

## Stress proteins co-expressed in suberized and lignified cells and in apical meristems

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### Abstract

We report the cloning of a small heat shock protein, Qs\_HSP17, and an osmotin like protein, Qs\_OLP, from cork oak phellem tissue (cork cells). Both genes are expressed in suberizing cells and in other cells subject to endogenous stress associated with free radicals. We provide evidence that smHSPs and OLPs accumulate in overwintering buds and speculate that their role is similar to that in seed dormancy. We also show that both stress proteins are mainly located in the region of the quiescent center in root apex and in central meristem in the shoot apex. We emphasize that smHSPs and OLPs are expressed in cells growing under endogenous stress or facing long life-span. We discuss a possible role of these stress proteins against oxidative stress. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords:* Cork-oak; Osmotin like proteins; Phellem; Small heat shock proteins; *Quercus suber*; Tissue specificity

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### 1. Introduction

The cellular stress response is a ubiquitous defense mechanism activated when cells are confronted with stress. The induction of the stress response leads to expression of a group of proteins referred to as stress proteins, thought to

protect the cell. The stress response is induced by heat shock and by many other agents, among them oxidative stress, osmotic stress and hypoxia, and also by some normal cellular events in embryonic development, cell division and differentiation and in programmed cell death [1]. In plants, stress genes are activated by developmental and environmental cues, hormonal stimuli and by microbial attack [2].

Most stress proteins are chaperones [3] and these include members of the major heat-shock

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<sup>1</sup> Equally contributed to this work.

protein (HSP) family and proteins involved in intracellular trafficking. Some chaperones (HSP27) have been implicated as regulators of actin cytoskeleton organization [4]; it has been proved that an OLP (osmotin like protein) from potato has actin-binding activity [5]. In plants, stress proteins include a great diversity of HSPs [2] in particular from the small heat-shock protein (smHSP) subfamily (for a review see [6]) and also include proteins that accumulate in response to cellular dehydration [7,8], OLPs among them [9]. Many dehydration-induced proteins, like the OLPs [9,10], also function in plant pathogen defense and are referred to as pathogenesis-related (PR) proteins [11]. Many plant stress proteins accumulate in the embryo when seeds are developing tolerance to desiccation [8,12] and belong to the group of the late embryogenesis abundant (LEA) proteins [13]. The plant hormone abscisic acid (ABA) is involved in most types of stress response [14–16].

For most woody perennials, the periderm replaces the epidermis and provides efficient radiation insulation protection against water loss and a barrier to diseases. In the periderm, the cork cambium (phellogen) produces exterior phellem or cork cells that become impervious and resistant to enzyme degradation. Cork cells deposit a layer of suberin and then undergo programmed cell-death [17]. Suberization requires the synthesis of suberin precursors and their polymerization by cell-wall laccases, a process associated with the presence of free radicals and oxidative stress [18]. Cell suberization is also a central event in plant defense reactions such as wound healing, microbial barrier formation and hypersensitive responses [19–22].

Here we report the cloning of two cDNAs from a cork oak phellem cells library encoding two new stress proteins, *Qs\_HSP17* and *Qs\_OLP*. Our interest was to characterize the gene expression and protein accumulation patterns of both stress genes in normal plant tissues, particularly in cells that, like cork cells, are subject to endogenous oxidative stress. On the other hand we have analyzed the induction capacity of these genes after the application to normal plants of oxidative as well as temperature and water stress.

## 2. Materials and methods

### 2.1. Plant material

*Quercus suber* phellem layer was obtained from actively growing shoots, during June, in the forest. Overwintering acorns were collected from the forest soil during the winter months. In these acorns the radicle usually protudes 1–2 mm. A description of cork-oak late embryo morphology and germination is in Molinas and Verdaguier [23,24]. Plants were obtained by germination of cork-oak acorns in water-soaked peat for 30 days to 3 months. The standard growth conditions were 22°C at 70% humidity in a light/dark cycle of 16/8 h for all experiments. Somatic embryos were obtained from embryo cultures as described in Puigderrajols et al. [25].

