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Developmentally and stress-induced small heat shock proteins in cork oak somatic embryos

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Abstract

The timing and tissue localization of small heat shock proteins (sHSPs) during cork oak somatic embryo development was investigated under normal growing culture conditions and in response to stress. Western blot analyses using polyclonal antibodies raised against cork oak recombinant HSP17 showed a transient accumulation of class I sHSPs during somatic embryo maturation and germination. Moreover, the amount of protein increased at all stages of embryo development in response to exogenous stress. The developmentally accumulated proteins localized to early differentiating, but not the highly dividing, regions of the root and shoot apical meristems. By contrast, these highly dividing regions were strongly immunostained after heat stress. Findings support the hypothesis of a distinct control for developmentally and stress-induced accumulation of class I sHSPs. The possible role of sHSPs is discussed in relation to their tissue specific localization.

Key words: Embryo development, HSP17, small heat shock proteins, somatic embryogenesis, tissue specificity.

Introduction

Small heat shock proteins (sHSPs), ranging in size from about 15–30 kDa, are molecular chaperones (Harndahl *et al*., 1999; Kuk *et al*., 2000; Lee and Vierling, 2000) whose expression has been widely reported in all organisms in response to stress (Nover and Scharf, 1997). In plants, sHSPs are the most abundant stress-induced proteins. They are also synthesized during some developmental processes such as pollen or seed maturation. In higher plants, sHSPs have been grouped into six classes based upon their amino acid homology, immunological cross reactivity and subcellular localization (for a review see Waters *et al*., 1996).

The expression of sHSPs during seed development is usually transient; the accumulation begins in mid–late phases of seed maturation, reaches a maximum in the dehydrated seed and declines during imbibition and germination (zur Nieden *et al*., 1995; Waters *et al*., 1996). However, only a very limited number of model plants have been studied and further investigation is needed.

Somatic embryogenesis is the process by which somatic cells undergo a developmental sequence closely resembling zygotic embryogenesis. Somatic embryogenesis is an excellent system for studying gene expression during embryo development, since high quantities of stage-specific embryos are easily obtained. However, the spatial and temporal patterns of sHSPs expression in somatic embryos are largely unknown. A few papers focus on sHSP accumulation at specific stages of somatic embryo development (Pitto *et al*., 1983; Hwang and Zimmerman, 1989; Zimmerman *et al*., 1989; Györgyey *et al*., 1991; Apuya and Zimmerman, 1992), but only one paper concerns the timing of sHSPs gene expression (Dong and Dustan, 1996). In a previous work, evidence of *hsp17* expression in cork oak (*Quercus suber*) zygotic and somatic embryos was reported (Pla *et al*., 1998).

Here, the temporal and spatial pattern of expression of class I sHSPs were investigated during cork oak somatic embryo maturation and germination. The aim was to discover the function of sHSPs in somatic embryos under normal growth conditions and in response to stress. Western blots and immunohistochemical analyses were

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performed using polyclonal antibodies raised against the cork oak recombinant protein HSP17 (Pla *et al.*, 1998). As class I sHSPs are immunologically related proteins, when HSP17 is mentioned, it refers to HSP17 and to immunorelated class I sHSPs. Results support the hypothesis that developmentally and stress-induced class I sHSPs are distinctly regulated. The possible role of sHSPs is discussed in relation to tissue specificity.

Materials and methods

Source, maturation and germination of somatic embryos

As a source of cork oak somatic embryos, a recurrent embryogenic line maintained on a plant growth regulator-free medium was used (Puigderrajols *et al.*, 1996). Macronutrients were those from SH (Shenck and Hildebrandt, 1972), and micronutrients, vitamins and Fe-EDTA were from MS (Murashige and Skoog, 1962), including 3% (w/v) sucrose. The culture medium was solidified with 0.6% (w/v) agar (type E, Sigma) and the pH was adjusted to 5.7 before autoclaving at 121 °C for 20 min. The cultures were incubated in a growth chamber at 25 ± 2 °C, and a 16 h photoperiod (50 μmol m^{-2} s^{-1}) was provided by cool-white plus Grolux fluorescent lamps.

In order to promote the embryo conversion into plantlets, translucent embryos of up to 5 mm in length, were accurately isolated and subcultured for 30 d in closed baby-food jars (60 mm diameter × 66 mm height) on 50 ml of the plant growth regulator-free medium. Embryos showing signs of secondary embryogenesis were discarded and the remaining embryos subjected to a specific treatment to induce germination (Fernández-Guijarro *et al.*, 1995). This treatment consisted of 7 d of partial dehydration at 25 °C followed by 30 d of cold. Partial dehydration was carried out by transferring each isolated embryo into an empty test tube (18 mm diameter) placed into a baby-food jar (60 mm diameter × 66 mm height) filled with 10 ml of distilled water (three test tubes per jar). Cold treatment was accomplished by subculturing the embryos into jars filled with 50 ml of freshly prepared solid medium at 4 °C in the dark. After the cold treatment, the culture vessels were placed into a growth chamber at 25 °C and 16 h photoperiod for germination. Young plantlets were harvested after 60 d.

