

Differences in DNA methylation, DNA structure and embryogenesis-related gene expression between embryogenic and non embryogenic lines of *Pinus radiata* D. don

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Abstract *Pinus radiata* is the most important conifer species for commercial forestry in countries such as Australia, New Zealand and Chile. Nowadays, SE (somatic embryogenesis) is considered the most promising in vitro method for large scale vegetative propagation of woody plants. The understanding of the molecular basis of SE is in its very beginning and a number of embryogenesis-related genes have been identified in conifers. Among the molecular mechanisms involved in regulation of SE, DNA methylation, which is an epigenetic modification associated with transcriptional silencing, has shown to be a pivotal factor controlling gene expression. In this work, we studied the morphological and molecular differences between cell lines previously characterized in terms of their embryogenic potential as embryogenic (E) and non embryogenic (NE), obtained from immature zygotic embryos of *P. radiata*. In

contrast to E lines, NE lines were composed of multicellular aggregates lacking polarity, and they were characterized by the presence of significantly lower transcript levels of embryogenesis-related genes and higher global DNA methylation. Furthermore, the detection of vibrational markers of DNA conformation indicated that DNA samples obtained from E lines presented the common B-DNA conformation, while NE samples presented Z-conformation. Taken together, our results highlight the role of epigenetic mechanisms such as DNA methylation in regulating the expression of embryogenesis-related genes, having impact on the embryo patterning and cell differentiation.

Keywords Somatic embryogenesis · DNA methylation · Embryogenesis-related genes · Z-DNA · Plant epigenetics

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Introduction

Pinus radiata D. is native of California and is widely grown in the Southern hemisphere for plantation forestry. Over the last decades, significant genetic gains have been made in *P. radiata* forestry using conventional breeding techniques (Bishop-Hurley et al. 2003). Now, procedures allowing rapid capture of the benefits of breeding are expected to play an important role in increasing the productivity, sustainability and uniformity of forest plantations. Clonal propagation by somatic embryogenesis (SE) is the primary enabling technology to achieve these objectives in conifers (Humánez et al. 2012). Also, because this system includes a large number of events such as changes in the gene expression and cell division patterns, and cell fate determination, SE has also become an appropriate method for studying the morpho-physiological and molecular aspects of cell differentiation (Rocha et al. 2016). Although protocols for

P. radiata SE have been considerably improved in recent years (Hargreaves et al. 2009; Montalbán et al. 2012), problems such as low SE initiation frequency, reduction or cessation of regeneration capacity, low genotype capture, among others, still limit the production that this technique offers (Lelu-Walter et al. 2016).

Somatic embryogenesis is a multistep regeneration process starting with initiation, which is the induction of pro embryogenic masses (PEMs). PEMs are cell aggregates which can pass through a series of three stages distinguished by cellular organization and cell number (Stages PEM I, PEM II, and PEM III) (von Arnold et al. 2002). In conifers, within the growing embryonic masses two different cell types can be distinguished based on their morphology. Small densely cytoplasmic cells composed the embryonic head of the filamentous embryos, whereas elongated cells with large vacuoles form the suspensor apparatus (Stasolla and Yeung 2003). In pines, SE is initiated most efficiently from immature zygotic embryos, most frequently at early to late cleavage polyembryony stage (Lelu-Walter et al. 2016). Cell lines can have quite variable potential of forming embryos (Palovaara and Hakman 2008), so embryogenic (E) and non embryogenic (NE) cell lines can be obtained and maintained.

