Androgenic switch in barley microspores

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Chapter 4

14-3-3 proteins in barley androgenesis

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

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Abstract

The members of the 14-3-3 isoform family have been shown to be developmentally regulated during animal embryogenesis, where they take part of cell differentiation processes. 14-3-3 isoform-specific expression patterns were studied in plant embryogenic process, using barley (*Hordeum vulgare* L.) microspore embryogenesis as a model system. After embryogenesis induction by stress, microspores with enlarged morphology presented higher viability than non-enlarged ones. Following microspore culture, cell division was only observed among 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 to 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 days 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.

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 et al., 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., 1996a; 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 signaling 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, fifteen 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 et al., 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 14-3-3ξ 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 positively regulate Ras-Raf signaling pathway, leading to MAPK cascade activation (Chang et al., 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 Heusden et al., 1996) indicates that 14-3-3 may take part of 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 efficiently induce 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 days of culture (Hoekstra et al., 1992). Barley androgenesis is a suitable model system to study plant embryogenesis, as embryo development can be easily 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 conditions described previously (Hoekstra et al., 1992). Barley anthers containing microspores at mid-late to late uninucleate stage were used for induction of androgenesis. Pre-treatment consisted of anther incubation in 0.37M mannitol solution for 4 days in the dark, at 25°C (Hoekstra et al., 1992). After pre-treatment, microspores were isolated manually from anthers and the number of enlarged and non-enlarged microspores was estimated in 8 independent experiments (n=8). Around 300 microspores were counted per experiment. Pre-treated microspores were loaded on a 15% (w/v) sucrose gradient in 0.37M mannitol solution and centrifuged at 125g for 10 minutes for separation of enlarged and non-enlarged cells. Microspores were used for protein isolation, cytological staining or cultured in order to develop embryo-like structures (ELSs). Microspore culture was done according to Hoekstra et al. (1993).

Due to heterogeneity of ELS development during microspore culture, representative stages of ELS development were purified from 0, 3, 8, 14 and 21 days-old cultures by filtration through appropriate mesh sizes. These fractions were used for both immunolocalization studies and western blot analysis. Enlarged microspores from 3 days-old cultures were separated from non-enlarged microspores by filtration through 45μm nylon

mesh, and the fraction bigger than 45μm was assayed. Eight days-old cultures were fractionated by filtration through a 110μm nylon mesh. The filtrate smaller than 110μm comprised enlarged non-dividing structures and dividing microspores inside the exine wall, while the retentate bigger than 110μm was mainly composed by ELSs that had just been released from the exine wall. The retentate bigger than 110μm was assayed. From 14 days-old cultures, ELSs retained by filtration through 500μm nylon mesh were collected. Embryos from 21 days-old cultures ranging 0.5mm-1.0mm were manually harvested from media by forceps.

Immature zygotic embryos

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

Protein isolation and Western analysis

Pre-treated microspores, purified enlarged and non-enlarged microspores and staged-ELSs from 0, 3, 8, 14 and 21 days-old cultures were ground with a glass pestle at room temperature in 60mM Tris pH 6.8, 10% glycerol, 5% β-mercaptoethanol and 2% SDS for total protein extraction. The extracts were boiled for 10 minutes at 95-100ºC and centrifuged two times at 15,000*g* for 10 minutes to collect supernatant. Soluble proteins (10μg) were separated on 15% (w/v) SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated overnight at 4ºC 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; Testerink et al., 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 days-old microspore cultures and 21 DAP zygotic embryos were fixed in 4% (w/v) paraformaldehyde in 10mM NaH_2PO_4, 120mM NaCl, 2.7mM KCl, pH 7.4 (Phosphate-buffered saline, PBS) containing 10mM dithiothreitol (DTT) overnight at 4ºC. 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 10mM. The resin was infiltrated through a graded series of ethanol: buthyl-methyl-methacrylate (BMM) 3:1, 1:1, 1:3 (v:v) containing 10mM DTT, overnight at 4ºC. The plant material was embedded in 100% (v/v) BMM containing 10mM DTT and the resin was polymerized in Beem capsules under UV light for 48 hours at -20ºC. Sections (5µm) were attached in 2% 3-aminopropiltriethoxy silane (Sigma) coated slides. After removal of the resin by acetone, proteins were denatured 20 minutes in 0.4% (w/v) SDS, 3mM β-mercaptoethanol, 12mM Tris pH 6.8 and blocked 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 4ºC in 0.01% acetylated BSA (Aurion) in PBS buffer (anti-14-3-3A diluted 1:1,000; anti-14-3-3B diluted 1:5,000 and anti-14-3-3C diluted 1:5,000). 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 1:5,000 in PBS buffer containing 0.01% acetylated BSA. 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 166g.l^{-1} polyvinylalcohol, 30% (v/v) glycerol in PBS buffer and visualized under 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 minutes at room temperature and observed under fluorescence microscope. The number of FDA positive and FDA negative cells was estimated in 8 independent experiments (*n*=8). Around 300 microspores were counted per experiment. Sections of 5µm from 3, 8, 14 and 21 days-old ELSs and 21 DAP zygotic

