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TISSUE-SPECIFIC EXPRESSION OF 14-3-3 ISOFORMS DURING BARLEY MICROSPORE AND ZYGOTIC EMBRYOGENESIS

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Conserved 14-3-3 proteins have been shown to play regulatory roles in eukaryotic cells, including cell cycle control and differentiation. We were interested in the possible involvement of 14-3-3 proteins in the embryogenic process of barley (*Hordeum vulgare* L.). Barley microspore-derived embryo development was used as a model system. Immunolocalization of three barley 14-3-3 isoforms, 14-3-3A, 14-3-3B and 14-3-3C, was carried out using isoform-specific antibodies. In immature microspore-derived embryos, 14-3-3C was specifically expressed underneath the L_1 layer of the shoot apical meristem and in the scutellum. Comparative studies showed that 14-3-3C was also expressed underneath the L_1 layer of the shoot apical meristem and in the scutellum of immature zygotic embryos. We further demonstrated that 14-3-3C expression is restricted to L_2 layer-derived cells of in vitro shoot meristematic cultures. Our results reveal that 14-3-3C isoform tissue-specific expression is closely related to defined events during differentiation processes in embryogenesis and in vitro meristematic cultures.

Key words: Barley, 14-3-3, embryogenesis, L_2 layer.

INTRODUCTION

The 14-3-3 proteins constitute a conserved family of 30 kD proteins present in all eukaryotic organisms studied so far. 14-3-3 dimers bind to phosphorylated motifs in a wide range of target proteins. Several biological functions have been attributed to members of the 14-3-3 protein family, as they are directly involved in transduction of signals related to stress, cell division and differentiation, activation of transcription and apoptosis, and other processes (Sehnke et al., 2002).

14-3-3 functions in eukaryotic cells are exerted by many isoforms, ranging from 2 in *Saccharomyces cerevisie* to 15 in *Arabidopsis thaliana* (Rosenquist et al., 2001). Though 14-3-3 activity is thought to be conserved among different 14-3-3 isoforms (van Heudsen et al., 1996), they appear to be spatially and temporally regulated during development in both plants and animals, suggesting isoform-specific developmental roles. Tissue-specific expression has been described for the 14-3-3χ isoform during *Arabidopsis thaliana* development (Daugherty et al., 1996), while the 14-3-3C isoform from barley (*Hordeum vulgare* L.) is known to be specifically expressed in the scutellum and in the L_{-2} layer of the shoot apical meristem (SAM) of germinating zygotic barley embryos (Testerink et al., 1999). In barley, 3 isoforms have been cloned so far: 14-3-3A, 14-3-3B and 14-3-3C (GenBank X62388, X93170 and Y14200). The developmentally regulated expression pattern of the 14-3-3C isoform in the SAM described by Testerink et al. (1999) prompted us to further investigate 14-3-3 isoform-specific patterns prior to embryo maturation, in embryogenesis both in vivo and in vitro. With a set of isoform-specific antibodies, we studied 14-3-3 immunolocalization in immature zygotic embryos and embryo-like structures

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(ELSs) derived from androgenic microspores. In addition, we investigated 14-3-3 immunolocalization following cell division of the shoot meristem using in vitro shoot meristematic cultures (SMCs).

MATERIALS AND METHODS

ANDROGENESIS INDUCTION AND MICROSPORE CULTURE

Barley (*Hordeum vulgare* L. cv Igri) androgenesis induction and microspore culture were performed according to Hoekstra et al. (1992). Embryo-like structures (ELSs) ranging in diameter from 0.5 to 1 mm were harvested from media by forceps after 21 days of culture for immunolocalization studies.

Immature zygotic embryos

Immature zygotic embryos developed in vivo were dissected under a binocular microscope from seeds 21 DAP (days after pollination) for immunolocalization studies.

Shoot meristematic cultures

Shoot meristematic cultures (SMCs) were obtained from mature grains of barley (*Hordeum vulgare* L. cv. Himalaya) germinated according to Louwerse (2002). The material for immunolocalization was obtained from a 16-month-old culture with high regeneration capacity.

Immunolocalization

Fixation and embedding of 21-day-old ELSs, 21 DAP immature zygotic embryos and 16 month-old SMCs were performed as described by Louwerse (2002). Isoform-specific antibodies raised against the C-terminal part of 14-3-3A, 14-3-3B and 14-3-3C isoforms were used for in situ immunolocalization (Testerink et al., 1999).

