Expression of the *QrCPE* gene is associated with the induction and development of oak somatic embryos

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Summary

Somatic embryogenesis is a powerful tool for plant regeneration and also provides a suitable material for investigating the molecular events that control the induction and development of somatic embryos. This study focuses on expression analysis of the *QrCPE* gene (which encodes a glycine rich protein) during the initiation of oak somatic embryos from leaf explants and also during the histodifferentiation of somatic embryos. Northern blot and in situ hybridization were used to determine the specific localization of *QrCPE* mRNA. The results showed that the *QrCPE* gene is developmentally regulated during the histodifferentiation of somatic embryos and that its expression is tissue- and genotype- dependent. *QrCPE* was strongly expressed in embryogenic cell aggregates and in embryogenic nodular structures originated in leaf explants as well as in the protodermis of somatic embryos from which new embryos are generated by secondary embryogenesis. This suggests a role for the gene during the induction of somatic embryos and in the maintenance of embryogenic competence. The *QrCPE* gene

was highly expressed in actively dividing cells during embryo development, suggesting that it participates in embryo histodifferentiation. The localized expression in the root cap initial cells of cotyledonary somatic embryos and in the root cap of somatic seedlings also suggests that the gene may be involved the fate of root cap cells.

Keywords: gene expression, glycine-rich protein, oak, QrCPE gene, somatic embryogenesis.

Introduction

Pedunculate oak (*Quercus robur* L.), which belongs to the Fagaceae family, is a valuable hardwood species with a wide distribution in Europe. In vitro propagation systems are highly desirable for this species, since oak trees are both socio-economically and environmentally important in agroforestry systems (Merkle and Nairn 2005).

Somatic embryogenesis (SE) is a powerful tool for plant regeneration and potentially provides an efficient system for clonal mass propagation and germplasm conservation. It is also the most suitable method of obtaining the target material for use in gene transfer techniques and for regenerating transgenic plants (Giri et al. 2004, Merkle and Nairn 2005). Moreover, SE has frequently been used to identify the genes that may be involved in the process (Chugh and Khurana 2002)

In *Q. robur*, SE has been induced from juvenile material, including stem and leaf explants excised from young seedlings (Cuenca et al. 1999). In species that are

recalcitrant to *in vitro* culture, such as *Q. robur*, the induction of somatic embryos from vegetative tissues obtained from mature trees is a difficult task. This is an important restriction for the *in vitro* cloning of individual trees with proven elite characteristics. However, oak plantlets have been regenerated by SE induced in leaf (Toribio et al. 2004, Valladares et al. 2006) and shoot apex explants (San-José et al. 2010) from mature trees, although the frequency of induction of somatic embryos in oak is still very low. Embryogenic lines have been established from somatic embryos isolated from original explants and maintained by secondary or repetitive embryogenesis (Sánchez et al. 2003, Valladares et al. 2006), which is a non-synchronous process as oak embryos of different sizes and at different stages of histodifferentiation occur simultaneously (Corredoira et al. 2006). This system can be used to isolate many somatic embryos of different developmental stages from the embryogenic lines, and it therefore provides a suitable source of material for investigating the molecular mechanisms underlying the development of somatic embryos. The original explants in which somatic embryos are initiated are also highly valuable for studying the molecular events that control the onset of SE.

Somatic embryogenesis is the process whereby certain somatic cells, under appropriate inductive conditions, undergo dedifferentiation and generate embryogenic cells with the capacity to differentiate into embryos (Quiroz-Figueroa et al. 2006). Somatic embryos develop through a sequence of several developmental stages associated with morphological and biochemical changes related to genomic activity. Although somatic embryos resemble zygotic embryos morphologically and physiologically, they are originated through a different pathway (von Arnold 2002). Cellular dedifferentiation and the acquisition of embryogenic competence by certain responsive somatic cells both require the current gene expression pattern to be reprogrammed to establish new

developmental programmes. This developmental switching involves activation and repression of specific genes that confer somatic cells with the ability to initiate the embryogenic pathway (Chugh and Khurana 2002). Further histodifferentiation of somatic embryos is also controlled by the temporal expression of specific genes during different developmental stages. However, the molecular mechanisms underlying the switch from the somatic cell fate to embryogenic competence and the regulation of embryo development remain unclear.

