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14-3-3 isoforms and pattern formation during barley microspore embryogenesis

Simone de F. Maraschin[1,3], Gerda E. M. Lamers[2], B. Sylvia de Pater[1], Herman P. Spaink[2] and Mei Wang[1]

[1] *Center for Phytotechnology LU/TNO, TNO Department of Applied Plant Sciences, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands*

[2] *Center for Phytotechnology LU/TNO, Institute of Molecular Plant Sciences, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands*

Abstract

The members of the 14-3-3 isoform family have been shown to be developmentally regulated during animal embryogenesis, where they take part in cell differentiation processes. 14-3-3 isoform-specific expression patterns were studied in plant embryogenic processes, using barley (*Hordeum vulgare* L.) microspore embryogenesis as a model system. After embryogenesis induction by stress, microspores with enlarged morphology showed higher viability than non-enlarged ones. Following microspore culture, cell division was only observed among the enlarged microspores. Western blot and immunolocalization of three barley 14-3-3 isoforms, 14-3-3A, 14-3-3B and 14-3-3C were carried out using isoform-specific antibodies. The level of 14-3-3C protein was higher in enlarged microspores than in non-enlarged ones. A processed form of 14-3-3A was associated with the death pathway of the non-enlarged microspores. In the early embryogenesis stage, 14-3-3 subcellular localization differed among dividing and non-dividing microspores and the microspore-derived multicellular structures showed a polarized expression pattern of 14-3-3C and a higher 14-3-3A signal in epidermis primordia. In the late embryogenesis stage, 14-3-3C was specifically expressed underneath the L_1 layer of the shoot apical meristem and in the scutellum of embryo-like structures (ELSs). 14-3-3C was also expressed in the scutellum and underneath the L_1 layer of the shoot apical meristem of 21 d after pollination (DAP) zygotic embryos. These results reveal that 14-3-3A processing and 14-3-3C isoform tissue-specific expression are closely related to cell fate and initiation of specific cell type differentiation, providing a new insight into the study of 14-3-3 proteins in plant embryogenesis.

Key words: Androgenesis, barley, embryogenesis, 14-3-3, pattern formation.

Introduction

The ubiquitous family of 14-3-3 proteins consists of dimeric α-helical pSer/Thr binding proteins that control cellular processes by mediating protein–protein interactions (Yaffe and Elia, 2001). In animal cells, 14-3-3 proteins have been reported to interact with a wide number of mitotic and apoptotic factors, functioning as mediators of signal transduction cascades involved in cell cycle, differentiation and apoptosis (for a review see Fu *et al.*, 2000). 14-3-3 homologues in plants have been found to interact with key enzymes of the carbon and nitrogen metabolism (Moorhead *et al.*, 1999), from which the best described is the 14-3-3 inhibition of nitrate reductase activity (Bachmann *et al.*, 1996*a*; Moorhead *et al.*, 1996). 14-3-3 proteins also take part of the plasma membrane H^+-ATPase regulation (Korthout and de Boer, 1994; Marra *et al.*, 1994; Oecking *et al.*, 1994). Their recent localization in the chloroplast (Sehnke *et al.*, 2000) and in the nucleus (Bihn *et al.*, 1997) demonstrates their putative involvement in interacting with other metabolic and signalling pathways.

In both animal and plant systems, 14-3-3 regulatory roles are exerted by a family of several highly similar yet distinct protein isoforms. All 14-3-3 isoforms are very conserved in their core region, however, the N-terminal

dimerization domain and the hyper-variable C-terminus show less homology (Wang and Shakes, 1996). In the inner groove of the conserved core lie the amino acids responsible for protein binding, thus different 14-3-3 isoforms interact with their targets probably by common mechanisms (Yaffe *et al.*, 1997). The apparent lack of 14-3-3 isoform-specificity (Lu *et al.*, 1994) seems to be compensated as 14-3-3 genes are differentially regulated at the expression level. In the *Arabidopsis* genome, 15 14-3-3 genes have been found (Rosenquist *et al.*, 2001). Tissue-specific expression has been demonstrated for the 14-3-3χ isoform during *Arabidopsis* plant development (Daugherty *et al.*, 1996). In barley (*Hordeum vulgare* L.), three 14-3-3 isoforms have been cloned: 14-3-3A (GenBank X62388; Brandt *et al.*, 1992), 14-3-3B (GenBank X93170) and 14-3-3C (GenBank Y14200). Upon germination of barley embryos, expression of the 14-3-3C isoform has been shown to be specific to the scutellum and the L_2 layer of the shoot apical meristem (Testerink *et al.*, 1999), whereas the 14-3-3A isoform has been reported to be processed by proteolytic cleavage of the unconserved C-terminus in a isoform- and tissue-specific manner (van Zeijl *et al.*, 2000; Testerink, 2001).

In other multicellular organisms, tissue-specific expression of 14-3-3 isoforms is a well-described phenomenon that has been often correlated with tissue differentiation during embryogenesis (Watanabe *et al.*, 1993a, b; Roseboom *et al.*, 1994; McConnell *et al.*, 1995; Luk *et al.*, 1998; Siles-Lucas *et al.*, 1998). In *Drosophila* and *Xenopus laevis,* for instance, 14-3-3ε and 14-3-3ζ expression were prior to the mitogen-activated protein kinase (MAPK) activation during tissue specification in early embryo development (Tien *et al.*, 1999; Kousteni *et al.*, 1997). The same isoforms have been reported to regulate the Ras-Raf signalling pathway positively, leading to MAPK cascade activation (Chang and Rubin, 1997; Kockel *et al.*, 1997). Though 14-3-3 proteins have not yet been reported to interact with cell-cycle regulators in plant cells, many cell-cycle regulators are shared by both animal and plant cells, such as MAPKs (Mironov *et al.*, 1997). Allied to this, the conserved 14-3-3 functional homology among eukaryotes (van Heudsen *et al.*, 1996) indicates that 14-3-3 may take part in the mechanisms involved in the control of plant cell cycle and differentiation. Since tissue differentiation involves the specific expression of 14-3-3 isoforms during animal embryogenesis, the first step towards elucidation of the role of 14-3-3 isoforms in plant differentiation processes is to understand how different 14-3-3 isoforms are regulated during the development of plant embryos. Therefore, barley (*Hordeum vulgare* L.) microspore embryogenesis was used as a model system to study 14-3-3 isoform-specific expression patterns.