embryos were incubated 1 minute at room temperature in 5.7mM iodine and 43.4mM 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 pre-treatment were correlated using the *r* correlation coefficient (*P*>0.95) in 8 independent experiments (*n*=8).

Results

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

Microspores were induced to enter the embryogenic pathway by pre-treating whole anthers in mannitol solution. The anthers were collected at the mid-late to late uninucleate stage of microspore development. After pre-treatment, 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; Fig. 1c). 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; Fig 1e). Microspore viability was assayed immediately after pre-treatment 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 pre-treatment (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 pre-treated microspores was studied using isoform-specific antibodies. In these samples, anti-14-3-3A recognized 2 bands, at 30 kD and 28 kD respectively. Anti-14-3-3B detected one band of approximately 31 kD, as well as anti-14-3-3C (Fig. 2a-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 analyzed 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 kD form (Fig. 2a; lane 2), while in the non-enlarged fraction a predominant band of 28 kD was present (Fig. 2a; lane 3).

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

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 analysis was done in dividing structures at 3, 8, 14 and 21 days 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 kD form of 14-3-3A was present (Fig. 3a). The 28 kD 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 days-old ELSs (Fig. 3b,c).

Immunolocalization of 14-3-3 isoforms during embryogenesis: 3 days-old microspores

Although the enlarged microspores were considered to have embryogenic potential, androgenic divisions were observed in only 7.1 $\pm$ 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 microspore population. Microspores directly isolated from pre-treated anthers (day 0) presented mainly cytoplasmic localization for the three 14-3-3 isoforms (data not shown). However, after 3 days of further culture, some enlarged microspores presented several division walls and multiple nuclei, while others did not divide. In dividing ones, strong 14-3-3A, 14-3-3B and 14-3-3C expression were mainly detected in the cytoplasm of cells (Fig. 4a-c). 14-3-3 signals in the nucleus were not higher than 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).

The non-dividing microspores degenerated and died up to 8 days 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 kD processed form of 14-3-3A (data not shown). This is in agreement with the appearance of the 28 kD processed form of 14-3-3A in non-enlarged microspores with decreased viability directly after pre-treatment (Fig. 2a).

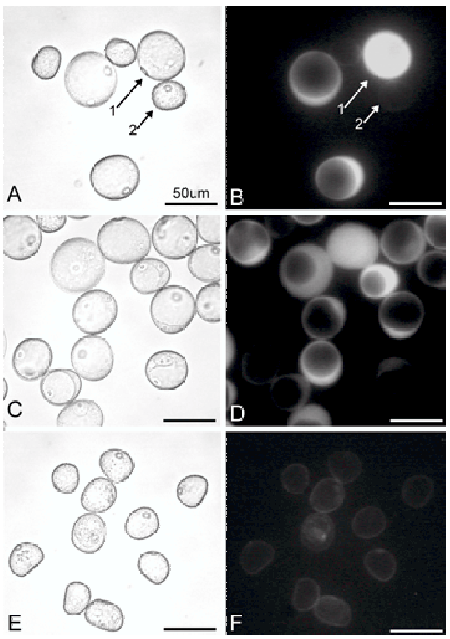

Figure 1. Brightfield image of isolated microspores after 4 days pre-treatment in mannitol solution (a) and under fluorescence microscope stained for FDA (b). The two types of microspores are indicated by arrows: *arrow 1* indicates enlarged microspore and *arrow 2* indicates non-enlarged microspore. The correlation coefficient *r* between cell enlargement and cell viability of the microspores after pre-treatment 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 fluorescence microscope stained for FDA (d). Brightfield image of purified non-enlarged microspores (e) and under fluorescence microscope stained for FDA (f).