The carrot model system of SE has often been used to identify genes involved in this process by comparing the gene expression patterns of non-embryogenic cultures with those of embryogenic cultures and/or somatic embryos at different developmental stages (Aleith and Ritcher 1990, Sato et al. 1995, Lin et al. 1996). Among these genes, Schmidt et al. (1997) identified a somatic embryogenesis receptor kinase (*SERK*), which marks the acquisition of embryogenic competence in carrot cells. Molecular markers of embryogenic potential have also been identified in other species, such as *Oriza sativa* (Jung et al. 1998) and *Hevea brasiliensis* (Charbit et al. 2004), by comparing the expression patterns of callus lines with different embryogenic capacity. The genes involved in the early stages and further development of somatic embryos have also been identified in herbaceous species other than carrot, such as *Medicago sativa*, *Chicorium*, *Dactylis glomerata* (Giroux and Pauls 1997, Helleboid et al. 2000a, 2000b, Somleva et al. 2000) and woody plant species (Charbit et al. 2004, Morcillo et al. 2007). Studies based on transcriptome analysis have shown that numerous genes are activated or differentially expressed during SE in various species (Thibaud-Nissen et al. 2003, Imin et al. 2008, Lin et al. 2009), including woody gymnosperms (Stasolla et al. 2004).

In forest species, most studies of gene expression during SE have been conducted on conifer embryogenic systems (Dong and Dunstan 1999, Stassola et al. 2004, Cairney et

al. 2006), whereas much less work has been done in angiosperms, including the Fagaceae. Among the SE-related genes identified in woody angiosperms, most are homologues of the members of the *SERK* and *LEC* gene families (Schellenbaum et al. 2008, Alemanno et al. 2008). In *Q. robur* several genes including dehydrin genes and *QrEm*, which belong to LEA genes (which encode late embryogenic abundant proteins), have been isolated (Šunderlíková et al. 2009a, 2009b).

We previously isolated a gene (*QrCPE*) that encodes a glycine-rich protein and that is expressed in tissues from embryonic to mature phases (Gil et al. 2003). Expression of this gene is progressively down-regulated during plant maturation in field-grown oak trees. In the present study, the somatic embryogenic system of *Q. robur* was used to investigate the involvement of the *QrCPE* gene in the initiation and development of oak somatic embryos. The results show that the gene is highly expressed in proembryogenic masses that appear in the initial explants as well as in the protodermis, shoot apical meristem and root pole of somatic embryos. In addition, expression of the gene in the root tissues also suggests a possible role in root development.

Materials and Methods

Plant material and culture conditions

The embryogenic lines used in this study, designated B17 and Sainza, were derived from two *Q. robur* trees over 100 years old. Embryogenic cultures were initiated from expanding leaf explants excised from forced shoots obtained from branch segments of the oak trees, following a tree-step culture procedure (Toribio et al. 2004, Valladares et al. 2006). Briefly, leaf explants were cultured on MS (Murashige and Skoog 1962) induction medium supplemented with 21.48 μ M naphthaleneacetic acid (NAA) and 2.22 μ M benzyladenine (BA) for 8 weeks. The explants were then cultured in a second

medium with a lower concentration of plant growth regulators (PGR) for further 4 weeks, and then in a PGR-free MS medium, in which somatic embryos began to arise from callus tissue developed in the initial explants. To determine the *QrCPE* expression pattern during early stages of somatic embryo initiation, leaf explants of the Sainza genotype exhibiting embryogenic nodular structures and somatic embryos just emerging from callus tissue were used.

Clonal embryogenic lines were established after isolation of primary somatic embryos that were proliferated by secondary embryogenesis on embryo proliferation medium consisting of basal medium supplemented with 0.27 μM NAA and 0.44 μM BA (Valladares et al. 2006). For germination, secondary embryos were cultured for 4 weeks on maturation medium consisting of basal medium with half-strength macronutrients and 6% sorbitol (Sánchez et al. 2003, Martínez et al. 2008) and then mature embryos (5-8 mm in length) were transferred to germination medium (i.e. medium with 0.44 μM BA, but no sorbitol) for plant recovery. To study the expression of the *QrCPE* gene during somatic embryo histodifferentiation, embryos at globular-, torpedo- and cotyledonary-stages were collected from proliferating cultures of the B17 and Sainza lines. A nodular callus line derived from embryogenic callus that had lost its embryogenic capacity was also evaluated for comparison with embryogenic tissue. Gene expression was also evaluated in sorbitol matured embryos, as well as in hypocotyls, roots and shoots (stem and leaves) sampled from somatic embryo derived plantlets.