### 2.2. Cloning and sequence analysis

Poly(A) + RNA was purified from cork-oak cork cells with polyAtract system 1000 (Promega) and used to construct a cDNA library in Uni-ZAP XR vector using the ZAP-cDNA synthesis kit (Stratagene). The lambda library was packaged in Gigapack II Gold packaging extract (Stratagene) and plated on the *Escherichia coli* cell line XL1-Blue MRF. Plasmids were rescued from two randomly chosen phages according to instructions from the supplier. Both strands of *Qs\_hsp17* and *Qs\_olp* cDNAs were completely sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer) and primers generated during sequencing. The sequencing device was Applied Biosystems ABI PRISM 310. Amino acid sequences were compared to SwissProt databank with the program FASTA. The nucleotide sequence data will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under accession numbers AJ000691 and AJ000692.

### 2.3. Protein purification and antibody production

EcoXho inserts of *pQs\_hsp17* and *pQs\_olp* were subcloned in pET28 vectors. Expression in *E. coli lysE* and purification of recombinant

proteins was done using the pET System (Novagen). Polyclonal antibodies were raised in rabbits. Goat anti-rabbit IgG alkaline phosphatase-conjugated (A-2556 Sigma) was used as a secondary antibody and NBT/BCIP (Boehringer Mannheim) was used as substrate for alkaline peroxidase (AP). Polyclonal antibodies anti any class I smHSP recognize a number of class I smHSP [6]. Cross-reaction between anti-OLPs has also been shown [10].

#### 2.4. Plant treatments and Western blots

Temperature treatments were performed by incubation of 30 day old seedlings in water at 42, 37, or 32 °C for 3 h or at 4°C for 7 h. Water stress was achieved with a constant stream of air for 3 h until seedlings have lost 10–15% fresh weight. For ABA or osmoticum, seedlings were incubated in 50 µM ABA for 4 h or in 342 mM NaCl for 6 days. UV irradiated plants were exposed to 312 nm UV light for 10 min. For H<sub>2</sub>O<sub>2</sub> treatment, plants were incubated for 24 h in 5% H<sub>2</sub>O<sub>2</sub>. After the end of each treatment plants were kept in water at standard growth conditions for 12 h before harvesting. Controls were performed by incubation in H<sub>2</sub>O. Plant material was homogenized in liquid nitrogen and suspended in 56 mM Na<sub>2</sub>CO<sub>3</sub>, 56 mM DTT, 2% SDS, 12% sucrose, 2 mM EDTA. Protein concentration was measured by Bradford. FA 30 µg mass of total protein were resolved in 12% SDS-PAGE and immunoblotted. A duplicate of each gel was stained with Coomassie.

#### 2.5. Immunohistochemistry and in situ hybridizations

Eight microgram sections were obtained from formalin-fixed paraffin-embedded specimens. For immunohistochemistry sections were rehydrated in phosphate-buffered saline pH 7.5 (0.01 M) and treated with the appropriate antibodies. In control sections, incubation with the primary antibodies was omitted. In situ hybridizations were performed as described in RPN 3300 (Amersham). Essentially DIG-labelled RNA was used as probe, and anti-DIG-AP antibody and NBT/BCIP

(Boehringer Mannheim) was the developing system. Controls were hybridized with sense probes.

#### 2.6. Peroxidase test

Unfixed free hand sections were incubated with H<sub>2</sub>O<sub>2</sub> and 3,3'-Diaminobenzidine as described in Sigma Tablet test Set (D-4418).

### 3. Results

From our cork oak phellem cells library we isolated, by serendipity, two cDNAs encoding two new stress proteins, Qs\_HSP17 and Qs\_OLP, representatives of the two major groups of plant stress proteins: the heat-shock proteins and dehydration-induced proteins.

Qs\_HSP17 (EMBL AC AJ000691) sequence shows 71.4% identity to that of 17.4 KDa class I HSP of *Arabidopsis thaliana* (EMBL AC P19036) and high sequence identity to all sequenced class I smHSPs.

Qs\_OLP (EMBL AC AJ000692) sequence shows 81% identity to that of soybean p21 protein (EMBL AC P25096) and 71–63% identity to that of OLPs, maize PR-5 proteins, alpha-amylase/trypsin inhibitor, osmotin and thaumatin.

From *Qs\_hsp17* and *Qs\_olp* cDNAs we produced recombinant proteins in *E.coli* and raised rabbit polyclonal antibodies against them.