To obtain a number of immature translucent embryos, a proliferating friable callus was first obtained (Puigderrajols *et al.*, 2001). Then, embryo formation was induced by subculturing small fragments of the proliferating callus (up to 5 mm) into vessels containing 50 ml of liquid plant growth regulator-free medium. The cultures were incubated in a rotatory shaker (100 rpm) at 25 °C under a light/dark cycle of 16/8 h. After 7 d, clusters of translucent embryos were harvested and subcultured.

Stress treatments

Immature embryos: Suspension cultures of clusters of early translucent embryos (up to 5 mm in lengh) were exposed to cold (4 °C) and elevated temperatures (30, 37, 42 °C), as well as to normal growth temperature (25 °C), for 3 h in the dark. Dehydration was carried out by placing the embryos between two layers of filter paper in Petri dishes for 1, 2 or 3 h at 25 °C. Osmotic stress was imposed by incubating the embryos for 3 h in 20 ml of the above culture medium with additional 3% or 6% (w/v) sucrose and with 2.5, 5 or 10% (w/v) polyethylene glycol (PEG 400, Sigma) at 25 °C, respectively. For UVC light (312 nm) treatment, embryos were deposited in Petri dishes filled with solid medium and irradiated for 1, 5, 10 or 20 min at 15 cm from the lamp (100 μW cm^{-2}). H_2O_2 treatment was performed by incubating the embryos in 20 ml of liquid culture medium containing 0.1, 0.5, 1 or 3% (v/v) H_2O_2 for 3 h at 25 °C in the dark.

Mid-maturation embryos and plantlets: Heat stress was administered by exposing the embryos and the plantlets to 42 °C for 3 h in the dark.

Immediately after all treatments, samples were either frozen in liquid nitrogen and stored at −80 °C or fixed in 4% buffered formalin pH 7.2.

Extraction, electrophoresis and immunological detection of proteins

Samples were homogenized in liquid nitrogen and suspended in 56 mM Na_2CO_3, 56 mM DTT, 2% (v/v) SDS, 12% (w/v) sucrose, 2 mM EDTA for one-dimensional electrophoresis or in 9 M urea, 2% (v/v) Triton X-100, 130 mM DTT, 2% (v/v) Pharmalyte 3–10 (Amersham Pharmacia Biotech), 2 mM PMSF, 2% (v/v) PVP for two-dimensional electrophoresis. Protein concentrations were determined (Bradford, 1976). One-dimensional SDS-PAGE was performed with a Mini-Protean II apparatus (Bio-Rad) using a 12% (w/v) resolving gel and a 3% (w/v) stacking gel. Two-dimensional electrophoresis was performed with a Multiphor II (Amersham Pharmacia Biotech) according to the instructions of the manufacturer using Immobiline DryStrips (pH 3–10 L) for the IEF and ExcelGel SDS gradient 8–18% and ExcelGel SDS buffer strips for SDS-PAGE. Proteins were transferred onto PVDF membranes (Millipore) and incubated with antisera (polyclonal antibodies raised against recombinant cork oak HSP17 protein in rabbits). Bound antibodies were detected using goat anti-rabbit IgG peroxidase conjugated (GAR-Po, Nordic Immunology) and a chemiluminiscent system (BM Chemiluminiscence Western Blotting Substrate, Boehringer Mannheim).

Immunohistochemistry

Formalin-fixed embryos were dehydrated in a series of ethanol and embedded in paraffin. Sections of 7 μm were rehydrated in distilled water and incubated with the appropriate antibodies. The rabbit anti-HSP17 antibodies were used as primary antibody and a goat anti-rabbit IgG alkaline phosphatase-conjugated (A-2556, Sigma) was used as a secondary antibody. The colour reaction was developed through a NBT/BCIP system (Boehringer Mannheim). In control sections, incubation with primary antibodies was omitted.