Cultures were maintained in a growth chamber with a 16-h photoperiod (50-60 $\mu\text{mol m}^{-2}\text{s}^{-1}$) provided by cool-white fluorescent lamps and 25°C day/20°C night temperature. The media, all of pH 5.7, were autoclaved at 115°C for 20 min.

RNA extraction, quantification and northern blot analysis

Plant material from all sources was harvested, immediately frozen in liquid nitrogen and stored at -70°C until use for RNA isolation. Total RNA from somatic embryos was extracted with Plant Concert RNA reagent (Invitrogen, Carlsbad, CA), in accordance with the manufacturer's instructions, and total RNA from somatic embryo-derived plantlets was isolated as described by Chang et al. (1993). Total RNA was quantified by detecting absorption at 260 and 280 nm, and RNA quality was checked by visualization of rRNA in 1.2% agarose gels before storage of the samples at -70°C.

For northern blot analysis, total RNA (20 µg) was denatured and electrophoresed in 1.2% agarose gels containing formaldehyde and then transferred to Bioblot-Nitrocellulose membranes (Costar, Corning-Life Sciences, Acton, MA), according to the manufacturer's instructions. Probes were directly labelled by PCR with 20 µCi of [α -³²P]-dATP and the previously cloned *QrCPE* cDNA (Gil et al. 2003). Prehybridization, hybridization and posthybridization washing steps were performed as described by Gil et al. (2003).

In situ hybridization

Somatic embryos at globular, torpedo and cotyledonary stages, were harvested from the B-17 and Sainza lines maintained on embryo proliferation medium. Primary roots excised from somatic plantlets obtained following somatic embryo germination were also used in this study. In the case of explants cultured on embryo induction medium, leaves of the Sainza genotype showing embryogenic response and developing embryos at different stages were collected. Samples were embedded and frozen in Jung Tissue freezing medium (Leyca Microsystems Nussloch GMBH, Heidelberg, Germany) in dry ice. The samples were cut into 10 µm longitudinal or transverse sections, placed on 3-aminopropyl-triethoxilan glass slides, dried and fixed. To generate *QrCPE* specific

probes, a 300-bp fragment corresponding to the 3'-untranslated region was cloned into the PCR II vector (Invitrogen) and amplified. The PCR fragment, flanked by the T7 and SP6 promoters, was used as template for synthesis of both sense and antisense DIG-labelled probes, with T7 or SP6 polymerase, respectively, according to the manufacturer's instructions (DIG RNA labelling kit SP6/T7, Roche Biochemicals). The probes were partially hydrolyzed to a mean length of 150 nucleotides by alkali treatment. In situ hybridization steps and detection of digoxigenin-labelled probes were performed as described by Sole et al. (2008). Photographs were taken with an Olympus digital camera attached to a Nikon microscope operating in bright-field illumination.

Results

Expression analysis of QrCPE in secondary somatic embryos by northern blot

We examined whether *QrCPE* would be expressed in oak somatic embryos at different developmental stages in two different embryogenic lines maintained by secondary embryogenesis. In both lines, *QrCPE* was expressed at higher levels in somatic embryos than in non embryogenic oak callus, in which a weak or undetectable signal was observed (Fig. 1). A comparison of mRNA abundance in embryos of both lines indicated that accumulation of mRNA was similar in globular- and cotyledonary- stage embryos of both lines (Figs 1A and B), whereas a stronger signal was detected in torpedo-stage embryos of the Sainza line (Fig. 1B) than those of the B17 line (Fig. 1A). However, mRNA accumulation was almost undetectable in cotyledonary embryos cultured in the maturation medium supplemented with sorbitol (line B-17, Fig. 1A). Northern blot analysis performed with RNA from different zones of plantlets derived from germinated somatic embryos revealed that the largest amounts of *QrCPE* transcripts were detected in roots excised from somatic seedlings raised from both

embryogenic lines (Fig. 1). *QrCPE* mRNA was undetectable in the hypocotyl of B17, although it was detected at moderate levels in the same region in the Sainza genotype, which in germination medium developed more secondary embryos on the hypocotyl than the B17 genotype.