In barley, a combination of starvation and osmotic stress is able to induce efficiently a microspore developmental switch from the gametophytic to the sporophytic pathway, a process called androgenesis. After acquisition of embryogenic potential, isolated barley microspores can develop into embryo-like structures after 21 d of culture (Hoekstra *et al.*, 1992). Barley androgenesis is a suitable model system to study plant embryogenesis, as embryo development can easily be monitored *in vitro* (Wang *et al.*, 2000). In the present study, western blot analysis and immunolocalization studies of three barley 14-3-3 isoforms were carried out using a set of isoform-specific antibodies that recognizes specifically 14-3-3A, 14-3-3B and 14-3-3C isoforms. This is the first report to show that 14-3-3 isoforms are differentially regulated and processed upon androgenesis induction and embryo pattern formation.

Materials and methods

Androgenesis induction and microspore culture

Donor plants of barley (*Hordeum vulgare* L. cv. Igri) were grown in a phytotron under the conditions described previously by Hoekstra *et al.* (1992). Barley anthers containing microspores at mid-late to late uninucleate stage were used for the induction of androgenesis. Pretreatment consisted of anther incubation in 0.37 M mannitol solution for 4 d in the dark at 25 °C (Hoekstra *et al.*, 1992). After pretreatment, microspores were isolated manually from anthers and the number of enlarged and non-enlarged microspores was estimated in eight independent experiments (*n*=8). About 300 microspores were counted per experiment. Pretreated microspores were loaded on a 15% (w/v) sucrose gradient in 0.37 M mannitol solution and centrifuged at 125 *g* for 10 min for the separation of enlarged and non-enlarged cells. Microspores were used for protein isolation, cytological staining or were cultured in order to develop embryo-like structures (ELSs). Microspore culture was done according to Hoekstra *et al.* (1993).

Due to the heterogeneity of ELS development during microspore culture, representative stages of ELS development were purified from 0, 3, 8, 14, and 21-d-old cultures by filtration through appropriate mesh sizes. These fractions were used for both immunolocalization studies and western blot analysis. Enlarged microspores from 3-d-old cultures were separated from non-enlarged microspores by filtration through 45 μm nylon mesh, and the fraction larger than 45 μm was assayed. 8-d-old cultures were fractionated by filtration through a 110 μm nylon mesh. The filtrate smaller than 110 μm comprised enlarged non-dividing structures and dividing microspores inside the exine wall, while the retentate bigger than 110 μm was mainly composed by ELSs that had just been released from the exine wall. The retentate bigger than 110 μm was assayed. From 14 days-old cultures, ELSs retained by filtration through 500 μm nylon mesh were collected. Embryos from 21-d-old cultures ranging 0.5–1.0 mm were manually harvested from media by forceps.

Immature zygotic embryos

In vivo developed immature zygotic embryos were dissected under a binocular microscope from 21 d after pollination (DAP) seeds and used for immunolocalization studies.

Protein isolation and western analysis

Pretreated microspores, purified enlarged and non-enlarged microspores and staged-ELSs from 0, 3, 8, 14, and 21-d-old cultures were ground with a glass pestle at room temperature in 60 mM Tris pH

6.8, 10% glycerol, 5% β-mercaptoethanol and 2% SDS for total protein extraction. The extracts were boiled for 10 min at 95–100 °C and centrifuged twice at 15 000 *g* for 10 min to collect the supernatant. Soluble proteins (10 μg) were separated on 15% (w/v) SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated overnight at 4 °C with isoform-specific anti-14-3-3 antibodies (1:20 000). The isoform-specific anti-14-3-3 antibodies were raised against the unconserved C-terminal region of the 14-3-3 proteins and the antibodies were demonstrated to show no cross-reaction (Testerink *et al.*, 1999). Two anti-14-3-3A antibodies, raised against synthetic peptides 237–250 and 251–261 were used (Testerink *et al.*, 1999, 2001). Unless mentioned otherwise, anti-14-3-3A raised against peptides 237–250 was used for western blot analysis and immunolocalization studies. Anti-14-3-3B and anti-14-3-3C antibodies were raised against peptides 248–262 and 251–262, respectively (Testerink *et al.*, 1999). Bands were visualized by goat anti-rabbit horseradish peroxidase conjugate (Promega), followed by enhanced chemoluminescent detection (ECL) (Amersham). Protein loading was checked by staining protein gels with 0.1% coomassie brilliant blue R-250 (Sigma) in 40% (v/v) methanol and 10% (v/v) acetic acid, followed by incubation in 20% (v/v) methanol and 10% (v/v) acetic acid.

Immunolocalization studies

ELSs from 0, 3, 8, 14, and 21-d-old microspore cultures and 21 DAP zygotic embryos were fixed in 4% (w/v) paraformaldehyde in 10 mM NaH_2PO_4, 120 mM NaCl, 2.7 mM KCl, pH 7.4 (phosphate-buffered saline, PBS) containing 10 mM dithiothreitol (DTT) overnight at 4 °C. The material was dehydrated at room temperature through a graded ethanol series as follows: 70%, 90%, 96%, and 100% (v/v). DTT was present in all dehydration steps at a concentration of 10 mM. The resin was infiltrated through a graded series of ethanol:buthyl-methyl-methacrylate (BMM) 3:1, 1:1, 1:3 (v:v) containing 10 mM DTT, overnight at 4 °C. The plant material was embedded in 100% (v/v) BMM containing 10 mM DTT and the resin was polymerized in Beem capsules under UV light for 48 h at –20 °C. Sections (5 μm) were attached in 2% 3-aminopropyl-triethoxy silane (Sigma) coated slides. After removal of the resin by acetone, proteins were denatured for 20 min in 0.4% (w/v) SDS, 3 mM β-mercaptoethanol, 12 mM Tris pH 6.8, and blocked for 30 min in 1% (w/v) BSA in PBS buffer. For immunolocalization studies, the antibodies were purified on an affinity column using the synthetic peptides according to Testerink *et al.* (1999). Primary antibody incubation was carried out overnight at 4 °C in 0.01% acetylated BSA (Aurion) in PBS buffer (anti-14-3-3A diluted 1:1000; anti-14-3-3B diluted 1:5000 and anti-14-3-3C diluted 1:5000). Control experiments were performed by omitting the first antibody and, in the case of 14-3-3C, preimmune serum was available which was used in a dilution of 1:5000 in PBS buffer containing 0.01% acetylated BSA (Aurion). Sections were developed with goat anti-rabbit alkaline phosphatase conjugate antibody (Promega). The signal was visualized by incubating sections in nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Promega). Slides were mounted in 166 g l^{-1} polyvinylalcohol, 30% (v/v) glycerol in PBS buffer and visualized under a light microscope.

