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ORIGINAL PAPER

Accumulation pattern of dehydrins during sugarcane (var. SP80.3280) somatic embryogenesis

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Abstract The objective of the present study was to determine dehydrin protein levels in sugarcane var. SP80-3280 during somatic embryogenesis. Dehydrins from embryogenic and non-embryogenic cell cultures were analyzed using western blot and in situ immunolocalization microscopy. Both techniques employ antibodies raised against a highly conserved lysine-rich 15-amino acid sequence termed the K-domain, which is extensively used to recognize proteins immunologically related to the dehydrin family. In embryogenic cultures, western blot analysis of the heat-stable protein fraction revealed eleven major bands ranging from 52 to 17 kDa. They were already visible on the first days, gradually increasing until reaching peak values around day 14, when organogenesis begins, to later decrease in concurrence with the appearance of green plantlets (around day 28). These fluctuations indicate that this pattern of accumulation is under developmental control. Dehydrins were mainly immunolocalized in the nuclei. A phosphatase treatment of protein extracts caused a mobility shift of the 52, 49, and 43 kDa dehydrin bands suggesting a putative modulation mechanism based on protein phosphorylation. In sugarcane embryogenic cultures, presence of dehydrins is a novel finding. Dehydrins were absent in non-embryogenic cultures. The novel findings regarding accumulation, nuclear localization, and phosphorylation of dehydrins provide a starting point for further research on the role of these proteins in the induction and/or maintenance of embryogenesis.
Key message The novel findings regarding accumulation, nuclear localization, and phosphorylation of dehydrins provide a starting point for further research on the role of these proteins in the induction and/or maintenance of embryogenesis.

Keywords Dehydrins · Dehydrin in situ immunolocalization · Dehydrin nuclear localization · Dehydrin phosphorylation · Dehydrin western blot · Embryogenic competence · Somatic embryogenesis · Sugarcane var. SP80-3280

Introduction

Group 2 LEA proteins, also known as dehydrins, are highly hydrophilic proteins characterized by the presence of a conserved lysine-rich 15-residue domain, EKKGIMDKIKEKLPG, named the K-domain (Close and Lammers 1993), which can be found in one or several copies. It has been proposed that the K-domain forms an amphipathic α-helix (Ismail et al. 1999a) that allows both hydrophilic and hydrophobic interactions to stabilize proteins in water-stress environments (Dure 1993). Other typical dehydrin features are: the presence of the Y-segment in the N-terminus, and a consensus sequence of [V/T]D[E/Q]YGNP, similar to the nucleotide binding site of plant and bacterial chaperones (Close 1996); the S-segment, a tract of Ser residues, which can be phosphorylated in certain proteins (Vilardell et al. 1990; Plana et al. 1991; Goday et al. 1994;

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Jiang and Wang 2004); and finally, the Φ-segments, regions usually rich in polar amino acids, only present in some dehydrins (Rorat 2006).

Dehydrins are constitutively expressed in the later stages of zygotic embryo development, i.e., during seed desiccation (Zimmerman 1993). In addition, in vegetative organs, they are constitutively present during normal growth conditions (Rorat et al. 2004), although they also accumulate in response to water stress provoked by drought, low temperature, or high salinity (Ismail et al. 1999b; Nylander et al. 2001). This is currently interpreted as indicating a role in the amelioration of different stress effects, even though their precise functions remain elusive (Battaglia et al. 2008).

The expression of certain dehydrin genes during seed development or in response to stress is mediated by ABA (Nylander et al. 2001). However, other genes are not responsive to ABA or may be regulated by it during seed development, but not in response to stress (Stanca et al. 1996; Giordani et al. 1999). Yet a third group of genes displays dual regulation (Welling et al. 2004).

Dehydrins accumulate in the cytoplasm, with some being additionally localized to the nucleus. Within the cytoplasm, dehydrins have been found in the vicinity of the plasma membrane, mitochondria, vacuole, and endoplasmic reticulum (Houde et al. 1995; Egerton-Warburton et al. 1997; Danyluk et al. 1998; Borovskii et al. 2002; Heyen et al. 2002). As for nucleus-directed SK2 proteins, the phosphorylated S-segment and the RRKK sequence have been postulated as nuclear localization signals (Plana et al. 1991). According to Battaglia et al. (2008), for some dehydrins, nuclear localization seems to be independent of the phosphorylation state of the S-segment, and what's more, proteins lacking the S-segment or RRKK motif have been localized to the nucleus (Riera et al. 2004). This suggests that the transport of different types of dehydrins to the nucleus occurs via different nuclear localization pathways.

To date, reports on the presence of dehydrins during somatic embryo development or in mature somatic embryos have been restricted to in vitro carrot and oak cultures. Studies in carrots have reported the presence of a phosphorylated dehydrin named ECPP44 during somatic embryogenesis and in mature somatic embryos (Tan and Kamada 2000). ECPP44 is also present, although not phosphorylated, in non-embryogenic cells. In their study, Tan et al. (2000) isolate and describe the cDNA encoding ECPP44, finding that the amino acid sequence contains a poly-serine consensus and nuclear targeting signal, both typical of dehydrin genes. Later, Ko et al. (2006) perform an ECPP44 western blot analysis and immunoprecipitation assay, concluding that this protein may play a role in the induction or maintenance of embryogenic competence. Another dehydrin, named B18, is described by Walkers et al. (1999) in ABA pretreated carrot somatic embryos. The authors associate B18 with the acquisition of desiccation tolerance during slow drying and suggest that this dehydrin, embedded in the glassy matrix, confers stability to embryos. Šunderlíková and Wilhelm (2002) study the expression patterns of two dehydrin-like homologues in somatic oak embryos under different maturation treatments, demonstrating that the regulation of their expression is under developmental control and can be influenced by manipulating the culture conditions.