QrCPE is expressed during the induction of somatic embryos in original explants

The possible role of the *QrCPE* gene during the early stages of initiation of somatic embryos from original leaf explants was investigated by the use of in situ hybridization. Cryosections obtained from Sainza explants which had generated calluses and embryogenic structures arising on their surface were compared with those obtained from explants with no embryogenic response. The *QrCPE* mRNA was not detectable in callus of non embryogenic explants (data not shown), which is consistent with the results of the northern blot analysis, in which a lack of signal was observed in non embryogenic callus (Fig. 1). In contrast, *QrCPE* mRNA was detected at very high levels in embryogenic cell aggregates or proembryogenic masses; the strongest signal was evident in the peripheral zone of these proembryogenic masses, which comprise actively dividing meristematic cells (Figs. 2A-B). However, no *QrCPE* transcripts were detected in the inner region of leaf callus tissues composed of parenchymatic cells. These cells were a pale pink colour (Figs. 2A-B), which indicates a non-specific signal as this background colour also appeared across all tissues in control sections hybridized with the sense strand probe under the same conditions (Fig. 2C). *QrCPE* mRNA was also present at abundant levels in globular- and oblong- stage embryos generated in original explants, with the highest accumulation being observed in the protodermis and subprotodermal cell layers (Fig. 2D). These tissues along with the root pole exhibited the strongest signal in torpedo and early cotyledonary embryos initiated from leaf explants (data not shown). In cotyledonary embryos at more advanced developmental

stage, abundant *QrCPE* mRNA transcripts were detected in the distal cell layers of root meristem, just below the quiescent centre, which includes both the cortical and root cap cell initials of the primary root (Fig. 2E, inset). A relatively high level of transcripts was also detected in cells located below the shoot apical meristem as well as in the axillary zone of cotyledons (Fig. 2F). The signal was absent in the control sections hybridized with the sense mRNA (data not shown).

Spatial accumulation of QrCPE transcripts in somatic embryos generated by secondary embryogenesis

The spatial accumulation of *QrCPE* transcripts in somatic embryos generated by secondary embryogenesis was characterized by *in situ* hybridization. In preglobular and globular embryos, the *QrCPE* gene was uniformly expressed throughout all cells (Figs. 3A and C), although a strong signal was also observed in protodermal cells undergoing cell divisions (Fig. 3C, inset). Histodifferentiation progressed towards late globular stages and the hybridization signal became more localized to the protodermis and the 2-3 cell layers of subprotodermal zone (Figs. 3D and G). As the embryo developed to more advanced stages (oblong) and polarization of the embryo was initiated, *QrCPE* transcripts were also localized in meristematic cells that possibly constitute the early stages of both, apical and radicular poles of the embryo (Figs. 3E-G). A strong signal was also observed in embryogenic structures generated by recurrent embryogenesis from the protodermis of globular embryos (Fig. 3E). At the globular-heart transition stages, abundant accumulation of transcripts was also detected at the incipient tips of the cotyledon primordia as well as in the shoot apical meristem (Fig. 4A). From torpedo to cotyledonary stages, *QrCPE* transcripts were preferentially localized in the protodermis, the outer cortical layers of the embryos and the root pole, as well as at the

shoot apical meristem, where the signal is also extended to the axillary region of cotyledons (Figs. 4C-G). A strong signal was observed in embryogenic structures and globular embryos generated by secondary embryogenesis in the hypocotyl zone of torpedo-stage embryos in the Sainza genotype (Fig. 4C). In this line, analysis of cotyledonary embryos at more advanced developmental stages showed that *QrCPE* transcripts were most abundant at the root pole, with specific localization in the root cap, than in the shoot apical meristem (Figs. 5A and B). In cotyledonary embryos of genotype B17, *QrCPE* mRNA was preferentially accumulated in the shoot apical meristem (Figs. 5E-G), although expression was also detected in the subprotodermal cell layers at the root pole, which will originate the root cap (Figs. 5F and H). A hybridization signal was also evident in procambial strands of embryos from both genotypes (Figs. 5 A and E). When sections were hybridized with the sense probe only a very low level of pink pale colour, which represents non-specific binding, was observed (Figs. 3B and H; 4B and H; 5C, D, I and J).