Cytological observations and starch staining

Isolated microspores after androgenesis induction were stained for viability with fluorescein diacetate (FDA). FDA can pass through the cell membrane whereupon intracellular esterases cleave off the diacetate group. The fluorescein accumulates in microspores which possess intact membranes so the green fluorescence can be used as a marker of cell viability. Microspores which do not possess an intact cell membrane or an active metabolism may not accumulate the fluorescent product. FDA was used in a final concentration of 0.04 μg ml^{-1} in acetone for 10 min at room temperature and observed under a fluorescence microscope. The number of FDA positive and FDA negative cells was estimated in eight independent experiments (n=8). Around 300 microspores were counted per experiment.

Sections of 5 μm from 3, 8, 14, and 21-d-old ELSs and 21 DAP zygotic embryos were incubated for 1 min at room temperature in 5.7 mM iodine and 43.4 mM potassium iodine in 0.2 N HCl (IKI) for staining of starch. Starch staining was observed under light microscope.

Experimental data

Mean values ±SD are presented unless stated otherwise. Cell enlargement and microspore viability after pretreatment were correlated using the *r* correlation coefficient (P >0.95) in eight independent experiments (n=8).

Results

14-3-3 expression in microspore developmental switch and androgenesis

Microspores were induced to enter the embryogenic pathway by pretreating whole anthers in mannitol salt solution. The anthers were collected at the mid-late to late uninucleate stage of microspore development. After pretreatment, 18.9±7% (n=8) of the microspores had acquired enlarged morphology (40–60 μm in diameter) and showed red/blue interference of the exine wall (Fig. 1A, arrow 1, C). The remaining non-enlarged microspore population was composed of cells with 35–40 μm in diameter, showing blue/black interference of the exine wall (Fig. 1A, arrow 2, E). Microspore viability was assayed immediately after pretreatment and only 19.2±5% (n=8) of the microspores were FDA positive, indicating that they were alive. While the enlarged microspores were usually positively stained for FDA, the non-enlarged microspores were negatively stained for FDA, indicating probably dying cells after pretreatment (Fig. 1A, B). Due to their difference in size, it was possible to separate these two populations by a sucrose gradient. FDA staining of the two separated microspore populations shows that only the enlarged population contains FDA positive cells (Fig. 1C–F).

The expression of 14-3-3A, 14-3-3B and 14-3-3C isoforms in the pretreated microspores was studied using isoform-specific antibodies. In these samples, anti-14-3-3A recognized two bands, at 30 kDa and 28 kDa, respectively. Anti-14-3-3B detected one band of approximately 31 kDa, as well as anti-14-3-3C (Fig. 2A, B, C, lane 1). In order to know whether there were any differences in 14-3-3 protein levels between enlarged and non-enlarged microspores, 14-3-3 protein expression was analysed in enlarged and non-enlarged microspores that were separated by a sucrose gradient. 14-3-3A in the enlarged fraction was present mainly as its 30 kDa form (Fig. 2A, lane 2), while in the non-enlarged fraction a predominant band of 28 kDa was present (Fig. 2A, lane 3).
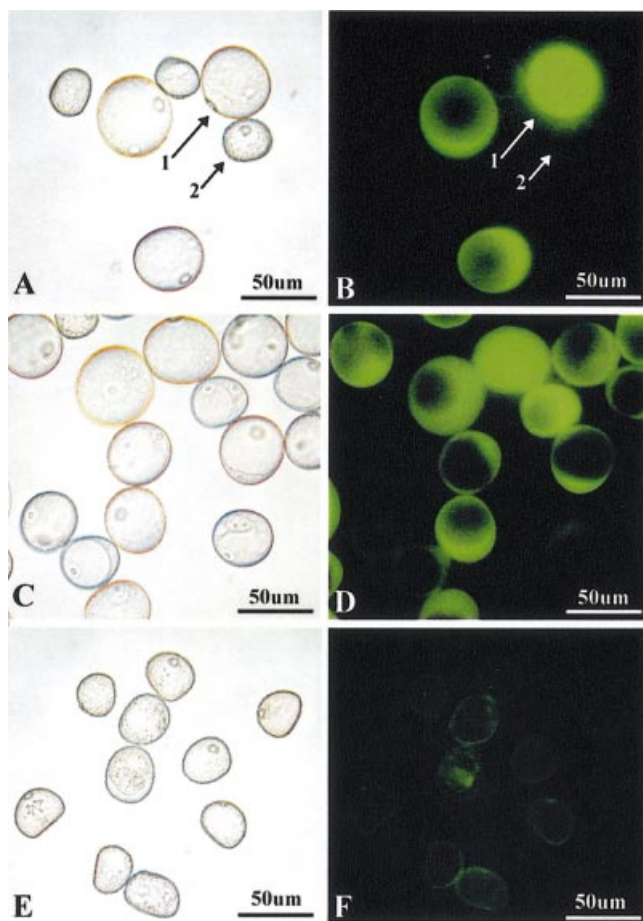
Fig. 1. Brightfield image of isolated microspores after 4 d pretreatment in mannitol solution (A) and under fluorescence microscope stained for FDA (B). The two types of microspores are indicated by arrows: arrow 1 indicates an enlarged microspore and arrow 2 indicates a non-enlarged microspore. The correlation coefficient *r* between cell enlargement and cell viability of the microspores after pretreatment was 0.74 (*n*=8, *P* >0.95). Enlarged and non-enlarged microspores were separated by a 15% sucrose gradient. Brightfield image of purified enlarged microspores (C) and under the fluorescence microscope stained for FDA (D). Brightfield image of purified non-enlarged microspores (E) and under the fluorescence microscope stained for FDA (F).