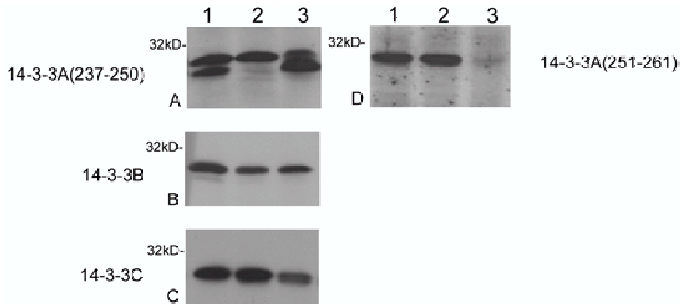

Figure 2. Western blot analysis of 14-3-3A, 14-3-3B and 14-3-3C proteins in microspores after androgenesis induction. 10μg per lane of total protein extracts were separated by SDS-PAGE, blotted onto nitrocellulose membranes and incubated with isoform-specific antibodies, followed by enhanced chemoluminescent 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 days pre-treatment in mannitol solution, *lane 2* enlarged microspores and *lane 3* non-enlarged microspores after separation by a 15% sucrose gradient. One representative blot from 4 independent experiments is shown for each 14-3-3 isoform.

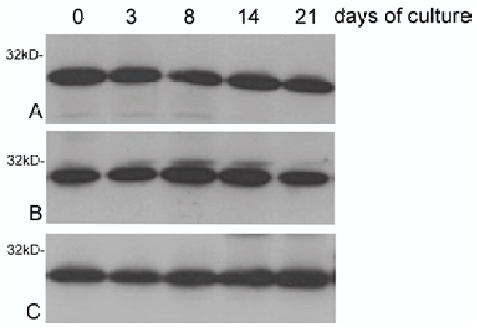

Figure 3. Western blot analysis of 14-3-3A, 14-3-3B and 14-3-3C proteins in ELSs from 0, 3, 8, 14 and 21 days-old cultures. Dividing structures were purified by filtration using appropriate mesh sizes and 10μg per lane of total protein extracts were separated by SDS-PAGE. Proteins were blotted onto nitrocellulose 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 4 independent experiments is shown for each 14-3-3 isoform.

Pattern formation in 8 and 14 days of culture

In 8 days-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 program. Some of the 14 days-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 days-old cultures. At day 8 of culture, 14-3-3A signal was distributed all over the multicellular structure, but it showed to be strongest in the outer layer, associated to the differentiation of the epidermis primordia (Fig. 5a). In a later stage of development (14 days-old ELSs), 14-3-3A isoform was detected in 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).

14-3-3B isoform exhibited a mosaic expression in 8 days-old multicellular structures, but no defined pattern was observed. Expression of 14-3-3B isoform in 14 days-old ELSs resembled that of 14-3-3 A, except that 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 days-old cultures, 14-3-3C was often higher expressed in one domain of the embryo, where it appeared associated with cytoplasmic dots. In other domains, 14-3-3C signal was less strong (Fig. 5e). In 14 days-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 days-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). We could not detect any signal in control experiments (Fig. 5g,h).

21 days-old ELSs and in vivo-*developed immature zygotic embryos*

At 21 days 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).

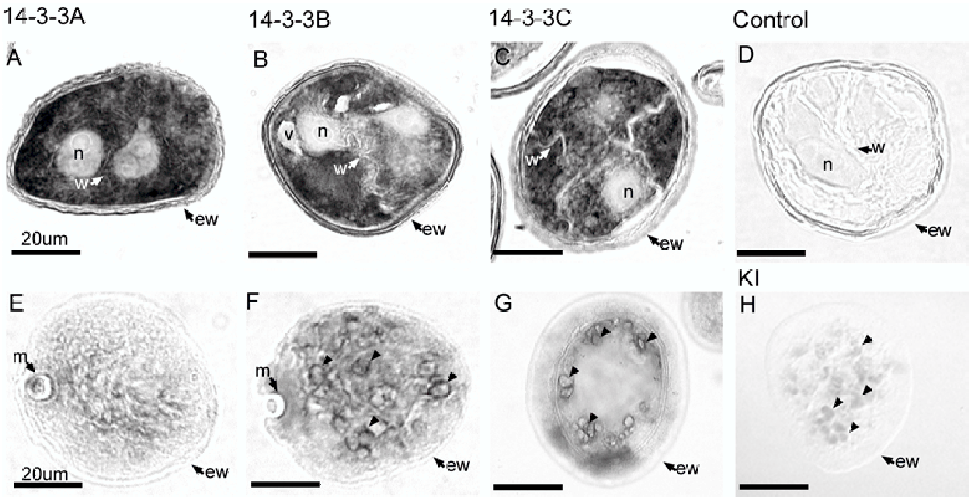

Figure 4. Brightfield image of 14-3-3 immunolocalization in enlarged microspores from 3 days-old cultures. Cross-sections of dividing (a) (b) (c) and non-dividing microspores (e) (f) (g) showing immunolocalization of 14-3-3A, 14-3-3B and 14-3-3C, respectively. Control section of dividing microspore incubated 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. *ew* exine wall, *m* micropore, *n* nucleus, *v* vacuole, *w* dividing wall. 14-3-3 immunolocalization was studied in 3 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.

