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ORIGINAL PAPER

Expression analysis of epigenetic and abscisic acid-related genes during maturation of *Quercus suber* somatic embryos

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Abstract Clonal propagation of *Quercus suber* via somatic embryogenesis is an alternative to conventional tree propagation methods; however, complete maturation of somatic embryos is considered the major bottleneck for mass propagation of *Quercus* species. During somatic embryogenesis, embryo development and maturation are controlled by signaling pathways that integrate information from genetic and epigenetic programs as well as hormonal signals. Therefore, in this study genes were identified related to epigenetic regulation and the abscisic acid (ABA) pathway during development and maturation of cork oak somatic embryos. A total of eight expressed sequence tags were obtained of genes encoding a 9-*cis*-epoxycarotenoid dioxygenase (NCED), two histone deacetylases (HDA6 and HDA19), two histone mono-ubiquitinases (HUB1 and HUB2), a histone H3 kinase (AUR3) as well as genes related to chromatin remodeling processes *PICKLE* and *VP1/ABSCISIC ACID INSENSITIVE 3-LIKE 1* (*VAL1*). The analysis of the expression patterns of selected genes during different developmental stages indicated that *QsNCED3* may play a role in ABA synthesis during embryogenesis. The change in the expression levels for all seven genes associated with epigenetic regulation showed that *QsHUB1* and *QsHUB2* may have a role in ABA signalling while *QsHDA6* and *QsHDA19* could act in different pathways than in Arabidopsis. Furthermore, expression levels of *QsAUR3* indicated that histone phosphorylation is an early epigenetic mark in *Q. suber* somatic embryos while *QsPICKLE* and *QsVAL1* may be necessary for the correct development of cork oak somatic embryos.

Keywords ABA · Epigenetic regulation · Gene expression · Maturation · Somatic embryogenesis

Introduction

Quercus spp. forests are valuable areas for the conservation of soil, climate and biodiversity; moreover, cork oak has a high economical and ecological impact in the Mediterranean area, contributing to the rural development in its geographical distribution range. The restricted option for classical reproduction via breeding or vegetative reproduction of *Quercus suber* trees are serious drawbacks for its conservation. Cork oak seeds are considered recalcitrant, which does not allow conventional conservation through dry storage. Additionally, successful vegetative propagation is limited because it is very difficult to root cuttings of adult trees (Vieitez et al. 2012). Important progress has been made in clonal propagation of *Q. suber* with somatic embryogenesis; a powerful technique used for large-scale propagation of selected material that is considered to be the most adequate tool for in vitro regeneration of woody species (Vieitez et al. 2012). Moreover, in vitro cell and tissue-based systems are

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valuable tools for fundamental research as well as commercial applications like genetic engineering and clonal propagation (Neelakandan and Wang 2012). However, an in-depth study of the molecular and cellular mechanisms that take place during the development of somatic embryos should be performed, especially during the embryo maturation phase that has been assessed as one of the major bottlenecks of this technique in cork oak species (Wilhem 2000; Vieitez et al. 2012). During somatic embryogenesis, embryo development and maturation are regulated by a complex signalling network that integrates genetic and epigenetic programs as well as hormonal and metabolic signals (Gutierrez et al. 2007; Gao et al. 2012). Abscisic acid (ABA) plays a key role in many developmental processes and more specifically endogenous ABA levels are correlated with the correct development and maturation of seeds and somatic embryos (Rai et al. 2011; Pérez et al. 2015). ABA forms a master regulator of the synthesis and deposition of storage proteins during somatic embryogenesis, as well as prevents precocious germination in maturing embryos. A key regulation step in the ABA biosynthesis pathway is the cleavage of 9-*cis* xanthophylls to xanthoxin that is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (*NCED*), a member of a large gene family (Seo and Koshiba 2002). Moreover, their activity is considered the rate-limiting step in ABA biosynthesis (Rodríguez-Gacio et al. 2009). It has also been reviewed that ABA controls the expression of many genes related to these processes (Suzuki and McCarty 2008; Rai et al. 2011) and that epigenetic regulation plays an important role in ABA-mediated processes (Chinnusamy et al. 2008). During embryogenesis, the embryo undergoes a complex series of morphological and cellular changes that require dedifferentiation and reprogramming of the cells in order to develop the embryo developmental program (Zhang and Ogas 2009). Cell differentiation and development are controlled by temporal and spatial activation and silencing of specific genes. These events are accompanied by complex modifications of chromosomal components and nuclear structures, including covalent modifications of DNA. These modifications of the chromatin are DNA methylation and posttranslational modifications (PTMs) of histones and are known as epigenetic marks, which are heritable and may be partly related to specific physiological states and stages (Lauria and Rossi 2011; Viejo et al. 2012). Posttranslational modifications of histones like acetylation, ubiquitination or phosphorylation play a major role in the epigenetic regulation of gene expression (Lauria and Rossi 2011) and have been related to the regulation of embryo development (Gao et al. 2012). Deacetylation of histone N-terminal tails by histone deacetylases (HDACs) has been correlated with gene silencing and chromatin condensation and the expression of two HDACs (HDA6 and HDA19) has been related to the regulation of genes expressed during embryogenesis in *Arabidopsis* seeds (Kim et al. 2012). In contrast, monoubiquitination of histone H2B has been associated with activation of gene transcription. *HISTONE MONOUBIQUITINATION 1* (*HUB1*) and its homologous *HUB2* act as E3 ligases responsible for monoubiquitination of histone H2B and chromatin remodeling in *Arabidopsis* seed development (Liu et al. 2007), and a possible role in the regulation of ABA levels has also been recorded (Chinnusamy et al. 2008). Histone phosphorylation has also been associated with active transcription (Kouzarides 2007). In *Arabidopsis thaliana*, three Aurora kinases (*AUR*) have been described to belong to the serine/threonine protein kinase family, acting as key regulators during mitosis by phosphorylating histone H3 at Ser 10 (Kawabe et al. 2005; Van Damme et al. 2011). During seed development ABA levels play a pivotal role in the expression of transcription factors essential for development-specific gene expression (Chinnusamy et al. 2008). The *PICKLE* and *VP1/ABSCISIC ACID INSENSITIVE 3-LIKE 1* (*VAL1*) genes are suggested to regulate the repression of seed transcriptional program after the completion of seed maturation via chromatin-remodeling processes (Suzuki et al. 2007; Zhou et al. 2013). PICKLE is a CHD3 chromatin remodelling factor that represses embryogenic traits after germination and it is thought to promote the repressive epigenetic mark H3K27me3 (Zhang and Ogas 2009; Zhou et al. 2013) while *VAL* genes (*VAL1*, *VAL2* and *VAL3*) belong to the LAV family and encode a CW domain and a putative plant homeodomain (PHD)-like zinc (Zn)-finger domain, frequently found in chromatin factors (Suzuki et al. 2007). Despite the importance of comprehensive knowledge of the molecular basis of the maturation program in *Q. suber* somatic embryos that has been assessed as one of the major bottlenecks of this technique in cork oak species (Wilhem 2000; Vieitez et al. 2012), only a few studies related to the genetic regulation of this process have been conducted in *Quercus* spp. (Puigderrajols et al. 2002; Šunderlíková and Wilhem 2002; Šunderlíková et al. 2009a, b). Differences in gene expression during somatic embryogenesis have not yet been reported. Therefore, the objective of the present study was to identify genes related to epigenetic processes and ABA network regulation involved in the early embryogenesis and maturation of *Q. suber* somatic embryos, and to describe their expression patterns.