Results

HSP17 accumulation during embryo maturation and germination

In somatic embryos, HSP17 accumulation depended on the developmental stage (Figs 1, 2). No protein was detected in the translucent embryos used as starting explants. HSP17 was first detected after 5 d of culture and its amount increased to a high level in mid-maturation embryos (30 d of culture) (Fig. 2A). The amount of protein remained at about the same level unless embryos

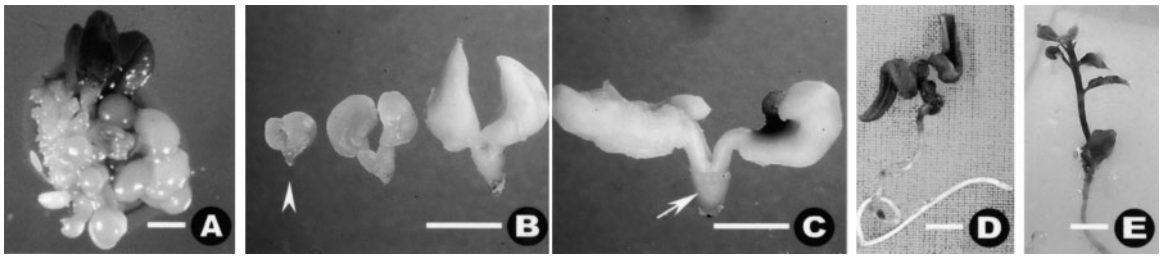

Fig. 1. Morphology of cork oak somatic embryo maturation and germination. (A) Mother embryo bearing translucent secondary embryos at the hypocotyledonary region. Bar = 2.5 mm. (B) Translucent embryo used as starting explant (arrowhead) and embryos after 5 d and 15 d of culture. Bar = 5 mm. (C) Mid-maturation embryo after 30 d of culture. Note the well-developed root cap (arrow) and the absence of secondary embryogenesis. Bar = 5 mm. (D) Germinating embryo 10 d after the inductive treatment for germination. Bar = 5 mm. (E) Plantlet 60 d after the inductive treatment for germination. Note the well-developed shoot. Bar = 10 mm.

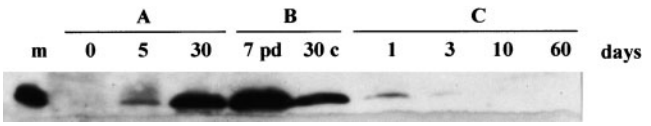

Fig. 2. HSP17 accumulation during cork oak somatic embryo maturation and germination. (A) Maturing embryos after 0, 5 and 30 d in normal growing culture conditions. (B) Embryos during the germination inductive treatment: after 7 d of partial dehydration (7 pd) and after 30 d of cold (30 c). (C) After the cold treatment, germinating embryos, kept at 25 °C, were harvested at 1, 3, 10 and 60 d (plantlet). Total protein extracts (15 μg) were resolved by one-dimensional gel electrophoresis and Western blots were probed with anti-HSP17 polyclonal antibodies. Only the HSP17 region is shown. Recombinant HSP17 expressed in *E. coli* was used as a marker (m).

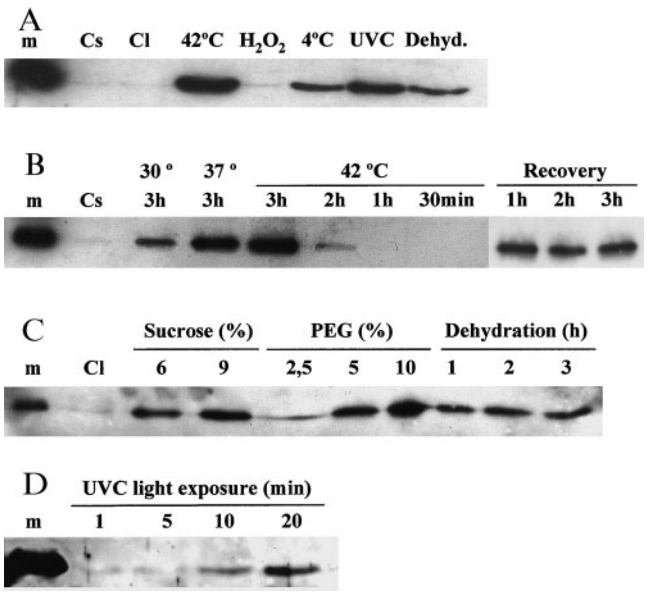

Fig. 3. Stress-induced HSP17 in early cotyledonary stage translucent somatic embryos. (A) HSP17 accumulation after high temperature treatment for 3 h (42 °C), 0.5% H_2O_2 for 3 h (H_2O_2), low temperature for 3 h (4 °C), ultraviolet C light for10 min (UVC) and dehydration at 25 °C for 3 h (Dehyd). (B) Accumulation in response to different heat stresses (30, 37 and 42 °C, respectively) and different times of recovery after a heat stress of 42 °C for 3 h (Recovery). (C) Water stress by osmotic (sucrose and PEG 400) and dehydration treatments. (D) Accumulation during UVC light exposure. Total protein extracts (15 μg) were resolved by one-dimensional gel electrophoresis and Western blots were probed with anti-HSP17 polyclonal antibodies. Only the HSP17 region is shown. Recombinant HSP17 expressed in *E. coli* was used as a marker (m). Controls were performed at 25 °C in the dark in 3% sucrose solid (Cs) or liquid (Cl) culture medium.