SE involves the reprogramming of gene expression patterns including cascades of genetic triggers turning on and off the expression of specific genes (Feher et al. 2003; Karami et al. 2009). Previous studies about the molecular mechanisms regulating the early phase of SE in gymnosperms have revealed genes with expression patterns regulated differentially between embryogenic and non-embryogenic tissue (Bishop-Hurley et al. 2003; Stasolla et al. 2003; Aquea and Arce-Johnson 2008). Recently, epigenetic mechanisms have emerged as being critical in control of both somatic and zygotic embryogenesis by ultimately determining gene expression patterns through modulation of access to DNA and definition of distinct chromatin states (Lelu-Walter et al. 2016). Among the epigenetic mechanisms, a special attention has been given to DNA methylation, which plays its role not only preventing binding of transcription factors and enzymes, but is also related to chromatin structure (Valledor et al. 2007). In plant and animal models, DNA methylation has become accepted as an essential epigenetic mechanism for maintenance of cell patterning and one of the most important alternatives of gene control during the progress of plant development (Viejo et al. 2010). DNA methylation has shown to be important in the induction and development of both zygotic and somatic embryogenesis (Santos and Fevereiro 2002; Nic-Can et al. 2013).

In this work, we described cellular and molecular differences between cell lines previously characterized in terms of their embryogenic potential (capacity to produce

embryos) as embryogenic (E) or non embryogenic (NE), obtained from immature zygotic embryos of *P. radiata*, and treated with identical protocols. Our results suggest a role of DNA methylation in regulating embryogenesis-related genes, influencing the embryo patterning and cell differentiation, and having impact in the embryogenic potential.

Materials and methods

Plant material

Plant material consisting in somatic embryogenic tissues were provided by the culture laboratory of the “Centro de Biotecnología CMPC Mininco S. A.”, Los Angeles, Chile. Cultures were initiated from immature zygotic embryos of *P. radiata* obtained by controlled pollination of elite trees. The megagametophytes containing immature embryos were excised under sterile conditions and placed in Petri plates. Somatic embryogenesis was performed with a method based in Smith et al. (1994). For the induction phase all megagametophytes were maintained in initiation EDM medium containing 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 μM BA (EDM6). Embryogenic lines with different genotypic background were used in this study. All the lines show embryogenic appearance after 1 month in induction phase. The lines were previously classified by the “Centro de Biotecnología CMPC Mininco S.A.” being defined as E for lines with full capacity of differentiate somatic embryos when they passed to maturation phase (higher than 20 somatic embryos per 100 mg of callus) and NE for lines that do not differentiate somatic embryos in maturation phase (<5 somatic embryos per 100 mg of callus). The embryogenic lines were maintained in culture (in darkness at 21 °C) for four multiplication cycles. After 10 days of the fourth cycle they were processed for the following assays. Both E and NE lines were previously studied in terms of their outer appearances (color and texture of the calli) and growing rates, and no differences were detected.

Microscopic analysis

A total of 10 E lines and 10 NE lines were studied by light microscopy. Three samples were obtained from each line. Tissues were fixed in FAA (ethanol, glacial acetic acid and formalin 85:5:10 v/v), dehydrated in an alcohol gradient and embedded in paraffin. Longitudinal sections were obtained with a glass knife on a microtome and stained with Fast Green. Cellular density was determined as the average number of cell aggregates of three fields from the same slide, considering one slide per sample. *T* student test was applied.

RNA extraction and gene expression profiling using conventional PCR and relative real-time qPCR

Total RNA was extracted from 3 E and 3 NE lines using CTAB buffer as in Chang et al. (1993). Total amount of recovery was determined using a Nanoquant Infinite Pro M 200 spectrophotometer (Tecan, Switzerland) and analyzed by 1% agarose gel electrophoresis. Reverse transcription was made using the commercial kit Thermo Scientific RevertAid First Strand cDNA Synthesis (Thermo Fisher Scientific #K1622, United States) following the manufacturer's indications. Conventional PCR amplification was conducted with a Mastercycler thermocycler (Eppendorf, Germany). For this was used a reaction containing 15 ng cDNA, 1X PCR buffer (Invitrogen # 11615-010, United States), 0.2 mM dNTPs, 2mM MgCl₂ (Invitrogen # 11615-010, United States), 0.5 μM forward and reverse primer and 1U Taq DNA polymerase recombinant (Invitrogen # 11615-010, United States). Primers used for detecting WOX2, SEPR9, SEPR39 and B-expansin were taken from conifer literature as it is detailed in Supplementary Table 1. For the detection of the other genes, primers were designed using the *Pinus taeda* genome as reference. Sequences of all primers are included in Supplementary Table 1. The thermal cycling conditions were as follows: 3 min at 94 °C, followed by 30 amplification cycles of 45 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C, with final extension to 72 °C for 10 min. Their identity was verified by automatized sequencing and bioinformatic analysis. For the real-time qPCR the amplification was conducted with a Rotor Gene thermocycler (Qiagen, Germany) (normal ramping) using the commercial kit 5× Hot FirePol EvaGreen qPCR Mix Plus (Rox) (Solis BioDyne, Republic of Estonia). The cycling parameters included 15 min at 95 °C, followed by 40 amplification cycles of 15 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C, with final extension to 72 °C for 2 min, a melting curve was obtained immediately after amplification. Raw fluorescence readings were imported into Minner software for determine the Ct and relative expression. T-student test was applied for comparing the relative transcription level of quantitative PCR results. Standard errors of the mean were incorporated in the graphs.