It has been reported that a 28 kDa 14-3-3A band is formed upon proteolytic cleavage of the unconserved C-terminal region of the 14-3-3A 30 kDa protein at position Lys 250/Ala 252, whereas 14-3-3B and 14-3-3C were not processed (van Zeijl *et al.*, 2000; Testerink, 2001). In order to confirm whether the 28 kDa form detected in non-enlarged microspores was due to the same post-transla-tional event, an anti-14-3-3A antibody that recognizes the amino acids 251–262 was used. The antibody detected only the 30 kDa 14-3-3A form in pretreated microspores (Fig. 2D). These results indicate that the 28 kDa 14-3-3A band observed in non-enlarged microspores was formed due to loss of the unconserved C-terminus of the protein.

14-3-3B protein expression did not differ between enlarged and non-enlarged microspores (Fig. 2B, lanes 2,

3). However, 14-3-3C protein was detected at higher levels in the enlarged fraction as compared to the non-enlarged population (Fig. 2C, lanes 2, 3).

The next step was to investigate whether microspore division and tissue differentiation during culture was also accompanied by a 14-3-3A post-translational event or differences in 14-3-3C protein levels. Western blot analy-sis was done in dividing structures at 3, 8, 14, and 21 d of microspore culture. At each time-point, multicellular structures were fractionated by filtration in appropriate mesh sizes in order to eliminate dead cells, degenerating microspores and dividing microspores with delayed development. This resulted in homogeneous populations that represented different embryo developmental stages during microspore culture. In these samples, only the 30 kDa form of 14-3-3A was present (Fig. 3A). The 28 kDa form of 14-3-3A was not detected in dividing structures. No changes in 14-3-3C levels were observed during microspore culture, however, anti-14-3-3B recognized an additional slightly higher molecular band in 8–14-d-old ELS (Fig. 3B, C).

Immunolocalization of 14-3-3 isoforms during embryogenesis

Microspores at 3 d of culture: Although the enlarged microspores were considered to have embryogenic poten-tial, androgenic divisions were observed in only 7.1±1 % (*n*= 3) of the enlarged microspores when cultured as isolated cells. Due to this heterogeneity in embryogenic potential, the subcellular localization of the 14-3-3 isoforms was further investigated in the enlarged micro-spore population.

Microspores directly isolated from pretreated anthers (day 0) presented mainly cytoplasmic localization for the three 14-3-3 isoforms, except that 14-3-3C was barely detectable in sections of non-enlarged microspores (data not shown). However, after 3 d of further culture, some enlarged microspores presented several division walls and multiple nuclei, while others did not divide. In dividing microspores, strong 14-3-3A, 14-3-3B and 14-3-3C expression was mainly detected in the cytoplasm of cells (Fig. 4A–C). 14-3-3 signals in the nucleus were not higher than the control background level (Fig. 4D). Incubation of sections with preimmune serum of the rabbit used for immunization with the 14-3-3C peptide revealed no background signal (data not shown). On the other hand, microspores that did not divide only presented a weak 14-3-3 signal in the cytoplasm. In these microspores, 14-3-3B and 14-3-3C isoforms appeared localized in a 'dotted' pattern (Fig. 4F, G, dots are indicated by arrowheads). This pattern was not pronounced for 14-3-3A (Fig. 4E). Concomitantly, the non-dividing microspores were also positively stained with IKI, indicating the presence of starch granules in the cytoplasm (Fig. 4H, starch granules are indicated by arrowheads).
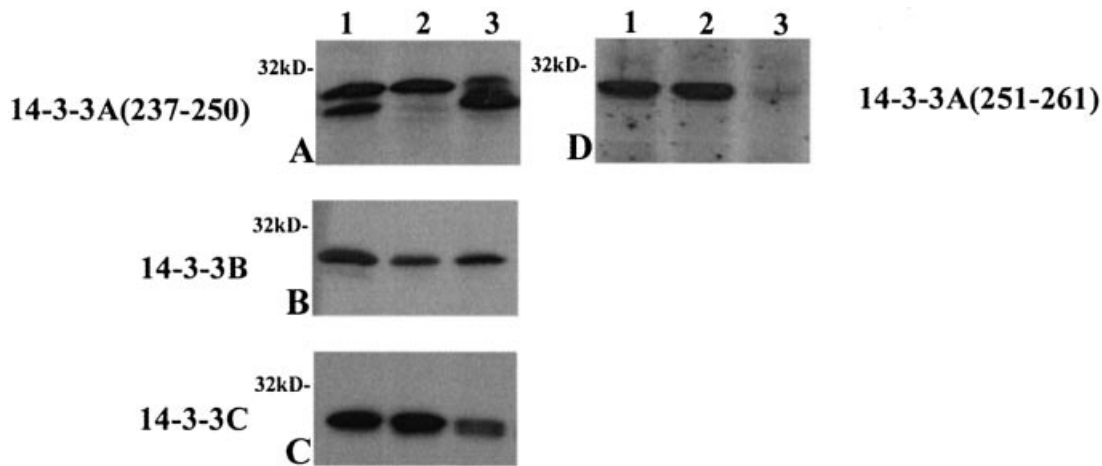
Fig. 2. Western blot analysis of 14-3-3A, 14-3-3B and 14-3-3C proteins in microspores after androgenesis induction. 10 μg per lane of total protein extracts were separated by SDS-PAGE, blotted onto nitrocellulose membranes and incubated with isoform-specific antibodies, followed by enhanced chemoluminescent detection. Lanes were equally loaded as checked by coomassie blue staining (data not shown). Blots were incubated with anti-14-3-3A raised against synthetic peptide 237–250 (A), anti-14-3-3B (B), anti-14-3-3C (C) and anti-14-3-3A raised against synthetic peptide 251–262 (D). Lane (1) microspores after 4 d pretreatment in mannitol solution, lane (2) enlarged microspores and lane (3) non-enlarged microspores after separation by a 15% sucrose gradient. One representative blot from four independent experiments is shown for each 14-3-3 isoform.
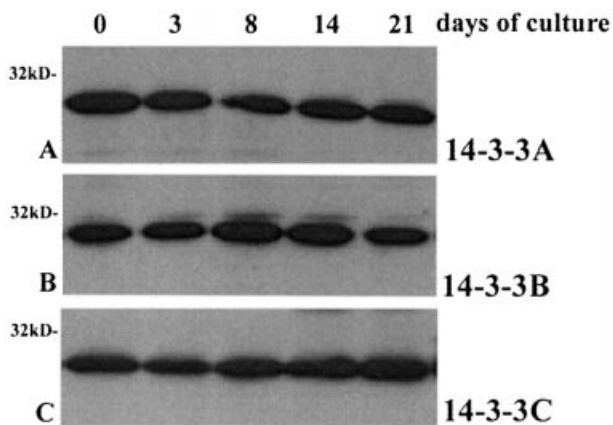
Fig. 3. Western blot analysis of 14-3-3A, 14-3-3B and 14-3-3C proteins in ELSs from 0, 3, 8, 14, and 21-d-old cultures. Dividing structures were purified by filtration using appropriate mesh sizes and 10 μg per lane of total protein extracts were separated by SDS-PAGE. Proteins were blotted onto nitrocellulose membranes and incubated with the isoform-specific antibodies. Signal was detected by enhanced chemoluminescent methods. Lanes were equally loaded as checked by coomassie blue staining (data not shown). Blots were incubated with anti-14-3-3A (A), anti-14-3-3B (B) and anti-14-3-3C (C). One representative blot from four independent experiments is shown for each 14-3-3 isoform.