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 days 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). 14-3-3B isoform seemed to be ubiquitously expressed at this stage, except that some regions within leaf primordia were not stained (Fig. 6j). 14-3-3C signal was detected in scutellum, mesocotyl and underneath the L_1 layer of the shoot apical meristem (Fig. 6k). No signal was detected when 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 days-old ELSs, both showed similar 14-3-3 isoform-specific expression patterns.

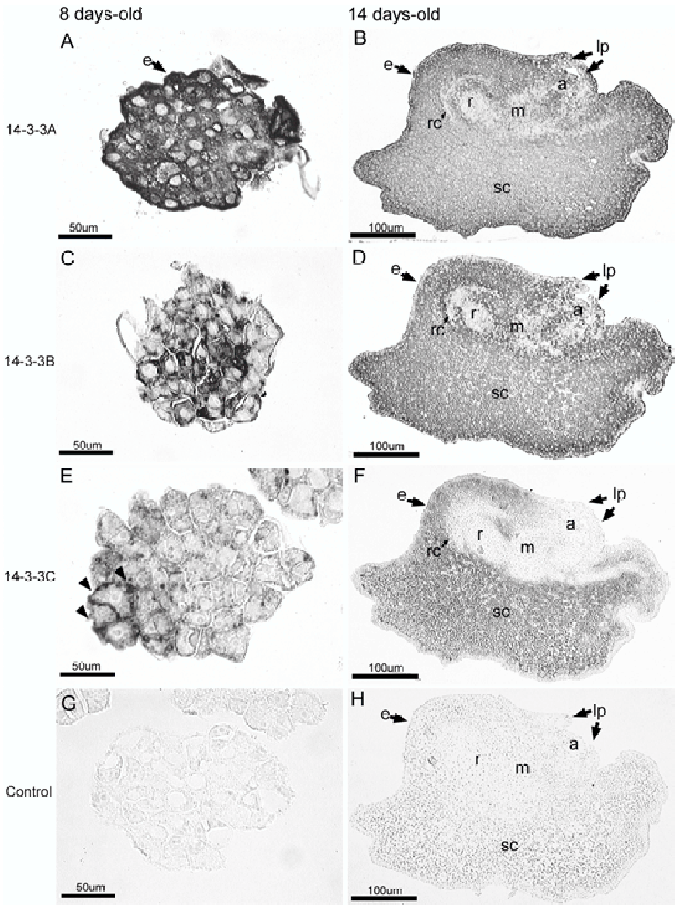

Figure 5. Brightfield image of 14-3-3 immunolocalization in 8 and 14 days-old ELSs. Cross-section of 8 and 14 days-old ELSs were incubated with anti-14-3-3A antibody (a/b), anti-14-3-3B antibody (c/d), anti-14-3-3C antibody (e/f). Control sections were incubated only with secondary antibody (g/h). Incubation 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. *a* shoot apical meristem, *e* epidermis, *lp* leaf primordial, *m* mesocotyl, *r* root meristem, *rc* root cap. 14-3-3 immunolocalization was studied in 3 independent experiments. Sections of at least 10 ELS were studied per experiment. Representative examples are shown for each 14-3-3 isoform.

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 to have enlarged morphology after pre-treatment.