#### 3.1. Induction of HSP and OLP in response to stress

The induction of Qs\_HSP17 and Qs\_OLP in response to stress was investigated by means of Western blots using the polyclonal antibodies raised against recombinant Qs\_HSP17 and Qs\_OLP. It has been described for other species that class I smHSPs [6] and OLPs [10] are immunogenically related. Since it is unknown whether or not Qs\_HSP17 and Qs\_OLP belong to a family of proteins in *Q. suber*, we usually refer to Qs\_HSP17 and Qs\_OLP.

Our results showed that smHSP and OLP proteins were induced in young cork-oak seedlings by different types of stress, oxidative

stress among them. The highest levels of induction were obtained in stem tissue by a heat shock treatment at 42°C (Fig. 1A and B). Heat shock treatments at 38 and 32°C also induced both proteins at progressively lower levels (Fig. 1A and B). Water stress and ABA induced both proteins (Fig. 1A for Qs\_HSP17, not shown for OLP). Cold markedly induced Qs\_OLP (Fig. 1B). High levels of Qs\_HSP17 were induced by H<sub>2</sub>O<sub>2</sub> treatment (Fig. 1A) and UV radiation induced Qs\_OLP (Fig. 1B).

It is well known that some smHSPs [12] and OLPs [26,9] are inducible by thermal stress, water stress or ABA. Recently a sHSP has been described to be transcriptionally induced by ozone [27].

### 3.2. Gene expression and protein accumulation patterns

To establish the tissue patterns of gene expression and protein accumulation in normal plant tissues, mRNA in-situ hybridizations and immunocytochemical reactions were performed. We examined non stressed young stems and their corresponding axillary buds, the root tips of overwintering acorns and mid maturation somatic embryos.

As predicted, *Qs\_olp* and *Qs\_hsp17* mRNAs (not shown) and the corresponding proteins (Fig. 2A and B) were expressed in the bark periderm

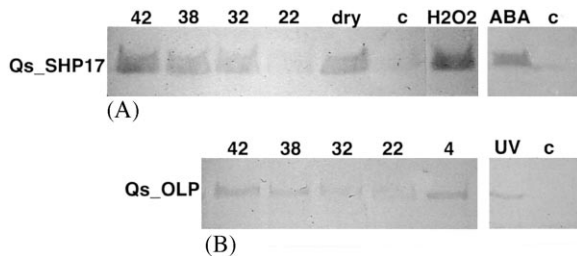


Fig. 1. Temperature, water and oxidative stresses induce Qs\_HSP17 and Qs\_OLP accumulation. Protein immunoblot analysis of stem total protein extracts with (A) anti-Qs\_HSP17 and (B) anti-Qs\_OLP specific antiserum. Both proteins are induced by heat shock at 42, 38 and 32°C compared to 22°C. Qs\_HSP17 is highly induced by water stress (dry) and by H<sub>2</sub>O<sub>2</sub> treatment. C is control. Qs\_OLP is induced by cold treatment at 4°C and by UV treatment.

cork cells committed to death. It is the first time that stress protein genes were shown to be constitutively expressed in bark periderm cells. But, moreover, Qs\_HSP17 and Qs\_OLP genes were co-expressed and proteins co-accumulated in other types of cells committed to death as well such as sclereids and fibers as also in meristematic stem cells.

The highest levels of expression of Qs\_HSP17 and Qs\_OLP was seen in cortical sclereids and fibers (Fig. 2A and B), in young xylem (Fig. 2D) and in periderm cells (Fig. 2A and B). Root endodermal cells (Fig. 2D) and some epidermal trichomes (not shown) were also labelled.

These cells gave positive in the test for endogenous peroxidase (not shown). All these cells have in common the synthesis of aromatic polymer species via free radical formation in the presence of H<sub>2</sub>O<sub>2</sub> [17,18,28] and most are committed to death. The abundance of some smHSPs or OLPs in lignified or cutinized cells has been reported in different plant organs [10,29,30].

However, an unexpected result was to find high levels of *Qs\_hsp17* and *Qs\_olp* mRNAs and proteins constitutively expressed in resting axillary buds. Both mRNAs were expressed in bud scale parenchyma and in internal bud tissues, as well as in the apical meristem cells (Fig. 3A and B). Both proteins were massively co-accumulated in the same bud tissues (not shown). This is the first time that two stress proteins are reported to be co-expressed and accumulated in resting buds.