Materials and methods

Plant material

Somatic embryogenesis procedure was accomplished following previous reports (Bueno et al. 2000; Pérez et al. 2015). Embryogenic cultures were initiated from a pool of immature embryos of *Q. suber* L. collected during the period

of fruit development according to Bueno et al. (2000). Once somatic embryogenesis was inducted, embryogenic lines were transferred to a medium supplemented with 3.4 mM glutamine and maintained in a proliferating phase by secondary embryogenesis and monthly subculture. Before each subculture, somatic embryos at a cotyledonary stage and without signs of recurrent embryogenesis were matured according to Pérez et al. (2015). Somatic embryos were cultured on a medium supplemented with 1 % activated charcoal for 1 month in darkness at 25 °C followed by 60 days in darkness at 4 °C. After that, in order to test if somatic embryogenesis was successfully accomplished; mature somatic embryos were germinated on a medium supplemented with 0.2 μM 6-Benzylaminopurine (BAP) and 0.5 μM indole-3-butyric acid (IBA) at a temperature of 25 ± 2 °C under lighting conditions (cool white fluorescent tubes 31, 36 watts providing a photosynthetic active radiation of 40 μmol m^{-2} s^{-1}, with 16 h photoperiod). All media were adjusted to pH 5.6 ± 0.1 and sterilized for 20 min at pressure conditions of 1 kg cm^{-2} at 120 °C. Glutamine was added to the medium after autoclaving by filter sterilization (0.22 μm). Semisolid cultures were accomplished on 20 mL of medium in Petri plates (9 cm in diameter).

Sample material

Five developmental stages were defined during somatic embryogenesis procedure as it was described in Pérez et al. (2015) and used as sample material (Online resource 1). In the proliferation phase three different types of samples were collected: immature translucent cotyledonary embryos (E1 stage); white opaque cotyledonary embryos (E2) and proliferative stages (PS) composed of white callogenic structures with globular embryos. E3 type somatic embryos (1–2 cm length) were collected after culturing cotyledonary somatic embryos onto maturation medium for 1 month in darkness at 25 °C. Subsequently, E3 embryos were stratified at 4 °C for 2 month in darkness and developed into E4 somatic embryos with bigger cotyledons and a longer embryonic axis.

RNA isolation and cDNA synthesis

Total RNA was isolated from 100 mg of frozen tissues on different developmental stages using NucleoSpin® RNA Plant (Macherey–Nagel) according to the manufacturer's instructions. RAP buffer with 1 % β-mercaptoethanol was used and DNA contaminants were removed with DNase (Macherey–Nagel) and RNA was eluted in 60 μL of RNase free-H2O. RNA concentration and quality was estimated using a Picodrop Microliter UV/Vis Spectrophotometer (Picodrop, UK) and by horizontal electrophoresis in agarose gels (1.4 % and 0.5 μg mL^{-1} ethidium bromide), using lambda DNA (New England Biolabs) as marker. The 28S/18S ratio was also determined with the KODAK 1 D Image Analysis software (KODAK) to verify high RNA integrity. For each sample, single-stranded cDNA was synthesized from 1 μg of total RNA with the RevertAidTM First Strand cDNA Synthesis Kit (Thermo Scientific) according to the supplier protocol and using 1 μL of oligo (dT) 18 primer. Samples were incubated at 25 °C for 10 min, 60 min at 37 °C, 60 min at 42 °C and a final step of 10 min at 70 °C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems).

Isolation of cork oak partial gene sequences

To isolate partial sequences from cork oak genes, degenerated primers were designed using the DNASTAR software package PrimerSelect (Lasergene). Genes were selected based on the existing bibliography related to embryo development and maturation as well as abscisic acid signaling (Chinnusamy et al. 2008; Gao et al. 2012). Coding sequences from model species were downloaded from the available databases of the National Center for Biotechnology (NCBI; http://www.ncbi.nlm.nih.gov/) and The *Arabidopsis* Information Resource (TAIR; http://www.Arabidopsis.org/) (Online resource 2) and sequence comparison was performed using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the MegAlign (Lasergene, DNASTAR) software. Degenerated primers were designed for the conserved domains of the target genes and PCR conditions were optimized for each primer pair (Table 1). PCR reactions were performed in 20 μL of final volume containing 1 μL of cDNA, reaction buffer, 0.2 mM dNTP, 0.25 μM of forward and reverse primer and 0.2 U of Taq DNA polymerase (Roche). DNA amplifications were performed as follows: initial denaturing at 94 °C for 2 min, an optimum number of cycles of denaturing at 94 °C for 30 s, annealing temperature for 30 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Obtained amplicons were ligated into pGEM-T Easy and transformed to JM109 high efficiency competent cells (Promega, UK). Transformed cells were plated onto LB medium plates supplemented with ampicillin. Plasmids were further isolated with the High Pure Plasmid Isolation Kit (Roche).

DNA sequencing and sequence analysis

Sequencing was performed at the DNA Synthesis and Sequencing Facility; Macrogen (Amsterdam, The Netherlands) on an ABI 3700 automated sequencer (Perkin-Elmer, Foster City, CA, USA). EST homologies were analysed with BLASTN and BLASTX, querying the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). To confirm that isolated ESTs were homologous, translated amino acid

Table 1 Degenerate primers sequences, annealing temperature and $MgCl_2$ concentrations used for the isolation of cork oak gene sequences

