNA replication and mitotic activity at the start of culture. In later stages, starting from 24 h, the FLN was comparable with values found in the surrounding parenchyma tissue in the top. High rates of labelled nuclei in vascular cells of the top region were mainly due to the cells that were located close to the scutellar

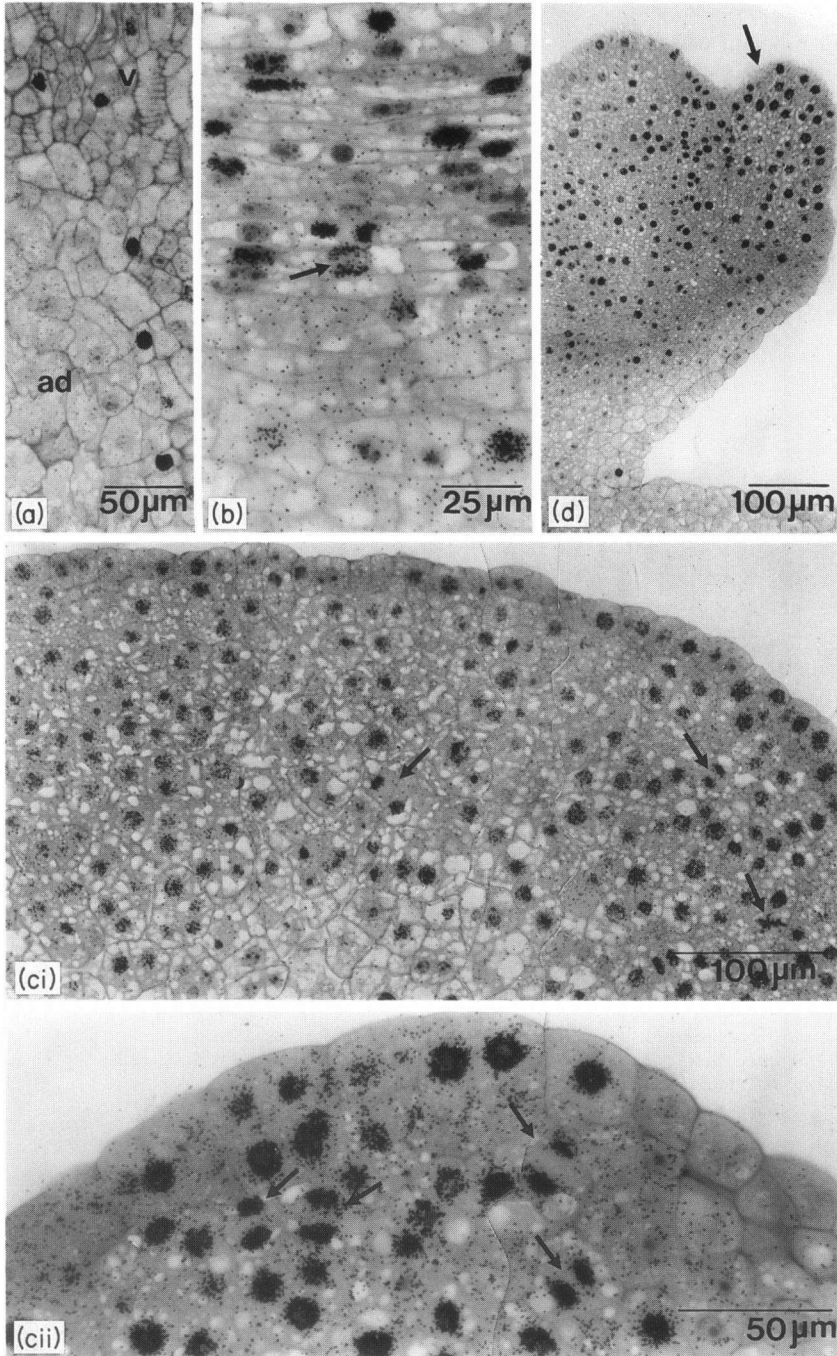


Fig. 4. (a) Micrograph showing the adaxial region in the top of the scutellum of an immature embryo of *Zea mays* L. at 24 h in culture, showing vascular cells (v) and enlarged adaxial parenchyma cells, ad = adaxial. (b) Light microscopical autoradiograph of procambium cells at 20 h in culture after a 4-h incubation with [3 H]thymidine. Note the labelled mitosis (arrow). (c) (i) Light microscopical autoradiograph of the abaxial middle and basal region of the scutellum, showing a zone of meristematic cells with labelled nuclei and mitoses (arrows) after 56 h in culture. (ii) Detail of the basal region. Note the periclinal division planes (arrows) of the labelled mitoses. (d) Light micrograph of the basal region of the scutellum at 72 h in culture, showing a protuberance (arrow).

node. This concurs with the observation that vascular cells differentiate during the early period of callus initiation (Fig. 4; see also Fransz & Schel 1987).

Epidermal cells also showed DNA replication activity in all regions, but the FLN in general was lower than in the parenchyma tissue. In the adaxial epidermis of the scutellum top, the mitotic activity disappeared completely as soon as culture was started. During further culture no mitotic figures were observed; this indicates that proliferating epidermal cells in the adaxial top became arrested in G2 during culture.