were subjected to the specific treatment to induce germination (see Materials and methods). In this case, the amount of HSP17 increased during the partial dehydration treatment (7 d, 25 °C) and then began to decline slowly over the cold phase (30 d, 4 °C) (Fig. 2B). When induced embryos were placed in the growth chamber for germination (25 °C), the protein disappeared very rapidly and no detectable amount remained after 3 d. Moreover, under normal growing culture conditions, no HSP17 was detected in germinating embryos and plantlets (up to 15 cm in length) (Fig. 2C).

HSP17 accumulation in response to stress

In translucent embryos, HSP17 was induced in response to different types of stress (Fig. 3). Under normal growing conditions (25 °C, in the dark), no HSP17 was found in embryos cultured in solid or in liquid medium. After exposure to high and low temperatures and water or oxidative stresses, variable amounts of HSP17 could be detected (Fig. 3A). In heat-stressed translucent embryos (Fig. 3B), the amount of HSP17 increased in correlation with temperature and duration of stress. At 42 °C, the protein began to be detected after 2 h and the highest levels were observed within 3 h. During recovery from 3 h at 42 °C, the amount of HSP17 remained at about the same level for at least 3 h. After 2–3 d of recovery, all heat-stressed embryos developed secondary embryogenesis. Water stress treatments using sucrose and PEG as osmoticum (Fig. 3C) resulted in a variable accumulation of HSP17 depending upon the osmotic concentration. In embryos stressed by dehydration, HSP17 reached its greatest amount within 1 h and remained at the same level after 2–3 h of treatment. After 2–3 d of recovery, all water-stressed embryos developed secondary embryogenesis. UVC light (Fig. 3D) induced detectable levels

of HSP17 after 5 min of irradiation and the amount of protein increased in correlation with the time of exposure. Exposures exceeding 20 min led to embryo necrosis. After 2–3 d of recovery from 10 min UVC light irradiation, all exposed embryos developed secondary embryogenesis. When used at relatively low concentrations (0.1, 0.5, 1%) H_2O_2 induced very low levels of HSP17 (data not shown). During recovery, the embryos showed a dark colour and a distorted morphology, but developed secondary embryogenesis. H_2O_2 concentrations over 1% caused embryo death.

Mid-maturation embryos (30 d of culture) showed a relatively high amount of HSP17 in the absence of stress, but the protein amount increased after exposure to elevated temperature (42 °C, 3 h) (Fig. 4A). Interestingly, the increase in HSP17 by high temperature occurred in the embryo body and did not occur in the cotyledons.

Plantlets did not show HSP17 accumulation under normal growing conditions. After heat stress (42 °C, 3 h), plantlets accumulated HSP17 in shoot and root tissues, but not in cotyledons (Fig. 4B).

Immunohistochemistry

In sections of translucent embryos under normal growing culture conditions (25 °C), no protein reacted with the anti-HSP17 antibodies (Fig. 5B). After stress, the dark-coloured signal appeared in all tissues, but the strongest signal was in the apical meristems and the procambial tissue (Fig. 5C–F). Except for the heat stress (Fig. 5C: 42 °C, 3 h), which resulted in a generally greater intensity, no differences were observed in relation to the stress sources (Fig. 5D–F: 10% (w/v) PEG, 3 h; UVC light, 10 min; 0.5% (v/v) H_2O_2, 3 h).

In sections of mid-maturation embryos a generally weak reaction was observed under normal growing conditions (25 °C) and a stronger signal localized in the shoot and root apical meristems. However, a closer examination of these meristems showed tissue specificity for HSP17. As seen in the root apical meristem (Fig. 6B–F), the absence of labelling was seen in regions consisting of smaller and more cytoplasmic cells. Interestingly, the most stained tissues were the early differentiating regions, consisting of somewhat larger and more vacuolated cells, but the more mature regions appeared only weakly or very weakly labelled. In the nearly median longisections (Fig. 6B), the procambial cylinder stood out from the surrounding pith and the cortical ground meristem for the absence of labelling. In cross sections at the distal end of the radicle (Fig. 6C), the root cap cells were positively stained, but the root meristem initial cells were unlabelled. In cross-sections taken at progressively more basal levels (Fig. 6D–F), the procambial cylinder was unlabelled whereas cells forming the future pith and the cortical cells were dark coloured. In the cortical cylinder, the inner cell layers consisting of larger and more vacuolated cells appeared darker in colour that the outer cell layers consiting of smaller and more vacuolated cells. At about 2 mm from the tip (Fig. 6G), the tracheary elements that began to differentiate in the procambial strands were strongly labelled (arrowheads). At this embryo level, the whole cortical tissue, consisting of highly vacuolated cells with relatively large intercellular spaces between them, appeared very weakly labelled, as were the highly vacuolated cells in the central region of the pith. By