Gene	Forward primer (5′3′)	Reverse primer (5′3′)	AT (°C)	PL (bp)	$MgCl_2$ (mM)
NCED3[a]	TGACCCCACCAGATTCCATHT	TGGAGTCTATGAAAGTGCCAT	52	500	4.5
HUB1	GAGARCSWGGWAGAAAA	AGCCCWWGCCTYRTATTC	48	380	8.25
HUB2	ATTGGYCAAGCWTATGAAGAT	TATTGCAGCCKCMCCASTYTC	46 30 s–53 30 s	513	1.5
ATAUR3	TCAYIGIGAYATMAAGCCWGAIA	GATRTGCATTCTTGATTATCCA	51	410	6
HDA19	GGGCTGGTGGNCTBCATC	CACCAGCARCGVGCRACRT	52	535	6
HDA6	ATCGGHRASTACTAYTAYGGYCAA	ATCKCCATGGTGGACATC	51	495	3.75
PICKLE	ATHCGDCCTGARTGGACHACTG	GKCCCATYTCATCMGCAAGKAT	50	403	1.5
VAL1	TTCAGATWTTGGCCCAAYAAYAA	TTCGCCATTTGGARCAATYATCAC	54	643	1.5

PL product length, *AT* annealing temperature

[a] Degenerated primers kindly provided by Victor Granda

sequences were aligned with truncated homologous sequences from *Arabidopsis* and other species with Clustal W2. Each EST was evaluated by scoring the chance of random alignment with a database entry [E(xpect) Value], and the highest percentage identity for a set of aligned segments to the same subject sequence [Max(imum) Identity]. Moreover, amino acid sequences were also aligned with MUSCLE (Edgar 2004) and grouped into phylogenetic trees using MEGA 5.2 software (Tamura et al. 2011).

Real time RT-PCR

Gene-specific primers were designed with Primer 3 web 4.0.0 (Rozen and Skaletsky 2000) (Table 2). Real time polymerase chain reactions were performed using a 7900 HT Fast Real-Time PCR system (Applied Biosystems) in 10 µL of final volume with 1× Fast SYBR® Green Master Mix (Applied Biosystems), 0.2 µM of each gene-specific primer and 1 µL of 15-fold diluted cDNA. For negative controls, 1 µL of water was used instead of cDNA. The cycling conditions comprised an initial 20 s polymerase activation at 95 °C followed by 50 cycles at 95 °C for 1 s and 60 °C for 20 s; this was followed by a final incubation of 15 s at 95 °C, 15 s at 60 °C and 15 s at 95 °C. Three biological replicates of each developmental stage and two technical replicates per biological replicate were analysed. PCR efficiency was tested using a standard curve for each gene with LinReg PCR 11.0 software (Ruijter et al. 2009). Analysis of dissociation curves was performed to check gene-specific amplification with the SDS 2.3 software (Applied Biosystems) and agarose gel electrophoresis (1.4 % and 0.5 µg mL^{-1} ethidium bromide) of the PCR products was used to verify amplicon size. To verify PCR products homology, amplicons of each gene were isolated and sequenced on an ABI 3700 automated sequencer (Perkin-Elmer, Foster City, CA, USA) and homologies were analysed with BLASTn, querying the NCBI database.

To select a constitutive gene as endogenous control for mRNA quantification, seven different genes were tested (Table 2) based on the existing bibliography (Soler et al. 2008; Chaves et al. 2011; Marum et al. 2012). The evaluation of their constitutive expression was performed with the geNorm software (Vandesompele et al. 2002; Marum et al. 2012) to select the two most stable genes. Subsequently the reference gene for normalizing data was selected based on the parameters of highest stability (lower standard deviation) and highest abundance (lower Ct) according to Soler et al. (2008). For each target gene, relative expression values were calculated and expressed as fold-change using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), normalized for the selected housekeeping gene and expressed relative to stage PS.

Statistical analysis

Changes in gene expression among developmental stages were analysed by analysis of variance (ANOVA) and post hoc Duncan test using SPSS software (SPSS Inc., USA). Spearman rank correlation was also applied for the comparison of *A. thaliana* and *Q. suber* gene expression levels. The level of significance was 0.05 in all the cases.

Results

Isolation of cork oak partial gene sequences

The use of degenerated primers designed for the conserved sequences of *NCED3*, *HUB1*, *HUB2*, *AUR3*, *HDA6*, *HDA19*, *PICKLE* and *VAL1* genes generated cDNA fragments in *Q. suber* samples. BLAST analysis of the nucleotide sequences allowed the identification of the ESTs (Online resource 3). Moreover, predicted amino acid sequences were aligned with homologous sequences of *A. thaliana* and other species

Table 2 List of primers used for real-time RT-PCR

Gene	GeneBank accession no	Forward primer (5′3′)	Reverse primer (5′3′)	PL (bp)
NCED3	JZ719306	ACAGGCTTGGAAGGAAATCCC	AAGGCTCACCACCAAACCTC	144
HUB1	JZ719307	TGGAATTCTGGAGAGGAAGG	AATCTTGGACCACAGCTTGG	101
HUB2	JZ719308	GCTGTTTCCAGGTTGACTGC	CTCTGAGAAGCATGCATTGG	165
ATAUR3	JZ719309	GAGGGTCGACTGAAAATTGC	CTTGTTCTCCACCATTTCTGG	111
HDA19	JZ719310	CACCATGGAGATGGTGTGG	TCGCGTATGTCACCTGTACC	107
HDA6	JZ719311	CCTCGCCGACGTTAAACCTCTTG	CGGCCAACCCCTCCGACAT	129
PICKLE	JZ719312	AGGTGGTGACGATGAGAAGG	TCTTTAGGGATGCTTTTCTGC	177
VAL1	JZ719313	TTAGAGGGTGTAACCCCTTGC	AAACCCCATCACAAGTTTGC	102
**18S rRNA*[a]	EE743696.1	TGATGTATTCAACGAGTTTATAGC	CGGGCAGGTACAAAGGG	168
**PSKR*[b]	–	GGGCCTTGGAGATTGTTGTAAC	TCGACAAGGTGGACCAATCTTA	150
**TUB*[c]	EE743717	AAGAACATGATGTGCGCTGCT	TCCACCTCCTTGGTGCTCA	100
**EF1*[a]	EE743684.1	ACAGCCTTATAGCCAGCAG	GGTTGAAGAGGAGGACATATTG	141
**EF2*[c]	ID2007208	TTGTGCCGTCCTCATTATTGACT	TCACGGGTCTGACCATCCTT	120
**ACTIN*[b]	–	GCCCCACGAGCTGTGTTC	TCTGGCCCATTCCAACCA	110
**HMG-CoA*[a]	EE743693.1	GCAAGCGAGTGATACTGTTC	TCCATACCTGTGCTCCATTAG	200

PL product length, *PSKR* phytosulfokine receptor, *TUB* tubulin, *EF* elongation factor, *HMG* HMG CoA synthase