Genomic DNA extraction

Genomic DNA was obtained from 3 E and 3 NE lines (the same lines used for gene expression profiling) powdering in liquid nitrogen, using a plant DNA extraction kit (DNeasy Plant Mini, Qiagen, Hilden, Germany), following the instructions of the manufacturer, with the exception of starting step, since in our conditions the best results were obtained using 200 mg of fresh tissue in presence of Polyvinylpyrrolidone. The resulting

DNA samples were analyzed for its integrity by agarose gel electrophoresis and total amount of recovery was determined using a Nanoquant Infinite Pro M 200 spectrophotometer (Tecan, Männedorf, Switzerland). Alternatively, DNA samples of additional lines, commercial kits and extraction protocols were evaluated, being DNeasy Plant the most efficient method for both E and NE lines. *T* student test was applied for comparing the total recovered DNA of 10 E and 10 NE lines. Standard errors of the mean were considered.

FT-IR microspectroscopy and multivariate analysis

FT-IR microspectroscopy was applied to 3 genomic DNA samples obtained from each of the 3 E (E1-E3) and 3 NE (NE1-NE3) lines. Each DNA sample was scanned in three independent experiments. Equal quantities of DNA (200 ng in 50 μl of nanopure water) were dried in a reflective glass Mirr IR (Kevey technologies, Chesterland, Ohio, United States) in the dark to prevent photo and atmospheric oxidation. IR spectra were collected with Spotlight 400 imaging system (Perkin Elmer, Middlesex, Massachusetts, United States), from 4000 to 600 cm⁻¹, in transmittance mode. For each DNA sample, scans of 15 points within an area of 100×100 μm were made, providing a spectral resolution of 2 cm⁻¹. Minimum and maximum normalization (extreme values taken from data within the interval of 1800–600 cm⁻¹) and linear baseline correction using the interval limits of 1800 and 750 cm⁻¹ were applied. The spectral pre-treatments were done using Spectrum 10.03 software (Perkin Elmer, Middlesex, Massachusetts, United States). Five representative spectra of each group (E and NE) were used to obtain main spectra and second derivative data (25 points). Band assignment was made as in Banyay et al. (2003).

DNA methylation assay

A total of 3 E and 3 NE lines (the same lines used for the previous assays) were included for the determination of the content of DNA methylation. Three samples per lines were analyzed in triplicate. Quantification of DNA methylation content was determined as % of methylated cytosines, by ELISA assay using the commercial kit MethylFlash (Epigentek), following the manufacturer's indications. Three independent experiments were performed. *T* student test was applied for comparing global methylation content of DNA from E and NE lines. Standard errors of the mean were incorporated in the graphs.