Particularly interesting are the results on the co-localization of *Qs\_hsp17* and *Qs\_olp* mRNAs and the corresponding proteins in the apical meristems. In overwintering acorn radicles we observed that cells in the quiescent center region and surrounding initials were strongly labelled by the in-situ hybridizations (Fig. 4) and immunohistochemical reactions (not shown). The same pattern of distribution was found in the root apex of mid maturation somatic embryos (Fig. 5). Moreover, in somatic embryos shoot apex, labelling was shown to be accumulated preferently in the central meristem (Fig. 5).

It has been demonstrated that some smHSPs are expressed at high levels in the apical meristems of maturing embryos [31,32]. Here, we re-

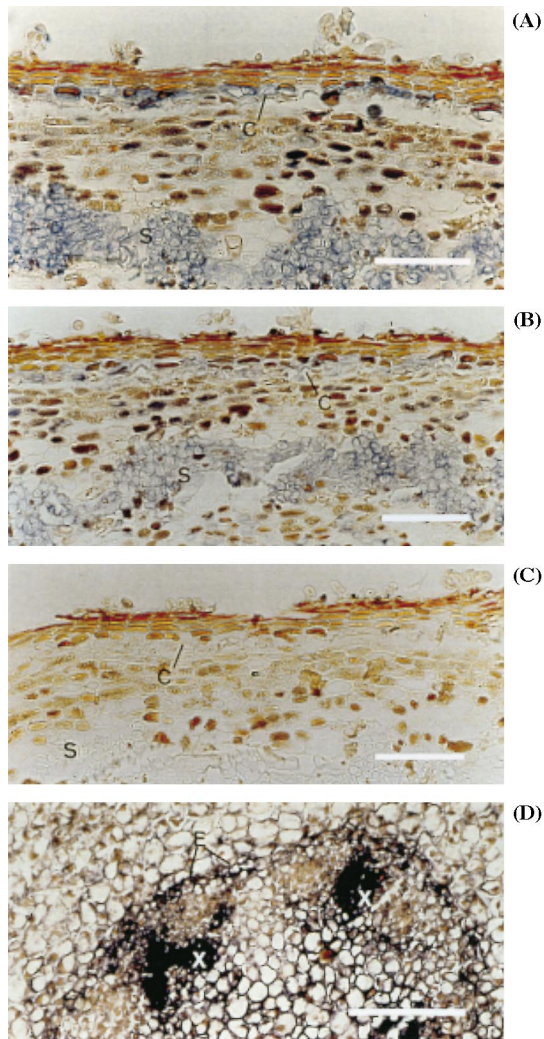


Fig. 2

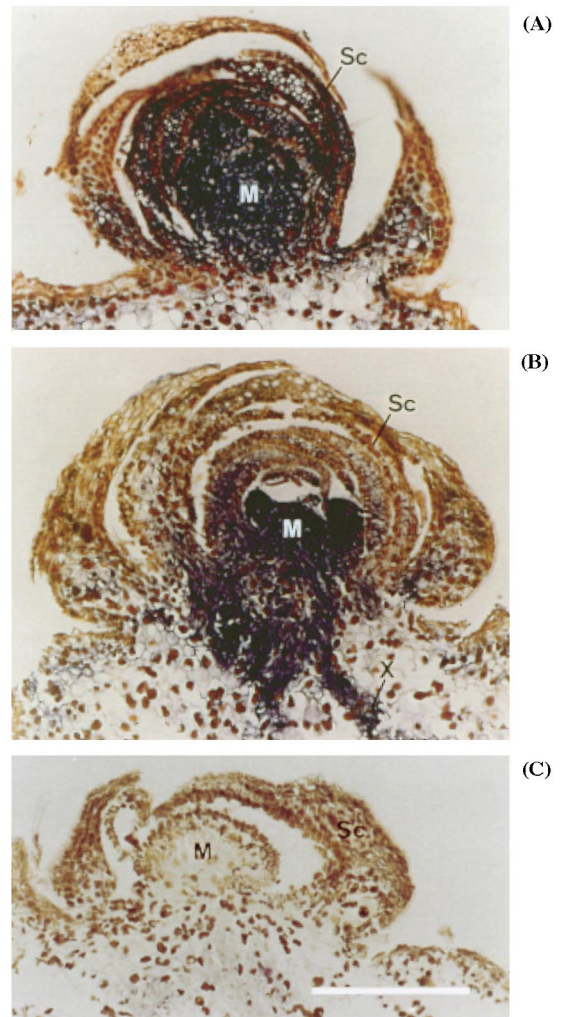


Fig. 3

Fig. 2. *Qs\_HSP17* and *Qs\_OLP* accumulation in cork cells and cortical sclerenchyma. *Qs\_olp* mRNA expression in endodermal and xylem cells. (A, B, and C) Shoot periderm with subjacent cortex, cross sections. (A) Immunodetection of *Qs\_HSP17*; (B) *Qs\_OLP*; and (C) control without primary antibody. Both proteins are shown in inner cork cells C and sclerenchyma cells S. (D) Hypocotyl cross section hybridized with probe specific for *Qs\_olp* mRNA showing expression in endodermal cells, E, and young xylem, X. Scale bar, 100  $\mu$ m.

Fig. 3. *Qs\_hsp17* and *Qs\_olp* mRNA expression in cork-oak resting buds. In situ hybridizations with probes specific for (A) *Qs\_hsp17* and (B) *Qs\_olp* mRNAs, (C) control. Both mRNAs are present in meristem cells M and in scales parenchyma Sc. Note that xylem elements, X, are labelled in (B). Scale bar, 200  $\mu$ m.

port evidence of co-expression of an smHSP (*Qs\_HSP17*) and an OLP (*Qs\_OLP*) in the region of the quiescent center. This cell specific localization is consistent with a possible protective function of quiescent cells.

#### 4. Discussion

The presence of stress proteins in cork-oak phellem suggests that these proteins could play a protective role against the stress caused by sube-

