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INITIATION OF LEAF SOMATIC EMBRYOGENESIS INVOLVES HIGH PECTIN ESTERIFICATION, AUXIN ACCUMULATION AND DNA DEMETHYLATION IN QUERCUS ALBA

Running head: Early markers of white oak somatic embryogenesis

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ABSTRACT

Somatic embryogenesis is considered a convenient tool for investigating the regulating mechanisms of embryo formation; it is also a feasible system for in vitro regeneration procedures, with many advantages in woody species. Nevertheless, trees have shown recalcitrance to somatic embryogenesis, and its efficiency remains very low in many cases. Consequently, despite the clear potential of somatic embryogenesis in tree breeding programs, its application is limited since factors responsible for embryogenesis initiation have not yet been completely elucidated.

In the present work, we investigated key cellular factors involved in the change of developmental program during leaf somatic embryogenesis initiation of white oak (Quercus alba), aiming to identify early markers of the process. The results revealed that pectin esterification, auxin accumulation and DNA demethylation were induced during embryogenesis initiation and differentially found in embryogenic cells, while they were not present in leaf cells before induction or in non-embryogenic cells after embryogenesis initiation. These three factors constitute early markers of leaf embryogenesis and represent processes that could be interconnected and involved in the regulation of cell reprogramming and embryogenesis initiation.

These findings provide new insights into the mechanisms underlying plant cell reprogramming, totipotency and embryogenic competence acquisition, especially in tree species for which information is scarce, thus opening up the possibility of efficient manipulation of somatic embryogenesis induction.

Keywords:
Auxin, cell reprogramming, cell totipotency, cell wall, DNA methylation, leaf in vitro culture, pectin esterification, somatic embryogenesis, white oak.
INTRODUCTION

To investigate the regulation of plant embryo formation, *in vitro* systems, such as somatic embryogenesis, constitute important tools. These *in vitro* embryogenesis systems are also very useful for biotechnological applications in plant breeding, propagation and conservation strategies (Germanà and Lambardi, 2016). In the cases of forest tree improvement, somatic embryogenesis (SE) is a powerful system whose major applications are large-scale propagation of selected material, genetic transformation and cryopreservation of elite genotypes. In woody species, several reports have dealt with the induction of SE in selected mature trees of economically relevant species, like *Quercus robur*, *Q. suber* and *Q. Ilex* (Blasco et al., 2013; Corredoira et al., 2014; (Barra-Jimenez et al., 2014), *Cyphomandra betacea* (Correia et al., 2011) and *Eucalyptus* sp. (Corredoira et al., 2015). *Q. alba* L. (white oak) and *Q. rubra* L. (red oak) are also of great relevance since they are widely distributed in North America (Steiner, 1993). White oak has had economic importance since colonial times; it was once extensively used in shipbuilding and is currently the major source of wood for cooperage. Induction of SE from leaf explants of shoot cultures derived from 6- to 7-year-old white oak (*Q. alba*) trees has been achieved. In this system, somatic embryos mainly originate from proembryogenic masses (PEMs), which are rounded/nodular structures of cellular aggregates that arise from leaf explants after induction, as the first morphological sign of embryogenic response (Corredoira et al. 2012). PEMs contain embryogenic cells that can give rise to somatic embryos or proliferate and produce more PEMs (Steiner et al., 2016).

Somatic embryogenesis has great potential in tree breeding; however, its application is limited since factors responsible for embryogenesis initiation (Ballester et al., 2016; Bonga, 2017), PEM formation and embryo development are still largely unknown. To date, the availability of structural details on the cellular origin and development of PEMs is scarce, especially from the leaf sections of woody plants. The identification of cellular markers during somatic embryogenesis initiation constitutes an important goal to (i) elucidate the mechanisms involved in totipotency acquisition and embryogenesis initiation to allow their efficient manipulation, and
(ii) to distinguish—after induction—responsive cells from non-responsive cells. Several reports have shown changes in cell activities and in the subcellular organization that occur concomitantly with cell reprogramming, totipotency and embryogenesis initiation in some herbaceous (Testillano et al., 2000; Testillano et al., 2005; Testillano et al., 2002) and woody species (Bueno et al., 2003; Germanà et al., 2011; Ramírez et al., 2004; Solís et al., 2008).

Increasing evidence has indicated the relevance of some cell wall components (Bárány et al., 2010a, b; El-Tantawy et al., 2013; Fortes et al., 2002; Solís et al., 2008), phytohormones (Prem et al., 2012; Rodríguez-Sanz et al., 2015) and epigenetic marks (Arnholdt-Schmitt, 2004; Costa and Shaw, 2007; El-Tantawy et al., 2014; Solís et al., 2012) in the *in vitro* development of organogenesis and embryogenesis in various plant species, but only a few reports have dealt with trees (Solís et al. 2008, Rodríguez-Sanz et al. 2014a). There is no information available about the dynamics of these factors during *in vitro* development in early somatic embryogenesis from leaf tissues of *Q. alba*.

Pectins are important components of primary plant cell walls. They are secreted into the wall as highly methylesterified forms that can be de-esterified *in muro* by pectin methylesterases (Pelloux et al., 2007). The proportion of esterified and non-esterified pectins and their distribution in the cell walls are two factors involved in many plant developmental processes (Dolan et al., 1997; Goldberg et al., 1986; Guillemin et al., 2005). The patterns of pectin esterification can be analysed using JIM5 and JIM7 monoclonal antibodies (Knox, 1997). JIM5 binds preferably to the relatively non-esterified pectin epitopes, whereas JIM7 binds to relatively highly methylesterified pectin epitopes (Clausen et al., 2003). The analysis of antigen distribution by JIM5 and JIM7 antibodies in different plant tissues and organs has revealed that changes in the ratio of esterified to non-esterified pectins, and their distribution in cell walls may influence several developmental processes (Dolan et al., 1997; Goldberg et al., 1986; Hasegawa et al., 2000). The modification of the degree of pectin methylesterification has been reported in young embryos formed *in vitro* from microspores of *Capsicum annuum* (Bárány et
al., 2010a), *Q. suber* (Ramírez et al., 2004), *Citrus clementina* (Ramírez et al., 2003), *Olea europaea* (Solís et al., 2008) and *Brassica napus* (Solís et al., 2016).