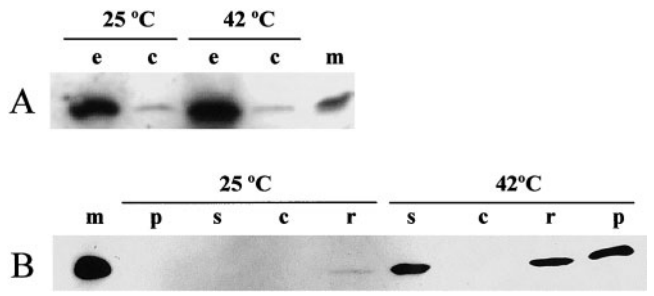

Fig. 4. HSP17 accumulation in mid-maturation somatic embryos and plantlets under normal growing conditions (25 °C) and after heat stress (42 °C, 3 h). (A) Mid-maturation somatic embryos. (B) Plantlets. Embryo body (e) and cotyledons (c) from mid-maturation embryos and shoot (s), root (r), and cotyledons (c) from plantlets (p) were dissected after the heat treatment and analysed separately. Total protein extracts (15 μg) were resolved by one-dimensional gel electrophoresis and Western blots were probed with anti-HSP17 polyclonal antibodies. Only the HSP17 region is shown. Recombinant HSP17 expressed in *E. coli* was used as a marker (m).

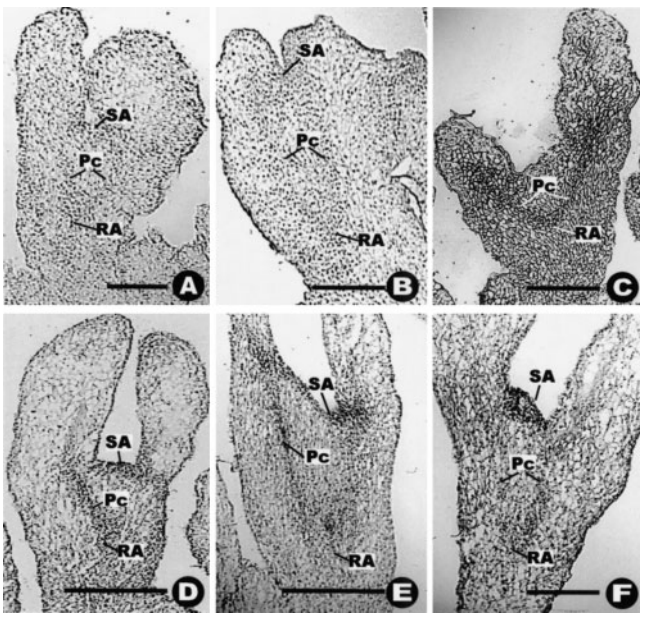

Fig. 5. Tissue immunolocalization of HSP17 in translucent somatic embryos (up to 5 mm in length). The presence of HSP17 is indicated by the dark-coloured end-product. (A) Control for specific binding by incubating without primary antibody. (B) Embryo under normal growing culture conditions (25 °C). (C–F) Embryos under stress. (C) Heat stress (42 °C, 3 h). (D) Osmotic stress (PEG 10%, 3 h). (E) UVC light (10 min). (F) H_2O_2 (0.5%, 3 h). Bars = 1 mm. Pc, procambium; RA, root apex; SA, shoot apex.

contrast, the smaller and more cytoplasmic cells at the pith and most peripheral cell layers were strongly stained. After exposure to heat stress (42 °C, 3 h), a general increase in labelling was shown specially in the previously unstained tissue (Fig. 6H–I).

Examination of the shoot apical meristem revealed a similar tissue specificity for HSP17. Under normal growing conditions, the procambial tissue was unstained or very weakly stained, but after heat stress it appeared strongly labelled (not shown).

