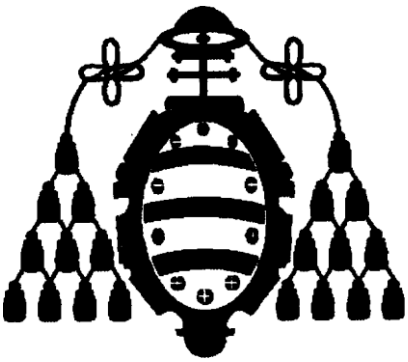


UNIVERSIDAD DE OVIEDO

Departamento de Biología de Organismos y Sistemas

Programa de Doctorado: "Biología aplicada a la sostenibilidad de recursos naturales (Mención de calidad)"

"Expresión Génica y Dinámica de Metilación del ADN en Procesos de Organogénesis Adventicia en *Pinus radiata* D. Don"

"Gene Expression and DNA Methylation Dynamics in Adventitious Organogenesis Processes in *Pinus radiata* D. Don"

TESIS DOCTORAL

José Luis Rodríguez Lorenzo

Oviedo 2013

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Expresión Génica y Dinámica de Metilación del ADN en Procesos de Organogénesis Adventicia en *Pinus radiata* D. Don	Inglés: Gene Expression and DNA Methylation Dynamics in Adventitious Organogenesis Processes in *Pinus radiata* D. Don

RESUMEN (en español)

Las coníferas representan más del 50 % de la masa forestal del planeta y debido a que los bosques juegan un papel crucial en el cambio climático, la multiplicación de genotipos élite a través del cultivo *in vitro* se considera la técnica más eficaz. Estas técnicas son una posible fuente de variación epigenética que regula selectivamente la activación o inactivación de la expresión génica, y la metilación del ADN se encuentra entre los diferentes mecanismos epigenéticos existentes.

Teniendo en cuenta estos antecedentes, en este estudio se aborda la regulación epigenética de la organogénesis adventicia inducida por la citoquinina bencil adenina (BA) en embriones zigóticos de *Pinus radiata*. El papel de la metilación del ADN y la acetilación de la histona H4 como reguladores de la organogénesis adventicia se comparó con otros programas de desarrollo como la rizogénesis adventicia o la formación de callo inducida por auxinas y la inhibición de la germinación por el ácido abscísico. Se evaluaron los niveles de 5-metil citosina global y la cantidad de Histona H4 acetilada y su distribución espacio-temporal mediante inmunolocalización. Se encontraron diferencias en las dos marcas epigenéticas en los embriones cultivados con auxinas y BA a lo largo del tiempo debido a un desarrollo diferencial inducido por los reguladores del crecimiento. En cuanto a la distribución de estas marcas en el tejido, los embriones cultivados en BA tienen una metilación uniforme en todo el embrión que va descendiendo según aumenta la acetilación de H4, especialmente en las nuevas yemas

en formación. En los embriones cultivados con auxinas se observan cambios muy drásticos en las marcas epigenéticas de los meristemos radicular y caulinar, regiones muy sensibles a la señalización por reguladores del crecimiento.

El análisis de la expresión diferencial de genes relacionados con la caulogénesis nos permitió observar una inducción de las funciones relacionadas con el metabolismo, encontrando diferentes genes inducidos y relacionados con estas funciones como RuBIsCO, LTP1 y α-tubulina 1, así como otros genes relacionados con la fase de adquisición de competencia y fotomorfogénesis inducida por este regulador del crecimiento. Debido al importante papel en la transmisión de señal de mecanismos de defensa y senescencia asociados a las citoquininas, varios genes relacionados con estrés tenían una expresión inducida en embriones cultivados en ABA, pero estaban reprimidos en BA.

Por último se constató una regulación mediante metilación del ADN en los genes identificados como diferenciales mediante un desenmascaramiento químico en cultivos celulares con 5-Aza-2-deoxicitidina mediante una inmunoprecipitación de ADN metilado (MeDIP). Así mismo se identificaron nuevos genes candidatos mediante la hibridación de un dot blot en esta misma situación experimental. Los resultados obtenidos mostraron la inducción de 25S ARNr y ERD15, secuencias relacionadas con la transducción de señal hormonal frente a estímulos externos, en el tratamiento con el agente demetilante. El enriquecimiento diferencial de las secuencias metiladas a través de MeDIP nos permitió identificar nuevas secuencias reguladas por metilación como un transposon y varios genes relacionados con estrés como LTP-1, PR-10 y XB3. En los cultivos celulares utilizados como control se identificaron dos grupos principales de

genes de acuerdo a su función biológica y un gen relacionado con desarrollo del meristemo como es *PROGRAMA DEL MERISTEMO ALTERADO 1 (AMP1)* que regula la formación del embrión en *Arabidopsis*. El primer grupo está relacionado con estrés en el retículo endoplasmático, cuya represión podría tener su significado con la inducción de ERD15 en el tratamiento con 5-Aza-2-deoxicitidina. El segundo grupo se compone de genes relacionados con fotosíntesis y transporte de electrones los cuales son reprimidos por 5-Aza-2-deoxicitidina probablemente debido a su posible acción en los genes nucleares del cloroplasto.

RESUMEN (en Inglés)

Conifers represent more than 50% of mass forest on the planet and because forests play a crucial role in climate change, the multiplication of elite genotypes by *in vitro* culture are considered the most effective techniques. These techniques are considered to be a possible source of epigenetic variation that selectively regulates epigenetic activation or inactivation of gene expression, and DNA methylation is among the existing different epigenetic mechanisms.

Keeping in mind this background, this study addresses epigenetic regulation of adventitious organogenesis induced by cytokinin benzyl adenine (BA) in *Pinus radiata* zygotic embryos. The role of DNA methylation and acetylation of histone H4 as regulators of adventitious caulogenesis was compared to other developmental programs such as auxin-induced adventitious rooting and callus formation, and inhibition of germination by abscisic acid. The levels of global 5-methyl cytosine, and the amount of acetylated histone H4 and their spatio-temporal distribution by immunolocalization were assessed. Several differences were found in both epigenetic marks on embryos cultured with auxin and BA along time due to differential development induced by

growth regulators. Regarding the distribution of these marks in the tissue, embryos cultured with BA showed a uniform methylation throughout the embryo that decreases at the same time that H4 acetylation show up, especially in new-formed buds. Those embryos cultured with auxins had differences mainly in root and shoot meristems whose cells are highly methylated in normal development but they are very sensitive to growth regulators.

A later expression analysis of different genes involved in caulogenesis allowed us to observe an induction of metabolism-related functions, finding different genes related to these functions such as RuBIsCO, LTP1 1 and α-tubulin, and other genes related to acquisition of competence and photomorphogenesis induced by this growth regulator. Because of the important role in defense and senescence signal transduction associated to cytokinins, several stress-related genes were induced in ABA cultured embryos, but they were repressed in BA.

Finally, it was found a regulation by DNA methylation in the genes previously identified as differential by chemical unmasking in cell cultures in the presence of demethylating agent 5-Aza-2-deoxycytidine by methylated DNA immunoprecipitation (MeDIP). In the same way candidate genes were identified by dot blot hybridization in the same experimental situation. The results showed the induction of 25S rRNA and ERD15, sequences related to hormonal signal transduction of external stimuli, in the treatment with the demethylating agent. Differential enrichment of methylated DNA assessed by MeDIP revealed new sequences like a transposon and various stress-related genes such as LTP-1, PR-10 and XB3 as regulated by methylation. In control cell cultures two main groups of genes, according to their biological function, and a gene

related to meristem development and organ formation patterns like AMP1 that regulates embryo formation in *Arabidopsis* were identified. The first group related to stress in the endoplasmic reticulum and their downregulation could be connected to ERD15 induction in 5-Aza-2-deoxycytidine mitigating the stress situation. The second group related to photosynthesis and electron transport both of them repressed by of 5-Aza-2-deoxycytidine because of its action in nuclear genes of the chloroplast.

Esta Tesis ha sido realizada en:

Universidad de Oviedo

Departamento de Biología de Organismo y Sistemas

Área de Fisiología Vegetal

Instituto Universitario de Biotecnología de

Asturias

Con la siguiente financiación:

Beca para la Formación de Personal Investigador:

BES-2005-7807

Proyecto AGL-2004-00810:

Impacto de la maduración-envejecimiento en la clonación de especies agroforestales: Sistemas de multiplicación, competencia-expresión de genes candidatos y validación de los regenerantes

Proyecto AGL2007-62907:

Código epigenético y regulación de subproteomas durante el envejecimiento-revigorización de especies agroforestales

Fondo Social Europeo

Fondo Europeo de Desarrollo Regional

Agradecimientos

Hace unos días enseñé a mi hermana un borrador de esta tesis y me comentó, ¡Ah! es eso. Me imagino que se esperaba un ejemplar encuadernado y con la portada definitiva, pero cuando vio el interior (aunque sólo fuera porque estaba escrita en inglés) y las gráficas y fotos, comprendió que había detrás un trabajo y un esfuerzo no sólo mío, sino de mucha otra gente que directa o indirectamente me ha ayudado a que se lleve a cabo.

En primer lugar, me gustaría agradecer a mis directores de Tesis, la Dra. María Jesús Cañal Villanueva y el Dr. Luis Valledor González, su apoyo y su guía además del tiempo compartido en el laboratorio. También me gustaría incluir al Dr. Roberto Rodríguez que formó parte de este proyecto durante todos estos años.

Quisiera también agradecer a todos los profesores del área de Fisiología Vegetal su amistad y su ayuda siempre que he necesitado algo, ya tuviera que ver con el desarrollo de esta tesis o fuera simple curiosidad.

A mis compañeros de laboratorio tanto los pasados como los presentes, y aquellos que estuvieron por aquí de manera temporal, que no han sido pocos, por su ayuda en el laboratorio, su amistad y esos buenos ratos con el Q3A, poniendo a punto el programa espacial, las noches de fiesta, las escapadas a "muestrear" y un largo etcétera de momentos que cruzan mi mente en estos momentos.

A los técnicos de laboratorio de Fisiología Vegetal y del resto de Áreas por su ayuda y remedios artesanales en algunos casos que acababan resolviendo el problema.

Al personal de Administración y Servicios y demás profesores del Departamento por su amabilidad y consejo cuando estaba en algún callejón sin salida gracias a su experiencia en otros campos.

Al personal de los Servicios Científico-Técnicos, tanto microscopía como secuenciación y espectrometría de masas que me ayudaron muchas veces más allá de sus obligaciones.

A mis amigos de la meseta de Medina y de Salamanca, que aunque eso de la tesis no les importara mucho siempre tenían en la boca, a ver si acabas ya.

A mi familia, mis padres y hermanos que siempre han creído en mí y me apoyaron cuando las dudas me asaltaban, mis tíos que siempre me preguntaban qué hacía y siempre se tragaban el rollo que les contaba.

A mi familia basauritarra, que llegaron al final del viaje pero siempre estuvieron expectantes de que llegara a buen puerto.

A mi tercera familia, la felina, y a Isora.

Y por último a Nuria, que aunque no vivió este trabajo desde el principio, si vivió la parte más intensa y su ayuda desde que la conocí ha mejorado, sin duda, esta tesis y a mí como persona, y espero que siga haciéndolo.

MUCHAS GRACIAS A TODOS.

Abbreviations

2, 4-D	2,4-Dichlorophenoxyacetic acid
5mC	5-methylcytosine
ABA	Abscisic acid
AcH4	Acetylated H4 histone
AMP1	*ALTERED MERISTEM PROGRAM 1*
ARR	*ARABIDOPSIS RESPONSE REGULATOR*
BA	Benzyladenine
BLASTx	Basic local alignment search tool
BSA	Bovine serum albumin
CER2	*ECERIFERUM2*
CHAPS	3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate
ChIP	Chromatin immunoprecipitation
CLV1-3	*CLAVATA 1-3*
CMT3	*CHROMOMETHYLASE 3*
CRE1	*CYTOKININ RESPONSE 1*
CRK1	*CYTOKININ REGULATED KINASE 1*
CUC1-2	*CUP SHAPE COTYLEDON 1-3*
DAPI	(4’, 6-diamidino-2-phenylindole)
DIG-UTP	Digoxigenin-Uridine-5'-triphosphate
DML3	*DEMETER-LIKE 3*
DRM2-3	*DOMAINS REARRANGED METHYLTRANSFERASE 2-3*
DTT	Dithiothreitol
E0	Mature zygotic embryo
EDTA	Ethylenediaminetetraacetic acid

EMP24/GP25L	*ENDOMEMBRANE PROTEIN PRECURSOR OF 24 KD*
ERD15	*EARLY RESPONSE TO DEHYDRATION 15*
ESR1	*ENHANCER OF SHOOT REGENERATION 1*
FDA	Fluorescein diacetate 6-isothiocyanate
HDAC	Histone deacetylase
HDM	Histone demethylase
HMT	Histone methyl transferase
HSP70	Heat shock protein 70
IBA	Indole-butyric acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KEGG	Kyoto encyclopedy of genes and genomes
KNAT1	*KNOTTED-1 LIKE*
KNOX	*KNOTTED-like homeobox*
LTP1	Lipid transfer protein 1
MeDIP	Methylated DNA immuno precipitation
MET1	*METHYLTRANSFERASE 1*
MS	Murashige-Skoog
NAM	*NO APICAL MERISTEM*
PBS	Phosphate-buffered saline
PCA	Principal Component analysis
PIN	*PIN-FORMED*
PMF	Peptide mass fingerprint
PMSF	Phenylmethylsulfonyl fluoride
PR-10	Pathogenesis-related 10
PRAABA	Pinus radiata abscisic acid library