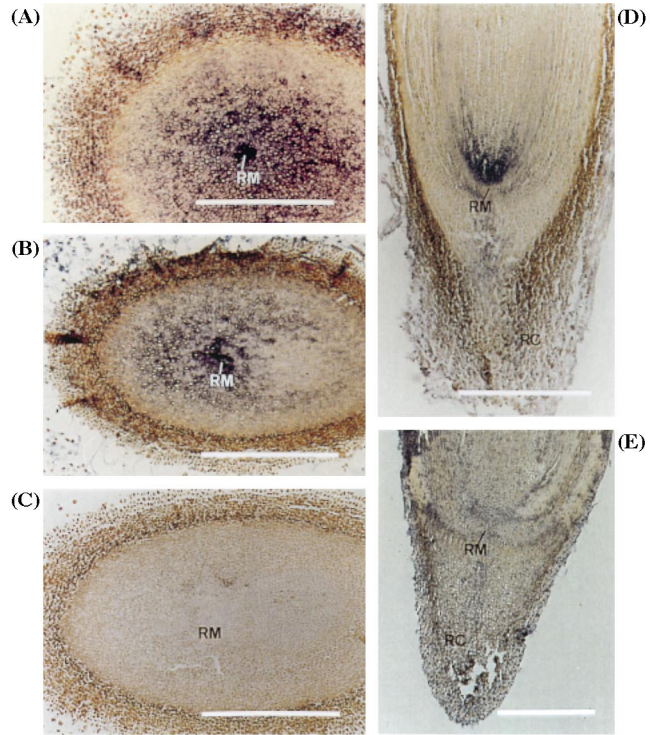


Fig. 4

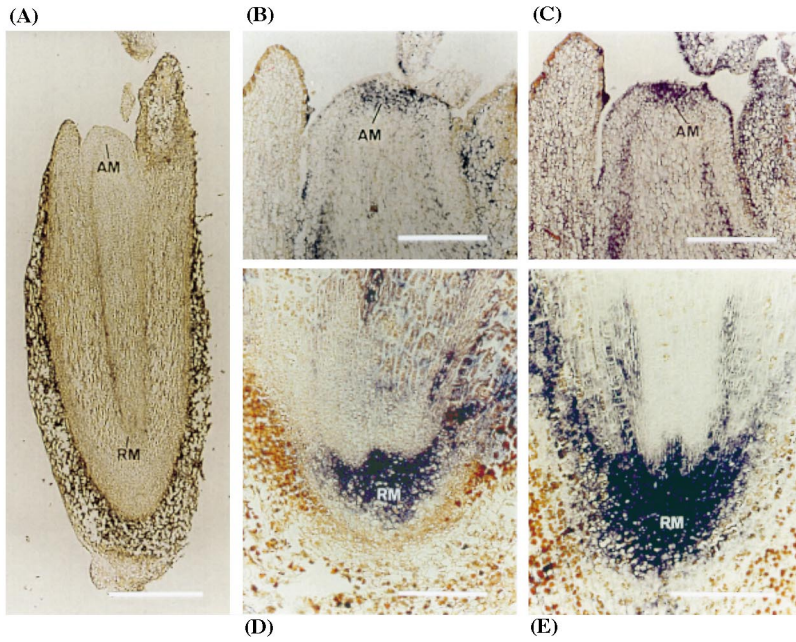


Fig. 5

rization. As shown by western analyses (Fig. 1), Qs\_HSP17 and Qs\_OLP were induced by factors such as temperature, water availability and UV radiation, factors known to have great influence on cork growth [33,34]. Another inductor, ABA, is known to stimulate suberization [35].

#### 4.1. Stress proteins and bud dormancy

Stress proteins may play a protective role in critical phases of plant development [6]. In most environments, perennial plants face multiple seasonal stresses and have short growing seasons. If organs preformed in buds are not protected, damage can occur. Buds are formed before active shoot growth ceases and remain in a reduced growth state until the active growth is reassumed. Factors that regulate the transition from quiescence to active growth are temperature, UV radiation, drought and several plant hormones [36]. Qs\_HSP17 and Qs\_OLP genes are responsive to all these factors (see above).

Entering bud dormancy seems homologous to seed maturation in which large amounts of stress proteins are accumulated, including smHSPs [12]. We can speculate that in resting buds these proteins play a role similar to that in late embryogenesis. smHSPs have been involved in cold, heat and drought tolerance in plants [37,38]. OLPs are important for low temperature and drought tolerance and have antifungal activity [9,10,39]. Many smHSPs and OLPs are inducible by ABA (see above) and one of the main functions of ABA seems to be the induction of drought, heat and chilling resistance [16]. Recently, two LEA proteins have been detected during desiccation and cold acclimation of overwintering grape buds [40].

#### 4.2. Qs\_HSP17 and Qs\_OLP, a protection against oxidative stress?

In plants, active oxygen species are involved in programmed cell death [41] and in most plant/pathogen interactions [42,43]. The specificity of Qs\_HSP17 and Qs\_OLP for cells subject to endogenous oxidative stress and their inducibility by oxidative treatments (see above) suggest a possible protective function against damage caused by oxidative stress. It has been reported that overexpression of HSP27 can protect mammalian cells against death caused by oxidative stress [44].