In sugarcane leaves, Wahid and Close (2007) investigate the presence of heat stress-induced dehydrin expression and its relationship with water availability. There are no previous studies on dehydrins in sugarcane during somatic embryogenesis, although Linacero et al. (2001) report differences in the expression of transcripts homologous to three LEA genes (pMA1049, pMA2005, and CM1) in two cultivars of sugarcane, one drought-resistant (JA-605) and the other drought-sensitive (C-8751), as well as in embryogenic and non-embryogenic tissues treated with abscisic acid (ABA) or after drought stress. The results show that: (i) the transcripts homologous to the pMA2005 and CM1 clones are differentially accumulated during somatic embryogenesis; (ii) the CM1-like transcript only appears in non-resistant somatic embryos; (iii) all the LEAs cDNAs are markedly increased in the embryogenic tissues treated with ABA; and (iv) there are differences among the accumulation of these mRNAs in the non-embryogenic tissues treated with ABA.

Here, we studied dehydrin accumulation in sugarcane var. SP80-3280 (*Saccharum officinarum* L. × *S. spontaneum* L.) during somatic embryo development, expecting to find that these proteins are constitutive and inherent in the embryo development program. In order to prove this hypothesis, we first studied somatic embryo development, followed by dehydrin expression in embryogenic and non-embryogenic cultures using western blot and in situ immunolocalization approaches.

Materials and methods

Plant material

Sugarcane var. SP80-3280 (*Saccharum officinarum* L. × *S. spontaneum* L.) was used in all experiments conducted. Immature nodal cane segments with axillary buds were collected from field-grown plants and put in plastic trays containing the commercial substrate PlantMax (DDL Agroindustria, Paulínia, SP, Brazil). Trays were maintained under environmental conditions for about three months. During this time, the new plants originated from sprouting axillary buds and grew approximately 45 cm high, while

their shoot apical meristems were used as explants after removing mature leaves.

Apical meristems were surface sterilized in ethanol 70 % during 1 min, immersed in 50 % commercial bleach (hypochlorite from 1 to 1.25 %) for 30 min, and then washed three times with sterilized water. At least ten apical apical meristems were transversely sectioned in 1-mm-thick slides, which were cultured in assay tubes (150 × 25 mm) containing 20 mL of MS medium (Murashige and Skoog) (Phytotechnology Lab, Shawnee Mission, KSA, USA), supplemented with 20 g L^{-1} of sucrose, 2 g L^{-1} of phytagel® (Sigma-Aldrich, St. Louis, MO, USA), and 10 μM of 2.4-D (Sigma-Aldrich). Tubes were maintained in the dark at 25 ± 1 °C, during about 45 days. After this time, the induced cultures were transferred to Petri dishes containing a maintenance culture medium. Maintenance medium is composed of MS medium supplemented with sucrose (20 g L^{-1}), phytagel (2 g L^{-1}), and 2.4-D (10 μM) pH 5.8, which were autoclaved at 121 °C during 15 min. Subcultures were made in the maintenance medium and renewed every 20–25 days. During this time, we did not observe a visible diminution of the regeneration capacity.

Maturation experiment

Four Petri dishes (90 × 15 mm) containing 20 ml of MS culture medium supplemented with sucrose (20 g L^{-1}), phytagel® (2 g L^{-1}), and activated charcoal (1.5 g/L) pH adjusted to 5.8, were inoculated with three colonies of 200 mg fresh matter (FM) (four per embryogenic and other four per non-embryogenic). The culture medium was sterilized by autoclaving at 121 °C for 15 min. Cultures were maintained in a growth chamber, at 25 ± 1 °C, in the dark for the first 7 days and thereafter in photoperiods of16 h of light (60 μmol m^2 s^{-1}).

Somatic embryo maturity was checked every day using a light stereomicroscope. Maturity was analyzed considering the presence or absence of green somatic embryos. Sections of calli (one per dish) were separated at 0, 7, 14, and 28 days and stored at −20 °C. For somatic embryogenesis studies, other intermediate stages were also analyzed.

Dehydrin extraction

Callus tissue samples (300 mg) were lyophilized and ground to a powder in liquid nitrogen and then homogenized for 90 min at 4 °C in extraction buffer containing 50 mM Tris–HCl, (pH 7.6), 500 mM NaCl, 5 mM $MgCl_2$, and Protease Inhibitor Cocktail (Sigma Chemical Company). After centrifugation at 10,000*g* (two times), the pellet was discarded and the supernatant was immersed in a bath at 96 °C for 10 min and then cooled at room temperature and centrifuged at 10,000*g* for 10 min at 4 °C in order to isolate thermostable proteins.

For quantification, a Qubit Quantitation Platform Protein Assay Kit (Invitrogen, Carlsbad, CA, USA) was used. All the assay were repeated at least three times in independent experiments.

Western blot assay

Proteins were resolved by 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970) at a constant voltage of 100 V for 120 min using a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Protein was loaded at 25 μg per lane and benchmark molecular-weight standards (Invitrogen) were used. Following electrophoresis, the fractionated proteins were transferred onto a nitrocellulose membrane (Hybon Amersham Pharmacia Biotech, Freiburg, Germany) at 100 V for 60 min using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad).