PRABA	Pinus radiata benzyl adenine library
PRACABA	Pinus radiata abscisic acid control library
PRACBA	Pinus radiata benzyl adenine control library
PsbQ	*PHOTOSYSTEM II SUBUNIT Q*
PTMs	Post-translational modifications
QC	Quiescent center
QLP medium	Quoirin Lepoivre medium macronutrients plus Murashige and Skoog medium micronutrients, vitamins and iron
RAM	Root apical meristem
RdDM	RNA-directed DNA methylation
RdRP	RNA-dependent RNA polymerase
RNAseq	Ribonucleic acid sequencing
RNP-CS	Ribonucleoprotein consensus sequence
RT-qPCR	Real time quantitative polymerase chain reaction
RuBIsCO small	Ribulose-1,5-bisphosphate carboxylase oxygenase small subunit
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSH	Suppressive subtractive hybridization
STM	*SHOOTMERISTEMLESS*
Tm	Melting temperature
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
WUS	*WUSCHEL*
XB3	Xa21-binding protein 3
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Index

Chapter 1. Introduction. 1

1.1. Economical and ecological importance of forests 1

1.2. Clonal propagation techniques in forestry species 2

1.3. Clonal propagation: Adventitious organogenesis 2

1.4. Epigenetic mechanisms of DNA regulation 4

1.4.1. DNA methylation 5

1.4.2. Post-translational modifications (PTMs) of histones. 6

1.4.5. Non-coding RNAs 8

1.4.6. DNA methylation and its relationship with other epigenetic modifications 9

1.5. DNA methylation in plants 10

1.5.1. DNA methyltransferases in plants 10

1.6. DNA methylation in plant development 11

1.6.1. DNA methylation in in vitro culture 12

1.7. Basic procedures for epigenetic analysis in plant cell and tissue culture 13

1.7.1. DNA methylation. 13

1.7.2. Chromatin modifications. 16

1.8. Objectives and experimental design 18

1.9. References 20

Chapter 2. DNA Methylation and H4 Histone Acetylation Landscapes Induced for Plant Growth Regulators in Pinus radiata D. Don Cotyledons 27

2.1. Introduction 27

2.2. Materials and methods 28

2.2.1. Plant material 28

2.2.2. Quantification of global DNA methylation 29

2.2.3. Quantification of total acetylated H4 Histone 30

2.2.4. Immunolocalization 31

2.2.5. Statistical analysis 32

2.3. Results 33

2.3.1. Macromorphological traits 33

2.3.2. Global DNA methylation during embryogenesis 35

2.3.3. Global DNA methylation during adventitious caulogenesis and other responses 36

2.3.4. Acetylated H4 Histone content and DNA methylation comparison 37

2.3.5. Immunohistochemistry 39

2.4. Discussion 48

2.4.1. Global methylation 48

2.4.2. AcH4 content 49

2.4.3. Immunolocalization 49

2.5. Conclusions 51

2.6. References 51

CHAPTER 3. Differential Expression Analysis and Identification of Candidate Genes Involved in Adventitious Organogenesis 55

3.1. Introduction 55

3.2. Methods 56

3.2.1. Plant Material. 56

3.2.2. In vitro culture 56

3.2.3. RNA extraction and amplification. 57

3.2.4. SSH library construction and sequencing. 57

3.2.5. Dot Blot Macroarray. 58

3.2.6. Real Time Expression 59

3.2.7. Statistical Analysis 59

3.3. Results 60

3.3.1. Characterization of suppressive subtractive hybridization libraries from P. radiata embryos 60

3.3.2. Dot Blot Data Analysis 62

3.3.3. Validation of dot-blot quantitations by RT-qPCR. 68

3.4. Discussion 71

3.4.1. Gene annotation and biological function 71

3.4.2. Transcriptional profile induced by BA and ABA 71

3.5. Conclusions 74

3.6. Appendix 75

3.7. References 88

Chapter 4. Chemical Unmasking and Involvement of Organogenic Candidate Genes Regulated by DNA Methylation in Undifferentiated Cells of Pinus radiata D. Don 91

4.1. Introduction 91

4.2. Methods 92

4.2.1. Plant Material. 92

4.2.2. In vitro culture and DNA methylation settings 92

4.2.3. Methylated DNA Immunoprecipitation 95

4.2.4. Quantitative PCR. 97

4.2.5. Dot blot macroarray. 97

4.2.6. Statistical analysis 98

4.3. Results 98

4.3.1. Cell suspension and DNA methylation settings 98

4.3.2. Methylation analysis by DNA immunoprecipitation 100

4.3.3. Dot blot data analysis 103

4.4. Discussion 105

4.4.1. Cell suspension culture and global DNA demethylation 105

4.4.2. Demethylated and upregulated genes by 5-Aza-2-deoxycytidine 106

4.4.3. Downregulated genes by 5-Aza-2-deoxycytidine 108

4.5. Conclusions 110

4.6. Appendix 111

4.7. References 121

5. **General discussion** 125

5.1. References 129

6. **Conclusions** 133

7. **Resumen** 135

8. **Conclusiones** 140

Chapter 1. Introduction.

1.1. Economical and ecological importance of forests

The world's total forest area was just over 4 billion hectares, corresponding to 31 percent of the total land area or an average of 0.6 ha per capita and five countries (the Russian Federation, Brazil, Canada, the United States of America and China) have more than half of the total forest area (Fig. 1.1) (Hansen et al. 2010). Thirty percent of the world's forests (1.2 billion hectares) are primarily used for production of wood and non-wood forest products, constituting conifers around the 50 % of these resources (FAO 2010). In addition, forests play a crucial role in climate change being included in Kyoto protocol because they contribute in the reduction of CO_2 emissions and they hold an amount of carbon greater than atmosphere (FAO 2011). Conifers represent more than 50 % of forestry area in the world and, in particular, in Spain the 94.4 % of this area belongs to *Pinus* genus being *Pinus radiata* D. Don the third most important species (http://wwwsp.inia.es/Investigacion/centros/CIFOR/redes/Selvired/Paginas/Introduccion.aspx).

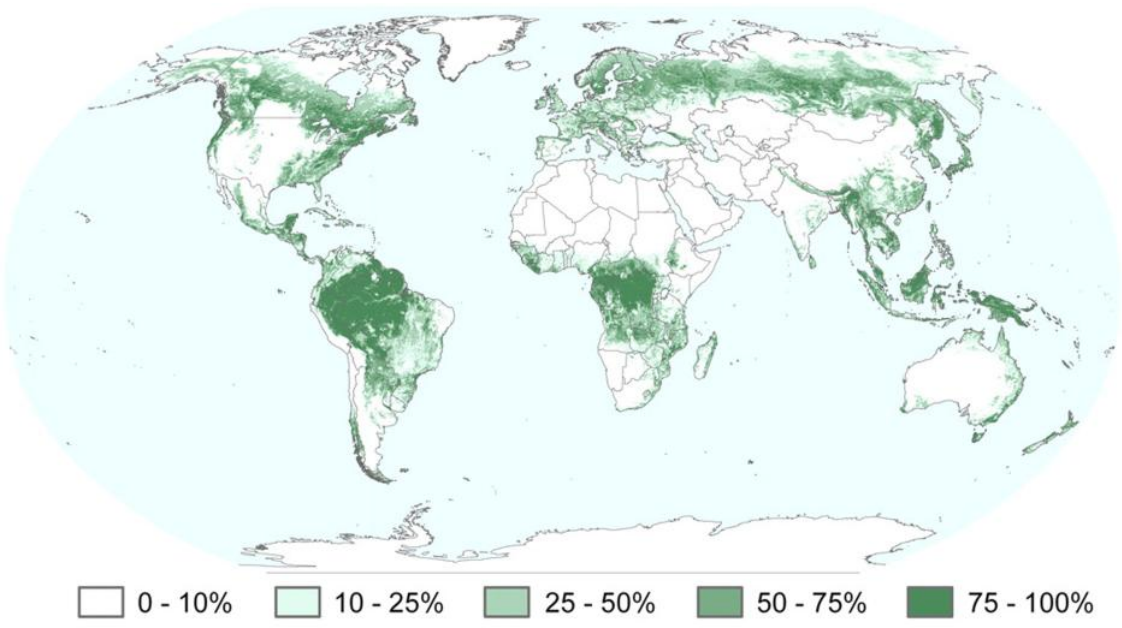


Figure 1.1: Estimated percent forest cover year 2005, from Hansen et al. (2010).

Given that *Pinus radiata* is characterized by its high cellulose and timber production, it is important to introduce this species in breeding programs to facilitate its selection and to clonally propagate elite individuals.

1.2. Clonal propagation techniques in forestry species

Up to now, clonal propagation of pinus species by *in vitro* adventitious organogenesis from zygotic embryos is the most efficient technique to get clonal individuals, considering that no high efficiency protocols have been developed from adult explants because of its low rooting rate and genotype-dependent response (Cortizo et al. 2009). Phase change, the transition from vegetative to reproductive growth, is greatly the main factor responsible for the low rooting and response of explants *in vitro* cultured and is considered the gate from juvenility to maturity (von Aderkas and Bonga 2000). *In vitro* rejuvenation protocols like chemical treatments, pruning or grafting, are considered an alternative to tackle the problem of explant response and rooting, and many efforts have been focused in this matter (Read and Bavougian 2012). Somatic embryogenesis as a source of mass embryo production is far from being considered the ultimate technique in clonal propagation. Mainly, its dependence of genotype during callus induction, proper embryo maturation or plantlet establishment, are issues that must be better known (Montalbán et al. 2010).

1.3. Clonal propagation: Adventitious organogenesis

Physiology and organ formation in clonal propagation are subjects of continuous investigation, and new molecular aspects of adventitious organogenesis are discovered constantly (DeYoung et al. 2005; Katayama et al. 2010), as well as developing of new induction protocols in the case of forestry species (De Diego et al. 2011).

In adventitious caulogenesis, somatic cells respond to hormonal stress by reprogramming processes that induce shoot formation, being cytokinins the most efficient growth regulators inducing caulogenesis. Cytokinin signal transduction has been deeply

studied and the identification of different genes involved in meristem formation make possible to propose an interplay model for its regulation and the interaction with the growth regulators auxin and gibberelic acid (Figure 1.2). Arabidopsis, as well as for many other developmental processes, has been the study model for indirect caulogenesis and most of the important findings belonging to this process have been found in mutants developed in this species.

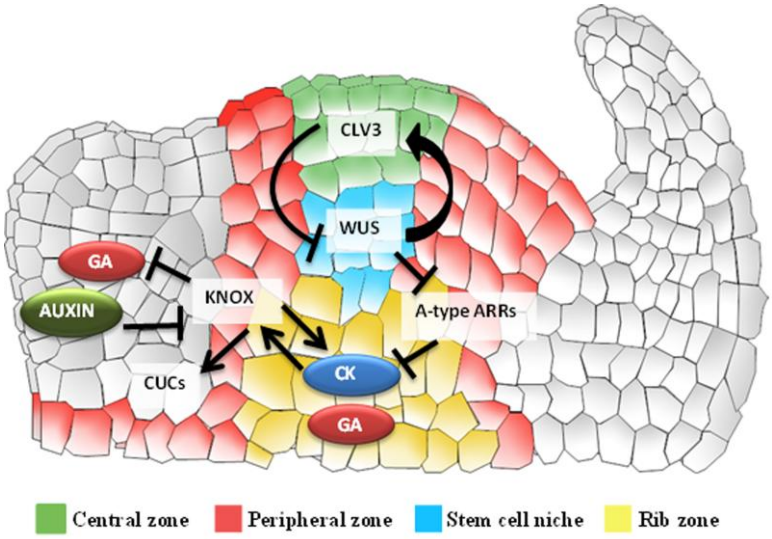


Figure 1.2: Schematic view of the interplay between multiple hormonal pathways and transcription factors at the shoot apical meristem (SAM).

WUSCHEL (WUS) is expressed at the base of the central zone (CZ) and interacts with the *CLAVATA (CLV)* receptor kinase pathway to establish a feedback loop between the stem cells and the underlying organizing center which regulates meristem size.

KNOTTED1-like homeoboxes (*KNOXs*) regulate meristem function, maintaining high cytokinin (CK) levels in the CZ to keep the cells in a meristematic state. *KNOXs* are downregulated at specific sites in the peripheral zone (PZ) to allow organ primordia formation. At the organ boundaries, KNOXs activate *CUP-SHAPED COTYLEDON (CUC)* expression to maintain the spatial separation of the meristem zones.

Undifferentiated cell fate at the SAM CZ is maintained by a high regimen of CK and a low concentration/signaling of auxin and gibberellins (GAs) that promote cell differentiation at the PZ and in the rib meristem.

ARABIDOPSIS RESPONSE REGULATORS (ARR) act as mediators between WUS and cytokinins as negative regulators.

Modified from Di Giacomo et al. (2013)

The Shoot Apical Meristem (SAM) is a structure where the relative expression and homeostasis of the different genes involved in its maintenance allow the existence of undifferentiated stem cells that will enable new organ formation and radial/longitudinal growth (Heisler and Jönsson 2007; Takeda and Aida 2011). The formation of the *de novo* shoot meristem involves a similar degree of patterning and cell organization to that of the embryonic shoot apical meristem (Gordon et al. 2007).