* Genes used for the evaluation of endogenous control

[a] From Chaves et al. 2011

[b] From Soler et al. 2008

[c] From Marum et al. 2012

to confirm that isolated ESTs were homologous (Fig. 1). The results indicated that the *Q. suber* EST had a query coverage percentage over 70 % in all the cases (Online resource 3). *NCED3* is a gene from the ABA biosynthesis pathway and belongs to the large 9-*cis*-epoxycarotenoid dioxygenase gene family with great similarity between the members; nevertheless, BLAST results as well as amino acid alignments (Fig. 1; Online resource 3) indicated that the sequence obtained from *Q. suber* samples was similar to *NCED3*. Moreover, the entire EST belonged to the conserved domain 9-*cis*-epoxycarotenoid dioxigenase NCED. A good bootstrap value was also observed in the phylogenetic tree (Fig. 2). Putative Histone monoubiquitination (*QsHUB1* and *QsHUB2*) also provided the best score with the nucleotidic sequences *HUB1* and E3 ubiquitin-protein ligase BRE1-like2 (*HUB2*) from *A. thaliana* (Online resource 3). Amino acid alignments showed high similarity, particularly for *HUB2* with a *Castanea sativa* EST (Fig. 1c). Although the ESTs obtained did not belong to a conserved domain, phylogenetic trees constructed showed a good grouping within the species analyzed (Fig. 2b, c). Moreover, *C. sativa* and *Q. suber* sequences group together with a bootstrap value of 100. For *QsAUR3*, the best hits in the BLAST analysis were obtained with *A. thaliana AtAUR3* and the predicted protein serine/threonine-protein kinase Aurora-3-like from *Vitis vinifera*. The protein alignment showed that the cork oak EST belonged to a catalytic domain of the AGC family Protein Serine/Threonine Kinases (Fig. 1d). This domain catalyzes the transfer of the gamma-phosphoryl group from ATP to serine/threonine residues on protein substrates and is related to many cellular processes including growth and differentiation. Phylogenetic analysis showed that all the *AUR3* sequences grouped together (Fig. 2d) and were separated from other members of the gene family (*AUR2* and *AUR1*). Both putative histone deacetylases (*HDA6* and *HDA19*) showed e-values of 3.00e−71 and 2.00e−142 with respect to *A. thaliana HDA6* and *HDA19* respectively. Protein alignments also exhibited high similarity with the sequences analyzed (Fig. 1e, f) and the *Q. suber* EST belonged to the conserved domain HDAC class I that catalyze the hydrolysis of N(6)-acetyl-lysine residues in histone amino termini to yield a deacetylated histone. Members of this class are involved in different processes including embryonic development. Moreover, *HDA6* and *HDA19* regulate the repression of key genes expressed during embryogenesis and the *HDA6/HDA19* double RNAi line showed arrested growth after germination and formation of an embryo-like structure (Kim et al. 2012). As *HDA6* and *HDA19* belong to the same gene family, a phylogenetic tree with truncated homologous sequences of histone deacetylases from the RPDE/HAD 1 histone deacetylase Class I family was produced for more stringent analysis (Fig. 2e). Branch grouping showed that the putative sequence of *Q. suber HDA19* grouped with homologous sequences of other


a
PLN02258,9-cis-epoxycarotenoid dioxygenase NCED,1.90e-42
Qs JZ719306, 3e-23
At NM_112304.2
Pt XM_002316835
Rc XM_002519619
Vv JQ319644
Cc DQ309332.1
Bn HQ260434.1
b
Qs JZ719307, 3e-42
At NM_130060.3
Pt XM_002302474
Rc XM_002511578.1
Vv XM_002274019.2
Gm XM_003517883.1
Mt XM_003622556.1
Qs JZ719307
At NM_130060.3
Pt XM_002302474
Rc XM_002511578.1
Vv XM_002274019.2
Gm XM_003517883.1
Mt XM_003622556.1
c
Qs JZ719308, 2e-66
At NM_001198304.1
Cs HO847187.1
Pt XM_002299092.1
Rc XM_002530823.1
Vv XM_002283381.2
Gm XM_003545557.1
Qs Jz719308
At NM_001198304.1
Cs HO847187.1
Pt XM_002299092.1
Rc XM_002530823.1
Vv XM_002283381.2
Gm XM_003545557.1
d
cd05123, STKc, 4.7e-50
Qs JZ719309, 4e-78
At NM_130111.3
Pt XM_002308997.1
Rc XM_002511696.1
Vv XM_002270426.2
Gm XM_003543887.1
cd05123, STKc
Qs Jz719309
At NM_130111.3
Pt XM_002308997.1
Rc XM_002511696.1
Vv XM_002270426.2
Gm XM_003543887.1
e
cd09991,HDAC classI, 3.23e-129
Qs JZ719310, 6e-113
At NM_119974.3
Pt XM_002328115.1
Rc XM_002531750.1
Vv XM_003633672.1
Br GQ253431.1
Gm XM_003543887.1
cd09991,HDAC classI
Qs JZ719310
At NM_119974.3
Pt XM_002328115.1
Rc XM_002531750.1
Vv XM_003633672.1
Br GQ253431.1
Gm XM_003543887.1
f
cd09991,HDAC classI, 8.45e-108
Qs JZ719311, 1e-91
At NM_125705.
Pt XM_002318625.1
Rc XM_002511291.1
Vv XM_002281281.2
Gm XM_003525508.1
cd09991,HDAC classI
Qs Jz719311
At NM_125705.3
Pt XM_002318625.1
Rc XM_002511291.1
Vv XM_002281281.2
Gm XM_003525508.1

◀**Fig. 1** continued

◀**Fig. 1** Translated amino acid alignments of *Quercus suber NCED3* (**a**), *HUB1* (**b**), *HUB2* (**c**), *AUR3* (**d**), *HDA19* (**e**), *HDA6* (**f**), *PICKLE* (**g**), and *VAL1* (**h**), with truncated homolog sequences from *Arabidopsis* and other species. GeneBank accessions are provided with species names. ClustalW2 was performed for doing the alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Below the alignment *asterisk* indicates identical residues, *colon* indicates conserved substitutions and *dot* indicates semi-conserved substitutions. For alignments with multiple conserved domains, residues in red font match the residue in the conserved domain in the first line of the alignment, while residues in *grey* highlight match the residue in a second conserved domain (Marchler-Bauer et al. 2011). Conserved domain name and sequence similarity of the conserved domain with the *Q. suber* sequence are also provided in the first line. Sequence similarity of *Q. suber* sequence with *A. thaliana* is also provided in *Q. suber* sequence line. Qs, *Quercus suber*; At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*; Vv, *Vitis vinifera*, Rc, *Ricinus communis*; Gm, *Glycine max*; Br, *Brassica rapa*; Mt, *Medicago truncatula*; Cs, *Castanea sativa*; Cc, *Citrus clementina*; Bn, *Brassica napus*; Fv, *Fragaria verna*