The integrity of blotted proteins was confirmed by Ponceau S staining. After overnight blocking at 4 °C with 5 % non-fat dried milk in TBS (50 mM Tris–HCl, 150 mM NaCl, pH 8) with shaking, membranes were incubated for 60 min at room temperature with the anti-dehydrin primary polyclonal antiserum at a 1:1,000 dilution in TTBS (TBS-0.05 % Tween 20). The rabbit antiserum was raised against the carboxy terminus of a consensus sequence present in angiosperm dehydrins: the K-domain (EKKGIMDKIKEKLPG) (Close and Lammers 1993). Membranes were washed three times (10 min each) in TTBS and incubated for 90 min with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a 1:4,000 dilution in TTBS. Following three consecutive washes carried out with TTBS as described above, the membranes were incubated with AP buffer (100 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) for 10 min. All incubations and washes were performed with gentle shaking at room temperature. For signal development, membranes were incubated in the dark in 10 ml of AP buffer with 66 μl of nitro blue tetrazolium and 33 μl of 5-bromo-4-chloro-3-indolylphosphate (Promega, Madison, WI, USA). Controls were treated as above, excluding the purified rabbit anti-dehydrin immune serum. The specificity of the dehydrin detection was confirmed by blocking the primary antiserum for 30 min with an equal volume of the K-domain peptide salt (5 mg/ml) prior to the first incubation of the duplicate membranes.

Dephosphorylation

25 μg of each thermostable protein extract corresponding to day 14, were diluted to 40 μl in 1X MULTI-CORE™

buffer (Promega) in the presence or absence of 10 units of Thermostable Shrimp Alkaline Phosphatase (Promega) for 6 h at 37 °C. For this analysis we used ECL™ Anti Rabbit IgG Peroxidase-linked whole antibody (donkey) (GE Healthcare UK Limited) as second antibody (1:4,000 in TTBS), which was revealed with Pierce® ECL Western Blotting Substrate (32109), and then exposed to X-ray film (Agfa).

Densitometry

The relative amounts of protein were quantified by densitometry, following Retamal et al. (1999), using a computational program from a TIFF image. The "gel perfect" program described by Bozzo and Retamal (1991) calculates the relative mobility of each stained band and its corresponding intensity, as well as producing a diagrammatic representation of the protein bands at their relative concentrations in relation to the total protein by lane.

Statistical analysis

Statistical analysis of differences was performed using one-way ANOVA test. A value of $P < 0.05$ was considered significant. Calculations were performed using the GraPhPadPrism software program (GraPhPad, San Diego, CA, USA).

Sample preparation for light and transmission electron microscopy (TEM)

For embryo developmental studies, samples were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (2 h at 4 °C), postfixed in 1 % osmium tetroxide, dehydrated in a graded ethanol series and embedded in paraplast. Sections (10 µm thick) were stained with toluidine blue O (Sigma T 3260 CI 52040). The presence of starch was determined with iodine potassium iodide (Sigma L 6146).

For immunological studies, samples were fixed in 4 % formaldehyde and embedded in London Resin White resin (Polyscience, Inc.; 17411) according to Harris et al. (1995). Semithin (1–5 µm thick) and ultrathin sections were obtained with an ultramicrotome (Reichert-Jung, Vienna, Austria) using a diamond knife.

Immunolabeling using differential interference contrast (DIC) microscopy

The distribution of dehydrins in tissues was observed with silver-enhanced LM. Semi-thin tissue sections were transferred to clean glass slides, hydrated on a drop of Milli-Q-grade water for 3 min, washed twice with rinsing buffer (0.1 % gelatin and 0.05 % Tween 20 in 10 mM PBS, pH 7.2) for 3 min, blocked for 90 min with 1 and 2 % gelatin in PBST (0.05 % Tween 20 in 10 mM PBS, pH 7.2) at room temperature, washed with rinsing buffer twice for 3 min, and incubated overnight at 4 °C with the purified rabbit anti-dehydrin primary antibody at a 1:200 dilution in the rinsing buffer. Next, sections were washed with three changes of rinsing buffer (5 min each), incubated for 1 h on a drop of colloidal gold (10 nm diameter)-conjugated goat antiserum to rabbit immunoglobulins (G-7402; Sigma Chemical Co.) diluted 1: 200 in rinsing buffer at 37 °C, and washed again with rinsing buffer (three changes, 5 min each). After a 45 min fixation with 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, sections were thoroughly washed with Milli-Q-grade water. Since individual gold particles are not visible at the LM level, a silver enhancement kit (Sigma SE-100) was used to develop a brown-to-black stain in immunoreactive areas. Finally, the silver deposition reaction was fixed with 2.5 % aqueous sodium thiosulfate followed by rinsing in Milli-Q-grade water. Sections were counterstained with 1 % (w/v) safranin in 95 % ethanol (Spence 2001) and viewed with an Olympus FluoView FV 300 confocal microscope with FluoView 3.3 software (Olympus, Tokyo, Japan). Control sections were treated as above, excluding the purified rabbit anti-dehydrin immune serum, and by blocking the primary antiserum with the recognized peptide sequence prior to the incubation step.