Auxin, and its predominant form indole-3-acetic acid (IAA), is a key regulator of plant growth and development (Friml, 2003; Friml et al., 2003). This phytohormone is a plant morphogenetic signal with a major role in the regulation of plant development. Auxin signalling controls cellular processes as important as cell division, expansion and differentiation (Grones and Friml, 2015; Mockaitis and Estelle, 2008). The action of auxin depends on its differential distribution within plant tissues, which is regulated partly by its local biosynthesis but mainly by its directional transport between cells (Petrasek and Friml, 2009). In several *in vitro* embryogenesis systems, there have been reports of stimulating effects of exogenous plant growth regulators, like the synthetic auxin 2,4-D (Bárány et al., 2005; Raghavan, 2004). Nevertheless, little is known about endogenous levels of these regulators at the initial stages of embryogenesis, or the localization of endogenous auxin in *in vitro* embryogenesis systems (Prem et al., 2012; Rodriguez-Sanz et al., 2015), especially in trees. To analyse auxin cellular accumulations during developmental processes, IAA antibodies have proven very useful in various plant species (Forestan et al., 2010; Krouk et al., 2010; Rodriguez-Sanz et al., 2014a; Schlicht et al., 2006).

Cell reprogramming, totipotency and somatic embryogenesis initiation involve changes in the developmental genetic program of the cell, which affects global genome organization; in this sense, epigenetic modifications constitute key factors of genome flexibility and may be involved in these genome organization changes (Arnholdt-Schmitt, 2004). DNA methylation is an epigenetic modification of the chromatin that leads to a transcriptionally inactive conformation and gene silencing. During microspore embryogenesis of *B. napus* and *Hordeum vulgare*, the level and distribution pattern of DNA methylation has been reported to change (El-Tantawy et al., 2014; Solís et al., 2012); these reports suggested the existence of an epigenetic reprogramming after *in vitro* induction of embryogenesis (Rodriguez-Sanz et al., 2014b).
In the present work, in order to characterize early markers of somatic embryogenesis, we have analysed changes in cellular structural organization, pectin esterification in cell walls, endogenous auxin accumulations and DNA methylation during the initiation and early stages of somatic embryogenesis in *Q. alba* leaf tissues, by means of a comparative study between embryogenic and non-embryogenic cells. The findings of this comparative study between embryogenic and non-embryogenic cells have provided new insights into the cellular processes that govern *in vitro* embryogenesis induction in white oak, a woody species.

**MATERIAL AND METHODS**

**Plant material and somatic embryogenesis induction**

Axillary shoot proliferation cultures were used as the source of leaf explants for initiation of somatic embryos. Stock shoot cultures were established *in vitro* from nodal explants excised from forced shoots in branch segments of a 7-year-old *Q. alba* tree designated WOQ-1 following the methodology described by (Vieitez et al., 2009). Induction of somatic embryogenesis was based on the procedure reported to initiate embryogenic systems in *Q. alba*, which consists in culturing leaf explants on embryo induction medium for 8 weeks and subsequent transfer to an expression medium without plant growth regulators for another 12 weeks (Corredoira et al., 2012). Taking into account the objectives of the present study only material cultured on induction medium was used. In brief, the most apical expanding leaves below the shoot apex (node 1) were excised from stock shoot cultures and, then leaves were cultured in induction medium consisting of MS (Murashige and Skoog, 1962) mineral salts and vitamins, 500 mg/L casein hydrolysate, 6 g/L Vitroagar (Pronadisa, Spain), 30 g/L sucrose, 21.48 µM naphthaleneacetic acid and 2.22 µM 6-benzylaminopurine. Ten leaf explants were placed (abaxial side down) in 90-mm Petri dishes containing 25 ml of induction medium. The cultures were incubated in darkness at 25 °C for 8 weeks.
Paraffin and resin embedding for light microscopy analysis

In order to capture the early stages of the embryogenic development only leaf explants (5-6 mm in length) cultured on induction medium (Fig. 1A) were analyzed. Approximately 240 leaf explants were initially cultured to be used in microscopy analysis and detection of somatic embryogenesis markers. Leaf samples were collected after 0, 2, 4, 6, and 8 weeks of culture on induction medium and 5 to 10 representative samples of each stage were processed for paraffin or plastic embedding to perform a microscopy analysis of the cellular structural organization.

Processing for paraffin sectioning

Leaf samples were fixed in FAA solution (formalin, glacial acetic acid and 50% ethanol [1:1:18 (v/v/v)], dehydrated through a graded n-butanol series and embedded in paraffin wax (Merck, Germany). Sections (8 µm) were stained with periodic acid-Schiff (PAS)-naphthol blue-black (Merck, Germany) to detect starch and other insoluble polysaccharides and total proteins, respectively (Feder and Obrien, 1968). Staining with toluidine O (0.05%) was also used.

Processing for semithin resin sectioning

Leaf samples were fixed at 4º C in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.05M sodium phosphate buffer (pH 6.8). After 48 h, samples were rinsed in the same buffer three times (20 min each), dehydrated in a graded aqueous ethanol series (30, 50, 70, 96, 100% 24h at 4ºC each step) and were progressively infiltrated with LR White resin (LRW; London Resin Company, England), according to the following schedule: 25, 50, 75% LRW/ethanol for at least 24 h each step, and two incubations in 100% LRW. Finally, samples were placed in plastic capsules containing 100% LRW and incubated for approximately 14 h at 60ºC. Two-µm thick sections were cut using a Reichert-Jung ultra-microtome (Leica, Heidelberg, Germany). Sections were stained with 0.05% Toluidine Blue (O'Brien and McCully, 1981).
The stained sections (paraffin and resin) were mounted with Eukit®, and photomicrographed with an Olympus DP71 digital camera (Japan) fitted to a Nikon-FXA microscope (Japan). 5 to 10 representative samples of each stage were analyzed.