In sections of cotyledons under normal growing conditions (Fig. 7), labelling was localized in the vascular strands, with the strongest signal in the tracheary elements, and no signal was shown in the cotyledonary

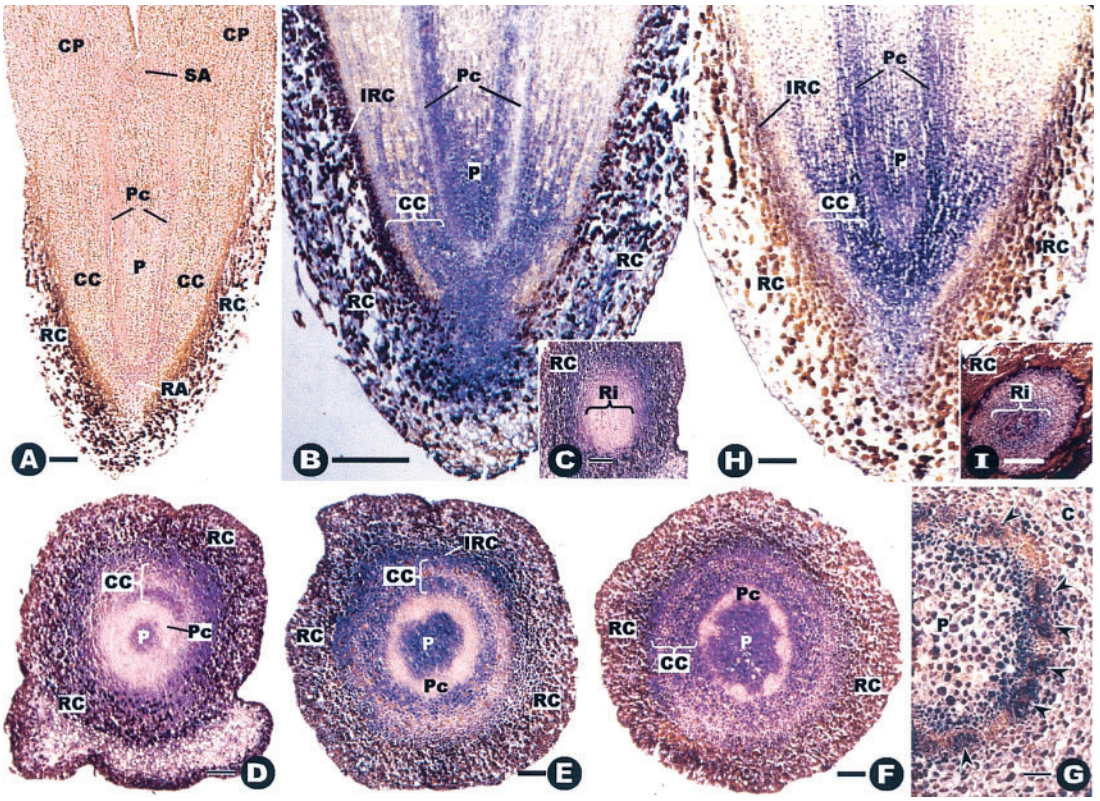

Fig. 6. Tissue immunolocalization of HSP17 in the root apical meristem of mid-maturation somatic embryos in normal growing conditions and after heat stress. The presence of HSP17 is indicated by the dark-coloured end-product. (A) Control for specific binding by incubating without primary antibody. Bar = 250 μm. (B–G) Embryos under normal growing culture conditions (25 °C). (B) Nearly median longitudinal section. The pith (p) and the cortical cylinder cells (cc) appear strongly labelled whereas the procambium (Pc) is unlabelled. Bar = 500 μm. (C–G) Cross-sections at different levels from the root tip showing tissue specificity of antibodies related to tissue differentiation. (C) Section at the root apex. The root initial cells (RI) are unlabelled. Bar = 250 μm. (D–E) Sections at upper levels. Note the absence of colour in the procambial cylinder (Pc) and the intense labelling of the early maturing pith (p) and cortical cells (cc). Note also the intense labelling of the early differentiating vascular elements (G, arrowheads). Bars = 250 μm. (H, I) Embryos after a heat stress treatment (42 °C, 3 h). (H) Nearly median longitudinal section showing procambial cylinder (pc) densely labelled. Bar = 250 μm. (I) Cross-section at the root apex. The root initial cells are labelled. Bar = 500 μm. C, cortex; CC, cortical ground meristem; CP, cotyledonary petiole; IRC, inner root cap cells; P, pith; Pc, procambial cylinder; RA, root apex; RC, root cap; Ri, root initial cells; SA, shoot apex.