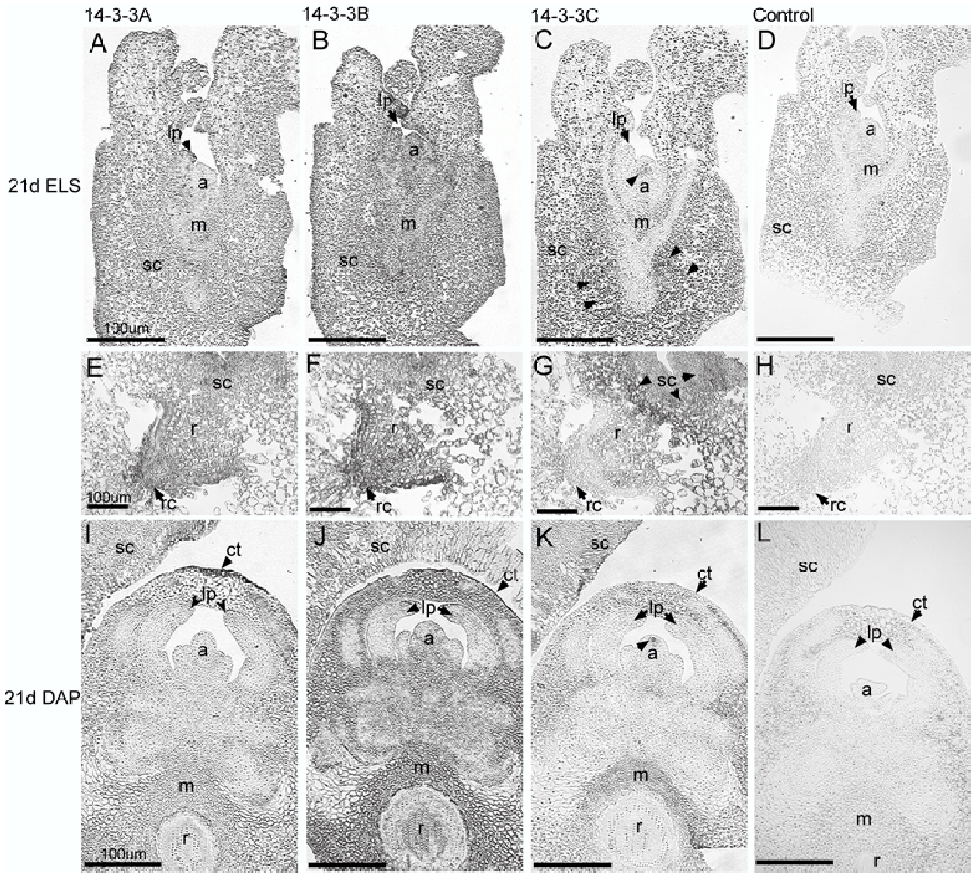

Figure 6. Brightfield image of 14-3-3 immunolocalization in 21 days-old ELSs and 21 DAP zygotic embryos. Sections of two different ELSs are shown in order to illustrate 14-3-3 expression in shoot (a, b, c, d) and root meristems (e, f, g, h). Sections of 21 days-old ELSs were incubated with anti-14-3-3A (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, j, k). Control section incubated only with secondary antibody (l). *Arrowheads* in (c) and (g) indicate 14-3-3C expression underneath L_1 layer of the shoot apical meristem and in scutellum cells and in (k) 14-3-3C underneath L_1 layer of the shoot apical meristem. *A* shoot apical meristem, *ct* coleoptile, *lp* leaf primordial, *m* mesocotyl, *r* root meristem, *rc* root cap, *sc* scutellum. 14-3-3 immunolocalization was studied in 3 independent experiments. Sections of at least 5 embryos were studied per experiment. Representative examples are shown for each 14-3-3 isoform.

In previous work, cell enlargement was found in 60% of the microspore population after pre-treatment of barley anthers (Hoekstra et al., 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 pre-treatment, however androgenic divisions were observed within only 7% of the viable cells. This indicates that only some of the enlarged, viable microspores have acquired embryogenic

potential after pre-treatment. 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 contain mainly the 30 kD form of 14-3-3A and have increased 14-3-3C expression level. Non-enlarged microspores have mainly the processed 28 kD form of 14-3-3A and 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 et al., 2001). Cell death has been reported to occur in barley scutellum (Lindholm et al., 2000) and 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 pre-treatment 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 30 kD and 28 kD forms were found to bind to the plasma membrane H^+- ATPase in *in vitro* western blot overlay assays (Testerink et al., 2001). Further characterization of the 14-3-3A proteinase will help elucidating the role of the 28 kD 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 pre-treatment is also related to cell death of these cells. However, later in culture, the meristematic regions of 14 days-old ELSs also showed 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 low 14-3-3C content is related to decreased viability of cells.

After 3 days of further culture of pre-treated 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 been recently 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 mitochondrial protein import (Alam et al., 1994) and subcellular compartmentalization of specific targets, by which they can affect cell fate (Muslin et al., 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 with the first tissue differentiation, the epidermis (Telmer et al., 1995). In barley androgenesis, globular masses of undifferentiated cells were released from exine wall after one 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 days-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 evidences 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 days-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 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 been so far described (Bachmann et al., 1996b; Rosenquist et al., 2000). In addition, it may reveal yet unexplored functions for 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 this expression patterns are also followed by activation of MAPK cascade, approximating 14-3-3 functions in animal and plant systems.

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