sequences with a bootstrap value of 100 and this group was separated from other members of the family. *Quercus suber HDA6* sequences also showed a high bootstrap value (99 %) with the homologous sequences and defined a stable and separated branch in the gene family phylogenetic tree. The putative *PICKLE* EST also showed a good e-value with *A. thaliana* sequences for both nucleotide (Online resource 3) and protein queries (Fig. 1g). In this sequence we found homology to two different conserved domains, a chromatin organization modifier domain which is involved in the binding activity of chromosomal proteins to methylated histone tails and also to a SNF2 family N-terminal domain, related to a variety of processes including transcription regulation. The grouping analysis showed that there is a differential grouping between *PICKLE* type genes and the related *PKR2*, a member of the same gene family (Fig. 2f). The *QsVAL1* EST showed an 80 % query coverage and the best e-value with the *A. thaliana* sequence; the protein alignment (Fig. 1h) provided evidence that the sequence has homology to a B3 DNA binding domain and also with a CW-type Zinc Finger that plays a role in eukaryotic processes including chromatin methylation status and early embryonic development. The phylogenetic tree showed that genes described as *VAL1* grouped together with a bootstrap of 94 (Fig. 2g) and cluster separately from other members of the *VAL* subgroup (*VAL2* and *VAL3*).

Selection of cork oak somatic embryogenesis housekeeping gene

To select a constitutive gene as reference for *Q. suber* somatic embryos, seven genes were selected based on the literature (Soler et al. 2008; Chaves et al. 2011; Marum et al. 2012) and successfully amplified in all cDNA samples from the different stages. Mean PCR amplification efficiency for each primer pair was between 1.8 and 2.0 (Online resource 4) for all the analysed situations and coefficient of determination (R^2) were within the range of 0.995–0.999 (data not shown) indicating the accurately amplification of primers (Ramakers et al. 2003). The stable expression of the different genes was analyzed (Fig. 3)

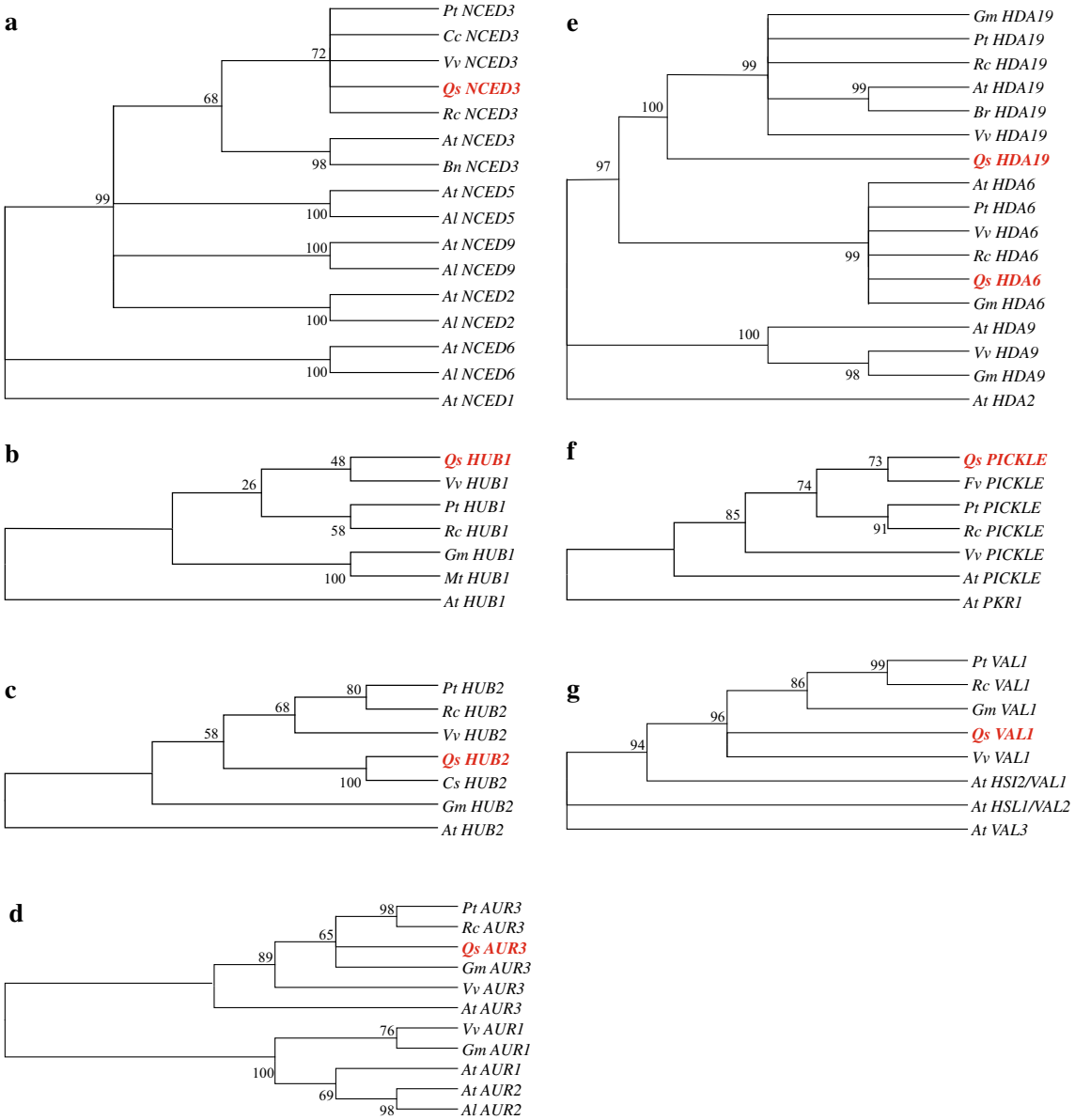

Fig. 2 Phylogenetic analysis of protein alignments of *Quercus suber NCED3* (**a**), *HUB1* (**b**), *HUB2* (**c**), *AUR3* (**d**), *HDA19* and *HDA6* (**e**), *PICKLE* (**f**), and *VAL1* (**g**) with truncated sequences of the gene family from Arabidopsis and other species. Neighbour-joining trees were constructed. *Numbers along branches* indicate bootstrap values from neighbour-joining analysis (2000 replicates). Qs, *Quercus suber* (*highlighted*); At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*; Vv, *Vitis vinifera*, Rc, *Ricinus communis*; Gm, *Glycine max*; Br, *Brassica rapa*; Mt, *Medicago truncatula*; Cs, *Castanea sativa*; Al, *Arabidopsis lyrata*; Cc, *Citrus clementina*; Bn, *Brassica napus* (see Online resource 2 for accession numbers)

using GeNorm algorithm and ranked according to their expression stability measure, calculated as the average pairwise variation for that gene with all other tested reference genes (Vandesompele et al. 2002). According to the data obtained (Fig. 3a), *18S* was the most unstable gene while *ACTIN* and *HMG-CoA synthase* (*HMG*) were the most stable genes within the reference genes analyzed in our experimental system. Additionally and similar to results by Soler et al. (2008), *ACTIN* and *HMG* showed the same stability value (1.1), Since *HMG* had the lowest standard deviation (highest stability) and a low Ct value (high transcript abundance) (Fig. 3b), *HMG* was selected as the reference gene for further normalization of data.