RESULTS

14-3-3 immunolocalization was first studied in 21 DAP immature zygotic embryos and in 21-day-old ELSs. Although 21 DAP immature zygotic embryos were at a further developmental stage compared to in vitro-developed 21-day-old ELSs, both showed similar 14-3-3 isoform-specific expression patterns

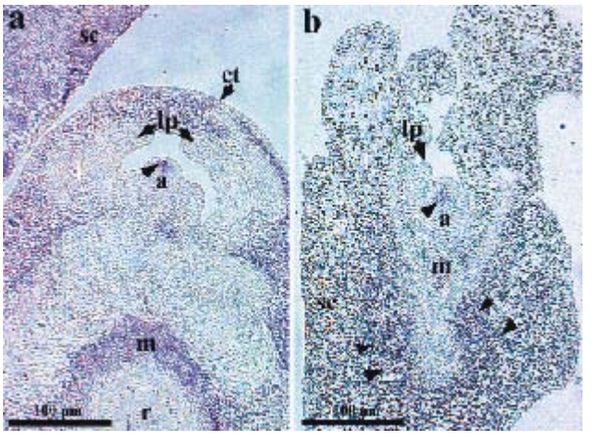

Fig. 1. 14-3-3C immunolocalization in 21 DAP zygotic embryos (**a**) and 21-day-old ELSs (**b**). Proteins were detected using a secondary alkaline-phosphatase-conjugated antibody followed by incubation in nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) in 5 µm sections. Arrowheads indicate 14-3-3C expression underneath the L_1 layer of shoot apical meristem and in scutellum cells. a – SAM; ct – coleoptile; lp – leaf primordia; m – mesocotyl; r – root meristem; rc – root cap; sc – scutellum.

(Fig. 1). 14-3-3A was immunolocalized in the scutellum, root and shoot meristems, root cap, leaf primordia, mesocotyl and epidermis, while 14-3-3B was found in all embryogenic tissues (data not shown). 14-3-3C expression was markedly different from that of 14-3-3A and 14-3-3B, being restricted to the scutellum, mesocotyl, coleoptile, and one region of the SAM (Fig. 1). Figure 2 shows a magnified view of 14-3-3 immunolocalization in the SAM. The 14-3-3A signal was detected in the leaf primordia and in some of the cells within the SAM, but no defined pattern could be observed (Fig. 2a,e). While 14-3-3B was ubiquitously expressed (Fig. 2b,f), 14-3-3C could only be detected in a group of cells underneath the L_1 layer of the SAM. No signal was detected in control sections incubated with only the secondary antibody (Fig. 2d,h).

L_2 layer-specific 14-3-3C expression in the SAM of mature barley embryos has been reported previously (Testerink et al., 1999). Our results suggest that high levels of 14-3-3C are already present underneath the L_1 layer of the SAM before the L_2 layer is morphologically differentiated. The question is whether the 14-3-3C-specific expression underneath the L_1 layer of developing SAMs is restricted to L_2 layer formation, or whether it is also a feature of further cell division of the L_2 layer during early organogenesis. To answer this, we studied

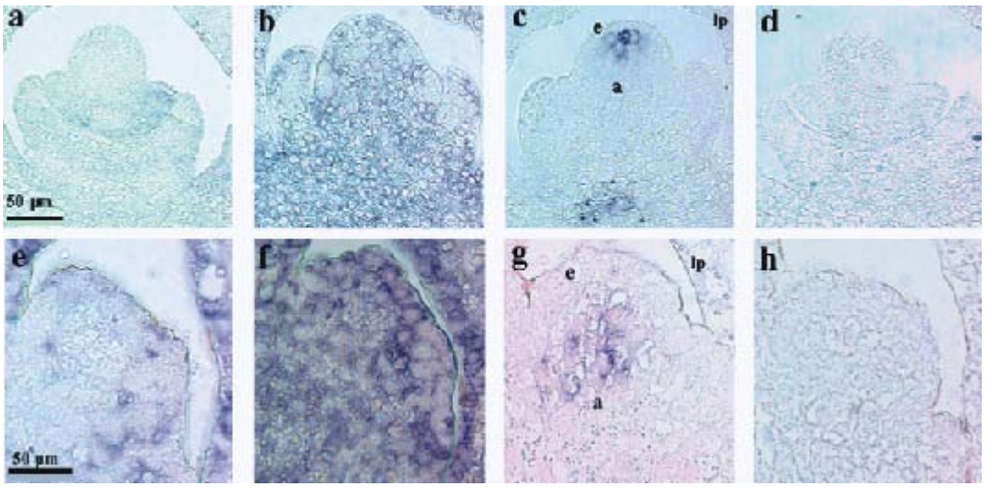

Fig. 2. Magnified view of developing SAM of 21 DAP zygotic embryos (**a–d**) and 21-day-old ELSs (**e–h**) showing immunolocalization of 14-3-3 proteins. 14-3-3A (**a,e**), 14-3-3B (**b,f**), 14-3-3C (**c,g**) and control incubated with only secondary antibody (**d,h**). a – SAM; e – epidermis; lp – leaf primordia.

14-3-3C immunolocalization in proliferating barley SMCs.