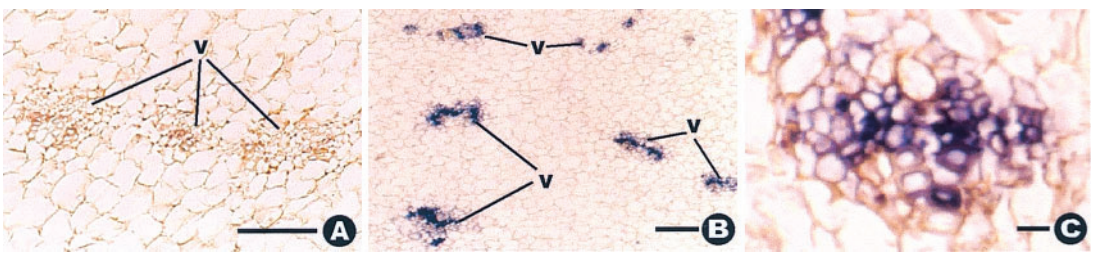

Fig. 7. Tissue immunolocalization of HSP17 in sections of mid-maturation somatic embryo cotyledons. The presence of HSP17 is indicated by the dark-coloured end product. (A) Control for specific binding by incubating without primary antibody. Bar = 250 μm. (B) Section through a cotyledon under normal growing culture conditions (25 °C) showing labelled vascular strands. Bar = 250 μm. (C) Detail of a vascular strand with labelled tracheary elements. Bar = 25 μm. V, vascular strand.

ground parenchyma cells. After exposure to heat stress (42 °C, 3 h), no changes were detected in the mid-maturation embryo cotyledons.

2-D electrophoresis of stressed and non-stressed somatic embryos

To investigate the complexity of HSP17 accumulation during embryo maturation and to characterize the changes induced by stress further, two-dimensional electrophoresis and immunoblot analysis was performed.

In both translucent and mid-maturation embryos under normal growing culture conditions, 2-D immunodetection assays showed the accumulation of a single polypeptide at 17 kDa (Fig. 8A, C, arrows). This single polypeptide coincided with the main immunospot of the recombinant HSP17 (Fig. 8G, arrow). However, whereas translucent embryos showed an extremely low amount of protein, a relatively high amount was present in mid-maturation embryos. After heat stress (42 °C, 3 h), in both translucent and mid-maturation embryos a set of 17 kDa protein species was induced (Fig. 8B, D). This set had the single protein species accumulated in the absence of stress. As demonstrated by the 2-D immunodetection of the recombinant HSP17 protein (Fig. 8G), at least two of the stress-induced 17 kDa protein species may correspond to post-translational modifications of HSP17.

In addition to the 17 kDa protein species, the polyclonal antibodies also detected other sets of proteins. Interestingly, conspicuous changes in the 9–15 kDa region were related to maturation and stress. Under normal growing conditions, four protein species accumulated in translucent embryos which could not be detected in mid-maturation embryos (Fig. 8A, C). By contrast, mid-maturation embryos showed a set of *c.* 10 kDa protein species not present in translucent embryos. Heat-stressed translucent embryos accumulated a new immunospot at *c.* 10 kDa (Fig. 8B, arrowhead) which coincides with one of the main spots identified in mid-maturation embryos in this region (Fig. 8C, arrowhead). Heat stress did not induce detectable changes in mid-maturation embryos in the 10 kDa region (Fig. 8D).

Interestingly, when the embryo body and the cotyledons of heat-stressed embryos were analysed separately, organ-related differences appeared (Fig. 8E–F). In the cotyledons, the *c.* 10 kDa protein species accumulated in greater amounts than those at the 17 kDa region. In the embryo body, both protein sets showed relatively similar levels.