Gene expression during cork oak somatic embryos maturation

In order to monitor the differential expression of selected genes during maturation of *Q. suber* somatic embryos, real-time RT-PCR analysis was performed. Prior to the data

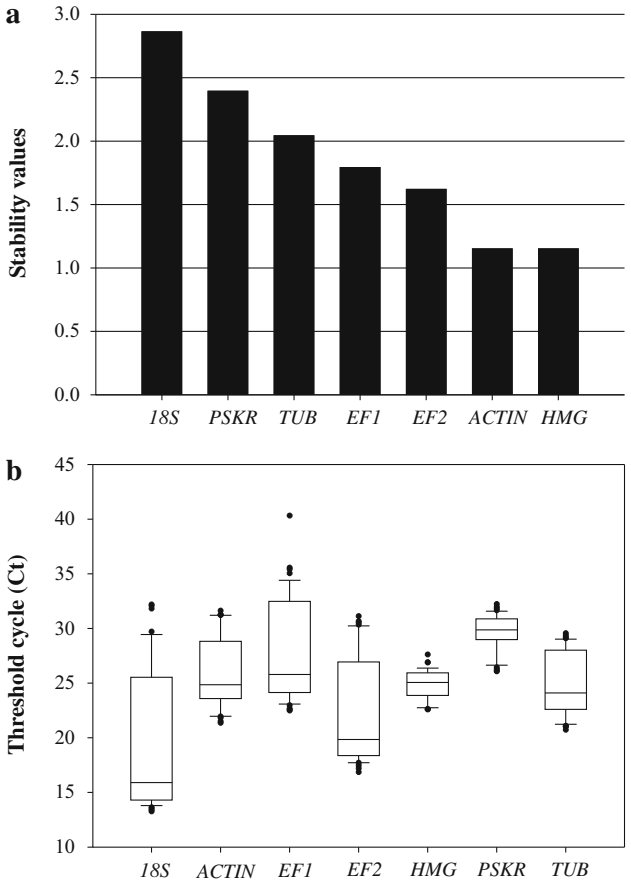

Fig. 3 Stability and constitutive expression of candidate reference genes. Stability values calculated using GeNorm software and all cDNA samples (**a**); *Box* and *whisker plot* of the variation of candidate reference gene expression (**b**). *Each box* indicates the 25/75 percentiles and the *horizontal line* inside *each box* indicates the median. *Whiskers* represent the maximum and minimum values and outliers are indicated by *dots*

analysis, PCR product identity was tested by sequencing and alignment with the ESTs previously obtained for *Q. suber* using Clustal W2 software. In all cases a query coverage of 100 % with *Q. suber* ESTs was achieved (data not shown). All the genes analyzed showed significant differences in transcript abundance between the development stages ($p < 0.05$; Fig. 4). The expression of *QsNCED3* indicated a transient increase from the PS and E1 to the E2 stage and a further gradual decrease recording similar expression levels at the end of the maturation phase as in the proliferative stage. Histone monoubiquitination genes (*QsHUB1* and *QsHUB2*) showed a transient increase after an initial decrease from the PS to the E1 stage reaching the highest values in the E2 embryos and a further decrease at the end of the maturation phase with similar levels observed in the proliferative stage. *QsAUR3* showed an early and a late peak in transcript abundance in stages E1 and E3. The early peak in the E1 stage was in contrast with the expression patterns observed in the rest of the studied genes. *QsHDA19* showed a transient decrease followed by a steady increase from the E2 stage to the E4 stage, with a higher expression level in the E4 stage than in the PS samples. Conversely, a transient decrease in gene expression was observed in *QsHDA6*, *QsPICKLE* and *QsVAL1* from the PS to the end of the maturation phase; while a lower expression level was recorded at the end of the maturation phase than in the proliferative stages. *Quercus suber* gene expression levels were compared with *A. thaliana* embryo expression patterns according to Le et al. (2010; Online resource 5) (also Winter et al. 2007; Bassel et al. 2008), and correlation between the data in the two species was performed. Only cork oak stages E1–E2 (Fig. 4) were suitable to be compared with Arabidopsis at the linear-cotyledon stage and mature-green stage, respectively. Physiologically and morphologically, *Q. suber* E2 somatic embryos resemble the mature green developmental stage in Arabidopsis embryos, while E1 somatic embryos can be compared with the linear-cotyledon stages in Arabidopsis embryos (Le et al. 2010).

An increase was recorded in *AtNCED3*, *AtHUB1* and *AtVAL1* (Online resource 5) gene expression when comparing linear cotyledon embryos with green-mature embryos; an increase in the expression was also observed in *QsNCED3*, *QsHUB1* and *QsVAL1* during development of somatic embryos. Additionally, a slight increase was observed in *AtPICKLE* embryos which positively correlated with the increase obtained in *QsPICKLE* from E1 to E2 somatic embryos ($r_s = 1$; $p \leq 0.01$). An increase was also observed in *QsHDA6* and *QsHUB2* gene expression; conversely, a slight decrease was observed in *AtHDA6* expression while no major differences were recorded for *AtHUB2* between the linear-cotyledon and green-mature stages (Online resource 5 f and c respectively). A negative correlation was also observed when comparing *QsHDA19* and *AtHDA19* ($r_s = -1$; $p \leq 0.01$) with a decrease in the gene expression between the linear cotyledonar and green-mature *Arabidopsis* embryos (Online resource 5e) while no statistical differences were obtained between E1 and E2 somatic embryos. Additionally, the high expression levels of *AtAUR3* observed in the linear cotyledonar embryos (Online resource 5d) resemble the peak of expression recorded in E1 stage of *Q. suber* somatic embryos ($r_s = 1$; $p \leq 0.01$).