The non-dividing microspores degenerated and died by 8 d of culture, while dividing cells increased their mass and ruptured the exine wall. Interestingly, western blot analysis in the non-dividing fraction at day 8 of culture revealed the presence of the 28 kDa processed form of 14-3-3A (data not shown). This is in agreement with the appearance of the 28 kDa processed form of 14-3-3A in non-enlarged microspores with decreased viability directly after pretreatment (Fig. 2A).

Pattern formation at 8 d and 14 d of culture: In 8-d-old cultures, multicellular structures had ruptured the exine wall and were characterized by a mass of undifferentiated cells. After exine wall release, they followed an intensive morphogenetic programme. Some of the 14-d-old multicellular structures started to differentiate tissues in the embryo body, with shoot and root meristem formation taking place. At this stage, the scutellum was present as a well-developed structure and the embryo-like structures (ELSs) were surrounded by a differentiated cell layer, the epidermis (Fig. 5).

14-3-3 immunolocalization was studied in multicellular structures derived from 8 and 14-d-old cultures. At day 8 of culture, 14-3-3A signal was distributed all over the multicellular structure, but it was shown to be strongest in the outer layer, associated with the differentiation of the epidermis primordia (Fig. 5A). At a later stage of development (14-d-old ELSs), the 14-3-3A isoform was detected in the scutellum, root and shoot meristems, mesocotyl, and in the epidermal tissue. Nevertheless, some cells within the root and shoot meristem showed no 14-3-3A signal (Fig. 5B).

The 14-3-3B isoform exhibited a mosaic expression in 8-d-old multicellular structures, but no defined pattern was observed. Expression of the 14-3-3B isoform in 14-d-old ELSs resembled that of 14-3-3A, except that a 14-3-3B signal was not detected in the epidermis (Fig. 5C, D).

It was quite clear that 14-3-3C expression differed significantly from that of 14-3-3A and 14-3-3B. In multicellular structures derived from 8-d-old cultures, 14-3-3C was often expressed more strongly in one domain of the embryo, where it appeared associated with

cytoplasmic dots. In other domains, the 14-3-3C signal was less strong (Fig. 5E). In 14-d-old ELSs, 14-3-3C signal was only detected in scutellum cells, while it was completely absent in all meristematic regions (Fig. 5F). IKI staining in sections of 14-d-old ELSs indicated the presence of starch granules in the cytoplasm of scutellum cells, where 14-3-3B and 14-3-3C were also localized in cytoplasmic dots (data not shown). No signal could be detected in control experiments (Fig. 5G, H).

21-d-old ELSs and in vivo-*developed immature zygotic embryos*: At 21 d of culture, most of the ELSs ranging 0.5–1.0 mm in culture are capable of germination when transferred to regeneration media (Hoekstra *et al.*, 1992). These ELSs were assayed for immunolocalization studies. At this stage, 14-3-3A was immunolocalized in the scutellum, root and shoot meristems, root cap, leaf primordia, mesocotyl and epidermis. (Fig. 6A, E). 14-3-3B signal was much stronger than that of 14-3-3A and it was found in all embryogenic tissues (Fig. 6B, F). 14-3-3C signal was restricted to scutellum and one group of cells underneath the L_1 layer of the shoot apical meristem (Fig. 6C, G). 14-3-3C was absent from the root meristem and root cap (Fig. 6G), as the signal was not stronger than that observed in control sections incubated only with the secondary antibody (Fig. 6D, H).

As ELSs were grown under *in vitro* conditions, 14-3-3 isoforms were studied during normal *in vivo* embryo development. To do so, immature zygotic embryos were assayed at 21 d after pollination (DAP) for 14-3-3 immunolocalization studies. In 21 DAP zygotic embryos, 14-3-3A was expressed in root and shoot meristems, mesocotyl, scutellum, coleoptile, and epidermis (Fig. 6I). The 14-3-3B isoform seemed to be ubiquitously expressed at this stage, except that some regions within the leaf primordia were not stained (Fig. 6J). The 14-3-3C signal was detected in the scutellum, mesocotyl and underneath the L_1 layer of the shoot apical meristem (Fig. 6K). No signal was detected when the first antibody was omitted (Fig. 6L) or, in the case of 14-3-3C, sections of both ELS and immature zygotic embryos were incubated with the preimmune serum (data not shown). Although 21 DAP immature zygotic embryos were at a further developmental stage compared to *in vitro*-developed 21-d-old ELSs, both showed similar 14-3-3 isoform-specific expression patterns.