Results and discussion

Aberrant cell aggregates and down-regulated expression of embryogenesis-related genes characterized NE cultures

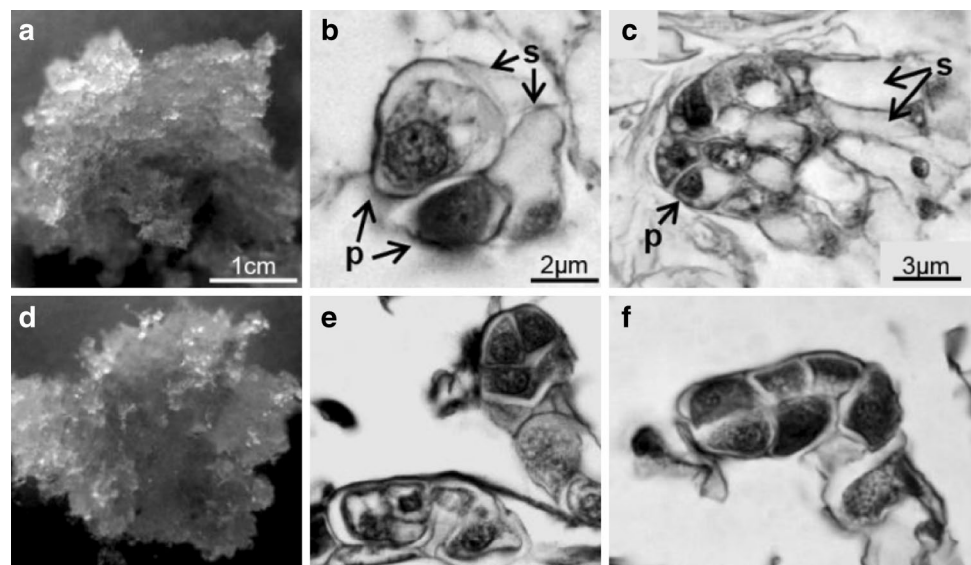
The dynamic and fate of cells committed to the somatic embryogenesis can be studied by microscopy techniques, and the cytological changes that have occurred during this process can also be of great value, by associating the observed cytological changes with the expression patterns of several genes (Rocha et al. 2016). The E and NE calli were indistinguishable by their outer appearance (Fig. 1a, d), both were composed by white, friable, and mucilaginous tissue. Microscopically, E lines were characterized by PEMs II-like structure (Fig. 1b, c), which are defined as cell aggregate composed of a small compact clump of densely cytoplasmic cells adjacent to more than one vacuolated cell (Filonova et al. 2000). Polarized clusters of different sizes were found in the same visual field, corresponding to early steps of embryogenesis. PEMs present in E lines were able to form proper cotyledonary embryos, which appear between 12 and 16 weeks of culture (Supplementary Fig. S1). Histological analysis of NE lines shown that in contrast to E lines, PEMs lack polarity, failing in differentiate the two cellular types characteristic of embryogenic PEMs (Fig. 1e, f). These lines do not produce cotyledonary embryos. No statistically significant differences between E and NE lines were detected in cellular density (data not shown).

The comparative analysis of E and NE material appeared critical to identify differentially expressed genes (Trontin et al. 2016). Based on conifer literature, we identified a set of embryogenesis-related genes as candidate for somatic

embryogenesis markers and a set of housekeeping genes potentially useful as normalizing gene during maintenance stage of *P. radiata* SE. The expression level of the selected genes was evaluated by detecting their transcript abundance in E and NE lines (Fig. 2).

The gene *WOX2* (WUS homeobox 2) codifies for a transcription factor highly expressed at early stage of SE (Mahdavi-Darvari et al. 2015). Conifer *WOX2* homolog expression has been shown to be highest at early stages of embryogenesis in response to auxin 2,4-D in *Picea* species (Klimaszewska et al. 2010), and it has been proposed as embryogenic potential marker, having a role in embryo patterning, since it is not present in NE lines (Palovaara and Hakman 2008). In Lodge pole pine, *WOX2* was expressed mainly in embryonal mass-like structures (formed by tightly packed meristematic cells adjacent to long vacuolated cells) and it has been suggested as early genetic marker to discriminate embryogenic cultures from non embryogenic callus (Park et al. 2010). We detected *P. radiata* *WOX2* homolog in E and NE lines by conventional (Fig. 2), and quantitative RT PCR (Fig. 3). Our results shown that in NE lines, levels of transcript are significantly lower than in E lines ($p=0.041$), in accordance to previous reports. *BABY BOOM* (BBM) is an AP2-L2 (Apetala 2-like 2) transcription factor preferentially expressed in developing embryos. Its overexpression promotes cell proliferation and morphogenesis, and it has been proposed as marker gene for embryo development (Boutilier et al. 2002; Passarinho et al. 2008; Nic-Can et al. 2013; Hand et al. 2016). In *Picea glauca* somatic embryos initiated from primordial shoots, AP2-L2 was present in embryogenic tissue but not in non embryogenic tissue (Klimaszewska et al. 2011). We detected *P. radiata* BBM homolog by conventional and