Immunolabeling for TEM

Ultrathin sections, corresponding to day 14, were mounted on Formvar-coated nickel grids (Polyscience, Inc., Warrington, PA, USA) for immunolabeling at the TEM level. Sections were first hydrated on a drop of Milli-Q-grade water for 3 min, washed with rinsing buffer twice for 3 min, blocked for 90 min with 1 % gelatin in PBS at room temperature, washed with rinsing buffer twice (3 min each), and incubated overnight at 4 °C with the purified rabbit anti-dehydrin primary antibody at a 1:100 dilution in the rinsing buffer. Next, sections were washed with rinsing buffer (5 changes, 5 min each), incubated for 1 h on a drop of colloidal gold (10 nm diameter)-conjugated goat antiserum to rabbit immunoglobulins diluted 1:150 in rinsing buffer at room temperature, and washed again with rinsing buffer (5 changes, 5 min each). After a 10 min fixation with 1 % glutaraldehyde in 0.1 M PBS, pH 7.2, sections were thoroughly washed with Milli-Q-grade water. Finally, grids carrying sections were counterstained with uranyl acetate followed by lead citrate (Prego et al. 1998), and examined under a Zeiss EM 109 turbo (Zeiss, Wiesbaden, Federal Republic of Germany) TEM operating at an accelerating voltage of 90 kV. Control sections were

treated as above, excluding the purified rabbit anti-dehydrin immune serum, and with the primary antiserum being blocked by the recognized peptide prior to the incubation step (as previously described).

Results

Embryogenic and non-embryogenic calli

In sugarcane var. SP80-3280 cultures, two morphological types of calli with different morphogenic potential were identified: (i) embryogenic, nodular, and compact calli (Fig. 1a–d) and (ii) non-embryogenic, mucilaginous, and translucent calli (Fig. 1e–h). Embryogenic calli eventually generated green embryos and were able to develop somatic plantlets (Fig. 1d). Non-embryogenic calli were unable to form embryos, instead presenting pigment production as a symptom of tissue oxidation (Fig. 1f–h).

Embryo development

During the first seven days, embryogenic cultures developed meristematic areas in the periphery, which later became globular structures (Fig. 2a). Somatic embryos (Fig. 2b–h) developed from each of these globular proembryos. First, around day 14, a lateral notch was formed, separating the scutellum from the shoot apical meristem (Fig. 2b). This was followed by the separation of the coleoptile (Fig. 2d) and later, the formation of primordial leaves (Fig. 2e–h), and axillary buds (Fig. 2h). Some embryos presented a monopolar structure (Fig. 2c), i.e., they were composed of a shoot apical meristem, without the root meristem. Bipolar embryos were observed around day 21. They presented an axis with shoot and root poles (Fig. 2e); procambia were detected in the axes (Fig. 2e, f). The scutellar tissues accumulated starch and lipids, distinguishing them from axis tissues (Fig. 2g, h).

Green plantlets could be observed around day 28 (Fig. 2e, f, i). By then, the starch of the scutellum had been consumed, and the coleoptile and first leaves had developed and unfolded.

Analysis of dehydrin accumulation

Western blot analysis of the heat-stable protein fraction was carried out in cultures prepared at 0, 7, 14, and 28 days. The corresponding dehydrin banding patterns are shown in Fig. 3a. Dehydrins were absent in non-embryogenic cultures (Fig. 3a, lanes 1–4). In embryogenic cultures, 11 dehydrin bands with molecular masses of 52, 49, 46, 43, 38, 35, 30, 26, 22, 19, and 17 kDa were visible from almost beginning (Fig. 3a, lane 5). Since dehydrins have putative serine phosphorylation sites, we assessed whether the dehydrins detected during development were indeed phosphorylated. Alkaline phosphatase (AP)-treated and mock-treated fractions of heat-stable and soluble proteins were analyzed by western blot. After AP-treatment, three dehydrin bands, specifically those of 52, 49, and 43 kDa, changed their mobility to 49, 47, and 41 kDa, respectively (Fig. 3b).

Total dehydrin accumulation reached its highest level at day 14 as revealed by both a stronger immunological reaction in western blot (Fig. 3a) and densitometry (Fig. 3c–e). The band of 49 kDa maintained a consistent level of accumulation from days 7 to 28. The remaining bands reached their highest levels of accumulation on day 14. These bands were also present in 28-day-old embryogenic cultures although their expression levels, except for