**Antibodies**

The antibodies used in this study were: JIM7 and JIM5 rat monoclonal antibodies (Plant Probes, Leeds, UK) that recognized highly and low-methyl esterified pectins respectively (Knox, 1997); anti-IAA mouse monoclonal antibody (Sigma, Cat. N. A 0855) (Rodríguez-Sanz et al., 2015); ant-5-methyl-deoxy-cytidine (anti-5mdC) mouse monoclonal antibody (Eurogentec, Cat. N. BIMECY-0100, Liege, Belgium) (Testillano et al., 2013).

**Immuno-dot-blot assay**

The assay was performed essentially as previously described (Bárány et al., 2010a; El-Tantawy et al., 2013), with minor modifications. Extracts were obtained from 60 mg samples collected at culture initiation (0 weeks) and after 8 weeks in culture (16-20 individual explants at 0 weeks, 10-12 samples at 8 weeks, collected from 10-12 Petri dishes of three *in vitro* cultures); they were homogenized in liquid nitrogen and afterwards in 50 ml of buffer solution containing 50 mMTris-HCl pH 7.2, 50 mM trans-1, 2-diaminocyclohexane- N,N,N#N#-tetraacetic acid (CDTA), and 25 mM dithiothreitol and centrifuged at 7000 rpm for 10 min. at 4°C. The supernatants were used for dot-blot after adjusting the concentration of all samples to 1 mg/ml. 5 μl aliquots of adjusted supernatants were applied to nitrocellulose membrane (Millipore; Bedford, MA) and left to dry for 1 h, according to (Bárány et al., 2010a), incubated overnight at room temperature, with the primary antibodies (JIM5 and JIM7), diluted 1:200 in the blocking buffer (2% powdered skimmed milk containing 0.05% Tween-20 in PBS), washed, and incubated for 1 h with alkaline phosphatase-conjugated anti-rat antibody diluted 1:1000 in the blocking solution. The signals were revealed by nitroblue tetrazolium and bromo-chloroindoly-phosphate (NBT-BCIP) mixture. Controls were performed by avoiding the first antibody. Three technical replicates and two biological replicates were performed.
Low-temperature processing and embedding for in situ localization assays

Samples at selected times of the in vitro culture, at culture initiation (0 weeks) and after 4 and 8 weeks in culture, were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, overnight at 4ºC, and processed as previously described (Solís et al., 2012). After washing in PBS, samples were dehydrated in an acetone series, infiltrated and embedded in Technovit 8100 (Kulzer, Germany) resin at 4ºC. Semithin resin sections (2µm thickness) were mounted on slides coated with APTES (3-aminopropyltriethoxysilane, Sigma), air-dried and stored at 4ºC until use for immunofluorescence assays. 5 to 10 representative samples of each stage from three independent cultures were processed and analyzed.

Immunofluorescence

Semithin sections washed in PBS. For 5mdC immunodetection, DNA of sections was denatured with 2N HCl for 45 min and washed in PBS. After that, in all cases, sections were blocked with 5% (w/v) bovine serum albumin (BSA) in PBS for 10min and incubated with the primary antibodies JIM5, JIM7, anti-IAA and anti-5mdC for 1h diluted 1:25 for JIM5 and JIM7, 1:100 for anti-IAA, and 1:50 for anti-5mdC, in 1% BSA in PBS. After washing in PBS, the signal was revealed by the Alexa Fluor 488-labelled anti-rat antibodies (for JIM5 and JIM7 antibodies), and by the Alexa Fluor 488-labelled anti-mouse antibodies (for anti-IAA and anti-5mdC antibodies) (Molecular Probes) diluted 1/25 in PBS for 45 min in the dark, as previously described (El-Tantawy et al., 2013; Testillano et al., 2013). Finally, sections were counterstained with 1mg/ml DAPI (4’.6-diamidino-2-phenylindole) for 10 min, washed with PBS, mounted in Mowiol and examined in a confocal microscope (Leica TCS-SP5-AOBS, Vienna, Austria). Optical sections and maximum projections images were obtained with software running in conjunction with the confocal microscope (Leica software LCS version 2.5). CLSM analysis was performed using the same laser excitation and sample emission capture settings for image acquisition in all immunofluorescence preparations, allowing an accurate comparison among signals from cells at different developmental stages. Sections from two biological replicates were used, and a minimum of three independent immunofluorescence
experiments were performed and analyzed under CLSM, for each antibody and sample (stage). Confocal images were analyzed and representative images, for each antibody and stage, were selected to illustrate the results.

Negative controls for JIM5 and JIM7 antibodies were obtained by replacing the primary antibody by PBS. For the anti-IAA antibody, negative control was obtained by immunodepletion assays; the anti-IAA antibody was incubated with a solution of 5 mg/ml synthetic IAA (1:4, v/v) at 4°C overnight. The pre-blocked antibody solution was used as primary antibodies for immunofluorescence, following the same protocol and conditions described above. For the anti-5mdC antibody, negative controls were performed by avoiding the denaturation step or eliminating the first antibody.

Quantification of DNA methylation by 5mdC ELISA-based immunoassay

Two types of samples were collected from 8 week-old cultures: embryogenic masses and non-embryogenic tissues. Embryogenic masses emerging from the explants were carefully excised and separated from non-embryogenic tissues; both samples were rapidly frozen by immersion in liquid nitrogen. Quantification of global DNA methylation was performed as previously described (Testillano et al., 2013). Total DNA was extracted from 100 mg of frozen material, (samples excised from 25-30 explants, collected from 10-12 Petri dishes of two in vitro cultures). DNA extraction was performed using a plant genomic DNA extraction kit (DNeasy Plant Mini, Qiagen) following the kit’s instructions. 100 ng of genomic DNA for each sample was used for global DNA methylation quantification by using a MethylFlash Methylated DNA Quantification Kit (Colorimetric, Epigentek, NY). The amount of methylated DNA was proportional to the optical density (OD) measured in an ELISA plate reader at 450 nm. The value of 5-methyl-deoxy-cytosine for each sample was calculated as a ratio of sample OD relative to the standard OD, after subtracting negative control readings. Results were expressed as the percentage of methylated deoxy-methyl-cytosines (5mdC) of total DNA. For the quantification, three technical replicates and two biological replicates for each sample were
used. Mean values and standard deviations were calculated and significant differences were assessed by Student’s $t$ test at $P \leq 0.05$.