Discussion

Role of 14-3-3 proteins in embryogenic potential acquirement

Androgenesis induction in barley is characterized by two types of morphologically distinct microspores, namely enlarged and non-enlarged cells (Hoekstra *et al.*, 1992). An average of 19% of the microspores showed enlarged morphology after pretreatment. In previous work, cell enlargement was found in 60% of the microspore population after pretreatment of barley anthers (Hoekstra *et al.*,
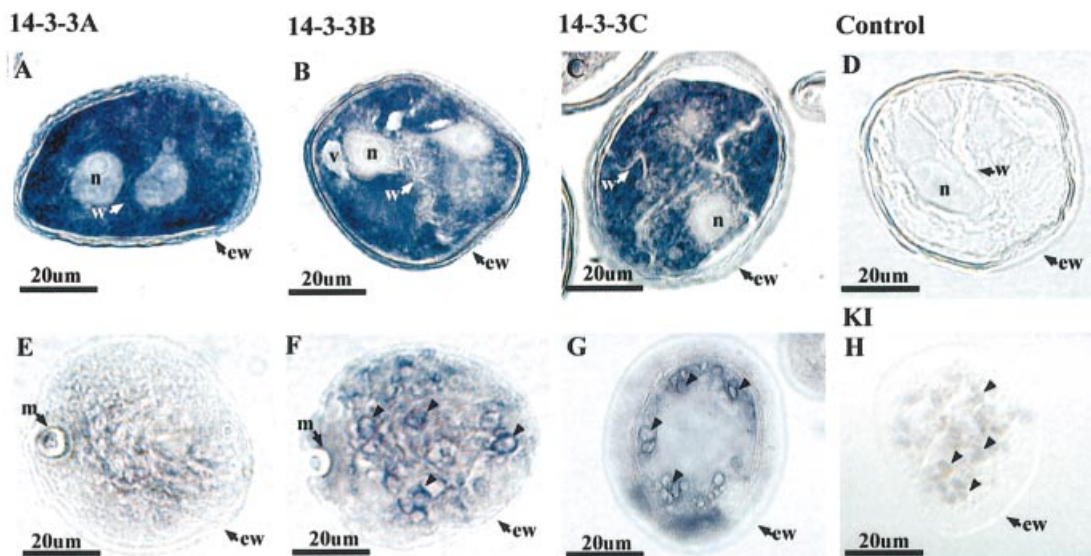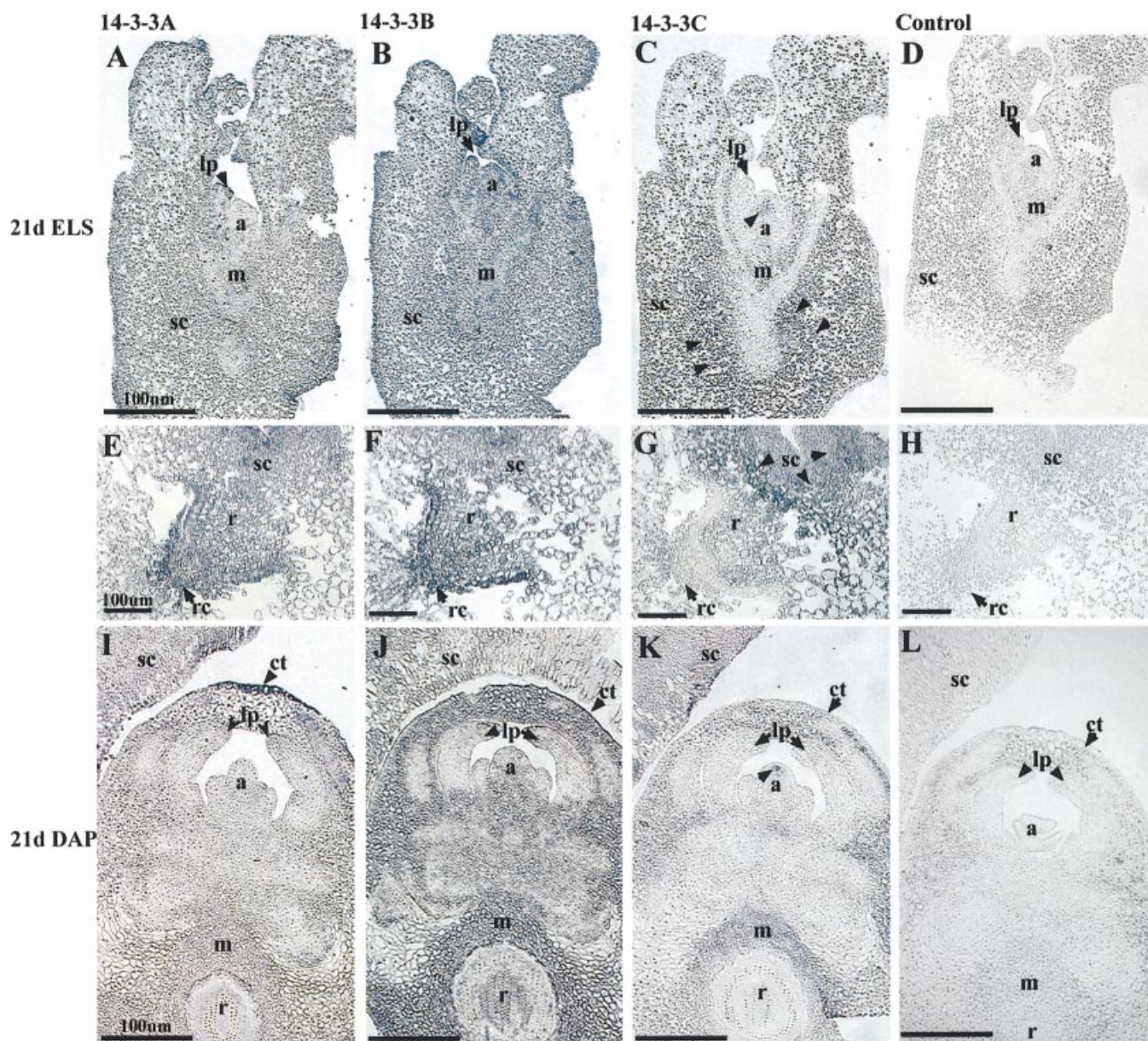