Fig. 1 Macro and micro-morphological features of E and NE cell lines. **a** and **d** macroscopic appearance of representative E and NE calli, respectively, in maintenance medium. **b** and **c** representative light micrographs of longitudinal sections of E calli, showing polarized embryo structures. **e** and **f** representative micrographs of longitudinal sections of NE calli, showing aberrant multicellular aggregates. P: proembryo forming-like cells characterized by its small size and round shape. S: suspensor-like cells distinguishable by its large size and vacuolated aspect



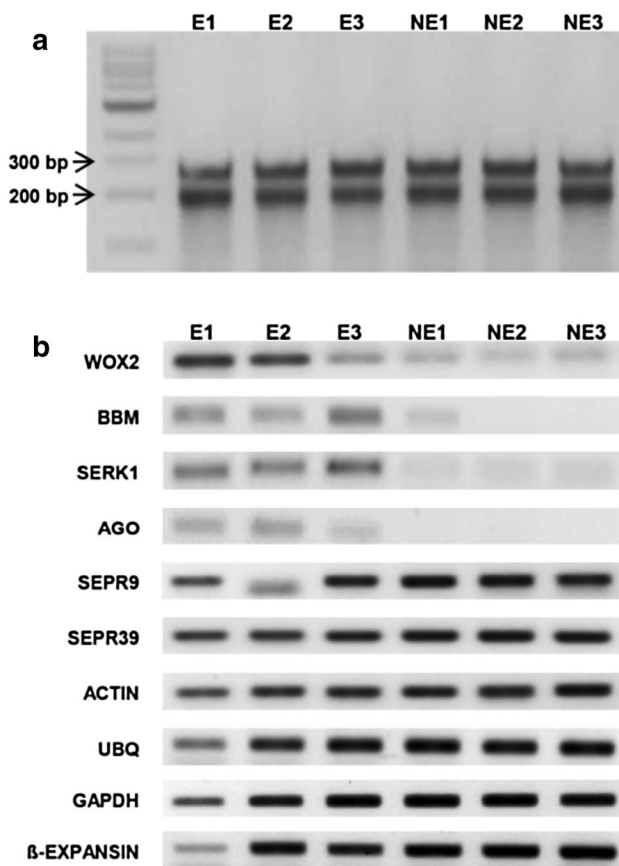


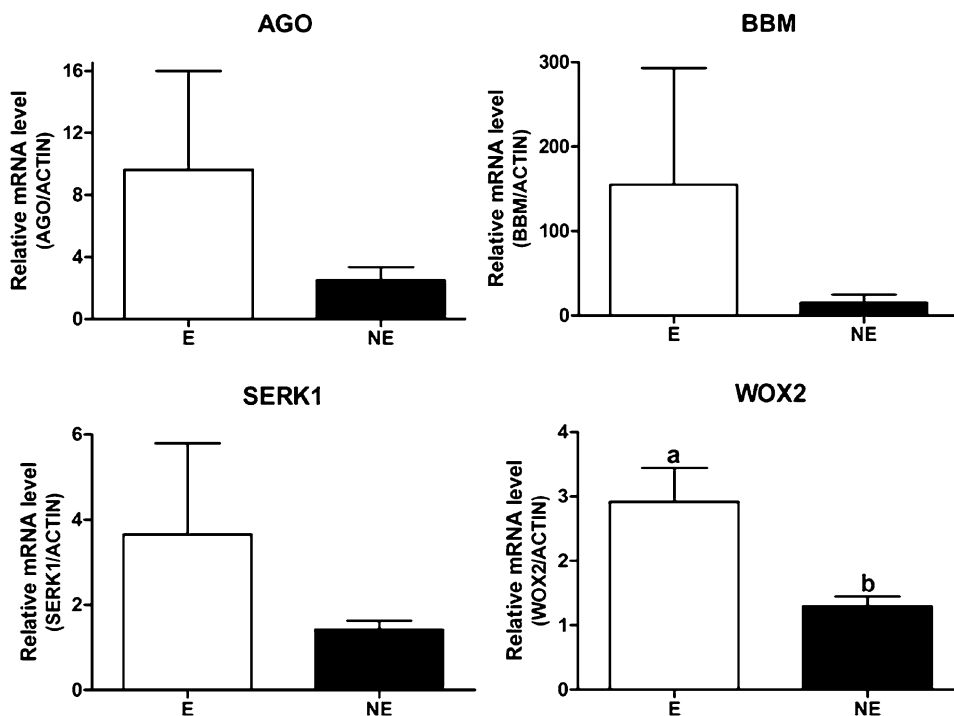
Fig. 2 Transcript detection of candidate genes by conventional RT-PCR. **a** Agarose gel electrophoresis of RNA extracted from E and NE calli. No qualitative or quantitative difference was detected between E and NE material. **b** Transcript detection of candidate genes. Embryogenesis-related genes showed differential expression pattern in E and NE lines. The figure is representative of three independent experiments

quantitative RT-PCR (Figs. 2, 3). The differences observed were not statistically significant, which can be explained by the genotypic heterogeneity of the E lines used. Nevertheless, a tendency to down-regulation was observed in NE lines, which is in accordance with literature. SERK1 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1) is a leucine-rich repeat receptor-like kinase that plays an important role in changing development fate of cells in SE (Hand et al. 2016), having a specific role in stem cell formation and maintenance, being a suitable marker of pluripotency/totipotency and embryogenic competence (Feher et al. 2003; Savona et al. 2012; Altamura et al. 2016). The expression of conifer homolog of SERK1 has been reported in *Araucaria angustifolia*, where it was found to be transcriptionally active in embryogenic cell clusters but not in non-embryogenic cell aggregates (Steiner et al. 2012) and in *Picea glauca*, where it was ubiquitously expressed in embryonal masses and non-embryogenic