RESULTS

Main developmental stages of *in vitro* leaf somatic embryogenesis of *Quercus alba*

Somatic embryogenesis (SE) was induced from leaf explants (Fig. 1A) of white oak WOQ-1 genotype which has shown a relatively high embryogenic capacity. After 2-3 weeks of culture, some morphological changes, such as thickening of the midvein and growth of the leaf, were evident. Initial development of small proembryogenic masses (PEMs) occurred in the petiole stump region and margins of the leaf blade (Fig. 1B) after 4 weeks of culture, although the first clearly visible PEMs and early globular-stage somatic embryos only became apparent in some explants after 5 weeks of culture on induction medium (Fig. 1C). During the 5th and 6th weeks of culture, PEMs increased in size and could be observed on the basal half of the explants (Fig. 1C, D). Extensive cell proliferation on the abaxial side curved leaf explants towards the adaxial side causing them to warp and deform. In subsequent weeks, the development of embryogenic masses increased, and numerous somatic embryos (not synchronized) could be observed after 8 weeks in culture (Fig 1E-F). PEMs and somatic embryos were mainly generated on the abaxial side of leaf explants, especially in the thickened midvein and at the margins of the leaf blade; somatic embryos usually developed from PEMs (Fig. 1E).

Microscopic analysis of culture samples at defined developmental stages permitted the characterization of the main changes in the cellular organization during somatic embryogenesis initiation and progression. On the day of explantation (day 0), transverse sections of leaves showed a low level of differentiation (Fig. 2A-B), as expected for young leaves from the first node. Developing leaves showed a developing structure in which the single-layered adaxial epidermis consisted of polygonal vacuolated cells with small nuclei. Adaxial epidermal cells were larger than the abaxial epidermal cells, which exhibited dense cytoplasm and a centrally located nucleus (Fig. 2B). The mesophyll comprised a single layer of palisade parenchyma on
the upper side and several layers of spongy parenchyma on the lower side of the blade (Fig. 2B). The spongy parenchyma was rather compact, with no evidence of intercellular spaces or starch grains. The midvein consisted of vacuolated sub-epidermis and inner parenchyma cells surrounding vascular bundles with no differentiation of sclerenchymatous fibres.

The changes observed after 2 weeks of culture included division of cells surrounding the vascular bundles of the leaves and enlargement of mesophyll cells. Cell proliferation produced cellular masses throughout the mesophyll, and protrusions especially on the abaxial side (Fig. 2C), where cell proliferation and growth increased the explant thickness over subsequent weeks. The appearance of the first meristematic embryogenic cells located in the abaxial cell layers was also noted.

After 4 weeks of culture (Fig. 2D-F), cell divisions in epidermal and subepidermal tissues of the abaxial side of explants gave rise to multicellular proembryogenic masses which, at this early stage of SE, displayed meristematic/embryogenic-like features consisting of small cell size, dense cytoplasm, few small vacuoles; and large nucleus with prominent nucleolus (Fig. 2D). Moreover, inside the proliferating mesophyll, meristematic-like cells were distinguishable associated with the vascular bundles of the leaf and at the explants surface (Fig. 2E). In the surface layers of the leaf explants, cells with features resembling a diffuse cambium-like tissue (as defined by Gautheret (1959)) were frequently observed. Small groups of embryogenic cells and multicellular proembryogenic masses (Fig. 2E-F) appeared dispersed among cells showing deposits of polyphenolic compounds characterized by intense toluidine blue staining (Fig. 2F). Many embryogenic-like cells also displayed intense accumulation of polyphenolic substances.

After 6-8 weeks, different phases of somatic embryo development were observed, including globular-, heart- and torpedo- stages (Fig. 2G). Occasionally, early cotyledonary-stage embryos were already differentiated after 8 weeks, that is, embryos in which the shoot and root meristems as well as a closed vascular procambium were observed (Fig. 2H). As embryo
development was not synchronized, proembryogenic masses and early embryos of different sizes were also observed at late stages of culture.

**Pectin esterification changes in cell walls during initiation and early stages of somatic embryogenesis**

To determine the changes in the proportion of pectin esterification in cell walls after somatic embryogenesis induction, immuno-dot-blot assays were carried out with the antibodies JIM7 and JIM5 recognizing highly and low methyl-esterified pectins respectively. The dot-blot analyses were performed in leaf explants before SE induction (0W) and after 8 weeks (8W) in induction medium, the stage in which explants showed numerous PEMs and somatic embryos at early developmental stages (Fig. 1E, F). Samples after 8 weeks in culture were used for immuno-dot-blot experiments since they provided a sufficient volume of embryogenic cells for pectin extraction and detection by this assay, while samples at earlier stages did not contain enough embryogenic cells. The differences in the signal intensities of immune-dot-blots, which were performed over equal weights of samples, revealed changes in the proportion of highly-esterified and non-esterified pectins of cell walls after somatic embryogenesis initiation; dot-blot signal intensities obtained in different technical and biological replicates of the experiment were similar for the two antibodies, variations among replicates were non-significant. Representative dot-blot results were illustrated in figure 3, which shows different levels of pectin esterification before and after SE initiation. The JIM5 immuno-dot-blot signal showed high levels of non-esterified pectins in leaf explants before induction (0W), whereas it diminished when somatic embryos were formed, at 8 weeks. In contrast, the JIM7 signal, corresponding to esterified pectins, was lower than the JIM5 signal in leaf explants before SE induction, but it increased with SE initiation and early somatic embryos development, at 8 weeks (Fig. 3). During somatic embryo formation, the proportion of esterified pectins significantly increased, moreover, the difference between non-esterified and esterified pectins in cell walls at this stage (8 weeks) became higher, since JIM5 signal significantly diminished.
while JIM7 signal increased. Controls without the first antibodies did not show significant signals in any developmental stage.