Previous studies have observed that histidine protein kinases act as cytokinin receptors, whereas histidine fosfotransferases transmit the signal to nucleus response regulators which activate or suppress the transcription (Zhang and Lemaux 2004). For example, genes such as *CYTOKININ RESPONSE 1 (CRE1),* an hormone receptor (Inoue et al. 2001) and *ENHANCER OF SHOOT REGENERATION (ESR1),* which over-expression allows shoot development (Fujimoto et al. 2000), or some family members of *NAM (NO APICAL MERISTEM)* and *CUC1 y CUC2 (CUP SHAPE COTYLEDON)* (Aida et al. 1997; Aida et al. 1999; Takada et al. 2001) are involved in new bud formation. The accumulation of endogenous cytokinins by transgenic plants or mutants like *ALTERED MERISTEM PROGRAM 1 (AMP1),* triggered the over expression of different genes such as *STM (SHOOTMERISTEMLESS) or KNAT1 (KNOTTED-1 LIKE)* that possess a domain induced by cytokinins and belongs to *KNOX* family, an important homeodomain transcription factors (Rupp et al. 2002; Hay and Tsiantis 2010).

WUSCHEL (WUS) is a homeotic transcription factor expressed in the cells in the organizing center, establishing and maintaining stem cell population (Mayer et al. 1998). It is thought to be a key gene given that its expression rockets during phase of shoot determination (Che et al. 2002). A family of genes named *ARABIDOPSIS RESPONSE REGULATOR (ARR)* act as mediators between *WUS* and cytokinins. There are two types of *ARR*, type A acting as negative regulators and they are proposed to be involved in adventitious organogenesis and type B acting as positive regulators (Buechel et al. 2010; Cortizo et al. 2010). Another family of genes regulating *WUS* is *CLAVATA (CLV)* by inhibiting *WUS* expression and controlling cell proliferation, keeping *WUS* expression in the stem cell niche (Durbak et al. 2012).

1.4. Epigenetic mechanisms of DNA regulation

Epigenetics is defined as “heritable changes in gene expression that occur without a change in DNA sequence” (Wolffe and Matzke 1999), and can be understood as a system to selectively regulate genome information through activating or inactivating gene expression.

At a molecular level, DNA methylation, post-translational histone modifications, chromatin remodelling factors, transcriptional factors, non-coding RNAs and chromosomal proteins cooperate together. Although the mechanisms of chromatin regulation are less studied in plants, advances in their research have shown they share similarities with other eukaryotes in chromatin remodelling factors, like histone acetylation/deacetylation and methylation/demethylation, and DNA methylation/demethylation at gene level as well (Li et al. 2002). Non-coding RNAs are players in epigenetic mechanisms that are still not completely understood, although RNAs were recently implicated in new mechanisms of genetic information transfer in yeast, plants and mice (Zhang et al. 2012).

Chromatin is not just a static method for packing DNA, but also a dynamic strategy that cells use to respond to environmental stimuli (Bräutigam et al. 2013). The precise control of chromatin modification in response to developmental and environmental cues determines the correct spatial and temporal expression of genes (Smith and Meissner 2013).

The differences among epigenetic modifications are a precise crosstalk. Variations in DNA methylation have an effect in histone deacetylation and methylation, affecting chromatin structure in animals (Okitsu and Hsieh 2007; Cedar and Bergman 2009) and plants (Tariq and Paszkowski 2004). To understand their influence in a specific physiological situation, an overview of the different modifications with a correct interpretation of the individual data must be done.

1.4.1. DNA methylation

Methylation of the fifth carbon of cytosine residues is one of the most extensively studied epigenetic modifications in both, plants and mammals. Cytosine methylation is mostly limited to CG dinucleotide sequence contexts in mammalian genomes, except for embryonic stem cells in which 25% of cytosine methylations are found in a non-CG context (Lister and Ecker 2009). In plants methylated cytosines can be additionally found in CNG and CNN sequence contexts (N = A, C or T) throughout their genomes (Cokus et al. 2008). The primary

role of cytosine methylation in higher eukaryotes was proposed as a genome defense system to protect genomes against both endogenous selfish DNA elements (predominantly transposable elements or TEs) and exogenous virus invasions (Penterman et al. 2007). Nowadays DNA methylation has been found that is involved in long-term expression stability in genes related to cell cycle and in many developmental processes (Smith and Meissner 2013). DNA methyltransferases are the enzymes that catalyze the transfer of a methyl group from S-adenosyl-L-methionine to cytosine or adenine bases in DNA. Several DNA methyltransferases have been found in bacteria, fungi, plants, and mammals with different functions depending on the organism (Table 1.1) (Jeltsch 2002).

Table 1.1: Typical DNA MTases from various organisms. Adapted from Jeltsch (2002)

	Recognition sequence	Classification
Prokaryotic enzymes		
M.HhaI	G<u>C</u>GC	cytosine-C5
M.HaeIII	GG<u>C</u>C	cytosine-C5
M.EcoRV	G<u>A</u>TAC	adenine-N^6 (α type)
E. coli dam	G<u>A</u>TC	adenine-N^6 (α type)
M.PvuII	CAG<u>C</u>TG	cytosine-N^4 (β type)
M.TaqI	TCG<u>A</u>	adenine-N^6 (γ type)
Eukaryotic enzymes		
Dnmt1 (mouse)	<u>C</u>G	cytosine-C5
Dnmt2 (mouse)	?	?
Dnmt3a (mouse)	<u>C</u>G	cytosine-C5
Dnmt3b (mouse)	<u>C</u>G	cytosine-C5
Met1 (Arabidopsis)	<u>C</u>G	cytosine-C5
CMT3 (Arabidopsis)	<u>C</u>NG	cytosine-C5
DRM2 (Arabidopsis)	?	cytosine-C5 ?
Masc1 (Fungus)	?	cytosine-C5
Masc2 (Fungus)	?	cytosine-C5

1.4.2. Post-translational modifications (PTMs) of histones.

Histone methylation plays an essential role in diverse biological processes, ranging from transcriptional regulation to heterochromatin formation. As one of the most complex modifications, it not only occurs at different residues (lysine and arginine) and distinct sites, but also differs in the number of methyl groups added. Unbiased mass spectrometry in combination with high-performance liquid chromatography (HPLC) separation, has been used to identify the histone modification profile in Arabidopsis revealing both, conserved and non-

conserved modifications compared to animals (Zhang et al. 2007). Studies on plant histone acetylation started in the 1960's when classical degradation protein sequencing was applied to purified histone H3 and H4 by Bonner et al. (1968). Histones were purified from germinating peas (*Pisum sativum*) and calf thymus (Fambrough et al. 1968), and sequencing of chromatographically purified proteolytic peptides, established for the first time the primary protein sequence of histone H4 (DeLange et al. 1969). H4 acetylation was discovered at lysine 16, 5, 8 and 12, and H4 methylation at lysine 20. Identification of acetylated lysines in histone H3 came next, and gradually there was the confirmation that lysine residues 4, 9, 14, 18, 23 and 27 were all targets for acetylation in plant H3 histones. Remarkably, plant histone H4 differed at only 2 residues from the animal H4 with I60 vs. V60 and R77 vs. K77, respectively. This extreme degree of sequence conservation, including functional side chain conservation, has been proven to be characteristic. The same holds true for plant and animal histone H3s, if one takes into account that in animals and in plants histone H3 developed into two distinct functional variants (Waterborg 2011). The replication-coupled (RC) H3 forms, like H3.1 and H3.2 in mammals, are produced predominantly or exclusively during the S phase of the cell cycle. These forms are used in assembling newly replicated DNA into nucleosomes using histone chaperones. In the same cells the replication-independent (RI) H3 variant, like H3.3 in animals, is produced constitutively and is incorporated into nucleosomes by specialized chaperones across transcribed DNA sequences. This process repairs the nucleosomal packaging of transcribed genes when transcription has caused loss of nucleosomes. The study of dynamic histone acetylation, synthesis and turnover in alfalfa (*Medicago sativa*) was instrumental in identifying the RI histone H3 function in plants. This discovery preceded the recognition that this inherent instability of transcribed chromatin and the linked turnover of the ‘replacement’ RI histone H3 also existed in animals (Ahmad et al. 2010).

The enzymes in charge of the processes of histone post-traslational modifications are Histone Methyl Transferases (HMT) and Histone Demethylases (HDM) regarding to

methylation and Histone methylation enzyme classification depends on the amino acid, being differentiated by arginine or lysine modification (Wang and Zhu 2008). Histone Acetyl Transferases (HAT) and Histone Deacetylases (HDAC) are the ones related to acetylation (Table 1.2) (Marmorstein and Roth 2001).

Table 1.2: Mammal histone deacetylases and acetyltransferases

Histone deacetylases		
Class I		HDAC1
		HDAC2
		HDAC3
		HDAC8
Class II	Class IIa	HDAC4
		HDAC5
		HDAC7
		HDAC9
	Class IIb	HDAC6
		HDAC10
Class III		SIRT family NAD-dependent (SIRT1-SIRT7)
Class IV		HDAC11

Histone acetyl transferases
GCN5/PCAF family
MYST family
TAF_{II} 250 family
CBP/p300 family
SRC family
ATF-2
HAT1
TFIIIC

1.4.5. Non-coding RNAs

The Small RNAs (sRNAs) constitute a family of regulatory non-coding RNAs (ncRNAs) of 19-28 nt in length derived from double-stranded RNAs (dsRNAs), and are highly conserved evolutionarily across species. There are two main classes of small RNAs involved in RNA silencing: microRNAs (miRNAs) and small interfering RNAs (siRNAs) and their classification depends on the biogenesis and not on their action (Reinhart et al. 2004; Kim 2005). Small double-stranded RNAs (dsRNAs) of 21–24 bp trigger RNA-directed DNA methylation (RdDM) of homologous DNA sequences leading to transcriptional gene silencing in plants. Involvement of dsRNAs due to RNA-dependent RNA polymerase (RdRP) and possibly small RNAs have been shown in paramutation of the *b1* locus in maize (Alleman et al. 2006).

These sRNAs come from the long single stranded RNA (ssRNAs) and are processed by RNAse III proteins. They are involved in several processes including DNA removing,

heterochromatin assembly, mRNA cleavage and translational repression, all of them related to developmental processes and response to stress (Mallory and Vaucheret 2006). It has been recently discovered several miRNAs involved in *in vitro* shoot regeneration regulating known specific genes like *WUS*, *CLV3* and *CUC 1* and *2* in *A. thaliana* (Qiao and Xiang 2013).

1.4.6. DNA methylation and its relationship with other epigenetic modifications

It has been shown a direct relationship between DNA methylation and histone modifications, especially in H3 modifications in several biological situations (Jackson et al. 2002; Johnson et al. 2002; Mathieu et al. 2005; Okitsu and Hsieh 2007), and in *in vitro* cell cultures of *S. tuberosum* epigenetic patterns of each different modification have been established (Law and Suttle 2005). Non-coding RNAs have been proposed as molecular interplayers as well, inducing changes in DNA methylation and in chromatin structure modifying histones (So et al. 2011; Olovnikov et al. 2012). An illustration of the main epigenetic factors is shown in Fig. 1.3.

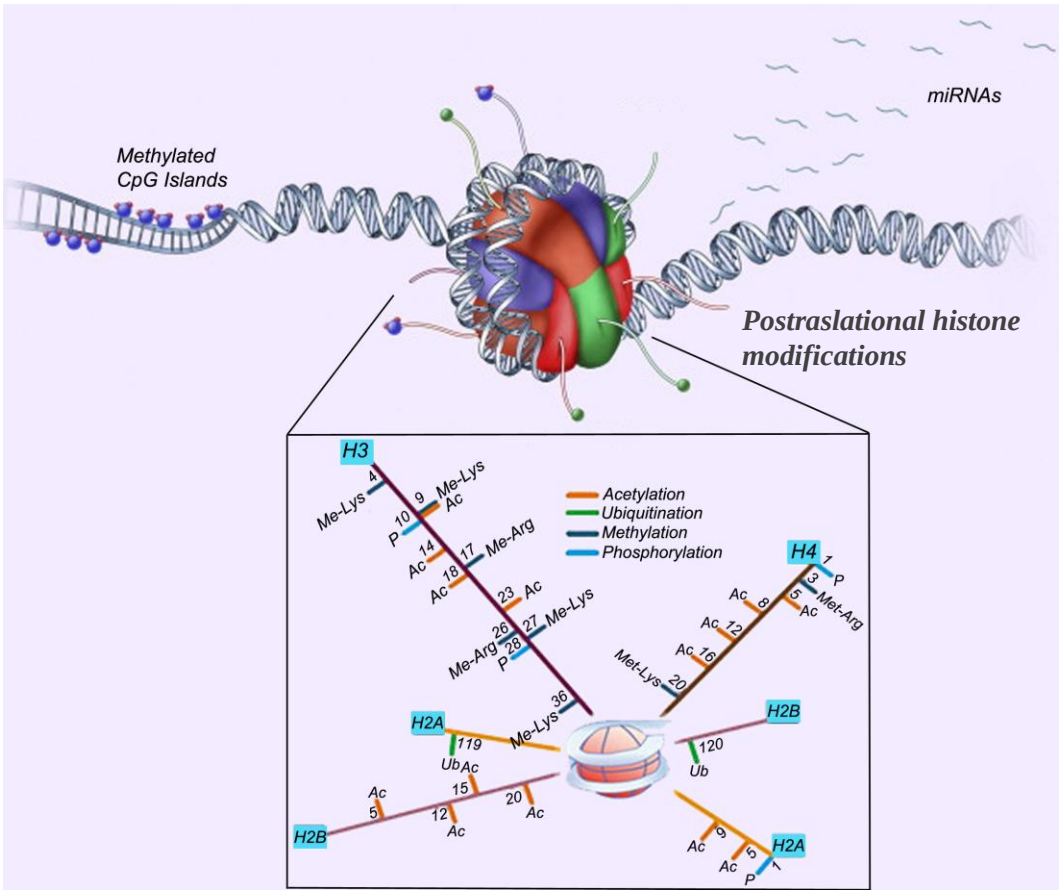


Figure 1.3: Summary of mayor epigenetic mechanisms. Adapted from McCleary-Wheeler et al. (2012).