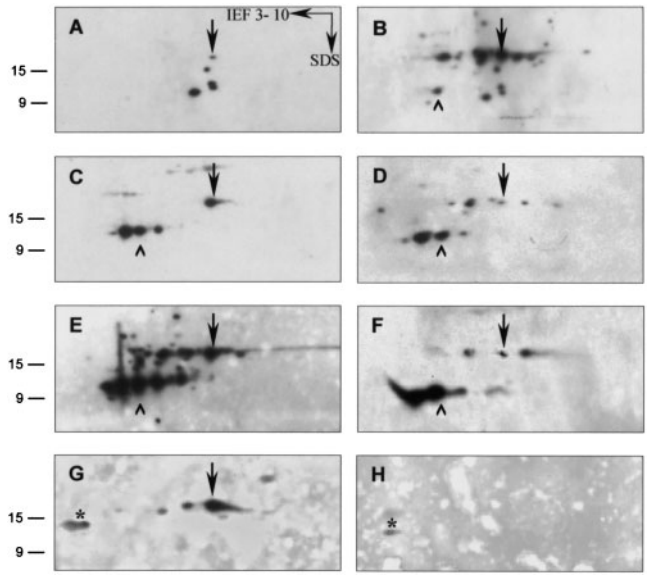

Fig. 8. Two-dimensional electrophoresis of translucent and mid-maturation somatic embryos protein extracts probed with anti-HSP17 polyclonal antibodies. (A, B) Translucent embryos under normal growing culture conditions (A) and after heat stress (42 °C, 3 h) (B). 130 μg of total protein extracts were loaded. (C–F) Mid-maturation embryos. 200 μg of total protein extracts were loaded. Non-stressed whole embryo (C); heat-stressed (42 °C, 3 h) whole embryo (D), dissected embryo body (E) and cotyledon (F). (G, H) *E. coli* cells transformed with a plasmid containing (G) or not containig (H) recombinant *hsp17*. The acidic end of the gels (pH 3) is on the left, basic end (pH 10) is on the right. Position of the molecular weight markers corresponding to 15 and 9 kDa is indicated. Arrows indicate the 17 kDa polypeptide accumulated during maturation. Asterisks indicate an *E. coli* specific protein that reacted with anti-HSP17 antibody.

Discussion

Experiments carried out in cork oak demonstrate a gradual increase in HSP17 during somatic embryo maturation and a decline during germination. A similar pattern was also reported in white spruce somatic embryos for two class II sHSPs genes (Dong and Dunstan, 1996). This pattern parallels that commonly described for cytoplasmic sHSP in zygotic embryo development. The expression of cytoplasmic sHSPs in maturing seeds has been attributed to endogenous rather than to environmental signals (Coca *et al.*, 1994; De Rocher and Vierling, 1994). The fact that, in cork oak under the same temperature (25 °C) and high humidity conditions, HSP17 accumulates in maturing but not in germinating somatic embryos supports the above hypothesis. It has been suggested that sHSP accumulation during embryo maturation is caused by the hydric stress generated by seed desiccation (Coca *et al.*, 1994; De Rocher and Vierling, 1994; Wehmeyer and Vierling, 2000). However, for several zygotic embryos, sHSP accumulation begins significantly before the onset of the seed desiccation programme (zur Nieden *et al.*, 1995). Furthermore, it has been reported that class I sHSPs are abundant in both high and low moisture content forest seeds (Collada *et al.*, 1997). In cork oak somatic embryos, HSP17 begins to

accumulate at a relatively early stage of embryo maturation and it should be noted that somatic embryos maintain a high hydric content throughout the maturation process (Livingston *et al*., 1992; Fernández-Guijarro, 1997). In addition, acorns are recalcitrant seeds which retain a high moisture content throughout seed development. Thus, desiccation does not seem to be a necessary condition for developmental HSP17 accumulation in cork oak somatic embryos.

As not all stress induced class I sHSPs are developmentally expressed during embryo maturation (Coca *et al*., 1996; Haukinen *et al*., 1996; Waters *et al*., 1996) and not all developmentally expressed class I sHSPs are induced by stress (Coca *et al*., 1996; Wehmeyer *et al*., 1996; Carranco *et al*., 1997) it is assumed that developmentally and stress-induced sHSPs may have distinct regulatory controls. Results in cork oak somatic embryos support this hypothesis. In mid-maturation embryos only one 17 kDa protein species, that matching the recombinant HSP17 main spot, is developmentally accumulated. However, heat stress induces an intense accumulation of 17 kDa isoforms, at least some of them corresponding to post-translational modifications of the *hsp17* gene product. A distinct developmental and stress regulation has been recently demonstrated in transgenic *Arabidopsis* (Wehmeyer and Vierling, 2000).

In the light of previous studies on the apical organization of the root apex in cork oak (Molinas and Verdaguer, 1993; Verdaguer and Molinas, 1999), the analysis of the immunostained somatic embryo sections revealed a close relationship between cell differentiation and the developmental expression of class I sHSPs. HSP17 is conspicuously absent from the undifferentiated highly dividing regions of the shoot and root apical meristems, consisting of small cytoplasmic cells without intercellular spaces between them. The highest level of protein accumulation is reached in early differentiating regions dividing at a much slower rate and consisting of more vacuolated larger cells with small intercellular spaces. Moreover, the HSP17 signal becomes weak or even disappears as cells reach a more mature stage showing a larger size, a fused central vacuole and larger intercellular spaces. Thus, function of developmentally accumulated HSP17 seems to be restricted to a specific stage of cell development, the cell division to differentiation transition. In animal cells, sHSPs expression during the transition from cell division to differentiation has been related to a preventive role in differentiating cells from undergoing apoptosis (Arrigo, 2000).

Although a further characterization is required, a specialized role for the *c*. 10 kDa protein species in the developing tracheary elements may be deduced. In the cotyledons of mid-maturation embryos in which these protein species were chiefly predominant, the only immunolabelled tissue was the tracheary elements. Moreover, no change was induced by stress in these embryo organs.

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