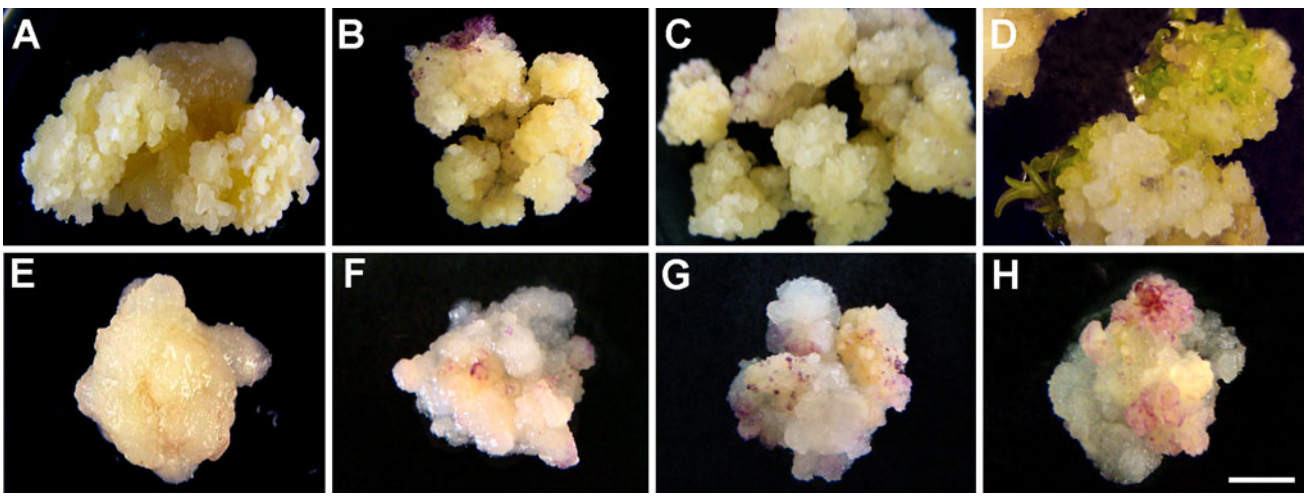

Fig. 1 Sugarcane var. SP80-3280 somatic embryogenesis. **a–d** Somatic embryogenic development in cultures at 0, 7, 14, and 28 days, respectively. **e–h** Non-embryogenic cultures at 0, 7, 14, and 28 days, respectively. *Bar* 5 mm

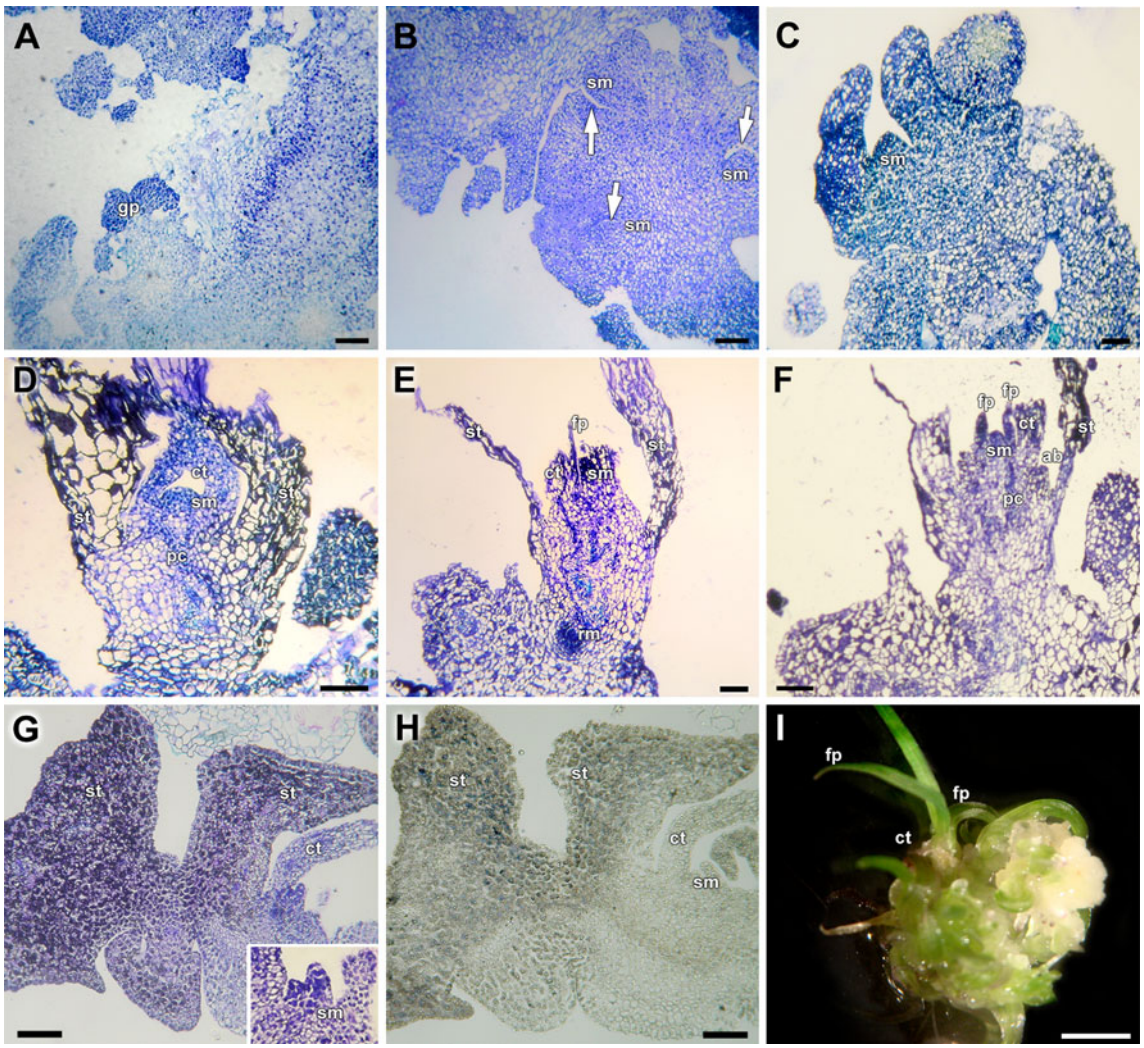

Fig. 2 Somatic embryogenesis in sugarcane var. SP80-3280 cultures. The figure is a representative of observation of at least 20 calli of each stage in three independent maturation experiments. **a** Globular proembryos (pe). **b** Lateral notches (*arrows*) define scutellum and shoot apical meristem. **c** Shoot apical meristem (sm) in a monopolar embryo. **d** Coleoptile (ct) is differentiated. **e.** Leaf primordial (lp) and root apical meristem (rm) in a bipolar embryo. **f** Axillary buds (ab) are observed. **g** Starch is detected in the scutellum by iodine potassium iodide. **h** Lipids are detected in the scutellum by osmium tetroxide, which is used as a second fixing agent. **i.** Plantlets (34 days old). *Bars* 200 μm (**a**–**c**), 100 μm (**d**–**h**), 2.5 mm (**i**). ab, axillary buds; ct, coleoptiles; gp, globular proembryo; pc, procambium; rm, root apical meristem; sm, shoot apical meristem, st, scutellum

the 52 kDa band, decreased significantly (Fig. 3e). In 28 day old cultures, as compared to 14-day-old cultures, densitometric analysis (Fig. 3c, e) revealed that the relative intensity of the17 kDa band had decreased by more than twice its intensity level.