callus regenerated from primordial shoots (Klimaszewska et al. 2011). In *Picea balfouriana*, SERK homolog among other genes has been suggested as putative molecular marker of the early stages of somatic embryogenesis (Li et al. 2014a). We detected *P. radiata* SERK 1 homolog by conventional and quantitative RT-PCR (Figs. 2, 3). The results shown a general tendency to down regulation in NE lines, as in the previous reports, but the differences were not statistically significant because of the variability among E lines. The same expression pattern was observed when *P. radiata* AGO (ARGONAUTE) homolog was detected by conventional and quantitative RT-PCR (Figs. 2, 3). AGO genes codify for effectors of RNA silencing, and are able to bind small RNAs and mediate mRNA cleavage, translational repression or DNA methylation (Havecker et al. 2010). The expression of AGO genes during SE has been studied in several gymnosperms (Altamura et al. 2016). In *Picea glauca* and in *A. angustifolia* AGO-like genes were upregulated at early embryo stages and its deregulation resulted in severe embryo abnormalities (Stasolla et al. 2003; Schlogl et al. 2012). The expression of homolog AGO1 is also required for embryo development in *Pinus pinaster* (de Vega-Bartol et al. 2013), *Pinus taeda* (Oh et al. 2008) and *Picea glauca* (Tahir et al. 2006). Our results shown a general tendency to down regulation in NE lines, but the differences were not statistically significant. Taken together, the embryogenesis-related gene expression profile presented by E lines of *P. radiata* is in agreement with the role attributed previously in conifer literature to WOX2, BBM, SERK1 and AGO. The general tendency to down regulation observed in NE lines can explain the manifestation of aberrant histological features. The variability among E lines might be reflecting the different embryogenic potential of each individual line, while NE lines were more homogenous.