1.5. DNA methylation in plants

The first comprehensive DNA methylation mapping of an entire genome of Arabidopsis thaliana showed that pericentromeric heterochromatin, repetitive sequences, and regions producing small interfering RNAs are heavily methylated (Zhang et al., 2006). Interestingly, over one-third of expressed genes are methylated within transcribed regions and only about 5% genes within promoter regions. Genes methylated in transcribed regions are highly expressed and constitutively active, whereas promoter-methylated genes show a greater degree of tissue-specific expression.

Specific changes in DNA methylation accompany the entire life of a plant, starting from seed germination up to the death programmed or induced by various agents and factors of biological or abiotic nature. In fact, the ontogenesis and the life itself are impossible without DNA methylation, because this genome modification in plants, like in other eukaryotes, is involved in a control of all genetic functions including transcription, replication, DNA repair, gene transposition and cell differentiation. Plant genes may be methylated at both adenine and cytosine residues; specific adenine DNA-methyltransferase was described. Adenine DNA methylation may influence cytosine modification and vice versa. Anyway, two different systems of the genome modification based on methylation of adenines and cytosines coexist in higher plants (Vanyushin and Ashapkin, 2011).

1.5.1. DNA methyltransferases in plants

In Arabidopsis, two maintenance type and one de novo type of DNA methyltransferase have been identified (Table 1.3). *DNA METHYLTRANSFERASE 1* (*MET1*), a homolog of the mammalian Dnmt1 methyltransferase, catalyzes methylation at a CG dinucleotide site, whereas *CHROMOMETHYLASE 3 (CMT3)*, a plant-specific DNA methyltransferase, catalyzes methylation at CNG sites. Both CG and CNG sites are symmetric sequences, therefore, the methylation patterns at these sites can be transmitted to and maintained in the sister strand during DNA replication by the activities of *MET1* and *CMT3*,

respectively. Asymmetric CNN methylation is maintained by de novo methylation that is catalyzed by *CMT3* and *DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)*, an ortholog of the mammalian *Dnmt3a/b de novo* methyltransferase (Cao and Jacobsen 2002; Chan et al. 2005). A recent study showed that *DRM3*, a catalytically mutated DNA methyltransferase paralog, also plays an important role in establishing *de novo* cytosine methylation in all sequence contexts in the process of RNA-directed DNA methylation (RdDM) by stimulating the activity of *DRM2* (Henderson et al. 2010). In mammals, it has been shown that the interaction between *Dnmt3a* and the catalytically mutated DNA methyltransferase *Dnmt3L* is required for establishing *de novo* DNA methylation during germline development (Jia et al. 2007).

Table 1.3: Predicted function of plant DNA methyltransferases from Zhang et al. (2010).

Classification	Gene name	Species	Function
MET1	AtMET1	*A. thaliana*	Maintenance: CG, probably CNG; single-copy DNA
	NtMET1	*N. tabacum*	Maintenance
	OsMET1-1	*O. sativa*	Maintenance
	OsMET1-2	*O. sativa*	Maintenance
	ZmMET1	*Zea mays*	Maintenance
CMT	AtCMT3	*A. thaliana*	Maintenance: CNG in repetitive DNA and transposons in heterochromatin
	OsCMTL	*O. sativa*	Putative enzyme, function unknown
	OsMET2a	*O. sativa*	Putative enzyme, function unknown
	ZMET2	*Z. mays*	Perhaps maintenance: CNG in transposons
	ZMET5	*Z. mays*	Perhaps maintenance: CNG in transposons
DRM	AtDRM1	*A. thaliana*	De novo: CG, CNG, CNN Maintenance: CNG, CNN
	AtDRM2	*A. thaliana*	De novo: CG, CNG, CNN Maintenance: CNG, CNN
	NtDRM1	*N. tabacum*	De novo: CNG, CNN, some CG
	ZMET3	*Z. mays*	Putative enzyme, function unknown
Dnmt2	AtDnmt2L	*A. thaliana*	Putative enzyme, RNA methylation
	ZMET4	*Z. mays*	Putative enzyme, function unknown
DNA glycosylase	DME	*A. thaliana*	Imprinted genes, FWA and MEA
	ROS	*A. thaliana*	mCpCpG of silent transgene and homologous endogene

1.6. DNA methylation in plant development

Although the role of cytosine methylation as a genome defense system to maintain genome integrity is well established, it is known that DNA methylation in plants is tissue- and age-specific. The tissue specificity of DNA methylation demonstrated that DNA methylation is associated with cellular differentiation and ageing in *P. radiata* needles (Valledor et al. 2010) and chestnut embryo determination (Viejo et al. 2010), and several developmental

processes like reinvigoration, bud dormancy or shoot transition from vegetative to floral state are strongly influenced by DNA methylation (Fraga et al. 2002c; Hasbún et al. 2007; Meijón et al. 2009; Santamaría et al. 2009). Plant growth and development are finely regulated by specific phytohormone levels, being a modulation of DNA methylation one of the tools used by plant growth regulators (Gehring and Henikoff 2007). It is still largely unclear how cytosine methylation is involved in the control of developmental patterning in plants. Nonetheless, substantial progress has been made in this area by using various loss-of-function methylation mutants. The first implication that cytosine methylation is important for plant development was obtained from a study using antisense MET1 Arabidopsis plants, which showed a number of striking phenotypes, including reduced apical dominance, alterations in flowering time, extensive floral abnormalities, and curled leaves (Finnegan et al. 1996).

1.6.1. DNA methylation in in vitro culture

Plants vegetatively produced in tissue culture may differ from the plants from which they have been derived. Two major classes of off-types occur: genetic and epigenetic ones.

In micropropagated plants important differences in DNA methylation have been examined (Monteuuis et al. 2009). Epigenetic changes are thought to underlie various well known tissue-culture phenomena including rejuvenation, habituation, and morphological changes such as flower abnormalities, bushiness, and tumorous outgrowths. They are often temporary and plants may revert to the normal phenotype relatively easy, but some of these changes can be long lasting and may even be transferred during sexual propagation (Brettell and Dennis 1991). These epigenetic changes have been assessed in *Doritaenopsis* and in oil palm, and they modify the capability of flowering of *in vitro* propagated plants (Park et al. 2009; Smulders and de Klerk 2011) finding these alterations during *in vitro* cell differentiation in *Rosa hybrida* as well (Xu et al. 2004). Regarding to *in vitro* shoot induction, new findings point out the more and more importance of DNA methylation in this process at a

global level and with specific genes involved in shoot apical meristem development (De-la-Pena et al. 2012; Huang et al. 2012).

1.7. Basic procedures for epigenetic analysis in plant cell and tissue culture

1.7.1. DNA methylation.

Recent advances in whole genome methylation profiling technologies have caused a great interest in analyzing cytosine methylation levels and distribution within the genome, allowing us to better understanding various developmental processes, like *in vitro* plant culture. Some reviews about DNA methylation analysis techniques have been done (Fraga and Esteller 2002; Bowler et al. 2004), and an increasing number of scientific research about DNA methylation has spread out in different plant development processes, including *in vitro* culture (Viejo et al. 2012).

1.7.1.1. Global DNA methylation.

It is widely accepted that DNA methylation controls differentiation maintaining cell status stability (Fraga et al. 2002b), and global methylcytosine content in genomic DNA varies widely across species, organs, and developmental states. Epigenetic modifications, and in particular DNA hypomethylation, are suspected of being responsible for somaclonal variation (Kaeppler et al. 2000) being growth treatments and environmental stress proposed as main factors in this variation (Jaligot et al. 2000). Although genetic stability is certified by conventional DNA markers, no uniform clone regenerants are often obtained being this variation caused by different DNA methylation levels. The analysis of global methylated DNA can be measured by the content of 5-mdC or by its distribution along the tissue.

HPLC-MS and HPCE-MS (High Performance Liquid Chromatography-Mass Spectrometry, High Performance Capillary Electrophoresis-Mass Spectrometry).

Quantification of 5-methyldeoxycytosine genomic DNA methylation establishes certain degrees of global DNA methylation which can be used as markers for developmental or physiological processes (Causevic et al. 2005). The analysis of global DNA methylation

can be performed by HPCE (Fraga et al. 2000; Hasbún et al. 2008) or by HPLC (Johnston et al. 2005; Magaña et al. 2008) both methods share the enzymatic hydrolysis of the DNA strands and a phosphatase alkaline treatment, to obtain deoxynucleosides, these deoxynucleosides are run in a HPCE or HPLC system and the peaks obtained are measured by the following formula % 5-mdC = 5-mdC peak area/(5-mdC + 5-dC peak areas).

It has been shown that different levels in global DNA methylation are essential for a correct response of plant material to *in vitro* culture (Valledor et al. 2007) and it seems to be that both, 5-mdC quantity and physiological state of the plant have a key influence in the success of the culture, even different tissues in the same organ (Fraga et al. 2002a; Hasbún et al. 2007; Viejo et al. 2010). Global DNA methylation has been proposed as an epigenetic marker in different developmental situations (Baurens et al. 2004; Marum et al. 2008) although there are some disagreement about the levels of DNA methylation and the age of the explants depending on the species (Bionti et al. 2002; Fraga et al. 2002c; Monteuuis et al. 2008).

Immunolocalization is a technique to quantify 5-methyldeoxycytosine that provides important information to see the general dynamics, and its distribution along the tissue shows us how these dynamics change and are differentially represented in the different tissues. It is based in the detection of 5-methyldeoxycytosine in the nuclei of the cells that form the tissue by an antibody against 5-methyldeoxycytosine and a later analysis by fluorescence or confocal microscopy (Meijón et al. 2009).

Differential patterns of acetylation-methylation were observed in floral and vegetative shoots in *Azalea japonica* by Meijón et al. (2009), and a specific cell and tissue pattern was detected during floral bud induction transition and vegetative development, as well as in dormant organs, where an important regulation mediated by DNA methylation took place (Santamaría et al. 2009).

1.7.1.2. Genome wide DNA methylation.

5mC marks can be selectively detected through the genome using specific antibodies and proteins that specifically bind 5mC (Rauch and Pfeifer 2005; Weber et al. 2005). These methods are relatively straightforward and do not require either digestion of genomic DNA or bisulfite treatment. The basis of these methods is the enrichment of a fraction of DNA in methylated DNA. This population of DNA fragments can follow two main analysis strategies, identifying the sequences of the enriched fraction by mass sequencing and hybridizing a microarray seeing the differential amount of each sequence.

MeDIP, MIRA, MBD columns (Methylated DNA Immuno Precipitation/ Methylated CpG Island Recovery Assay).

For this kind of analyses, DNA is fragmented in a size range about 200-1000 bp by enzyme restriction or by mechanical methods. For MeDIP analysis a later precipitation is done using a 5-methyl cytosine antibody and in the other two methods a methylated-CpG binding domain (MBD) protein is used. The shorter protein isoform of MBD2 family MBD2B, which possesses the highest affinity to methylated DNA among the MBD proteins, is the most used (Rauch and Pfeifer 2005; Weng et al. 2009). PCR amplification with specific primers to determine whether DNA sequences of interest were precipitated prior to a comparison of the signal obtained from RT-qPCR must be done. In the case of MeDIP a comparison between the immunoprecipitated template (IP) and the input DNA (IN), a DNA without modifications except fragmentation step, is performed giving to us the enrichment (relative amount of a specific gene in both samples starting with the same amount of genomic DNA) of the target sequence.

1.7.1.3. Sequencing DNA methylation.

Bisulfite sequencing, Pyrosequencing.

Direct bisulfite sequencing was first time used by Frommer et al. (1992) to identify the nucleotides resistant to bisulfite conversion. After DNA conversion a suitable couple of primers must be designed free of CpG dinucleotides (Kovacova and Janousek 2012), however all programs for bisulfite primer design are reduced exclusively to mammal DNA and

therefore to CpG dinucleotide, so primer design for plant DNA could be a cumbersome process sometimes. This technique requires cloning of the PCR product prior to sequencing for adequate sensitivity, because a mixture of different populations of differential methylated cytosines along the sequence is present. In the chromatogram results all sites of unmethylated cytosines are displayed as thymines in the resulting amplified sequence whereas methylated ones remain as cytosines. This technique has been widely used in plant tissue culture to control transposon activation and see specific promoter methylation status of interest genes (Berdasco et al. 2008; Ngezahayo et al. 2009).

After DNA bisulfite modification and PCR amplification of the region of interest, pyrosequencing is used to determine the bisulfite-converted sequence of specific CpG sites in the region. The technique detects the amount of C and T incorporated during the sequence extension being very precise with the relative amount of each one (Dupont et al. 2004). The main limitation of this method is that only around fifty nucleotides can be sequenced accurately, so a low number of CpGs are analyzed.

1.7.2. Chromatin modifications.

Chromatin is the state in which DNA is packaged within the cell. The core histones present in the nucleosome are predominantly globular, except for their N-terminal tails. Chromatin remodelling factors in histone modifications act by covalently altering the charge of hydrophobicity of specific amino acids on these histone tails. These modifications alter cis-regulatory elements binding to DNA and act by modifying other chromatin regulators (Wagner 2003). There is a myriad of possible modifications carried out by a gene complex network (Jarillo et al. 2009). However, because the different response they cause, they cannot be present at the same time and their variations along a process determine a specific chromatin state (Kouzarides 2007). To study chromatin there are several protocols depending on the nucleosome distribution, proteins involved or their modifications (Bowler et al. 2004).

1.7.2.1. Genomic profile of histone PTMs.

A general overview of a determined histone modification is essential to know its possible role along a process and along the tissue. Up to date, these techniques of global quantification allow us to identify from a single histone modification to a group of modifications in different histones (Freitas et al. 2004; Valledor et al. 2010).