QrCPE is preferentially expressed in the root tip of the primary root

To further assess the expression pattern of *QrCPE* during root development in germinating embryos, in situ hybridization experiments were performed in sections of primary roots formed from somatic embryo-derived plantlets of the Sainza genotype. Strong hybridization signals were observed in the zone of active cell division of the root (Figs. 6A, C and E), but disappeared in the mature region of the root (Fig. 6B). A longitudinal section of the developing root, including the elongation zone, cell division zone and root cap, is shown in Fig. 6E. In the elongation zone, a moderate signal was detected in the procambial cells and no expression was detected in the vascular tissues and cortical parenchyma cells (Fig. 6F). The levels of *QrCPE* transcripts increased

gradually from the elongation zone towards the cell division zone, where important differences in gene expression are shown in both longitudinal (Fig. 6E) and transverse sections (Figs. 6F vs H). A high level of expression was observed in the inner ground meristem and developing cortex of the cell division zone, whereas no *QrCPE* transcripts were detected in the central region of the root apical meristem, which includes the quiescent centre (Figs. 6C, E and H). In the root cap, high levels of mRNA were observed in the columella (Fig. 6J), and levels decreased towards the more distal and outermost cell layers of the lateral root cap, where cells sloughed off together with cells from the outside of the lateral region (Fig. 6E).

No signals above background were detected in the control sections hybridized with the sense probe (Figs. 6D, G, I and K). The brownish background colour that appeared in the epidermal cell layers of the cross sections hybridized with the antisense probe represents a staining artefact, possible due to the presence of polyphenols, as this brownish background colour also appeared in control sections.

Discussion

In the present study, a detailed molecular analysis of the *QrCPE* gene was performed in two oak embryogenic lines to investigate the putative role of the gene during the induction of primary somatic embryos and during the histodifferentiation of secondary somatic embryos. The *QrCPE* gene was differentially expressed during embryo development (Fig. 1), and its expression was downregulated in tissues of somatic embryo-derived plantlets, in which mRNA was most abundant in roots, as previously reported for oak rooted microcuttings (Gil et al. 2003). The results demonstrated that *QrCPE* expression is developmentally regulated during SE, and that it is affected by the genotype and is also tissue dependent. Previous studies have reported that the

embryogenic lines used in this study exhibited different capacities for embryo proliferation, maturation and germination (Valladares et al., 2006; Martínez et al. 2008). The different expression level of *QrCPE* in these embryogenic lines, as well as the higher accumulation of *QrCPE* transcripts in both torpedo-stage embryos and hypocotyl of germinated Sainza embryos than in B17 embryos, may be related to their different embryogenic potential for recurrent embryogenesis. The *QrCPE* expression was almost undetectable in non-embryogenic proliferating callus (Fig. 1), indicating that its expression in proliferating embryos is not only related to cell proliferation, but that the gene also plays a role in somatic embryo development. Similarly, four carrot genes encoding GRPs were found to be highly expressed in all stages of somatic embryos, but showed relatively low expression in cells of callus suspension before induction of somatic embryos (Lin et al. 1996). According to the classification of Mangeon et al. (2010), the characteristic features of the predicted QrCPE protein (Gil et al. 2003) indicate that it belongs to the class III GRPs. These proteins have been proposed to be expressed in response to abiotic stresses (Kar et al. 2012), as occurs during the induction and development of somatic embryos. The expression of genes encoding GRPs is highly regulated during plant development as well as under the influence of several external stimuli, and it also shows tissue-specific localization (Bocca et al. 2005). In the present study, *QrCPE* expression was detected in embryogenic masses and somatic embryos growing either on medium with (Figs. 3, 4 and 5) or without PGR (Fig. 2) indicating that gene expression is associated with SE and not only related to the presence of these PGR. Furthermore, the lack of a hybridization signal in cotyledonary embryos after culture in maturation medium (without PGR) also suggests that *QrCPE* may participate in embryo histodifferentiation, prior to the maturation stage. However, a negative regulation of the gene by sorbitol cannot be ruled out.

GRPs are thought to interact with other proteins and/or molecules playing a role in cell-cell signalling through these interactions (Fusaro and Sachetto-Martins 2007). The involvement of GRPs in signalling events was proposed by Park et al. (2001) who suggested that the interaction of WAK1 with AtGRP3 regulates the function of WAK1 in the plant-pathogen interaction responses. The possible role of GRPs in plant-microbe interactions and plant defence mechanisms has also been proposed in other studies (Mousavi and Hotta 2005). Two nodule-specific GRPs from *Alnus glutinosa*, which showed high degree of similarity with the mature QrCPE protein (Gil et al. 2003), were specifically expressed in the zone of root nodules infected with *Frankia* hyphae (Pawlowski et al. 1997). Furthermore, the grapevine homologue of the QrCPE protein was suggested to have an important function in the defence response against pathogen attack (Perazzoli et al. 2010). Therefore, in addition to the *QrCPE* function during SE, a possible role for the gene in plant defence cannot be ruled out. Several pathogenesis-related proteins and genes with roles in the defence response are also expressed during embryogenesis, suggesting that they may play a role in both plant development and defence (Helleboid et al. 2000a, 2000b).