Subcellular localization of sugarcane dehydrins

Western blot analysis was complemented by in situ immunolocalization of dehydrins in embryogenic and non-embryogenic cultures, in order to establish the subcellular localization of these proteins (Figs. 4, 5). At the subcellular level, dehydrins showed mainly nuclear localization. In the nuclei, labeling was associated with chromatin and the nucleoplasm was devoid of staining (Figs. 4a, 5). In non-embryogenic cultures, labeling was absent in nuclei (Fig. 4b). Control sections, i.e., sections that had not been incubated with the purified rabbit anti-dehydrin immune serum, as well as those that had been incubated with primary antiserum previously blocked with purified lysine-rich peptide derived from the K-domain present in all dehydrins, were free of labeling (not shown).

Discussion

The objective of the present study was to identify dehydrins from sugarcane var. SP80-3280, during somatic

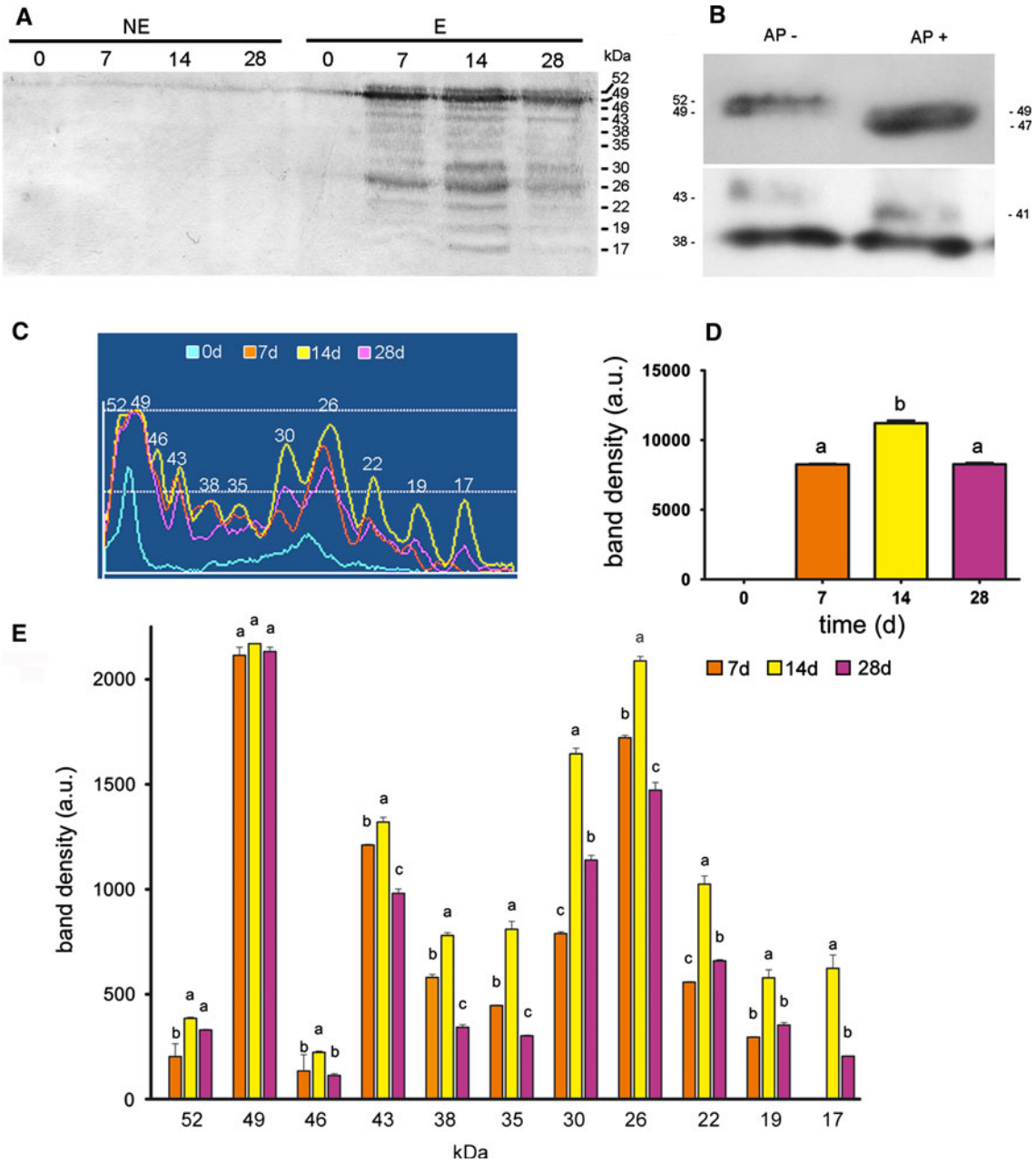

Fig. 3 **a** Western blot analysis of dehydrins in sugarcane var. SP80-3280 during somatic embryogenesis**.** Heat-stable proteins were separated by 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The first antiserum was a purified anti-dehydrin antiserum directed against the K-domain present in all dehydrins at a 1:1,000 dilution. The second antiserum was an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G at a 1:4,000 dilution. Protein molecular masses were calculated by the Gel Perfect Software and are indicated on the right. Lanes 1–4, Non-Embryogenic (NE) and Lanes 5–8 Embryogenic(E) cultures at 0, 7, 14, and 28 days. **b** Western blot analysis on the heat-stable fraction (dehydrin-enriched fraction) of proteins extracted from cultures after14 days. At this time, samples were diluted in buffer, with (+) or without (−) alkaline phosphatase (AP) treatment, an ECL™ Anti Rabbit IgG Peroxidase-linked whole antibody (from donkey) was used as second antibody, which was revealed