Discussion

ABA regulates key events during seed development like the deposition of storage proteins and preventing precocious germination (Kermode 2005). A key step during ABA synthesis pathways in plants is catalysed by 9-*cis*-

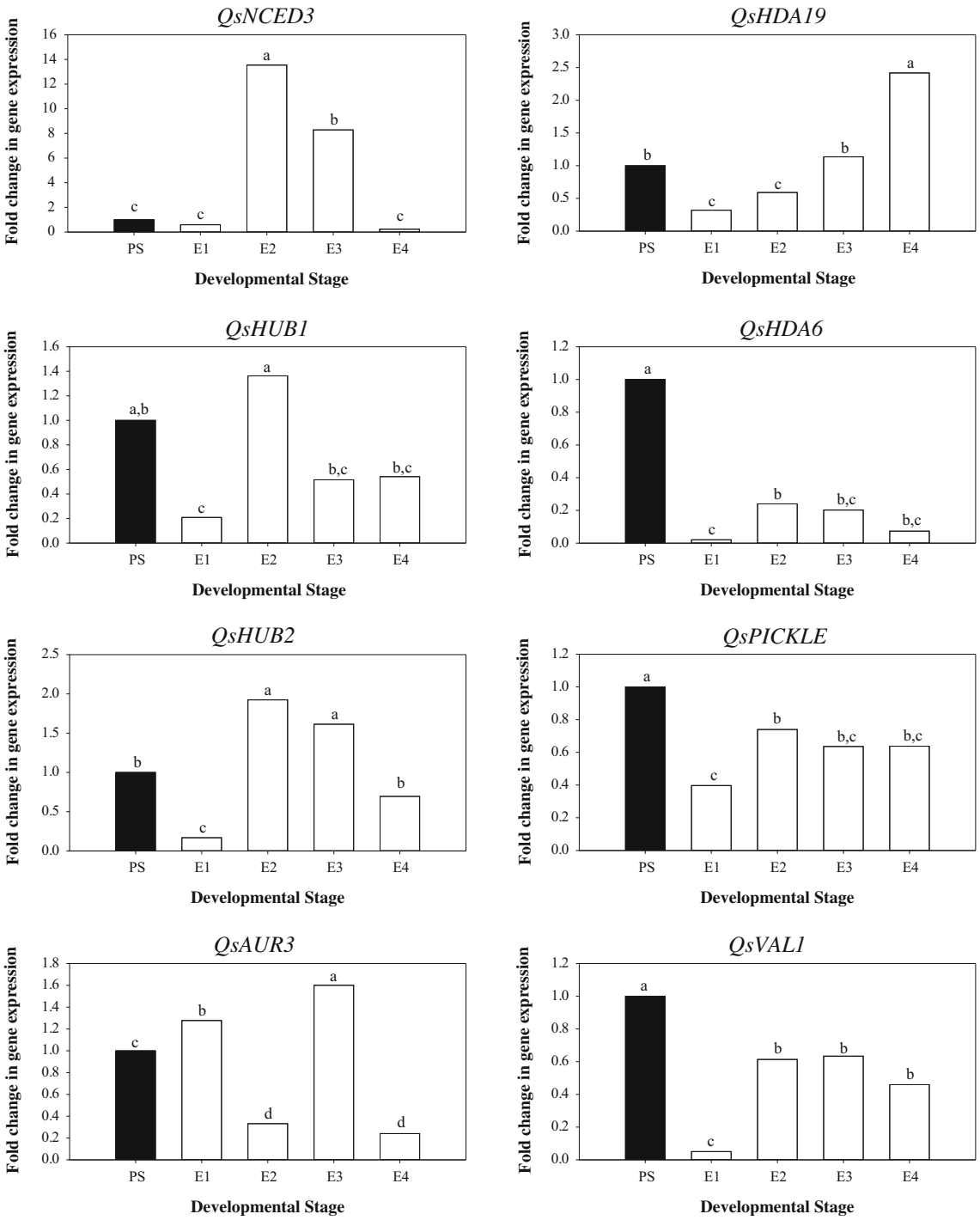

Fig. 4 Relative gene expression of *NCED3*, *HUB1*, *HUB2*, *AUR3*, *HDA19*, *HDA6, PICKLE* and *VAL1* during the maturation stages of cork oak somatic embryos, assessed by real time RT-PCR. Expression is relative to HMG and normalized for PS stage (in *black*). *Different letters* above *bars* significant differences between stages. ($p \leq 0.05$, ANOVA and post hoc Duncan tests)

epoxycarotenoid dioxygenase (*NCED*) genes that have been cloned from several species and conform a gene family that plays distinct roles in the regulation of ABA synthesis during seed development and germination (Seo and Koshiba 2002; Rodríguez-Gacio et al. 2009). In our experimental system, *QsNCED3* showed a differential expression pattern along the developmental stages analysed with a transient increase from the PS to the E2 stage and a further gradual decrease until the E4 stage; a similar pattern was observed for ABA levels (Pérez et al. 2015). A similar expression pattern was reported for *Arabidopsis* zygotic seed development (Tan et al. 2003), with high