In *P. radiata* somatic embryos, β -expansin, Uridilate kinase (SEPR9) and Enolase (SEPR39) have been reported as differentially expressed between E lines and NE lines (Bishop-Hurley et al. 2003; Aquea and Arce-Johnson 2008). Our results showed (Fig. 2b) that these genes are equally expressed in E and NE lines. The discrepancy with previous report can be explained by the use of different sources of explant, since NE lines used in the present work were obtained from immature zygotic embryos, which can be closer to the embryogenic pattern of expression than the NE lines derived from needle of *P. radiata* by Aquea and Arce-Johnson (2008). In *Pinus* species adult tissues are considered as recalcitrant with regard to the initiation of somatic embryogenesis (Bishop-Hurley et al. 2003). The genes ACTIN, UBIQUITIN, and GAPDH were stably expressed in E and NE lines and any of them can be used as normalizing gene for qRT-PCR assays.

Fig. 3 Transcript levels of embryogenesis-related genes detected by qRT-PCR. The transcript abundance of embryogenesis-related genes AGO, BBM, SERK1 and WOX2, tend to be lower in NE compared with E lines, although this difference was statistically significant for WOX2. The figure is representative of three independent experiments



Differences in genomic DNA conformation and in global DNA methylation between E and NE lines

As result of the application of DNA extraction protocol, mean values of extracted DNA for E lines were three times higher than NE lines ($p=0.001$). These differences were confirmed using a larger number of samples and other methods for DNA extraction (data not shown). To further investigate these unexpected differences, equal amount of genomic DNA were subjected to FT-IR microspectroscopy, a well-validated technique to detect subtle structural changes occurring at chemical level in DNA molecules (Taillandier and Liquier 1992; Banyay et al. 2003; Whelan et al. 2011). The spectra obtained in the range between 1800 and 800 cm^{-1} represent the IR vibration peaks of the bonds present in DNA samples of E and NE lines (Supplementary Fig. S2). Band assignment was made for each peak (Supplementary Table 2). The comparison between E and NE spectra showed main differences detected at 1408, 1220 and 964 cm^{-1} , related to different DNA conformation. Spectra obtained from E lines presented 2 markers of B-form of DNA, that are absent in NE samples. In turn, NE samples only presented a marker of Z-form of DNA (Table 1). These differences in DNA structure could explain the unexpected differential output to the extraction protocol between E and NE lines, since it was designed for the most common conformation, which is B-DNA. The biological significance of Z-DNA (the left handed double helical version) has not yet been clarified (Li et al. 2014b), but is believe to play important roles in gene expression and regulation (Bothe

Table 1 DNA conformation markers in E and NE samples

Conformation marker	Wave number (cm^{-1})	E	NE
Z-form	1408	-	+
B-form	1220	+	-
B-form	964	+	-

et al. 2012). In human cancer, the epigenetic regulation by Z-DNA silencing has been described as occurring by a mechanism that involves methyl-CpG-binding protein (Ray et al. 2013). Transcription factors that bind specifically to sequences that form Z-DNA have been described in plant model *Arabidopsis* (Gangappa et al. 2013). To our knowledge, this is the first report describing Z-DNA conformation in conifers.

It has been previously reported that the formation of Z-DNA is enhanced by DNA methylation (Zacharias et al. 1990; Temiz et al. 2012). Methylation of DNA has been associated with transcriptional silencing and is considered a determining factor in the imposition and maintenance of the ontogenic developmental stages, being essential for correct plant development to such extent that variation in DNA methylation profile lead to abnormal development patterns, such as non-differentiating callus (Lambé et al. 1997; Viejo et al. 2010). Global content of DNA methylation was quantified in 3 E and 3 NE lines by ELISA assay. By using this technique, NE lines DNA was shown to be hypermethylated (Fig. 4). This difference