with ECL and exposed to X-ray film. **c** Densitometric analysis of the dehydrin bands shown in embryogenic cultures, using Gel Perfect software. The program calculates the relative mobility of each stained band and its corresponding intensity. **d** Total dehydrin accumulation at 0, 7, 14, and 28 days. *Columns labeled with the same letters* are not significantly different ($P < 0.05$). **e** Accumulation levels of each of the eleven dehydrin bands as detected at 0, 7, 14, and 28 days. For each band, *columns labeled with the same letters* are not significantly different ($P < 0.05$). *a.u.* arbitrary units

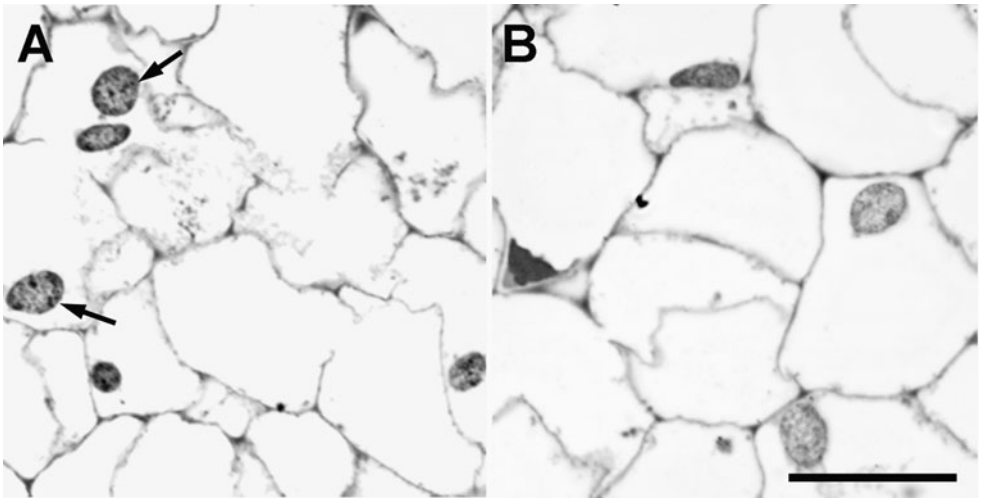

Fig. 4 **a**, **b** Dehydrin in situ immunolocalization in semithin sections of sugarcane var. SP80-3280 cultures using the K-domain specific antiserum and observed under differential interference contrast (DIC) microscopy. Since individual gold particles are not visible at this magnification level, a silver enhancement kit was used to develop a brown-to-black stain in the immunoreactive areas. In order to detect chromatin, sections were counterstained with 1 % (w/v) safranin in 95 % ethanol. **a** Embryogenic cultures (14 days old). **b** Non-embryogenic culture (14 days old). *Bars* 20 μm. *Arrows* indicate silver deposits at the site of gold labeling. In all cases, labeling was mainly observed in nuclei

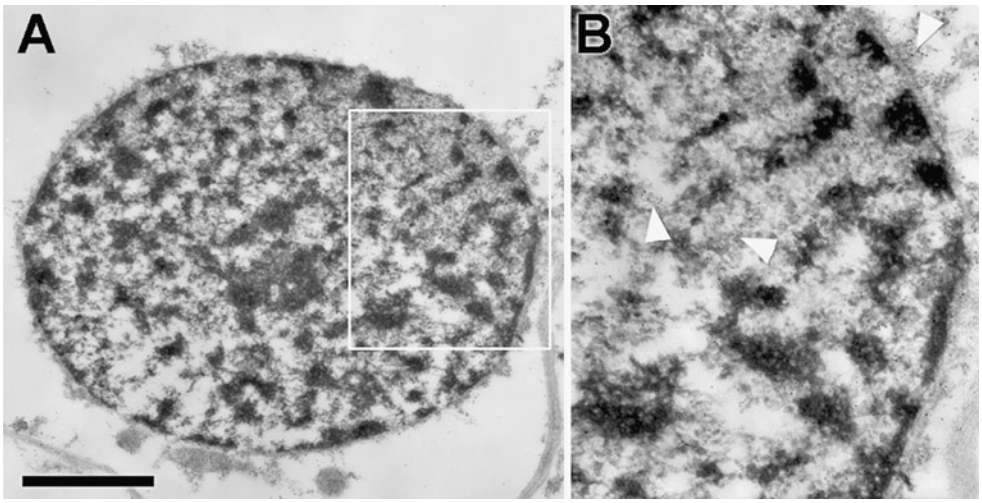

Fig. 5 **a**, **b** Dehydrin in situ immunolocalization in ultrathin sections of embryogenic sugarcane var. SP80-3280 cultures (14 days old), using the K-segment specific antiserum. Ultrathin sections were incubated with the purified rabbit anti-dehydrin primary antibody and then on a drop of colloidal gold-conjugated goat antiserum to rabbit immunoglobulins. *Arrowheads* indicate colloidal gold particles (10 nm), which are mainly associated with chromatin. Images were obtained using TEM. Panel **b** corresponds to the area circumscribed in panel **a**. *Bar* 1 μm