levels of transcript in developing siliques and a further increase in maturing siliques. Moreover, concurrent with the increase observed in *QsNCED3* expression levels in the stages E1–E2, gene expression levels during *Arabidopsis* embryo development showed an increase from the linear-cotyledon stage to the green-mature stage (Le et al. 2010) related to embryo growth and storage reserves accumulation. Additionally, high expression levels o *AtNCED3* were observed in cells of the entire root tip in seeds while the signal was absent from the emerging radicle of the growing seedling. Moreover, in ripening avocado fruits the expression of two *NCED* mRNAs (*PaNCED1* and *PaNCED3*) correlated with the ABA endogenous levels (Chernys and Zeevaart 2000) supporting our conclusion that *QsNCED3* plays a role in ABA synthesis during somatic embryogenesis. Gene transcription in eukaryotic cells is controlled through temporal and spatial activation and silencing of specific genes; a specific interaction between the signaling pathways and developmental programs is required and this gene regulation is controlled in part by epigenetic mechanisms (Viejo et al. 2012). Of these, histone modifications play a major role in the epigenetic regulation of gene expression including ubiquitination, phosphorylation and histone acetylation (Lauria and Rossi 2011). Several of the genes studied here are associated with abscisic acid signaling as outlined below. The change in the expression levels observed for all seven genes associated with epigenetic marks studied between the proliferative structures and E1 somatic embryos in *Q. suber* supports the idea that the transition to E1 concurs with major changes in gene expression. Between E1 and E2 embryos a change in gene expression patterns was also recorded in most of the genes associated with epigenetic marks. It has been reviewed that the accumulation of storage proteins during the maturation phase is regulated by specific transcription factors (Zhou et al. 2013) and many of these are related to epigenetic processes (Chinnusamy et al. 2008) supporting our results. Monoubiquitination of histone H2B is associated with actively transcribed genes (Liu et al. 2007). The highest expression of *QsHUB1* and *QsHUB2* was observed in the E2 stage, which was associated with the highest ABA levels previously observed in the same stage in *Q. suber* somatic embryos (Pérez et al. 2015). Those results were in agreement with the model proposed by Chinnusamy et al. (2008) that described an epigenetic regulation of seed maturation by ABA signaling, hypothesizing that *HUB1* and *HUB2* were related to ABA levels and ABA sensitivity in seeds, thus allowing seed maturation. Additionally, an increase in the *AtHUB1* levels was recorded once the maturation processes started (Le et al. 2010) as it occurred from E1 to E2 embryos supporting the hypothesis that E2 embryos are in the onset of the maturation. In addition, high expression levels of both histone monoubiquitination were recorded in the proliferative tissues. It has been proposed that H2B ubiquitination is a fundamental process that affects the expression of many different genes involved in various cellular processes (Liu et al. 2007); moreover, it has been described that *HUB1* affects the cell regulation cycle, more specifically in proliferative tissues (Fleury et al. 2007). Taken together, *QsHUB1* and *QsHUB2* gene expression levels may indicate not only a role in ABA signaling pathway but also a differential epigenetic regulation through chromatin activation during *Q. suber* somatic embryos development and maturation. In contrast with other gene expression patterns, *QsAUR3* showed an early expression peak in the E1 stage. *AtAUR3* encodes a member of a family of Ser/Thr Kinases and it has been described to play a major role in the phosphorylation of serine 10 of histone H3 (Kawabe et al. 2005); moreover, high expression levels were also reported for the maturing embryo (Le et al. 2010), more specifically in the linear-cotyledon embryo which indicates that histone phosphorylation is an early epigenetic mark in cork oak somatic embryos in the E1 stage. Additionally, a late expression peak in transcript abundance was observed in the E3 stage, an actively growing development phase. The three *Aurora* genes described in *Arabidopsis* have a peak expression during cell division and are defined as key regulators of cell division (Demidov et al. 2005; Van Damme et al. 2011). This supports our observed peaks in gene expression in the E1and E3 stages characterized by active growth of the embryo. Moreover, in *Castanea sativa* buds Santamaría et al. (2011) found that an *AtAUR3* orthologue showed higher expression during bud burst with active growth than during bud set. In our experimental system we have described two histone deacetylases (*HDACs*), *QsHDA6* and *QsHDA19*, which are expressed differentially during development of somatic embryos. HDACs catalyze the removal of acetyl groups from acetylated lysine residues in the N-termini of histone proteins that are active epigenetic marks (Kim et al. 2012) and their function is related to transcription repression. These genes have been described as master regulatory genes of the transcription factors *LEC1*, *FUS3* and *ABI3* that are active during seed maturation (Tanaka et al. 2008). The high expression level of *QsHDA6* observed in the proliferative stage could be related with its function as inhibitor of the maturation program in early embryo formation (Kim et al. 2012). Additionally, *HDA6* and *HDA19* have been proposed to play an important role during plant development, embryogenesis and environmental stress response (Chinnusamy and Zhu 2009; Kim et al. 2012). *QsHDA6* and *QsHDA19* showed differences in mRNA transcript abundance between the analyzed developmental stages; however, they have different expression levels with a slight

increase in *QsHDA6* at the beginning of the maturation phase and no major changes along embryos maturation while *QsHDA19* showed a steady increase from E2 to E4 stage, with the higher expression levels at the end of the maturation phase. This differential expression might be caused by the role of *HDA19* on the regulation of the shoot apical pole observed in *Arabidopsis* seeds (Long et al. 2006) by repressing root promoting genes in the top half of the embryo to allow a proper differentiation of the root pole; moreover, elevated expression levels have been recorded in all the tissues that form the shoot apical meristem in *Arabidopsis* seeds (Yadav et al. 2009). Furthermore, as was previously recorded for *QsHUB2*, a differential expression pattern could be observed in *A. thaliana* embryos development (Le et al. 2010). This supports the idea that various different epigenetic regulation mechanisms might occur during the maturation phase in *Q. suber* somatic embryos. The major role described for *PICKLE* and *HSI2* (*VAL1*) in *Arabidopsis* seed development is to act as repressor of the seed developmental program after germination by repressing master regulatory genes of the maturation phase (Tsukagoshi et al. 2007; Tanaka et al. 2008; Zhang and Ogas 2009; Zhou et al. 2013). Nevertheless, *PICKLE* has also been described as a repressor of *LEC1* (a master regulatory gene related to embryo maturation) during embryogenesis and preventing the maturation program in early embryos (Verdier and Thompson 2008) which could explain the higher expression levels recorded in *Quercus* proliferative structures for *QsPICKLE*. The strong decline in *QsVAL1* expression suggests a similar role during this development stage. In addition, *PICKLE* acts as an important factor during cell differentiation and is required for the maintenance of the meristem activity (Aichinger et al. 2011), showing high expression levels in all the tissue layers of the shoot apical meristem (Yadav et al. 2009), while the analysis with double and triple mutants of *VAL* genes indicated that they are required for development and maintenance of a functional apical meristem (Suzuki and McCarty 2008). During *Arabidopsis* seed development, an increase in *AtPICKLE* and *AtVAL1* was shown at the beginning of the maturation phase (Le et al. 2010) resembling the expression pattern recorded in E1–E2 *Q. suber* somatic embryos. Moreover, double knockouts of *HSI2* and *HSL1* (*VAL2*) in *Arabidopsis* seeds showed defects during embryogenesis (Tsukagoshi et al. 2007) indicating that *VAL* genes as well as *PICKLE* are necessary for the proper development of embryos prior to germination.

In summary, current results provide novel information on expression of genes associated with epigenetic regulation of development and maturation of embryos during somatic embryogenesis of *Q. suber*. The differential expression levels observed during the developmental program of *Q. suber* somatic embryos showed that ABA appears to play an important role in this regulation and the expression of *QsNCED3* seems to be a precursor of the ABA biosynthesis pathway during the development. Even though *PICKLE* and *VAL1* have been mainly described during germination processes, our results have shown that both genes also have a role during cork oak somatic embryos development. Additionally, observed changes in expression levels of genes involved in epigenetic control *QsHDA6*, *QsHDA19*, *QsHUB1*, *QsHUB2* and *QsAUR3* indicate that there is a complex epigenetic control of development and maturation of *Q. suber* somatic embryos and that these genes could act differently than in Arabidopsis. Understanding how somatic embryos development and maturation are regulated at the molecular level forms a key first step for further application to cork oak mass propagation. It has been demonstrated that changes in global DNA methylation levels as well as the dynamic ABA endogenous content during somatic embryo maturation play an important role for the correct maturation and further germination in this species (Pérez et al. 2015). Moreover, since cold stratification seems to be necessary for the correct maturation of somatic embryos, the use of ABA inhibitors or demethylating agents during the maturation process might reduce the need for stratification as well as enhance the quality of embryos. In spite of the current results, further studies are required to create a deeper knowledge on the different signaling networks that regulate development and maturation of cork oak somatic embryos.

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Conflict of Interest We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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