Much of the injury caused by environmental stresses is associated to oxidative damage at the cellular level [45,46] and the ability of plants to detoxify radicals is probably the most critical requirement under water deficit [47]. Aging [48] and plant senescence [49] have been related to cumulative damage generated by oxygen radicals. Quiescent cells are exposed to oxidative stress for long periods, if quiescent cells were not protected plant survival could be threatened. The localization of Qs\_HSP17 and Qs\_OLP in the quiescent centers in root and shoot apical meristems could be related with a possible protection of the quiescent cells from oxidative damage.

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Fig. 4. Qs\_HSP17 and Qs\_OLP expression and accumulation in meristem from 1–2 mm overwintering acorn radicles. In situ hybridization in cross sections with probes specific for (A) *Qs\_hsp17* and (B) *Qs\_olp* mRNAs; (C) control. Immunodetection in medial longitudinal sections of (D) Qs-HSP17 and (E) Qs\_OLP. Both mRNAs and proteins are abundant in the region of quiescent center and meristematic initials RM. Qs\_OLP is detected in root cap RC. Scale bar, 500  $\mu$ m.

Fig. 5. Qs\_HSP17 and Qs\_OLP expression and accumulation in cork-oak somatic embryo tissues. Analysis of nearly medial longitudinal sections of mid-maturation somatic embryos. (A, B, and C) immunodetection of (B) *Qs\_HSP17*, (C) *Qs\_OLP* and (A) control. (D and E) in situ hybridization with probes specific for (D) *Qs\_hsp17* and (E) *Qs\_olp* mRNAs. Qs\_HSP17 and Qs\_OLP are accumulated in the central shoot apical meristem AM. Both mRNAs are shown in the region of quiescent center and meristematic initials RM. Scale bar, (A) 500  $\mu$ m, (B, C, D, and E) 200  $\mu$ m.

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