embryogenesis. According to Gandonou et al. (2005), Cidade et al. (2006), and Lakshmanan (2006) in sugarcane cultures, the influence of genotype on the morphogenic response necessitates adjustments that adapt different experimental protocols to each variety. Therefore, we strictly consider the present results in relation to the variety studied herein.

Ho and Vasil (1983a, b) establish that the regeneration of plants from calli, as well as from cell suspension cultures in sugarcane, takes place because of the formation of somatic embryos, and that the terms meristemoids, shoot apex, and somatic embryos may have been used previously to describe the same structures, i.e., monopolar embryos. Falco et al. (1996) distinguish two regeneration pathways in sugarcane culture: somatic embryogenesis and organogenesis, which may correspond to monopolar and bipolar structures, respectively. In accordance with Ho and Vasil (1983a, b), Guiderdoni (1986a, b, 1988) and Falco et al. (1996), here we demonstrated the simultaneous occurrence of monopolar and bipolar embryos in the same culture, but

we also managed to identify the scutellum as a starch-storing organ, in contrast to zygotic scutella, where starch is absent.

Little is known about the presence of dehydrins during somatic embryogenesis. Until now, studies of dehydrins during somatic embryogenesis were limited to carrot and oak. In carrot, Tan and Kamada (2000) identify one a specific phosphoprotein named ECPP44, which shows partial homology to two *Arabidopsis* dehydrins (ERD10 and ERD14) (Brini et al. 2007). In fact, ECPP44 is found in embryonic cells, in both stressed- and non-stressed-tissues, and somatic embryos, but not in non-embryogenic cells, suggesting that they may play a role in the induction or maintenance of embryonic competence (Ko et al. 2006). A high accumulation of transcripts of two dehydrin-like homologues designated as Dhn1a and Dhn1b are found in oak somatic embryo cultures by Šunderlíková and Wilhelm (2002), specifically in cultures with a high frequency for conversion into plantlets; these authors conclude that the regulation of these proteins is under developmental control but can be influenced by manipulating the culture conditions. In embryogenic cultures of sugarcane var. CP 5243, Blanco et al. (1997) perform a SDS-PAGE electrophoresis based on soluble protein content and describe the presence of a marker protein (not a dehydrin), suggesting a potential association between this protein and a culture's regenerative ability. The only study on proteins associated with hydric stress in sugarcane reports the accumulation of mRNAs of three LEA genes and the presence of three LEA cDNAs, which markedly increase in the embryogenic tissues treated with ABA, in contrast to non-embryogenic tissues (Linacero et al. 2001). Here, we report the presence of dehydrins in embryogenic cultures of sugarcane var. SP80-3280. They were first present in the first days, gradually increasing until reaching peak values around day 14 and then gradually decreasing. The fall coincided with the appearance of green shoots (around day 28). This pattern of accumulation seems to be under developmental control.

Data on subcellular localization in vegetative (non-seed) tissues reveal that dehydrins are primarily localized in the cytoplasm and nucleus, but can also be found in various other cell compartments, such as in the vicinity of the plasma membrane (Danyluk et al. 1998), mitochondria (Borovskii et al. 2002), chloroplasts (Wisniewski et al. 1999), endoplasmic reticulum (Neven et al. 1993), vacuoles (Heyen et al. 2002), and protein bodies and amyloplasts (Rinne et al. 1999). In this study, we show that dehydrins were mainly found in nuclei.

Possible mechanisms by which some dehydrins are translocated to the nucleus are relevant issues that remains to be studied. For nucleus-directed SK2 proteins, the phosphorylated S-domain and the RRKK sequence have been postulated as nuclear localization signals (Plana et al. 1991). However, Battaglia et al. (2008) advert that the nuclear localization of some dehydrins seems to be independent of the phosphorylation state of the S-segment, and furthermore, proteins lacking the S-domain or RRKK motif have nevertheless been localized to the nucleus (Riera et al. 2004). This suggests that the transport of different types of dehydrins to the nucleus occurs via different nuclear localization pathways. In somatic embryogenesis, Ko et al. (2006) find that ECPP44 dehydrins present a different phosphorylation pattern in embryogenic versus non-embryogenic cultures. In this study, we report the mobility shifts of three dehydrin bands (52, 49, and 43 kDa) following alkaline phosphatase treatment, which suggests that these bands are phosphorylated.

Conclusion

In sugarcane (var. SP80.3280), during somatic embryogenesis, dehydrin levels present an accumulation pattern that accompanies both the development and emergence of green plantlets. Novel observations regarding dehydrin accumulation, nuclear localization, and phosphorylation provide a starting point for further studies on the role of these proteins in the induction and/or maintenance of embryogenesis. Now we are interested in establishing the sequence of sugarcane dehydrins during somatic embryogenesis, specifying which (if not all) dehydrins accumulate in nuclei, and determining if this accumulation pattern is correlated with their phosphorylation status.

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Conflict of interest The authors declare that they have no conflict of interest.

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