HPLC-MS, Antibody based detection.

Genome wide histone analysis, as well as global DNA methylation levels, reveals a general behaviour of the studied characteristic and uses it as a marker in the physiological process. Mass spectrometry is a chemical analysis technique that exploits the physical properties of ions to determine their mass to charge ratio (m/z), and a variety of methods can be used to make the analysis after HPLC procedure (Garcia et al. 2007). Histones can be easily extracted from cells and a reproducible separation of hte histone family members can be achieved on C8- or C18-based columns using reverse-phase high-performance liquid chromatography (RP-HPLC) (Gurley et al. 1983). Individual fractions of the histones are enzymatically digested and identified by PMF (Peptide Mass Fingerprint), MS/MS (tandem MS), CAD (Collisionally Activated Dissociation) and ETD (Electron Transfer Dissociation) (Olsen and Mann 2004; Thiede et al. 2005; Siuti and Kelleher 2007).

Western blot histone PTMs have been widely analyzed and in the field of plant *in vitro* culture and we have many references mainly devoted to monitor chromatin remodelling (Law and Suttle 2005; Causevic et al. 2006). On the other hand, to see the distribution in the different tissues of an organ, immunolocalization is an easy and an informative technique (Meijon et al. 2010; Valledor et al. 2010).

Genome wide analysis.

Chromatin immunoprecipitation (ChIP) is one of the most powerful tools in the characterization of protein modifications and *in vivo* DNA-protein interactions (Saleh et al. 2008). ChIP procedure consists of discrete steps including *in vivo* cross-linking of proteins to DNA with formaldehyde, isolation of chromatin from isolated nuclei, shearing of chromatin

into small fragments from 200 pb to 800 pb by ultrasound or micrococcal nuclease (MNase) digestion, immunoprecipitation with specific antibodies to DNA-bound proteins, release of the co-precipitated DNA and finally, PCR amplification with specific primers to determine whether DNA sequences of interest were precipitated. The latter steps involve comparison of the signal obtained from qPCR signals from the precipitated template with positive and negative controls (Haring et al. 2007; Saleh et al. 2008; Reimer and Turck 2010; Ricardi et al. 2010).

1.8. Objectives and experimental design

In vitro culture techniques are a source of epigenetic variation provoking transposable element activation and modifications in specific genes. In the same way, genes involved in cell reprogramming and tissue differentiation undergo these epigenetic modifications. These modifications imply new patterns of genic expression and changes in cell plasticity, and are considered appropriated experimental systems to study the loss of competence regarding developmental state as well as the study of epigenetic changes that take place. Adventitious caulogenesis in *P. radiata* cotyledons involves the response of somatic cells to a growth regulator, which induces a change in cellular fate because of variations in specific gene expression by modifications in previous epigenetic marks among other modifications. Unravelling the processes that regulate this cell plasticity is very important both, from an applied point of view regarding biotechnological importance and from a basic point of view, given that development processes are connected to epigenetic modifications. DNA methylation and histone acetylation as inductive or repressive epigenetic marks are related to cell reprogramming processes that take place during adventitious bud induction. Their dynamics together with differential gene will allow us to identify the molecular mechanisms in this process.

Epigenetic mechanisms regulating chromatin rearrangement, control cell plasticity and reprogramming process of ectopic regeneration. *In vitro* adventitious organogenesis involves

changes in cell fate combined with a reactivation of cell cycle, an euchromatin/heterochromatin balance and a genic reprogramming of somatic cells.

This thesis try to answer questions related to epigenetic regulation by DNA methylation associated with adventitious caulogenesis induced in zygotic embryos of *Pinus radiata* D. Don and other organogenic processes associated to cell reprogramming.

Taking into account adventitious caulogenesis as a cell reprogramming process, it is proposed a main objective, **the study of DNA methylation as chromatin remodeling mechanism and its role in the regulation of candidate genes**. To carry out this objective, several secondary objectives are proposed.

1.- Global and spatio-temporal dynamics of DNA methylation in adventitious caulogenesis and other organogenic processes that get involved cell reprogramming. The organogenic responses in zygotic embryos induced by different growth regulators will be analysed and the different responses correlated with global DNA methylation profiles and the distribution along the tissue of 5mC and AcH4 by immunolocalization.

2.- Differential expression analysis and identification of candidate genes involved in adventitious organogenesis. A suppressive subtractive hybridization technique will allow us to identify genes related to each organogenic response and the design of a macroarray to analyse gene expression in the different experimental systems. A RT-qPCR of candidate genes will be necessary to validate macroarray data.

3.- Epigenetic regulation of candidate genes by DNA methylation and chemical unmasking of other candidate genes. The performance of an immunoprecipitation of methylated DNA (MeDIP) and the quantification by qPCR of sequence enrichment will let us determine which previously selected candidate genes are regulated by DNA methylation in their sequence. A chemical treatment in cell cultures with demethylating agent 5-aza-2–deoxycytidine and a hybridization of the macroarray developed in the previous objective will allow us to identify other candidate genes.

1.9. References

Ahmad A, Zhang Y, Cao XF. 2010. Decoding the Epigenetic Language of Plant Development. *Molecular Plant* **3**: 719-719.

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. 1997. Genes involved in organ separation in Arabidopsis: an analysis of the *cup-shaped cotyledon* mutant. *The Plant Cell Online* **9**: 841-841.

Aida M, Ishida T, Tasaka M. 1999. Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the *CUP-SHAPED COTYLEDON* and *SHOOT MERISTEMLESS* genes. *Development* **126**: 1563-1563.

Alleman M, Sidorenko L, McGinnis K, Seshadri V, Dorweiler JE, White J, Sikkink K, Chandler VL. 2006. An RNA-dependent RNA polymerase is required for paramutation in maize. *Natur* **442**: 295-298.

Baurens FC, Nicolleau J, Legavre T, Verdeil JL, Monteuuis O. 2004. Genomic DNA methylation of juvenile and mature *Acacia mangium* micropropagated *in vitro* with reference to leaf morphology as a phase change marker. *Tree Physiol* **24**: 401-407.

Berdasco M, Alcázar R, García-Ortiz MV, Ballestar E, Fernández AF, Roldán-Arjona T, Tiburcio AF, Altabella T, Buisine N, Quesneville H. 2008. Promoter DNA hypermethylation and gene repression in undifferentiated Arabidopsis cells. *PLoS One* **3**: e3306.

Bionti MB, Cozza R, Chiappetta A, Giannino D, Castiglione MR, Dewitte W, Mariotti D, Van Onckelen H, Innocenti AM. 2002. Distinct nuclear organization, DNA methylation pattern and cytokinin distribution mark juvenile, juvenile-like and adult vegetative apical meristems in peach (*Prunus persica* (L.) Batsch). *J Exp Bot* **53**: 1047-1054.

Bonner J, Roger Chalkley G, Dahmus M, Fambrough D, Fujimura F, Huang R-cC, Huberman J, Jensen R, Marushige K, Ohlenbusch H. 1968. Isolation and characterization of chromosomal nucleoproteins. *Methods Enzymol* **12**: 3-65.

Bowler C, Benvenuto G, Laflamme P, Molino D, Probst AV, Tariq M, Paszkowski J. 2004. Chromatin techniques for plant cells. *Plant J* **39**: 776-789.

Brettell RIS, Dennis ES. 1991. Reactivation of a silent Ac following tissue culture is associated with heritable alterations in its methylation pattern. *Molecular and General Genetics MGG* **229**: 365-372.

Cao X, Jacobsen SE. 2002. Role of the Arabidopsis DRM Methyltransferases in De Novo DNA Methylation and Gene Silencing. *Curr Biol* **12**: 1138-1144.

Catala C, Rose JKC, Bennett AB. 1997. Auxin regulation and spatial localization of an endo-1, 4-D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls. *Plant J* **12**: 417-426.

Causevic A, Delaunay A, Ounnar S, Righezza M, Delmotte F, Brignolas F, Gagège D, Maury S. 2005. DNA methylating and demethylating treatments modify phenotype and cell wall differentiation state in sugarbeet cell lines. *Plant Physiol Biochem* **43**: 681-691.

Causevic A, Gentil MV, Delaunay A, El-Soud WA, Garcia Z, Pannetier C, Brignolas F, Hagège D, Maury S. 2006. Relationship between DNA methylation and histone acetylation levels, cell redox and cell differentiation states in sugarbeet lines. *Planta* **224**: 812-827.

Cedar H, Bergman Y. 2009. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* **10**: 295-304.

Černý M, Dycka F, Bobal'ova J, Brzobohaty B. 2011. Early cytokinin response proteins and phosphoproteins of Arabidopsis thaliana identified by proteome and phosphoproteome profiling. *J Exp Bot* **62**: 921-937.

Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE. 2008. Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Natur* **452**: 215-215.

Cortizo M, Álvarez JM, Rodríguez A, Fernández B, Ordás RJ. 2010. Cloning and characterization of a type-A response regulator differentially expressed during adventitious shoot formation in *Pinus pinea* L. *J Plant Physiol* **167**: 1023-1026.

Cortizo M, de Diego N, Moncaleán P, Ordás RJ. 2009. Micropropagation of adult Stone Pine (*Pinus pinea* L.). *Trees-Structure and Function* **23**: 835-842.

Chan SWL, Henderson IR, Jacobsen SE. 2005. Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat Rev Genet* **6**: 351-360.

Che P, Gingerich DJ, Lall S, Howell SH. 2002. Global and Hormone-Induced Gene Expression Changes during Shoot Development in Arabidopsis. *Plant Cell* **14**: 2771-2785.

De-la-Pena C, Nic-Can G, Ojeda G, Herrera-Herrera JL, Lopez-Torres A, Wrobel K, Robert-Diaz ML. 2012. KNOX1 is expressed and epigenetically regulated during in vitro conditions in Agave spp. *BMC Plant Biol* **12**: 203.

De Diego N, Montalbán IA, Moncalean P. 2011. Improved micropropagation protocol for maritime pine using zygotic embryos. *Scandinavian Journal of Forest Research* **26**: 202-211.

DeLange RJ, Fambrough DM, Smith EL, Bonner J. 1969. Calf and pea histone IV. *J Biol Chem* **244**: 319-334.

Dupont J, Tost T, Jammes H, Gut IG. 2004. De novo quantitative bisulfite sequencing using the pyrosequencing technology. *Anal Biochem* **333**: 119-127.

Durbak A, Yao H, McSteen P. 2012. Hormone signaling in plant development. *Curr Opin Plant Biol* **15**: 92-96

Fambrough DM, Fujimura F, Bonner J. 1968. Quantitative distribution of histone components in the pea plant. *Biochemistry* **7**: 575-585.

FAO. 2010. Global forest resources assessment. in *FAO forestry paper*. Food and Agriculture Organization of the United Nations, Rome, Italy.

FAO. 2011. *State of the World's Forests*. Food and Agriculture Organization of the United Nations, Rome, Italy.

Finnegan EJ, Peacock WJ, Dennis ES. 1996. Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc Natl Acad Sci U S A* **93**: 8449-8454.

Fraga MF, Cañal MJ, Aragones A, Rodriguez R. 2002a. Factors involved in *Pinus radiata* D. Don. Micrografting. *Ann For Sci* **59**: 155-161.

Fraga MF, Cañal MJ, Rodríguez R. 2002b. Phase change related epigenetic and physiological changes in *Pinus radiata D Don*. *Planta* **215**: 672-676.

Fraga MF, Esteller M. 2002. DNA methylation: A profile of methods and applications. *BioTechniques* **33**: 632-649.

Fraga MF, Rodríguez R, Cañal MJ. 2000. Rapid quantification of DNA methylation by high performance capillary electrophoresis. *Electrophoresis* **21**: 2990-2994.

Fraga MF, Rodríguez R, Cañal MJ. 2002c. Genomic DNA methylation-demethylation during ageing-reinvigoration of *Pinus radiata*. *Tree Physiol* **22**: 813-816.

Freitas MA, Sklenar AR, Parthun MR. 2004. Application of mass spectrometry to the identification and quantification of histone post translational modifications. *J Cell Biochem* **92**: 691-700.

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A* **89**: 1827-1831.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M. 2000. Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *The Plant Cell Online* **12**: 393-393.

Garcia BA, Shabanowitz J, Hunt DF. 2007. Characterization of histones and their post-translational modifications by mass spectrometry. *Curr Opin Chem Biol* **11**: 66-73.

Gehring M, Henikoff S. 2007. DNA methylation dynamics in plant genomes. *Biochim Biophys Acta* **1769**: 276-286.

Gordon SP, Chickarmane VS, Ohno C, Meyerowitz EM. 2009. Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. *Proc Natl Acad Sci U S A* **106**: 16529-16534.

Gordon SP, Heisler MG, Reddy GV, Ohno C, Das P, Meyerowitz EM. 2007. Pattern formation during de novo assembly of the Arabidopsis shoot meristem. *Development* **134**: 3539-3548.

Gregersen PL, Culetic A, Boschian L, Krupinska K. 2013. Plant senescence and crop productivity. *Plant Mol Biol*.

Gurley LR, Valdez JG, Prentice DA, Spall WD. 1983. Histone fractionation by high-performance liquid chromatography. *Anal Biochem* **129**: 132-144.

Hanson J, Smeekens S. 2009. Sugar perception and signaling-an update. *Curr Opin Plant Biol* **12**: 562-567.

Haring M, Offermann S, Danker T, Horst I, Peterhansel C, Stam M. 2007. Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Methods* **3**: 11.

Hasbún R, Valledor L, Berdasco M, Santamaría E, Cañal MJ, Rodríguez R. 2007. Dynamics of DNA methylation during chestnut trees development, Application to breeding programs. *Acta horticulturae* **760**: 563–566.