The in situ localization patterns of esterified and non-esterified pectins were analysed by immunofluorescence with JIM7 and JIM5 antibodies followed by confocal microscopy analysis at three developmental stages: before induction (0 weeks), after 4 weeks in culture, when the first signs of embryogenic response appeared as thickening of leaf tissues with few-cell proembryos but no embryos yet visible, and after 8 weeks, when somatic embryos at different early stages were formed. To allow an accurate comparison among signals from cells of different developmental stages, the analyses were performed in the confocal microscope after standardization of settings, i.e. using the same laser excitation and sample emission capture settings for image acquisition in all immunofluorescence preparations. JIM5 and JIM7 immunofluorescence assays revealed specific changes in the distribution pattern of esterified and non-esterified pectins in the walls of embryogenic cells from early developmental stages.

In leaf explants before SE induction (0 weeks), JIM7 immunofluorescence signal was low and mainly localized in cell walls of the peripheral cell layers and inner parenchyma cells of the midvein thickening (Fig. 4A, A’). After induction, in 4-week samples, small groups of proliferating cells began to arise from the abaxial surface of the leaf as protuberances or proembryogenic masses (PEMs) (Fig. 4B, arrows). These PEMs, formed by cells smaller and denser than the parenchyma cells, exhibited an intense JIM7 immunofluorescence signal, whereas the larger and vacuolated cells of the leaf parenchyma showed a much less intense signal (Fig. 4C, C’). In various explants at 4 weeks, small proembryos could also be found as isolated rounded structures emerging from PEMs (Fig. 4D); JIM7 signal was very high on the cell walls of proembryos and much lower in the non-embryogenic neighbour cells of the explants (Fig. 4D, D’). At later culture stages, in 8-week culture samples, cells of the somatic embryos at different early developmental stages were clearly distinguished by the typical organization of proliferating cells, with dense cytoplasm and large nuclei; SE cells were
intensely labelled by JIM7 immunofluorescence in contrast with the clear and vacuolated non-embryogenic cells which did not show JIM7 labelling (Fig. 4E, E’).

Immunofluorescence assays with JIM5 antibody revealed very different distribution patterns to those observed with JIM7 antibody. Leaf explants before SE induction (0 weeks) showed labelling in most cells; JIM5 labelling was more intense in cell walls of the vascular region, in the interior of the midvein (Fig. 5A, A’). After induction, at 4 weeks, neither PEMs that emerged from the leaf surface, nor non-embryogenic cells of the explants showed significant JIM5 labelling (Fig. 5B, B’). At later stages, somatic embryos formed at 8 weeks did not exhibit significant fluorescence signal with JIM5 antibody, and this was also the case for the rest of the non-embryogenic cells present in the culture at this stage (Fig. 5C, C’).

Immunofluorescence control experiments replacing the primary antibody (either JIM7 or JIM5) with buffer did not show labelling in any cellular structure at any developmental stage.

**Localization of endogenous auxin accumulations during initiation and early stages of leaf somatic embryogenesis**

Immunofluorescence with anti-IAA specific antibodies was performed to evaluate changes in the *in situ* intracellular auxin accumulation after induction and at early developmental stages of somatic embryogenesis. Before SE induction, very low IAA immunofluorescence signal was observed in leaf explants (Fig. 6A, A’). After embryogenesis induction, in 4-week samples, cells of the PEMs, located at the periphery and emerging from the explants (Fig. 6B, arrows), showed an intense IAA immunofluorescence labelling (Fig. 6C, C’), whereas no IAA signal appeared over the non-embryogenic cells adjacent to the intensely labelled PEMs (Fig. 6C, C’). Labelling was localized in the cytoplasm of embryogenic cells while the nuclei, cell walls and vacuoles were negative (Fig. 6C, C’). Later, after 8 weeks in culture, somatic embryos at early stages of development were observed and displayed intense IAA immunofluorescence in the cytoplasm of every embryo cell (Fig. 6D, D’, E, E’). Non-embryogenic cells were clearly
recognized by their larger size and high vacuolation and located either in direct contact or near PEMs and early somatic embryos (Fig. 6C, D, E). Despite their proximity to embryo cells, non-embryogenic cells did not show labelling with anti-IAA antibodies (Fig. 6C, C’, D, D’, E, E’), indicating that endogenous auxin was differentially accumulated in cells of developing PEMs and somatic embryos.

Control experiments performed by omitting the IAA antibody and by immune-depletion did not show labelling over any type of cell—embryogenic or non-embryogenic (Fig. 6F, F’, F’’) — at any developmental stage, supporting the specificity of the IAA immunofluorescence signal.

**Global DNA methylation changes during initiation and early stages of leaf somatic embryogenesis**

In the present work, changes in global DNA methylation levels have been analysed in early somatic embryogenesis. The percentage of global DNA methylation was quantified by an ELISA-based immunoassay with anti-5-methyl-deoxy-cytidine (5mdC) antibodies (Testillano et al., 2013). Samples were collected from 8-week-old cultures which, due to their asynchronic development, contained embryogenic masses and embryos at different early stages. These embryogenic masses and embryos were clearly visible facilitating manual excision from the rest of the explants, which mainly contained non-embryogenic cells. Global DNA methylation was quantified in these two types of samples, embryogenic and non-embryogenic masses. Although the presence of some embryogenic cells in the interior of the explants at 8 weeks could not be completely ruled out, it can be assumed that their proportion would be very low in comparison with the non-embryogenic cells which form the majority. Therefore, the contribution of the embryogenic cells to the global DNA methylation analysis should not be significant in the excised non-embryogenic explants.