DISCUSSION

It has been proposed that two phases can be distinguished during early callus formation in cultured maize embryos (Fransz & Schel 1987). The first phase or shock response takes about 1 day and is unaffected by genotype or the presence of 2,4-D. The second phase or growth response starts after 1 day in regions that will give rise to callus. This phase is dependent on the genotype and on the presence of 2,4-D. In this study the presence of a two-phasic process during callus initiation in maize embryos is confirmed. The shock response comprises two events: (i) a decrease in mitotic activity and probably also in DNA replication, and (ii) a recovery of both activities. This shock response takes place in the whole scutellum regardless of its further development, e.g. into embryogenic callus, non-embryogenic callus, or no callus at all. During the growth response only regions that are capable of producing callus remain mitotically active and replicate DNA, whereas in the other regions these activities decline and cells differentiate.

In cultures of the Jerusalem artichoke the tuber cells also show a two-phasic process during callus initiation (Macleod *et al.* 1979). The ultrastructural changes are quite similar to those in cultured maize embryos (Yeoman & Street 1977; Fransz & Schel 1987). However, a discrepancy exists between the cultured tuber slices and the scutellum tissue of cultured maize embryos with respect to their cell cycle changes. The tuber slices were mitotically inactive before culture and showed a nearly synchronous first-cell division after 1 day of culture (Yeoman *et al.* 1966). Prior to this event DNA was replicated only in the cells that were induced to divide and subsequently produced callus (Yeoman & Evans 1967; Yeoman & Mitchell 1970). On the contrary, the scutellum of the immature maize embryo is mitotically highly active before culture (Fig. 3; see also Springer *et al.* 1979). Moreover, the first cell divisions do not take place synchronously and DNA is replicated in all cells, including those that do not produce callus. A reason for the difference between the artichoke tuber and the maize embryo might be that in immature maize scutellum cells the cell cycle is a stress-sensitive process, as discussed for other plants by Rost (1977). In the tuber cells such a stress-sensitive activity has not yet been shown.

The cell cycle is strongly disturbed during the shock response when ultrastructural changes and protein synthesizing activities take place (Fransz & Schel 1987; Fransz & Boersma 1988). This deregulation of the cell cycle is registered by a decrease in the mitotic index and the low fraction of labelled nuclei. The observed cell cycle arrest in either G1 or G2 is in agreement with the principle control point hypothesis of Van't Hof & Kovacs (1972), which implies that energy-consuming processes cause cell-cycle arrest in G1 or G2. The *in-vitro* situation might provoke similar processes in the differentiated tuber as in the undifferentiated embryo. These processes, e.g. increased protein synthesis and organelle production, probably disturb stress-sensitive activities like the cell cycle. The observed DNA replication, followed by a nearly synchronous cell division, then reflects the start of the growth response (Fig. 3).

The results further confirm the site-specific origin of embryogenic callus development. High rates of DNA replication and mitotic activity were observed only in the middle and basal part of the scutellum. It has already been shown that only the scutellum cells towards the coleorrhiza become mitotically active (Springer *et al.* 1979). In fact, only the epidermal and subepidermal layers give rise to embryogenic callus or somatic embryos (Vasil *et al.* 1985; McCain & Hodges 1986; Fransz & Schel 1987). Such a difference in embryogenic potential, between the abaxial and adaxial regions, is possibly reflected by the difference in timing of the cell cycle changes and in the fraction of labelled nuclei between both regions. It gives rise to a polarity, probably induced by the flow of nutrients and 2,4-D from the embryo axis towards the subepidermal layers of the scutellum. The fact that cultured embryos with the scutellum against the medium are unable to produce embryogenic callus might be related to this phenomenon (Green & Phillips 1975).

A similar polarity is recognized during organogenesis and direct somatic embryogenesis. In cultured maize embryos shoot apices develop in the epidermal layer of the scutellum (Springer *et al.* 1979), whereas root primordia originate in the adaxial scutellum (Vasil *et al.* 1985). During direct somatic embryogenesis from zygotic embryos, the position of the somatic embryo axis is as follows: the root primordium is located adaxially, towards the zygotic embryo axis, whereas the shoot apex develops at the opposite site (Vasil *et al.* 1985). A correlation between embryo polarity and cell cycle events is also found during seed maturation in *Triticum durum*, where during G1 the accumulation of proliferative cells starts at the shoot apex and proceeds in the root (Avanzi *et al.* 1969). The difference in timing of the cell cycle changes in the abaxial and adaxial subregions reflects the polarity in the scutellum and thereby might contribute to somatic embryo development.

ACKNOWLEDGEMENTS

We would like to thank Dr R. Deltour (Université de Liège, Liège, Belgium) for his advice concerning autoradiography and for critically reading the manuscript; Professor M.T.M. Willemsse for valuable comments; S. Massalt, P. van Snippenburg and A. Haasdijk for preparing the photographs and the drawings; and J. Cobben-Molenaar and T. van de Hoef-van Espelo for the preparation of the typescript.

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