Fig. 4. Brightfield image of 14-3-3 immunolocalization in enlarged microspores from 3-d-old cultures. Cross-sections of dividing (A–C) and non-dividing microspores (E–G) showing immunolocalization of 14-3-3A, 14-3-3B and 14-3-3C, respectively. Control section of a dividing microspore incubated only with secondary antibody (D). Section of a non-dividing microspore stained with IKI observed by differential intereference contrast (H). Arrowheads in (F) and (G) indicate 14-3-3B and 14-3-3C 'dotted' cytoplasmic localization. Arrowheads in (H) indicate starch granules. Abbreviations: ew, exine wall; m, micropore; n, nucleus; v, vacuole; w, dividing wall. 14-3-3 immunolocalization was studied in three independent experiments. Sections of at least 10 dividing and non-dividing microspores were studied per experiment. Representative examples are shown for each 14-3-3 isoform.
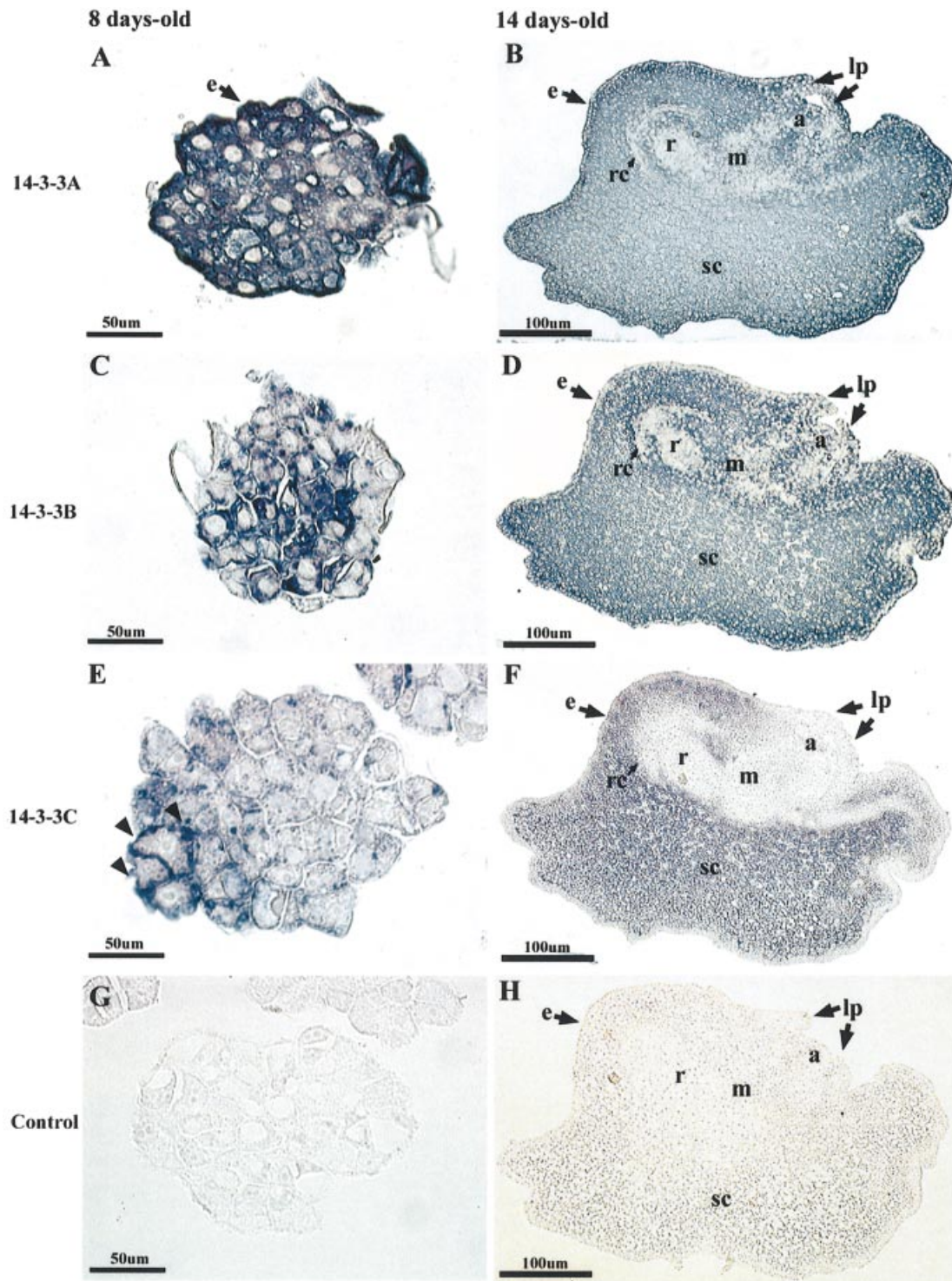
Fig. 5. Brightfield image of 14-3-3 immunolocalization in 8 and 14-d-old ELSs. Cross-section of 8 and 14-d-old ELSs were incubated with anti-14-3-3A antibody (A, B), anti-14-3-3B antibody (C, D), anti-14-3-3C antibody (E, F). Control sections were incubated only with secondary antibody (G, H). Incubation of sections with preimmune serum of the rabbit used for immunization with the 14-3-3C peptide revealed no background signal (data not shown). Arrowheads in (E) indicate domain of ELS with increased 14-3-3C expression. Abbreviations: a, shoot apical meristem; e, epidermis; lp, leaf primordia; m, mesocotyl; r, root meristem; rc, root cap. 14-3-3 immunolocalization was studied in three independent experiments. Sections of at least 10 ELS were studied per experiment. Representative examples are shown for each 14-3-3 isoform.

Fig. 6. Brightfield image of 14-3-3 immunolocalization in 21-d-old ELSs and 21 DAP zygotic embryos. Sections of two different ELSs are shown in order to illustrate 14-3-3 expression in shoot (A–D) and root meristems (E–H). Sections of 21-d-old ELSs were incubated with anti-14-3-3A (A, E), anti-14-3-3B (B, F) and anti-14-3-3C (C, G) antibodies, respectively. Control sections incubated only with secondary antibody (D, H). Sections of 21 DAP zygotic embryo incubated with anti-14-3-3A, anti-14-3-3B and anti-14-3-3C antibodies, respectively (I–K). Control section incubated only with secondary antibody (L). Arrowheads in (C) and (G) indicate 14-3-3C expression underneath the L_1 layer of the shoot apical meristem and in scutellum cells and in (K) 14-3-3C underneath the L_1 layer of the shoot apical meristem. Abbreviations: a, shoot apical meristem; ct, coleoptile; lp, leaf primordia; m, mesocotyl; r, root meristem; rc, root cap; sc, scutellum. 14-3-3 immunolocalization was studied in three independent experiments. Sections of at least five embryos were studied per experiment. Representative examples are shown for each 14-3-3 isoform.

1993). This variation could be due to the different microspore isolation techniques used, as previously reported by Ritala *et al*. (2001). Cell enlargement was further demonstrated to correlate with cell viability after stress pretreatment, however androgenic divisions were observed only within 7% of the viable cells. This indicates that only some of the enlarged, viable microspores have

acquired embryogenic potential after pretreatment. This is supported by the observations made by single-cell tracking experiments in barley microspores (Bolik and Koop, 1991; Kumlehn and Lörz, 1999).