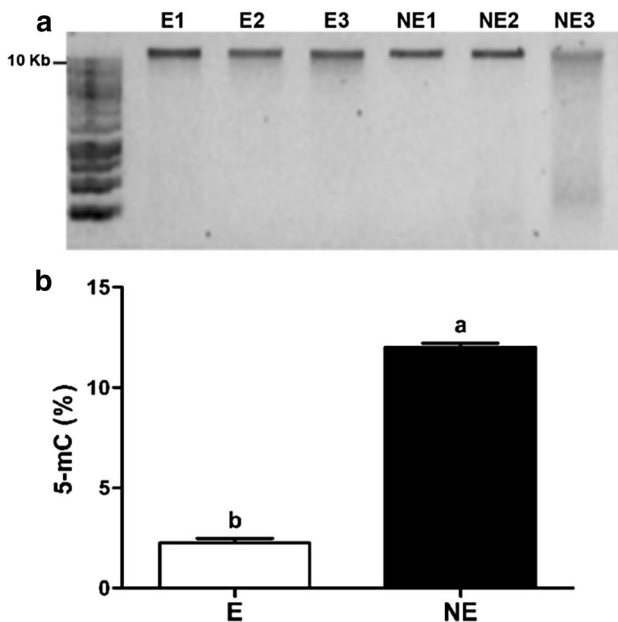


Fig. 4 Global DNA methylation of E and NE samples. **a** Agarose gel electrophoresis of genomic DNA samples used as input material to detect the percentage of methylated cytosines (5-mC%) in E and NE lines. The quantity of DNA was equalized as much as possible. The same samples were used for FT-IR assays. **b** Global DNA methylation expressed as 5-mC%. Compared with E lines, the levels of DNA methylation were statistically significantly lower in NE samples. The figure is representative of three independent experiments. Bars represent the mean of three E and 3 NE lines with their respective standard error

was statistically significant ($p=0.0001$). Several recent reports in SE of different conifer species have suggested causal relationships between global DNA methylation and in vitro morphogenetic competence and proliferation (LoSchiavo et al. 1989; Causevic et al. 2005; Klimaszewska et al. 2009; Noceda et al. 2009; Tyssier et al. 2014; Fraga et al. 2016). In *Pinus nigra*, by quantification of global genomic DNA methylation levels, it was revealed the existence of specific DNA methylation levels for particular embryogenic potentials, where the line considered embryogenic showed the lowest methylation levels (Noceda et al. 2009). Hypermethylation of DNA has been also associated with a fail in expression of key genes, impairing embryo formation and embryogenic potential (Valledor et al. 2007). Recently, it has been reported that the changes in DNA methylation patterns are associated with the regulation of genes involved in SE such as WUS and BBM among others, indicating that DNA hypomethylation is involved in the signal that leads to the induction of SE (De la Peña et al. 2015). Therefore, our results are in agreement with the previously assigned role to DNA methylation in early embryogenesis.

Concluding remarks

Deciphering the molecular determinants for SE can contribute to revealing the genetic program underlying the phenomenon of stem cell totipotency and pluripotency, and somatic embryo formation (Altamura et al. 2016). Understanding these fundamental molecular events could guide the improvement of the rate of success in the first stages of SE, which is not only important for developing varietal lines, but is critical for the management of genetic diversity, genetic engineering and molecular tree breeding (Park et al. 2006).

Taken together, our results highlight the contribution of epigenetic mechanisms such as DNA methylation to regulate the expression of key developmental-related genes, which is critical for the formation of proper embryos in SE. Besides, our work may offer valuable molecular tools for evaluating embryogenic potential of *P. radiata* SE cultures.

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Authors contributions S. B. and R. H. planned and designed the research, analyzed and interpreted the results and wrote the manuscript. A. B did the DNA methylation experiments. A.T. extracted DNA and acquired FT-IR data. F.S. was responsible for conventional RT-PCR assays. P.J. performed qRT-PCR analysis. M. J. P. obtained micro-morphological data. All the authors participated in drafting or revising the work and approved the final manuscript in agreement with all its aspects.

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