Hasbún R, Valledor L, Rodríguez JL, Santamaría E, Ríos D, Sánchez M, Cañal MJ, Rodríguez R. 2008. HPCE quantification of 5-methyl-2´-deoxycytidine in genomic DNA: Methodological optimization for chestnut and other woody species. *Plant Physiol Biochem* **46**: 815-822.

Hay A, Tsiantis M. 2010. KNOX genes: Versatile regulators of plant development and diversity. *Development* **137**: 3153-3165.

Heisler MG, Jönsson H. 2007. Modelling meristem development in plants. *Curr Opin Plant Biol* **10**: 92-97.

Henderson IR, Chan SR, Cao X, Johnson L, Jacobsen SE. 2010. Accurate sodium bisulfite sequencing in plants. *Epigenetics* **5**: 141-143.

Howell SH. 2013. ER Stress, ERAD, and the Unfolded Protein Response in Plants. *Annu Rev Plant Biol* **64**.

Huang LC, Hsiao LJ, Pu SY, Kuo CI, Huang BL, Tseng TC, Huang HJ, Chen YT. 2012. DNA methylation and genome rearrangement characteristics of phase change in cultured shoots of *Sequoia sempervirens*. *Physiol Plant* **145**: 360-368.

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T. 2001. Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Natur* **409**: 1060-1063.

Jackson JP, Lindroth AM, Cao X, Jacobsen SE. 2002. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Natur* **416**: 556-560.

Jaligot E, Rival A, Beule T, Dussert S, Verdeil JL. 2000. Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. *Plant Cell Rep* **19**: 684-690.

Jarillo JA, Piñeiro M, Cubas P, Martínez-Zapater JM. 2009. Chromatin remodeling in plant development. *The International Journal of Developemntal Biology* **53**: 1581-1596.

Jeltsch A. 2002. Beyond Watson and Crick: DNA Methylation and Molecular Enzymology of DNA Methyltransferases. *ChemBioChem* **3**: 274-274.

Jia D, Jurkowska RZ, Zhang X, Jeltsch A, Cheng X. 2007. Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. *Natur* **449**: 248-251.

Johnson LM, Cao X, Jacobsen SE. 2002. Interplay between Two Epigenetic Marks DNA Methylation and Histone H3 Lysine 9 Methylation. *Curr Biol* **12**: 1360-1367.

Johnston JW, Harding K, Bremner DH, Souch G, Green J, Lynch PT, Grout B, Benson EE. 2005. HPLC analysis of plant DNA methylation: a study of critical methodological factors. *Plant Physiol Biochem* **43**: 844-853.

Joyce CM, Villemur R, Snustad DP, Silflow CD. 1992. Tubulin gene expression in maize *Zea mays* L.: Change in isotype expression along the developmental axis of seedling root. *J Mol Biol* **227**: 97-107.

Kaeppler SM, Kaeppler HF, Rhee Y. 2000. Epigenetic aspects of somaclonal variation in plants. *Plant Mol Biol* **43**: 179-188.

Katayama N, Koi S, Kato M. 2010. Expression of SHOOT MERISTEMLESS, WUSCHEL, and ASYMMETRIC LEAVES1 homologs in the shoots of Podostemaceae: Implications for the evolution of novel shoot organogenesis. *The Plant Cell Online* **22**: 2131-2131.

Kim VN. 2005. Small RNAs: classification, biogenesis, and function. *Mol Cells* **19**: 1-15.

Kouzarides T. 2007. Chromatin modifications and their function. *Cell* **128**: 693-705.

Kovacova V, Janousek B. 2012. Bisprimer--a program for the design of primers for bisulfite-based genomic sequencing of both plant and Mammalian DNA samples. *J Hered* **103**: 308-312.

Lafon-Placette C, Faivre-Rampant P, Delaunay A, Street N, Brignolas F, Maury S. 2013. Methylome of DNase I sensitive chromatin in *Populus trichocarpa* shoot apical meristematic cells: a simplified approach revealing characteristics of gene-body DNA methylation in open chromatin state. *New Phytol* **197**: 416-430.

Law RD, Suttle JC. 2005. Chromatin remodeling in plant cell culture: patterns of DNA methylation and histone H3 and H4 acetylation vary during growth of asynchronous potato cell suspensions. *Plant Physiol Biochem* **43**: 527-534.

Li G, Hall TC, Holmes-Davis R. 2002. Plant chromatin: development and gene control. *BioEssays* **24**: 234-243.

Lister R, Ecker JR. 2009. Finding the fifth base: genome-wide sequencing of cytosine methylation. *Genome Res* **19**: 959-966.

Magaña AA, Wrobel K, Caudillo YA, Zaina S, Lund G. 2008. High-performance liquid chromatography determination of 5-methyl-2'-deoxycytidine, 2'-deoxycytidine, and other deoxynucleosides and nucleosides in DNA digests. *Anal Biochem* **374**: 378-385.

Mallory AC, Vaucheret H. 2006. Functions of microRNAs and related small RNAs in plants. *Nat Genet* **38**: S31-S36.

Mankessi F, Saya AR, Favreau B, Doulbeau S, Conéjéro G, Lartaud M, Verdeil J-L, Monteuuis O. 2011. Variations of DNA methylation in Eucalyptus urophylla×Eucalyptus grandis shoot tips and apical meristems of different physiological ages. *Physiol Plant* **143**: 178-187.

Marmorstein R, Roth SY. 2001. Histone acetyltransferases: function, structure, and catalysis. *Curr Opin Genet Dev* **11**: 155-161.

Marum LM, Hasbún R, Rodriguez R, Oliveira MM, Miguel C. 2008. Epigenetic Studies in Embryogenic Culture of *Pinus pinaster*. in *Cryoperservation of crop species in Europe* (eds. J Laamanen, M Uosukainen, H Häggman, A Nukari, S Rantala), p. 69. COST Action 871, Oulu, Finland.

Mathieu O, Probst AV, Paszkowski J. 2005. Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. *The EMBO Journal* **24**: 2783-2791.

Mayer KFX, Schoof H, Haecker A, Lenhard M, Jürgens G, Laux T. 1998. Role of WUSCHEL in Regulating Stem Cell Fate in the Arabidopsis Shoot Meristem. *Cell* **95**: 805-815.

Meijón M, Feito I, Valledor L, Rodriguez R, Canal MJ. 2010. Dynamics of DNA methylation and Histone H 4 acetylation during floral bud differentiation in azalea. *BMC Plant Biol* **10**: 10.

Meijón M, Valledor L, Santamaría E, Testillano PS, Risueño MC, Rodríguez R, Feito I, Cañal MJ. 2009. Epigenetic characterization of the vegetative and floral stages of azalea buds: Dynamics of DNA methylation and histone H4 acetylation. *J Plant Physiol* **166**: 1624-1636.

Meng HX, Hackett JA, Nestor C, Dunican DS, Madej M, Reddington JP, Pennings S, Harrison DJ, Meehan RR. 2011. Apoptosis and DNA Methylation. *Cancers* **3**: 1798-1820.

Montalbán IA, De Diego N, Moncaleán P. 2010. Bottlenecks in *Pinus radiata* somatic embryogenesis: improving maturation and germination. *Trees-Structure and Function* **24**: 1061-1071

Monteuuis O, Baurens FC, Goh DKS, Quimado M, Doulbeau S, Verdeil JL. 2009. DNA Methylation in Acacia mangium In vitro and Ex-vitro Buds, in relation to their Within-shoot Position, Age and Leaf Morphology of the Shoots. *Silvae Genet* **58**: 287-292.

Monteuuis O, Doulbeau S, Verdeil JL. 2008. DNA methylation in different origin clonal offspring from a mature *Sequoiadendron giganteum* genotype. *Trees-Structure and Function* **22**: 779-784.

Ngezahayo F, Xu C, Wang H, Jiang L, Pang J, Liu B. 2009. Tissue culture-induced transpositional activity of mPing is correlated with cytosine methylation in rice. *BMC Plant Biol* **9**: 91.

Okitsu CY, Hsieh CL. 2007. DNA methylation dictates histone H3K4 methylation. *Mol Cell Biol* **27**: 2746-2757.

Olsen JV, Mann M. 2004. Improved peptide identification in proteomics by two consecutive stages of mass spectrometric fragmentation. *Proc Natl Acad Sci U S A* **101**: 13417-13417.

Park SY, Murthy HN, Chakrabarthy D, Paek KY. 2009. Detection of epigenetic variation in tissue-culture-derived plants of Doritaenopsis by methylation-sensitive amplification polymorphism (MSAP) analysis. *In Vitro Cellular & Developmental Biology-Plant* **45**: 104-108.

Penterman J, Zilberman D, Huh JH, Ballinger T, Henikoff S, Fischer RL. 2007. DNA demethylation in the Arabidopsis genome. *Proc Natl Acad Sci U S A* **104**: 6752-6752.

Rauch T, Pfeifer GP. 2005. Methylated-CpG island recovery assay: a new technique for the rapid detection of methylated-CpG islands in cancer. *Lab Investig* **85**: 1172-1180.

Rauch TA, Pfeifer GP. 2010. DNA methylation profiling using the methylated-CpG island recovery assay (MIRA). *Methods*

Read PE, Bavougian CM. 2012. In vitro rejuvenation of woody species. *Methods in molecular biology (Clifton, NJ)* **11013**: 383-395.

Reimer J, Turck F. 2010. Genome-Wide Mapping of Protein-DNA Interaction by Chromatin Immunoprecipitation and DNA Microarray Hybridization (ChIP-chip). Part A: ChIP-chip Molecular Methods in *Plant Epigenetics: Methods and Protocols* (eds. I Kovalchuk, F Zemp), pp. 139-160. Springer, Hatfeld.

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. 2004. MicroRNAs in plants. *Genes Dev* **16**: 1616–1626.

Ricardi MM, González RM, Iusem ND. 2010. Protocol: fine-tuning of a Chromatin Immunoprecipitation(ChIP) protocol in tomato. *Plant Methods* **6**: 11.

Saleh A, Alvarez-Venegas R, Yilmaz M, Le O, Hou G, Sadder M, Al-Abdallat A, Xia Y, Lu G, Ladunga I. 2008. The highly similar Arabidopsis homologs of trithorax ATX1 and ATX2 encode proteins with divergent biochemical functions. *Plant Cell* **20**: 568-579.

Santamaría ME, Hasbún R, Valera MJ, Meijón M, Valledor L, Rodríguez JL, Toorop PE, Cañal MJ, Rodríguez R. 2009. Acetylated H4 histone and genomic DNA methylation patterns during bud set and bud burst in *Castanea sativa*. *J Plant Physiol* **166**: 1360-1369.

Scott RJ, Spielman M. 2004. Epigenetics: Imprinting in Plants and Mammals-the Same but Different? *Curr Biol* **14**: 201-203.

Siuti N, Kelleher NL. 2007. Decoding protein modifications using top-down mass spectrometry. *Nat Meth* **4**: 817-821.

Smulders MJM, de Klerk GJ. 2011. Epigenetics in plant tissue culture. *Plant Growth Regul* **63**: 137-146.

Takada S, Hibara K, Ishida T, Tasaka M. 2001. The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. *Dev Biol* **128**: 1127-1135.

Takeda S, Aida M. 2011. Establishment of the embryonic shoot apical meristem in *Arabidopsis thaliana*. *J Plant Res* **124**: 211-219.

Tariq M, Paszkowski J. 2004. DNA and histone methylation in plants. *Trends Genet* **20**: 244-251.

Taylor JC, Markham GD. 2000. The Bifunctional Active Site of S-Adenosylmethionine Synthetase. *J Biol Chem* **274**: 32909-32914.

Thiede B, Höhenwarter W, Krah A, Mattow J, Schmid M, Schmidt F, Jungblut PR. 2005. Peptide mass fingerprinting. *Methods* **35**: 237-247.

Tusnády GE, Simon I, Váradi A, Tamás A. 2005. BiSearch: primer-design and search tool for PCR on bisulfite-treated genomes. *Nucleic Acids Res* **33**: e9.

Uribe X, Torres MA, Capellades M, Puigdomenech P, Rigau J. 1998. Maize α-tubulin genes are expressed according to specific patterns of cell differentiation. *Plant Mol Biol* **37**: 1069-1078.

Valledor L, Hasbún R, Meijón M, Rodríguez JL, Santamaría E, Viejo M, Berdasco M, Feito I, Fraga MF, Canal MJ et al. 2007. Involvement of DNA methylation in tree development and micropropagation. *Plant Cell Tissue Organ Cult* **91**: 75-86.

Valledor L, Meijón M, Hasbún R, Jesús Cañal M, Rodríguez R. 2010. Variations in DNA methylation, acetylated histone H4, and methylated histone H3 during *Pinus radiata* needle maturation in relation to the loss of *in vitro* organogenic capability. *J Plant Physiol* **167**: 351-357.

Vanyushin BF, Ashapkin VV. 2011. DNA methylation in higher plants: past, present and future. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms* **1809**: 360-368.

Viejo M, Rodríguez R, Valledor L, Pérez M, Cañal MJ, Hasbún R. 2010. DNA methylation during sexual embryogenesis and implications on the induction of somatic embryogenesis in *Castanea sativa* Miller. *Sex Plant Reprod* **23**: 315-323.

von Aderkas P, Bonga JM. 2000. Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiol* **20**: 921-928.

Wagner D. 2003. Chromatin regulation of plant development. *Curr Opin Plant Biol* **6**: 20-28.

Wang X, Zhu WG. 2008. Advances in histone methyltransferases and histone demethylases. *Chin J Cancer* **27**: 296-301.