The high levels of *QrCPE* expression in embryogenic cell aggregates and embryogenic nodular structures induced in initial leaf explants, and the lack of signal in parenchymatic cells of callus surrounding these aggregates (Fig. 2) also indicates that transcription is associated with the first stages of somatic embryos and that the gene may be involved in somatic embryo induction. Several genes encoding GRPs have been associated with early embryogenic development *in vitro* (Sato et al. 1995, Magioli et al. 2001). The predicted QrCPE protein also exhibits significant amino acid sequence identity with two GRPs encoded by cDNAs, which were isolated from embryogenic cell suspensions of *Daucus carota* L. (Aleith and Ritcher 1990). Gil et al. (2013)

suggested that the higher expression of *QrCPE* in mature microshoots than in juvenile microshoots, could be related to the low morphogenetic capacity (rooting ability) of crown-derived mature shoots. However no evidence was presented for gene expression or mRNA localization in specific cells during the rooting process. Moreover, different signaling pathways can be involved in the two processes, SE and adventitious rooting.

Independently of the origin of somatic embryos (primary or secondary embryos), at early stages of embryo formation, the *QrCPE* antisense probe produced a strong signal in all of the actively dividing embryogenic cells (Figs. 2A and B, 3A and C). In embryos with differentiated protodermis the strongest signal was detected in this tissue and in dividing protodermal cells (Figs. 2D, 3 and 4) from which new somatic embryos can be formed directly by secondary embryogenesis (Figs. 3E, 4C), indicating that *QrCPE* participates in the initiation of somatic embryos and also appears to be associated with the maintenance of embryogenic competence. The protodermis is a tissue with high embryogenic ability, as shown during the formation of somatic embryos via a direct pattern of development (Kurczyńska et al. 2007, Vieitez et al. 2012). The *AtGRP5* promoter was also found to be very active in protodermal derived tissues in *Arabidopsis* transgenic plants (Sachetto-Martins et al. 1995) and in specific peripheral areas of embryogenic callus tissues originated during secondary SE (Magioli et al. 2001).

The spatial distribution of *QrCPE* changes during embryo development, from uniform expression in proembryogenic aggregates and early globular embryos to specific localization in areas undergoing cell divisions at different stages of embryo development, which indicates that *QrCPE* expression is associated with actively dividing cells that appear to be in a meristematic condition. Likewise, the preferential

expression of the *AtGRP2* in tissues undergoing cell division (embryos, root tip) has also been reported (Fusaro et al. 2007).

QrCPE transcripts were also abundant at the root tip region, especially in the cell division zone and root cap (Fig. 6). Similarly, it has been suggested that three maize GRP genes that display tissue- specific patterns restricted to the root tips participate through a cooperative action in the development of the maize root tip (Woll et al. 2006). Although *QrCPE* is not a root-specific gene, the localized expression of *QrCPE* in the putative initial cells of the root cap (located below the quiescent centre) of cotyledonary embryos, also suggests a role for *QrCPE* in specification of the fate of root cap cells.

In conclusion, the results show that the expression of *QrCPE* is associated with the initiation of somatic embryos both during the process of embryo induction from the original leaf explants, and during secondary embryogenesis, which suggests that the gene plays a role in the embryogenic competence. The *QrCPE* expression pattern changes during embryo transition phases in a developmentally regulated manner, which indicates that the gene is involved in embryo development. Further studies on responses to different stresses, pathogen infection, protein localization and functional analysis by genetic transformation will help clarify the gene functions.

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Data Archiving Statement

The *QrCPE* cDNA sequence was deposited in the European Molecular Biology Laboratory (EMBL) database, Accession number AJ271778.