The results of the quantification showed changes in DNA methylation associated with initiation and early somatic embryogenesis. The DNA methylation percentage was significantly lower
(p≤0.05) in embryogenic cells (2.60 ± 0.02 SE) than in non-embryogenic cells (3.27 ± 0.14 SE), indicating that DNA methylation decreased with SE induction.

We also analysed the nuclear localization pattern of methylated DNA by immunofluorescence assays with anti-5-methyl-deoxy-cytidine (5mdC) antibodies. These assays were performed in samples of 8-week-old cultures containing embryogenic masses and early embryos emerging from masses of non-embryogenic cells (Fig. 8A). Confocal analysis of 5mdC immunofluorescence assays showed specific signals over nuclei with different patterns of distribution and intensity depending on the cell type, embryo cells showed less fluorescence signal than non-embryogenic cells (Fig. 8A, A’). Early embryo cells showed 5mdC immunofluorescence signals of low intensity over most of their nuclei, with a few small brighter spots, corresponding with small heterochromatin regions (Fig. 8B, B’), and some nuclei showed a particularly faint fluorescence signal, or none at all, indicating very low levels of DNA methylation. By contrast, the 5mdC signal on nuclei of non-embryogenic cells was much higher in all cells, with a distribution pattern of numerous intensely fluorescent spots that formed a thick reticulum in the whole area of the nucleus (Fig. 8C, C’), corresponding to a higher degree of chromatin condensation. Controls omitting the DNA denaturation step or in the absence the first antibody did not provide any signal.

DISCUSSION
The efficiency of somatic embryogenesis in many woody species is still very low. The aim of the present work was to investigate if the change of developmental program of leaf cells towards embryogenesis involved changes in key cellular processes and components, which would provide information on the mechanisms underlying leaf somatic embryogenesis initiation and constitute early markers of the process. The results revealed specific changes in the degree of pectin esterification in cell walls, endogenous auxin accumulation and global DNA methylation. These changes were induced with embryogenesis initiation, and occurred in embryogenic cells at very early stages after leaf embryogenesis induction while they were not
present either in leaf cells before induction or in non-embryogenic cells. Despite some previous reports of cellular rearrangements associated with in vitro embryogenesis progression in herbaceous species (El-Tantawy et al., 2013; Feher, 2015; Fortes et al., 2002; Smertenko and Bozhkov, 2014; Testillano and Risueño, 2009), the information on woody plants is scarce and has been obtained mainly from in vitro systems in which somatic embryogenesis is induced from immature zygotic embryo cells or microspores (Ramírez et al., 2003; Ramírez et al., 2004; Rodriguez-Sanz et al., 2014a). In the present work, white oak somatic embryogenesis is not induced from immature embryo or reproductive tissues but from vegetative organs, such as leaves, a more differentiated system with possibly less reprogramming potential.

**Pectin esterification increase in cell walls identifies leaf embryogenesis initiation**

The modifications in pectin residues and other cell wall components have been related to initiation of cell responses and changes in cell fate and development (Willats et al., 2001). The esterification status of pectins has a direct effect on the biophysical properties of plant cell walls that highly influence the processes of cell elongation and growth in differentiation events (Goldberg et al., 1986; Micheli, 2001). In the present work, the comparative analysis between embryogenic and non-embryogenic cells, has allowed the identification of the increase in pectin esterification as a differential cell wall feature of early leaf embryogenesis, and therefore associated it with cell totipotency and embryogenesis initiation. This finding is consistent with the fact that newly-formed walls contain a higher proportion of esterified pectins, as reported in proliferating cells of young microspore-derived embryos and root tip meristematic cells, in some herbaceous species (Bárány et al., 2010a, b; Solis et al., 2016).

Changes in cell wall mechanics are reported controlled by the esterification level of pectins which underlies organogenesis initiation and phyllotaxis in Arabidopsis (Peaucelle et al., 2011; Peaucelle et al., 2008). Biochemical changes in the cell wall have been found at early embryo stages, and are related to cell wall loosening and remodelling, which are processes that are crucial for proper embryo growth and embryogenesis progression (Levesque-Tremblay et al.,
The higher proportion of esterified pectins that we found in embryogenic cells at early SE could be a key feature for cell wall loosening to facilitate the growth that is characteristic of this early stage.

In various plant species such as Capsicum annuum, Q. suber, Citrus clementina and Olea europaea, changes in pectin esterification have been found in microspores and microspore-derived embryos as well as in meristematic and differentiating cells of Allium cepa root tips (Bárány et al., 2010a; Ramírez et al., 2003; Ramírez et al., 2004; Rodriguez-Sanz et al., 2014a; Solís et al., 2008). The present results in leaf embryogenesis of Q. alba showed a higher proportion of esterified pectins in embryogenic cells of proembryogenic masses and early embryo cells, which are active cycling cells, indicating that high pectin esterification would be not only a marker of the switch of developmental program and totipotency during leaf embryogenesis initiation of Q. alba, but also a marker of active proliferation. Moreover, these findings provide new insights into the possible role of pectin esterification in cell wall remodelling during plant cell totipotency acquisition and leaf embryogenesis initiation.

**Auxin differentially accumulates in embryogenic cells during leaf embryogenesis initiation**

Auxin immunofluorescence assays showed the accumulation of endogenous auxin in embryogenic cells after embryogenesis induction. Auxin action depends on its differential distribution within plant tissues, which is mainly regulated by its local biosynthesis and its directional transport between cells (Petrasek and Friml, 2009). This balance between synthesis and transport leads to higher auxin concentrations in specific regions of the plant, e.g. apical meristems of shoots, root meristems and lateral root initiation sites (Himanen et al., 2002; Prasad and Dhonukshe, 2013).