14-3-3 expression analysis showed that enlarged microspores mainly contain the 30 kDa form of 14-3-3A and have an increased 14-3-3C expression level. Non-enlarged

microspores mainly have the processed 28 kDa form of 14-3-3A and a decreased level of 14-3-3C expression. 14-3-3A processing by proteolytic cleavage at positions Lys 250/Ala 252 has been previously described in the germination of mature barley embryos (van Zeijl *et al.*, 2000; Testerink, 2001). Cell death has been reported to occur in barley scutellum (Lindholm *et al.*, 2000) and the aleurone layer during the onset of germination (Wang *et al.*, 1996). In the *in vitro* system studied, 14-3-3A processing was observed in non-enlarged microspores with decreased viability after pretreatment and in non-dividing cells in culture. The isoform-specific proteolytic cleavage of 14-3-3A may represent the beginning of a total global degradation of the 14-3-3A protein, being related to the cell death pathway of these cells. However, the possibility that processing affects 14-3-3 subcellular localization (van Zeijl *et al.*, 2000) and the functionality of the 14-3-3A isoform cannot be excluded. Recently, both the 30 kDa and 28 kDa forms were found to bind to the plasma membrane H^+-ATPase in *in vitro* western blot overlay assays (Testerink, 2001). Further characterization of the 14-3-3A proteinase will help elucidating the role of the 28 kDa processed form of 14-3-3A in relation to cell death. The question raised is whether the low 14-3-3C content in non-enlarged microspores after pretreatment is also related to the death of these cells. However, later in culture, the meristematic regions of 14-d-old ELSs also showed a decreased 14-3-3C signal in immunolocalization assays. Developing meristems are known to have high division ability (Goldberg *et al.*, 1994). Thus, it is not likely that the low 14-3-3C content is related to the decreased viability of cells.

After 3 d of further culture of pretreated enlarged microspores, two sub-populations could be distinguished, dividing and non-dividing cells. In non-dividing cells, starch granules were observed and 14-3-3B and 14-3-3C were mainly localized in cytoplasmic dots. Starch accumulation in pollen amyloplasts marks the commitment to the gametophytic pathway (McCormick, 1993). In the amyloplasts, 14-3-3 proteins have recently been found associated with starch granules and starch synthase III has been proposed as a putative 14-3-3 interacting target (Sehnke *et al.*, 2001). It is possible that 14-3-3B and 14-3-3C signals detected in these dots are associated with starch granule formation in the non-dividing microspores. Dividing cells, however, did not accumulate starch and 14-3-3 isoforms were mainly localized in the cytoplasm. The allocation of different 14-3-3 isoforms inside the cell may play an important role in global 14-3-3 protein interactions. In plants, 14-3-3 proteins form a guidance complex with chloroplast precursor proteins (May and Soll, 2000), facilitating protein import into the chloroplast. In animal cells, 14-3-3 proteins have been reported to be involved in the import of mitochondrial protein (Alam *et al.*, 1994) and in the subcellular compartmentalization of

specific targets, by which they can affect cell fate (Muslin and Xing, 2000). This suggests that the subcellular differences in the localization of the 14-3-3 proteins in dividing and non-dividing microspores may have implications in their different developmental pathways.

Tissue-specific expression of 14-3-3 proteins during *in vivo* and *in vitro* embryogenesis

The shift from radial to bilateral symmetry is a crucial event in plant embryogenesis, as it comprises the events involved in the establishment of the scutellum and the embryonic axis (Souter and Lindsey, 2000). During microspore embryogenesis, pattern formation is delineated after the release of undifferentiated multicellular structures from the exine wall of microspores (Yeung *et al.*, 1996). The periclinal divisions of the outer cell layer of globular embryos have been demonstrated to culminate in the first tissue differentiation, the epidermis (Telmer *et al.*, 1995). In barley androgenesis, globular masses of undifferentiated cells were released from the exine wall after 1 week of culture. The uniformly cytoplasmic localization of 14-3-3 isoforms in dividing microspores was progressively changed after exine wall rupture. Prior to epidermis differentiation, 14-3-3A signal was highest in the outer layer of multicellular structures, while 14-3-3C expression was polarized. During further development, 14-3-3C expression was tissue-specific to the scutellum of 14-d-old ELSs, while the epidermis expressed only the 14-3-3A isoform. In other androgenic systems, expression of polarity prior to tissue differentiation has been reported only at the structural and morphological level. Multicellular structures were shown to polarize according to cell size while still inside the exine wall of microspores of *Zea mays* L. and *Triticum aestivum* L. (Magnard *et al.*, 2000; Bonet and Olmedilla, 2000). After exine wall rupture, polarization has been demonstrated by means of spatial differences in starch accumulation in globular embryos of *Triticum aestivum* L. and *Brassica napus* L. (Indrianto *et al.*, 2001; Hause *et al.*, 1994). The spatial differences described here for the expression of 14-3-3 isoforms are among the first biochemical evidence to show pattern formation in androgenesis. Later in development, 14-3-3C isoform was specifically expressed in the scutellum and in one group of cells underneath the L_1 layer of the shoot apical meristem of 21-d-old ELSs. As the same 14-3-3C expression pattern was observed in 21 DAP immature zygotic embryos, this indicates a feature of both *in vivo* and *in vitro* embryogenesis. Testerink *et al.* (1999) have described L_2 layer-associated 14-3-3C expression in mature barley embryos. These results suggest that the re-expression of 14-3-3C in the shoot apical meristem is likely to occur during L_2 layer specification, and reinforce the idea that 14-3-3C specific expression is prior to the formation of tissues during the embryogenic process of barley.

The highly developmentally regulated expression patterns observed for 14-3-3A and 14-3-3C may be involved in conferring isoform-specific functions *in vivo*, as little isoform-specificity has so far been described (Bachmann *et al*., 1996b; Rosenquist *et al*., 2000). In addition, it may reveal as yet unexplored functions for the plant 14-3-3 isoforms. Spatial and temporal regulation of 14-3-3 isoforms are known to be involved in MAPK activation and tissue differentiation during pattern formation in animal embryogenesis (Tien *et al*., 1999). It will be a challenge to determine which are the binding partners of 14-3-3A and 14-3-3C during pattern formation and L_2-layer specification in plant embryogenesis, and whether these expression patterns are also followed by activation of the MAPK cascade, approximating 14-3-3 functions in animal and plant systems.

Acknowledgements

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