Waterborg JH. 2011. Plant histone acetylation: In the beginning…. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* **1809**: 353-359.

Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schübeler D. 2005. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* **37**: 853-862.

Weng YI, Huang TH, Yan PS. 2009. Methylated DNA Immunoprecipitation and Microarray-Based Analysis: Detection of DNA Methylation in Breast Cancer Cell Lines. in *Methods Mol Biol* (ed. N (Clifton), pp. 165-176

Wolffe AP, Matzke MA. 1999. Epigenetics: Regulation Through Repression. *Sci* **286**: 481-481.

Wright WE, Leavitt SW. 2005. Needle cell elongation and maturation timing derived from pine needle cellulose δ18O. *Plant Cell Environ*: 28-28.

Xu M, Li X, Korban SS. 2004. DNA-methylation alterations and exchanges during *in vitro* cellular differentiation in rose (*Rosa hybrida* L.). *Theor Appl Genet* **109**: 899-910.

Zhang K, Sridhar VV, Zhu J, Kapoor A, Zhu JK. 2007. Distinctive core histone post-translational modification patterns in Arabidopsis thaliana. *PLoS One* **2**.

Zhang M, Kimatu JN, Xu K, Liu B. 2010. DNA cytosine methylation in plant development. *Journal of Genetics and Genomics* **37**: 1-12.

Zhang S, Lemaux P. 2004. Molecular Analysis of *in vitro* Shoot Organogenesis. *Crit Rev Plant Sci* **23**: 325-335.

Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SWL, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE. 2006. Genome-wide high-resolution mapping and functional analysis of DNA methylation in Arabidopsis. *Cell* **126**: 1189-1201.

Chapter 2. DNA Methylation and H4 Histone Acetylation Landscapes Induced for Plant Growth Regulators in *Pinus radiata* D. Don Cotyledons

2.1. Introduction

Morphogenesis and adventitious organogenesis are complex processes of cell plasticity and, according to Christianson and Warnick (1985), they can be divided in three stages: acquisition of competence, induction, and morphological differentiation.

In adventitious caulogenesis, somatic cells respond to the hormonal stimulus with a reprogramming process achieving a new cell fate. Cytokinins are the most efficient plant growth regulators in the induction of this process (Biondi and Thorpe 1982). Previous studies have observed that histidine protein kinases act as cytokinin receptors, whereas histidine fosfotransferases transmit the signal to nucleus response regulators which activate or suppress the transcription (Zhang and Lemaux 2004). For example, some genes such as *CYTOKININ RESPONSE 1 (CRE1),* an hormone receptor (Inoue et al. 2001) and *ENHANCER OF SHOOT REGENERATION (ESR1),* which over-expression allows shoot development (Fujimoto et al. 2000), or some family members of *NAM (NO APICAL MERISTEM)* and *CUC1 y CUC2 (CUP SHAPE COTYLEDON)* (Aida et al. 1997; Aida et al. 1999; Takada et al. 2001) are involved in new bud formation in Arabidopsis. In other plant species like *Paulownia kawakamii*, *PkMADS1* gene has an expression only observed in the apex and in tissues producing adventitious shoots (Prakash and Kumar 2002).

New organ formation involves changes in cell fate and a different gene expression pattern as well, regulated among others by epigenetic processes (Bhalla and Singh 2006; Costa and Shaw 2007). Regulation of gene expression has an important role to generate a correct caulogenic pattern, and genomic DNA methylation represents one of the most studied regulatory mechanisms in animals and plants (Vanyushin and Ashapkin 2006).

The aim of this work is to investigate the correlation among the levels of DNA methylation and the response of *Pinus radiata* D. Don embryos in presence of an inductor of adventitious caulogenesis and other inductors of different developmental processes. The importance of these methylation levels lies in the tissue where they are present, for that reason, patterns of 5-mdC and acetylated H4 Histone in mature zygotic embryos and along the process of adventitious shoot induction and seedling development in *P. radiata* embryos were determined as well.

2.2. Materials and methods

2.2.1. Plant material

Pinus radiata cones (Basque Country Coast ES06, batch 2614 producer 00949/05) were taken during three consecutively years from the Breeding and Conservation Service of Forest Genetic Resources (Spanish Sea, Rural and Environment Ministry). Embryos used for epigenetic analyses and *in vitro* culture were isolated from immature and mature seeds. Seed coats from mature seeds were removed and mega gametophytes were surface sterilized by immersion in a 33 % (v/v) H_2O_2 solution for 20 min followed by three rinses in sterile double distilled water. For embryo culture, dissected embryos under sterile conditions, were vertically placed upside down in petri dishes containing 20 mL of QLP medium (QL medium macronutrients plus MS medium micronutrients, vitamins and iron), supplemented with sacarose (30 g L^{-1}) and with 0.8 % (w/v) agar (Roko S.A., Oleiros, Spain). Before autoclaving, the pH of all media was adjusted to 5.8. Mature whole embryos were cultured for 3, 6, 10, 14 and 21 days on QLP medium and in QLP medium plus BA, ABA, IBA and 2, 4-D 22.19 µm. All plant growth regulators belong to Duchefa Biochemie (B.V. Harleem, The Netherlands). Cultures were maintained in a growth chamber at 25 ± 2 ºC with a 16 h photoperiod at a photon flux of 80 ± 5 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58W/33, Philips, France).

2.2.2. Quantification of global DNA methylation

2.2.2.1. DNA extraction

A fresh weight of 75-100 mg of *in vitro* cultured embryos as well as from mature and immature embryos was used for DNA extraction with DNeasy Plant Mini Kit (QIAGEN N.V. Venlo, The Netherlands) according to manufacturer´s instructions. DNA was concentrated with a DyNA Vap (Labnet Edison, NJ, USA) connected to a vacuum pump (Heto Guang Dong, China) and resuspended in 10 µL of ddH_2O (0.75 µg μL^{-1}).

2.2.2.2. DNA hydrolysis

DNA samples with at least 5 µg of genomic DNA were denatured for 5 min at 95 ºC and then chilled on ice. Enzymatic hydrolisis was carried out by adding 1.25 µL of 10 mM $ZnSO_4$ and 2.5 µL of 200 u mL^{-1} Nuclease P1 in 30 mM $C_2H_3O_2Na$ (Sigma-Aldrich St. Louis, MO, USA) mixture. Samples were incubated for at least 16 h at 37 ºC, under moist conditions to avoid desiccation. 2.5 µL 0.5 M, pH 8.3 Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (Sigma-Aldrich St. Louis, MO, USA) and 0.5 µL 50 u mL^{-1} in 2.5 M $(NH_4)_2SO_4$ alkaline phosphatase (Sigma-Aldrich St. Louis, MO, USA) were added later on, and samples were incubated another 2 h at 37 ºC to remove phosphate group. After 20 min at 14 000 rpm centrifugation, supernatant was stored at -20 ºC until analysis.

2.2.2.3. Capillary electrophoresis procedure

Global methylation quantification was carried out by High Performance Capillary Electrophoresis technique as previously described by Hasbún et al. (2008). An eCAP capillary tubing (Beckman, Palo Alto, CA, USA) 75 µm ID, 375 µm OD and 60 cm long in an Agilent CE system device was used and data was obtained with Agilent 3D-CE Chemstation software (Agilent Technologies Santa Clara, CA, USA). Five analytical samples were measured for

each hormone treatment and time of induction. The percentage of DNA methylation was calculated as follows: mdC peak area x 100/ (dC peak area + mdC peak area).

2.2.3. Quantification of total acetylated H4 Histone

Whole mature embryos cultured in the presence of BA, ABA, IBA, 2, 4-D and basal medium QLP without growth regulators after 3 and 6 days were picked up and frozen in liquid nitrogen for protein quantification. Acetylated H4 Histone (AcH4) was quantified by Western blot and image analysis techniques.

2.2.3.1. Protein Extraction.

Whole protein extraction was carried out according to Valledor et al. (2010). An amount of 500 mg of each sample was ground to a fine powder with liquid nitrogen using a mortar and a pestle. The powder was suspended in 10 mL of extraction buffer (100 mM Tris-HCl pH 8, 10 % Sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 % β-mercaptoethanol). Proteins were denatured by boiling for 10 min and then centrifuged at 8 000 g for 10 min. Supernatant was transferred to a new tube containing 25 mL of acetone with 0.07 % (w/v) Dithiothreitol (DTT) and proteins were allowed to precipitate at -20 °C for 1 h; the precipitate was recovered after centrifugation at 35 000 g for 30 min, cleaned with 8 mL of cold (-20 °C) acetone containing 0.07 % (w/v) DTT and sonicated twice more, 30 s each, keeping the extract at -20 °C for 30 min, and then centrifuged at 20 000 g for 30 min. The cleaning process was repeated once. The final pellet was air-dried and solubilized in 400 µL of 8 M urea, 2 % (w/v) 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 20 mM DTT, 0.5 % (v/v) Biolytes pH range 3 -10 (Bio-Rad), and 0.0001 % (w/v) bromophenol blue. Insoluble material was removed by centrifugation at 20 000 g for 15 min. Protein concentration was determined according to the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (BSA) as standard. Samples were stored at -80°C until SDS-PAGE.

2.2.3.2. Protein blot

Proteins and standard Rainbow Low Range (Bio-Rad, Hercules, CA, USA) were loaded in Laemmli buffer (1970), separated by electrophoresis in 15 % acrylamide SDS gels and then transferred by electroblotting (350 mA for 2 h) to immobilon membranes (Millipore Corp., Bedford, MA, USA). For the immunodetection, the membranes were blocked overnight in 2 % powdered skimmed milk in phosphate-buffered saline (PBS) containing 0.5 % Tween 20 at 4 ºC. Then, the membranes were incubated for 2 and 1 h with primary and secondary antibodies diluted 1/2000 and 1/1000, respectively, in blocking solution. The primary antibodies used were polyclonal anti-acetyl-histone H4 (Upstate, Millipore Corp., Bedford, MA, USA ref. 06-866) and polyclonal anti-Actin (Chemicon, Millipore Corp., Bedford, MA, USA ref. AB978); the latter antibody was used as a control. Secondary goat anti-Rabbit IgG antibody was coupled to phosphatase alkaline (Calbiochem, La Jolla, CA, USA ref. 401312). The membranes were washed three times with 0.2 % powdered skimmed milk in PBS and 0.05 % Tween 20 for 5 min. The secondary antibodies were revealed by treatment with 5 % nitroblue tetrazolium 2.5 % bromo-chloro-indolyl-phosphate in NN-Dimethylformamide. Densitometric measurements were taken after immunodetection using the Kodak Gel Logic 100 and Kodak 1D v 3.6 (Kodak, Rochester, NY, USA). AcH4 abundance index was calculated as follows: AcH4 band intensity/Actin band intensity. Band analysis was carried out by Image J v 1.43u (US National Institutes of Health, Bethesda, Maryland, USA).

2.2.4. Immunolocalization

Whole mature embryos and embryos cultured for 3 and 6 days in the presence of the aforementioned growth regulators, and cotyledons from embryos cultured at 10 days with BA were picked up and fixed in 4 % paraformaldehyde in PBS in vacuum conditions for immunolocalization

Overnight fixed embryos were washed three times in PBS and stored in 0.1 % paraformaldehyde at 4 ºC. Tissue was sectioned at 50 µm thickness using a cryomicrotome CH1510-1 (Leica Microsystems GmbH Wetzlar, Germany). Samples were mounted on slides coated with APTES (3-aminopropyltriethoxysilane (Sigma-Aldrich St. Louis, MO, USA). Immunolocalization was performed following the procedure described by Meijón et al. (2009) with some modifications. Embryos were dehydrated in an ascending series of ethanol and rehydrated through a descending series of the same solvent. Then, embryo sections were rendered permeable to the antibodies by incubating them for 45 min at 30 ºC in 2 % cellulose in PBS and denatured in 2N HCl for 15 min. Permeabilized embryo sections were incubated 1 hour with mouse anti-5-methylcytidine (Ref. BI-MECY-0100; Eurogentec Liège, Belgium) or with rabbit anti-acetyl-Histone H4 (Ref. 06-866 Upstate Millipore, Billerica, MA, USA) antibody diluted 1/50 in 1 % blocking solution. Non bounded antibody was washed with 0.1 % Tween 20 in PBS. Alexa Fluor 488-labelled anti-mouse or anti-rabbit polyclonal antibody (Cat. Nº A-11001; Molecular Probes Eugene, OR, USA) diluted 1/25 was used as secondary antibody. Finally slides were counterstained with DAPI (4', 6-diamidino-2-phenylindole; Fluka Sigma-Aldrich St. Louis, MO, USA). Negative controls were obtained replacing the primary antibody by PBS. Fluorescence was visualized using a confocal microscope TCS-SP2-AOBS (Leica Microsystems GmbH Wetzlar, Germany) connected to a workstation and images were processed with Leica Confocal Software v2.5.

2.2.5. Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the R version 2.9.2 software (R Foundation for Statistical Computing, Vienna). Residuals normality for all analysis was evaluated by Shapiro–Wilk normality test (Shapiro and Wilk 1965) without transformation. However, homocedasticity was analyzed by Levene's test (Brown and Forsythe 1974). Multiple comparisons were performed by significant Tukey's HSD test (Steel

and Torrie 1980) after ANOVA. A probability level of $p < 0.01$ was considered significant for all statistical analysis.

2.3. Results

2.3.1. Macromorphological traits

Regarding to macromorphological aspect, QLP, BA, ABA, IBA and 2,4-D treated embryos showed remarkable differences from day 6 (Table 2.1; Fig 2.1). An absence of elongation and growth, both hypocotyl and cotyledons, in the embryos cultured with BA, IBA and 2,4-D was observed. A callus formation previous to adventitious organogenesis also occurred in BA treatment, and later on was observed in embryos cultured with IBA (adventitious root induction) and 2,4-D (callus induction). ABA showed few differences along time regarding normal development pattern, except for a delay in germination time until day 10 where values were similar to QLP.