Recent reports in another system of somatic embryogenesis—microspore embryogenesis of B. napus—have shown that auxin accumulations were present in embryo cells from the very early stages. This would indicate that auxin biosynthesis is activated with microspore reprogramming.
and embryogenesis initiation (Prem et al., 2012; Rodriguez-Sanz et al., 2015). In zygotic embryogenesis of *Arabidopsis*, auxin signalling has been localized in embryo cells, from the 2-cell until the 8-cell stage (Moller and Weijers, 2009; Robert et al., 2013). The present results on auxin accumulation during leaf embryogenesis initiation are consistent with the auxin signalling distribution during early zygotic embryogenesis (Rademacher et al., 2012) and microspore embryogenesis (Prem et al., 2012; Rodriguez-Sanz et al., 2014a; Rodriguez-Sanz et al., 2015). They also indicate that both leaf embryogenesis and microspore embryogenesis follow a similar pattern to that of zygotic embryogenesis.

The involvement of auxin in cell division induction and cell cycle progression control has been reported, and its biosynthesis related to dividing cells and growing tissues. Several reports have shown that auxin starvation causes cell division arrest in suspension cell and tissue cultures of various species (Adamowski and Friml, 2015; Chen et al., 2014; Himanen et al., 2002; Perrot-Rechenmann, 2010; Schaller et al., 2015). The embryogenic cells, derived from leaf explants, are totipotent cells, acquire a new cell fate and initiate embryogenesis, which is a developmental pathway that begins with proliferation. Therefore, our findings revealing differential auxin accumulation in embryogenic cells suggested that, in *Q. alba* leaves, endogenous auxin biosynthesis may be involved in the activation of proliferation, as one of the initial events after the switch of the embryogenic program.

**Global DNA demethylation is induced with leaf embryogenesis initiation**

The acquisition of cell totipotency by differentiating plant cells is accompanied by the remodelling of gene expression programs which likely involves large-scale chromatin reorganization (Tessadori et al., 2007). The level and distribution patterns of DNA methylation change during plant cell differentiation and proliferation, as do other epigenetic marks. Changes in global DNA methylation have been reported in the regulation of global gene expression programs (Kohler and Villar, 2008).
We analysed whether changes in global DNA methylation levels and distribution patterns occurred during leaf embryogenesis initiation. The analysis performed by quantification of the percentage of methylated DNA revealed a decrease in global DNA methylation in proembryogenic masses and early embryos in comparison with non-embryogenic cells. 5mdC immunofluorescence results agreed with the quantification results and are in line with the lower DNA methylation of early embryo cells. Furthermore, embryogenic cell nuclei exhibited a 5mdC distribution pattern compatible with discrete foci of heterochromatin masses, while non-embryogenic cell nuclei showed a more condensed chromatin pattern. A few reports in tree species showed that low levels of DNA methylation were associated with morphogenic and embryogenic capacities (Rodriguez-Sanz et al., 2014a; Valledor et al., 2007). In Castanea sativa, a transient DNA demethylation has been found in the ovules after pollination (Viejo et al., 2010). At early stages of microspore embryogenesis of B. napus and H. vulgare, a decrease in DNA methylation that correlates with chromatin decondensation has also been reported (El-Tantawy et al., 2014; Solís et al., 2012) Testillano and Risueño (2016). More recently, the promoting effect of the demethylating agent 5-azacytidine on microspore embryogenesis induction has been described (Solis et al., 2015). After microspore reprogramming, in microspore-derived proembryos of several species, a decondensed pattern of chromatin has been reported, as a characteristic feature of proliferating cells (Seguí-Simarro et al., 2011; Testillano et al., 2000; Testillano et al., 2005). Also in microspores, DNA hypomethylation and histone H3 and H4 acetylation have been associated with transcriptional activation and totipotency acquisition (El-Tantawy et al., 2014; Rodriguez-Sanz et al., 2014b; Solís et al., 2012). The results presented here are consistent with those reports and revealed—for the first time in Q. alba—that DNA demethylation and chromatin decondensation are induced with the initiation of leaf embryogenesis, also illustrating that epigenetic reprogramming accompanies the change of developmental program of leaf cells.
Conclusions

The present study is the first to show specific cellular changes that are induced concomitantly with leaf embryogenesis initiation in a tree species of economic interest, *Q. alba*. The results are of particular relevance since they were obtained in a woody species, which are extremely recalcitrant to *in vitro* embryogenesis from non-zygotic material, such as leaf tissues. The identified changes can be considered as cellular markers of somatic embryogenesis initiation, providing information about the metabolic processes involved in the induction and control of plant cell reprogramming, totipotency and embryogenic competence acquisition.

The three factors identified—highly esterified pectins, endogenous auxin accumulation, and DNA hypomethylation—were specifically found in embryogenic cells. Some of these factors had been individually found in other *in vitro* embryogenesis systems and plant species (Rodriguez-Sanz et al., 2015; Solis et al., 2015; Solís et al., 2012); in the present report these three features have been identified for the first time at early leaf embryogenesis in a tree species. The findings further support the notion that these factors are early embryogenic markers that reflect general mechanisms involved in the process that could be exploited to improve *in vitro* embryogenesis induction in other species.

Since pectin esterification, auxin accumulation and DNA demethylation were induced with embryogenesis initiation, they represent processes that may be interconnected in the regulation of cell reprogramming and embryogenesis initiation. Recent studies have revealed that auxin, among other factors, contributes to the cell wall remodelling during organogenesis, and the de-esterification of pectins is required during the process (Braybrook and Peaucelle, 2013; Peaucelle et al., 2011). The initiation and progression of *in vitro* embryogenesis is associated with increasing auxin biosynthesis, action and polar transport (Rodriguez-Sanz et al., 2015). Increasing evidence has revealed the involvement of different phytohormones, including auxin, in signalling processes of chromatin remodelling by epigenetic mechanisms to activate specific gene expression programs of development. Further work would be necessary to investigate if
auxin may be involved in pectin-related cell wall remodelling and epigenetic reprogramming during *in vitro* embryogenesis initiation.

