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Original article

Initiation of leaf somatic embryogenesis involves high pectin esterification, auxin accumulation and DNA demethylation in *Quercus alba*

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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.

1. 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 (Germaná 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).

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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, 2010b; El-Tantawy et al., 2013; Fortes et al., 2002; Solís et al., 2008), phytohormones (Prem et al., 2012; Rodriguez-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; Rodriguez-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.

2. Material and methods

2.1. 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.

2.2. 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 5–10 representative samples of each stage were processed for paraffin or plastic embedding to perform a microscopy analysis of the cellular structural organization.