Figure 3a illustrates shoot formation and an adventitious shoot meristem (boxed) in a cross section of a 16-month-old SMC of barley. We observed the presence of L_2-derived cells as a thick differentiated layer underneath L_1 and above the corpus. While 14-3-3A (Fig. 3b) and 14-3-3B (data not shown) were expressed in all layers within the shoot apical meristem, 14-3-3C expression could be observed mainly in L_2 layer-derived cells (Fig. 3c). No 14-3-3 signal was observed when sections were incubated with only the secondary antibody (Fig. 3d).

DISCUSSION

The developmental programs that lead to embryo formation are thought to be common to both androgenesis and zygotic embryogenesis (Mordhorst et al., 1997). Here we describe similar 14-3-3 isoform-specific expression patterns during barley zygotic and microspore-derived embryogenesis. Our results are clear evidence for biochemical similarities between these two processes, indicating that the same spatial expression of members of the 14-3-3 family of regulatory proteins might be needed during the formation of barley embryos.

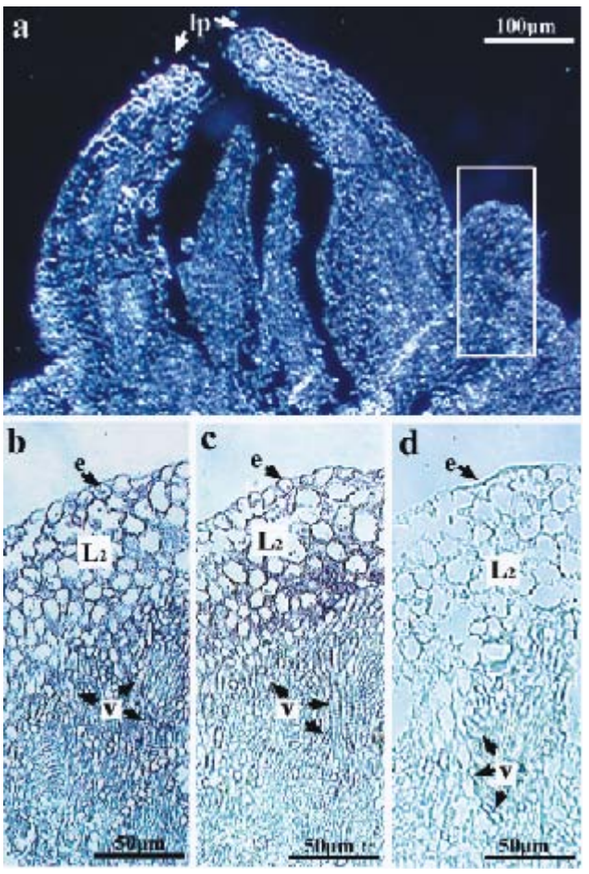

Fig. 3. Structure and 14-3-3 immunolocalization in SMCs; 5 µm section of 16-month-old SMC observed by darkfield (**a**). The equivalent area in the white box is presented at higher magnification (**b–d**). Proteins were detected using a secondary alkaline-phosphatase-conjugated antibody followed by incubation in NBT/BCIP. 14-3-3A (**b**), 14-3-3C (**c**) and control incubated with only secondary antibody (**d**). e – epidermis; L_2 – L_2 layer-derived cells; v – vascular bundles.

We demonstrate that L_2 layer specification and cell division from the L_2 layer are both accompanied by specific L_2 layer 14-3-3C expression in immature barley embryos and during early organogenesis of SMCs. The L_2 layer of the shoot apical meristem probably contributes to mesophyll formation in adult plants (Jenik and Irish, 2000). This expression pattern of the 14-3-3C isoform in the SMC points to specific functions for this isoform associated with the differentiation and function of the L_2 layer. In other multicellular organisms, tissue-specific expression of 14-3-3 isoforms during embryogenesis is a known phenomenon. For example, in *Drosophila*, 14-3-3 ε tissue-specific expression was prior to mitogen-activated protein kinase (MAPK) activation in the same tissue during early embryo development (Tien et al., 1999). 14-3-3 ε and 14-3-3 ξ have been reported to positively regulate the Ras-Raf signaling pathway, leading to MAPK cascade activation in *Drosophila* (Chang and Rubin, 1997; Kockel et al., 1997). Recently, Pnueli et al. (2001) reported the specific expression of at least one 14-3-3 isoform in the SAM of tomato seedlings (*Lycopersicum esculetum* L.). They showed that the same isoform takes part in the signaling system involved in determining SAM vegetative growth potential, a signaling system that is analogous to Raf-1 in animal cells. Further work is needed to address the possible 14-3-3C targets in the L_2 layer. In this regard, SMCs seem to be a suitable system to study 14-3-3C interacting proteins in the shoot meristem of barley.

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