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Fig. 1 Expression pattern of *QrCPE* in oak somatic embryos and in different organs of plantlets derived from somatic embryos of the B-17 (A) and Sainza (B) embryogenic lines. RNA gel blot analysis of *QrCPE* mRNA levels in samples of non embryogenic callus (nec) and globular-(g), torpedo-(t), cotyledonary- (c) stage embryos, cotyledonary embryos matured in sorbitol containing medium (cm), and from hypocotyl (h), root (r) and shoot (s) tissues excised from germinated somatic embryos. The gels at the bottom show ethidium bromide-stained rRNA as a loading control.

Fig. 2 Pattern of *QrCPE* mRNA accumulation during the induction of SE process in oak. Sections were hybridized with the antisense (A, B, D, E, F) or sense (C) *QrCPE*-specific probes. (A, B) callus tissue (c) developed on leaf explants with the presence of embryogenic cell aggregates (arrows) and proembryos (arrowheads) formed in peripheral layers of the callus. Strong expression is observed in the embryogenic structures. (C) Control section of A, B. (D) A oblong-heart shaped embryo attached to callus tissue showing expression in the protodermis and outer layers. Note the absence of expression in the callus tissue (c). (E) Cotyledonary shaped embryo showing strong expression in the distal cell layers of the root meristem that will give rise to the root cap. The high expression in these layers can be seen in the inset (bar represents 100 µm). (F) In a further tangential section of the embryo shown in E, *QrCPE* expression is localized in the sam and axillary meristem (arrows).

Fig. 3 In situ localization of *QrCPE* mRNA on sections of somatic embryos proliferated by secondary embryogenesis of Sainza (A-F) and B-17 (G-H) embryogenic lines. The sections were hybridized with antisense (A,C, D, E, F, G) or sense (B, H) *QrCPE*-specific probes. (A-B) Preglobular embryos. Note the absence of hybridization in the control (C). Early globular embryos showing cell division generating the protodermis (inset C). Bar in inset represents 50 µm. (D, E, G, H) Late globular embryos with a clearly differentiated protoderm (pd). Arrow in E indicates a proembryogenic structure. (F) Transition from globular to oblong stage showing polarized meristematic areas (arrows).

Fig. 4 In situ localization of *QrCPE* mRNA on longitudinal sections of somatic embryos from Sainza (A, B, C, E and G) and B-17 (D, F, H) genotypes. Sections were hybridized with antisense (A, C, D, E, F,G) or sense (B,H) *QrCPE*-specific probes. (A-D) Somatic embryos at the heart-(A, B) and torpedo- stages (C, D) showing preferential transcript accumulation at the protodermis (pd) and outer cortical layers. A strong signal was observed in the globular embryo originated by secondary embryogenesis on the hypocotyl of the torpedo stage embryo (C). (E, F, G, H) Somatic embryos at the cotyledonary stage showing strong signal in the shoot apical meristem (sam), axillary region of cotyledons (arrows in G) and root pole (rp). Note the absence of hybridization in the controls (B, H).

Fig. 5 Localization of *QrCPE* mRNA in longitudinal sections of late cotyledonary stage somatic embryos of the Sainza (A-D) and B-17 (E-J) lines. Sections were hybridized with the antisense (A, B, E, F, G, H) or sense (C, D, I, J) *QrCPE*-specific probes. (A, E) In late cotyledonary embryos, *QrCPE* expression was detected in the root cap (rc), shoot apical meristem (sam) and procambium (pc). (F) A further tangential section of the embryo shown in E, evidencing the signal in sam and root pole (rp). (B, D, G) High magnification of sam of embryos shown in A, C and E, respectively. (H) High magnification of the root pole (rp) of the embryo shown in F. No signal was observed in the sam (D, I) and the rp (J) hybridized with the sense probe.

Fig. 6 Pattern of *QrCPE* mRNA accumulation in the primary root originated from germinated somatic embryos of the Sainza genotype. Sections were hybridized with the antisense (A, B, C, E, F, H, J) or sense (D, G, I, K) *QrCPE*-specific probes. (A, E) Longitudinal sections of the primary root including the elongation zone (ez), cell division zone (cdz) and the root cap (rc). (B) No signal was observed in the mature region of the root. (C, D) High magnification of the root tip shown in A. (F, H, J)

Transverse sections of the root zones indicated in E, showing an increasing signal from the elongation zone towards to the root tip. cor, cortex; col, columella; gm, ground meristem; lrc, lateral root cap; pc, procambium; qc, quiescent centre; ram, root apical meristem; vc, vascular cylinder.