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FIGURE LEGENDS

Figure 1. Induction of somatic embryos from leaf explants of *Quercus alba* excised from axillary shoot cultures. A. Leaf explant at initiation of culture in induction medium, with the adaxial side (ad) up. B. Leaf explant after 4 weeks of culture in induction medium showing initial formation of embryogenic masses in the stump petiole (sp) and leaf margin (lm). C, D. Oblong-stage somatic embryo (C) and proembryogenic masses, PEMs (D; arrows) formed after 5 weeks (C) and 6 wk (D) in induction medium. E. Numerous proembryogenic masses and somatic embryos developed on the abaxial leaf margin after 8 weeks culture. Arrow indicates somatic embryo (se) formed from a PEM. F. Early cotyledonary-stage somatic embryos (arrow) generated on the abaxial leaf margin after 8 weeks culture in induction medium. Scale bars: 1 mm.
Figure 2. Cellular organization during leaf embryogenesis initiation of *Q. alba*. A. Transverse section of a leaf at initiation of culture in induction medium. B. Detail at high magnification of the leaf blade region of a leaf explant at initiation of culture in induction medium, note the different structure of the epidermal cells of adaxial (ad) and abaxial (ab) sides. C. Transverse section of leaf explant after 2 weeks of culture in induction medium showing the presence of meristematic-like cells in the abaxial mesophyll. D. Cell proliferation of the abaxial side of leaf explant after 4 weeks in culture in which embryogenic cells (arrows, e) can be observed. E. Cell proliferation on the abaxial side of the midvein after 4 weeks of culture showing the formation of a diffuse cambium (dc) which may be the origin of embryogenic cells (arrows). Note the presence of meristematic zones associated with vascular bundles (arrowheads). F. First divisions of embryogenic cells giving rise to embryogenic aggregates. The presence of small proembryos, some of them with accumulations of polyphenol compounds (arrows) is noted. G. Globular (gl) - and heart (h) - stages embryos attached to proembryogenic masses formed on abaxial side of leaf explant after 6 weeks of culture. H. Longitudinal section of cotyledonary-stage embryo showing differentiation of shoot and root meristems after 8 weeks of culture. Double staining with PAS-naphthol (blue-black reaction) was used, except in figures D, E and F, which show toluidine blue staining. Bars represent: A: 200µm, B, C: 50µm, D: 20µm, E, F, G: 100µm, H: 500µm.
Figure 3. Detection of non-esterified (JIM5) and highly-esterified (JIM7) pectins by immuno-dot-blot in leaf embryogenesis initiation. Immuno dot-blot assays with JIM5 and JIM7 antibodies on extracts of equal weight of samples from leaf embryogenesis cultures before embryogenesis induction (0W) and after 8 weeks in culture (8W) in induction medium, when somatic embryos at early developmental stages were formed.
Figure 4: *In situ* localization of esterified pectins during initiation and early stages of leaf embryogenesis. Immunofluorescence with JIM7 antibody to esterified pectins and confocal microscope analysis at different developmental stages. Panoramic views of transverse sections of leaves before induction (A, A’) and after 4 weeks in culture (B). Details at high magnification of explants after 4 weeks (C, C’, D, D’) and 8 weeks (E, E’). Confocal images of JIM7 immunofluorescence signal in green (A’-E’) and phase contrast images of the same sections (A-E). Arrows point to embryogenic cells at the periphery of proembryogenic masses and early embryos. Bars represent: A, A’, B: 200µm, C, C’, D, D’, E, E’: 20µm.
Figure 5: *In situ* localization of non-esterified pectins during initiation and early stages of leaf embryogenesis. Immunofluorescence with JIM5 antibody to non-esterified pectins and confocal microscope analysis at different developmental stages. Panoramic views of transverse sections of leaves before induction (A, A’). Details at high magnification of explants after 4 weeks in culture (B, B’) and 8 weeks (C, C’). Confocal images of JIM5 immunofluorescence signal in green (A’-C’) and phase contrast images of the same sections (A-C). Arrows point to embryogenic cells at the periphery of proembryogenic masses and early embryos. Bars represent: A, A’: 200µm, B, B’, C, C’: 20µm.
**Figure 6:** *In situ* localization of auxin (indolacetic acid, IAA) during initiation and early stages of leaf embryogenesis. Immunofluorescence with anti-IAA antibody and confocal microscope analysis at different developmental stages. Panoramic views of transverse sections of leaves before induction (A, A’) and after 4 weeks (B). Details at high magnification of explants after 4 weeks (C, C’) and 8 weeks (D, D’, E, E’). Confocal images of IAA immunofluorescence signal in green (A’-E’) and phase contrast images of the same sections (A-E). F, F’, F”’: Control by immunodepletion with pre-blocked antibody in 4 weeks explants; phase contrast (F), confocal image of IAA immunofluorescence signal in green (F’) and DAPI signal for nuclei in blue (F’’). Arrows point to embryogenic cells at the periphery of proembryogenic masses and early embryos. Bars represent: A, A’, B: 200µm, D, D’, E, E’, F, F’, F”’: 20µm.
Figure 7: Quantification of global DNA methylation in early leaf embryogenesis. Histogram represents the mean values of 5-methyl-deoxy-citidine (5mdC) percentage of total DNA in embryogenic masses and early embryos (left column) and non-embryogenic cells (right column) dissected from explants after 8 weeks, quantified by ELISA-based immunoassay. Bars on columns represent the standard errors of the means. Asterisk on column indicate significant differences according to Student-\( t \) test at \( P \leq 0.05 \).
Figure 8: *In situ* localization of 5mdC (methylated DNA) during early stages of leaf somatic embryogenesis. Immunofluorescence with anti-5mdC antibody and confocal microscope analysis of leaf explants and early embryos, after 8 weeks in culture. A, A’: Detail of a sample showing an early embryo (emb) and a mass of non-embryogenic cells (Non-emb). Phase contrast image (A) and 5mdC immunofluorescence signal (A’) of the same region. B, B’, C, C’: Higher magnification micrographs of nuclei of early embryo cells (B, B’) and non-embryogenic cells (C, C’). Confocal images of DAPI staining (blue) for DNA (B, C) and 5mdC immunofluorescence signal (green) (B’, C’) of the same sections. Bars represent 20µm.