Besides the lack of embryo elongation and growth of both, hypocotyl and cotyledons, a little swelling of the hypocotyls and visible adventitious buds came up from day 10 in BA treated embryos. Callus formation in the presence of the auxins IBA and 2, 4-D was observed from day 10 and remained in the hypocotyl, only cotyledons remained intact along time in IBA treatment. Meanwhile the whole embryos transformed into a callus mass in the case of 2, 4-D due to the loss of meristem identity, in both Root Apical Meristem (RAM) and Shoot Apical Meristem (SAM).

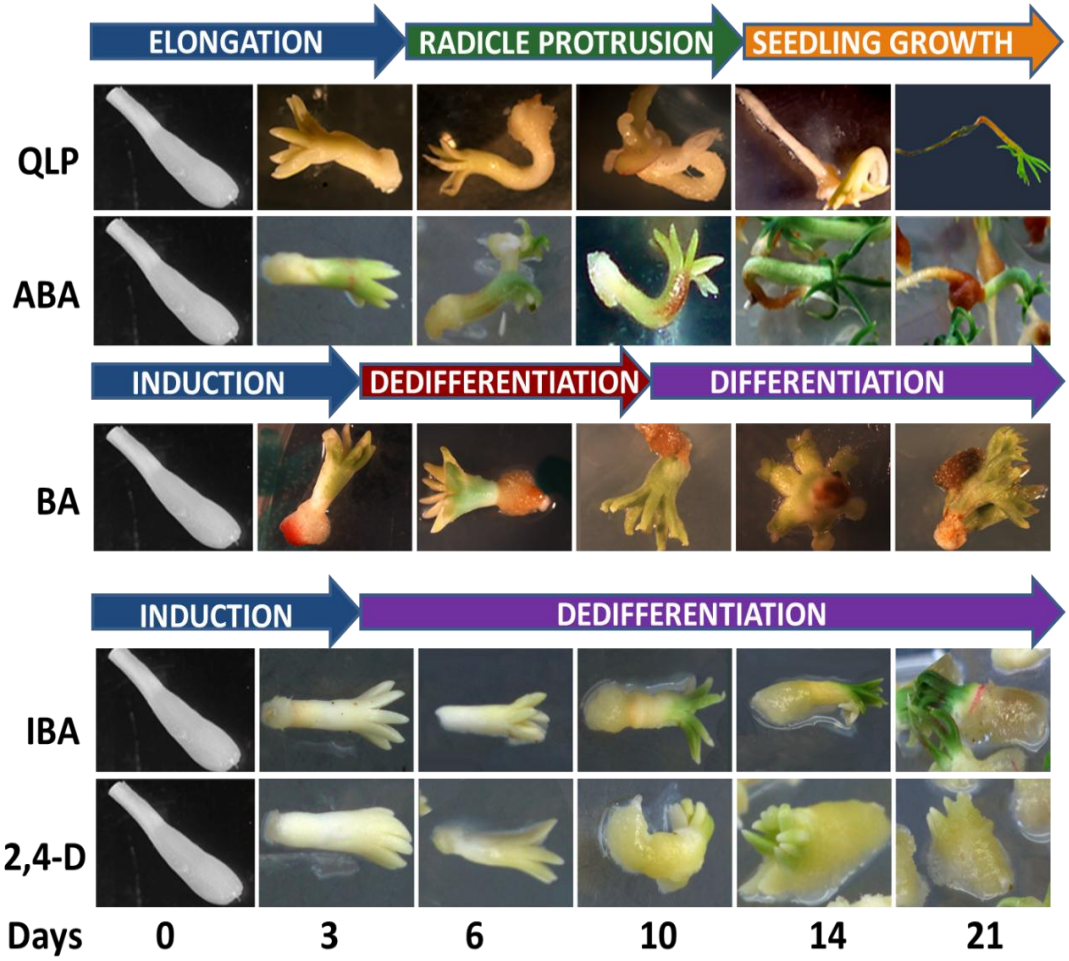


Figure 2.1: Macromorphological aspect of *P. radiata* mature embryo and embryos cultured during 3, 6, 10, 14, and 21 days in QLP basal medium and with BA, ABA, IBA and 2, 4-D growth regulators at a concentration of 22.19 uM.

Table 2.1: Macromorphological response of *P. radiata* embryos/seedlings cultured in basal medium QLP, and in basal medium QLP plus growth regulators BA, ABA, IBA and 2, 4-D (22.19 μM).

	Days of culture	Number of explants	Survival (%)	Germination (%)	Hypocotyl larger than 1 cm (%)	Cotyledons larger than 1 cm (%)
QLP	0	15	100	0	0	0
	3	15	100	0	0	0
	6	15	100	66.6	0	53.3
	10	15	93.3	73.3	53.3	66.6
	14	15	93.3	80	80	86.6
	21	15	86.6	86.6	80	86.6
	Days of culture	**Number of explants**	**Survival (%)**	**Germination (%)**	**Hypocotyl larger than 1 cm (%)**	**Cotyledons larger than 1 cm (%)**
ABA	0	15	100	0	0	0
	3	15	93.3	0	0	0
	6	15	93.3	13.3	0	0
	10	15	93.3	86.6	86.6	80
	14	15	93.3	93.3	86.6	86.6
	21	15	93.3	93.3	93.3	93.3
	Days of culture	**Number of explants**	**Survival (%)**	**Adventitious shoot induction (%)**	**Basal callus (%)**	**Cotyledons larger than 1 cm (%)**
BA	0	15	100	0	0	0
	3	15	100	0	86.6	0
	6	15	100	0	86.6	0
	10	15	93.3	40	86.6	0
	14	15	86.6	66.6	86.6	20
	21	15	86.6	80	86.6	73.3
	Days of culture	**Number of explants**	**Survival (%)**	**Indirect root induction (%)**	**Basal callus (%)**	**Cotyledons larger than 1 cm (%)**
IBA	0	15	100	0	0	0
	3	15	100	0	0	0
	6	15	100	0	0	0
	10	15	100	66.6	93.3	6.66
	14	15	100	100	100	33.4
	21	15	100	100	100	93.3
	Days of culture	**Number of explants**	**Survival (%)**	**Cell dedifferentiation (%)**	**Basal callus (%)**	**Cotyledons larger than 1 cm (%)**
2,4-D	0	15	100	0	0	0
	3	15	93.3	0	26.6	0
	6	15	93.3	53.3	53.3	0
	10	15	93.3	93.3	93.3	0
	14	15	93.3	93.3	93.3	13.3
	21	15	93.3	93.3	93.3	13.3

2.3.2. Global DNA methylation during embryogenesis

DNA methylation was quantified in three different development stages, Embryo sac (Immature megagametophyte), Immature and Mature embryo. In spite of embryo macromorphological differences between the Embryo sac and Immature embryo, there were no significant differences in the percentage of methylation (Fig 2.2). However not

only there were macromorphological differences between Immature and Mature embryos, but also a statistically significant increment in global DNA methylation.

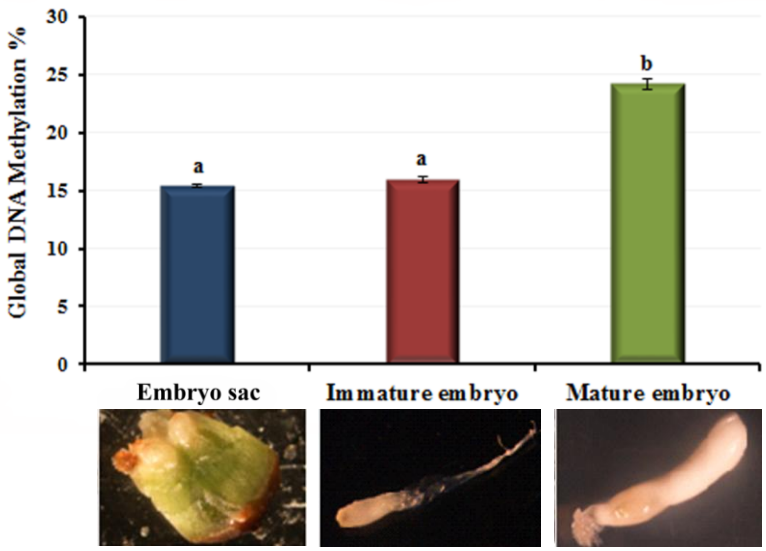


Figure 2.2: 5-mdC content in *P. radiata* embryos in the following stages: Embryo sac, Immature embryo and Mature embryo. Different letters indicate significant differences by Tuckey test after ANOVA α= 0.01%. Macromorphological aspect of embryos of *P. radiata* at different stages of maturation is also shown.

2.3.3. Global DNA methylation during adventitious caulogenesis and other responses

DNA methylation was quantified along time in QLP basal medium and in BA, ABA, IBA and 2, 4-D treatments to monitorize the differences in global methylation during cell reprogramming (Fig 2.3). ANOVA showed an interaction between the two variables, culture time and type of growth regulator (Table 2.2).

Comparing results along time after 3 days of culture IBA and 2, 4-D induced a significant decrease in methylation levels regarding the other essays. However after 6 days embryos in QLP, BA and ABA decreased their methylation level, being this decrease more marked in the case of ABA. IBA and 2, 4-D remained with almost the same values finding no differences among treatments. The auxins IBA and 2, 4-D kept their methylation levels at day 10, and ABA treatment increased its level close to auxin ones. In the meantime DNA methylation decreased in QLP and BA, but still no significant differences were found. A marked different tendency was observed at day 14 between the control QLP and the rest of treatments, with significant differences regarding to IBA and 2, 4-D. This

tendency was maintained at day 21 with an increase in DNA methylation in the control and a decrease in BA treatment. Significant differences between the control and growth regulators BA, IBA and 2, 4-D were found.

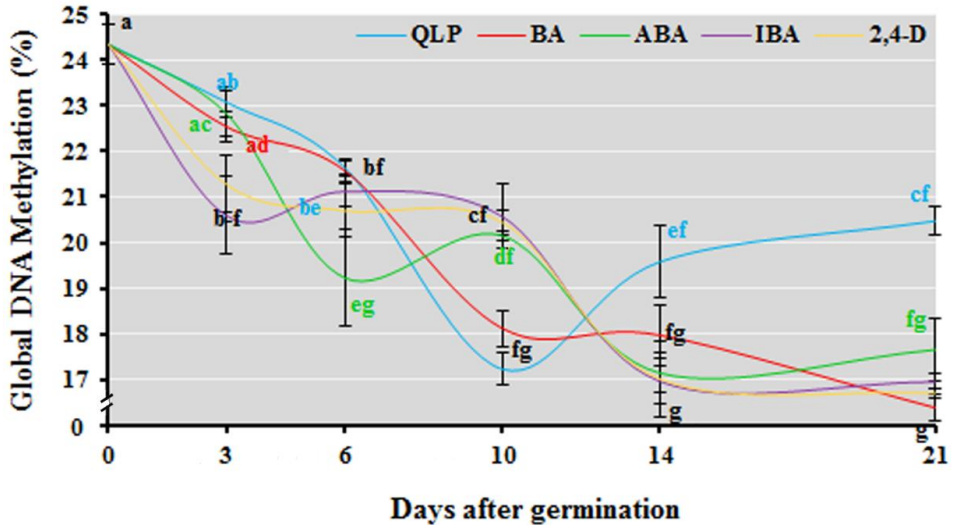


Figure 2.3: 5-mdC content in *P. radiata* embryos cultured in QLP basal medium and QLP plus BA, ABA, IBA and 2, 4-D 22.19 µM and for 3, 6, 10, 14 and 21 days. Different letters indicate significant differences by Tuckey test after ANOVA D= 0.01%. Common statistic range share leters in black.

Table 2.2: Analysis of variance ANOVA for Global DNA methylation of *Pinus radiata* zygotic embryos cultured on QLP and supplemented with BA, ABA, IBA and 2,4-D.

	Df	Sum Sq	Mean Sq	F Value	P-value
Days	5	353.19	70.64	95.405	***
Treatment	1	12.09	12.09	16.331	***
Days*Treatment	5	38.98	7.79	10.520	***
Residual	48	35.54	0.74		

2.3.4. Acetylated H4 Histone content and DNA methylation comparison

Acetylated H4 Histone (Ac H4) was quantified for 0, 3 and 6 days in QLP basal medium and in BA, ABA, IBA and 2, 4-D treatments to support the data of immunolocalization experiments. ANOVA showed an interaction between the two variables (Table 2.3).

Table 2.3. Analysis of variance ANOVA for Ac H4 amount of *Pinus radata* zygotic embryos cultured on QLP and supplemented with BA, ABA, IBA and 2,4-D 22.19 µM.

	Df	Sum Sq	Mean Sq	F value	p-value
Days	5	828.60	165.72	157.6067	***
Treatment	4	20.70	5.18	4.9217	**
Days*Treatment	20	148.07	7.40	7.0412	***
Residual	120	126.18	1.05		

According to obtained results, there was a marked difference between auxins and the rest of the treatments including control, and they were the only treatments with significant statistical differences regarding to embryos at 0 days (Fig 2.4 A). QLP remained stable along time and ABA had a drop at day 3 and increased its levels at day 6. BA treatment did not vary at day 3, but increased at day 6. Comparing with DNA methylation profiles during the same culture period we can see that, IBA and 2, 4-D had an opposite pattern to AcH4 meanwhile ABA and BA followed an inverse tendency at day 6, and at day 3 ABA had a light decrease (Fig 2.4 B).

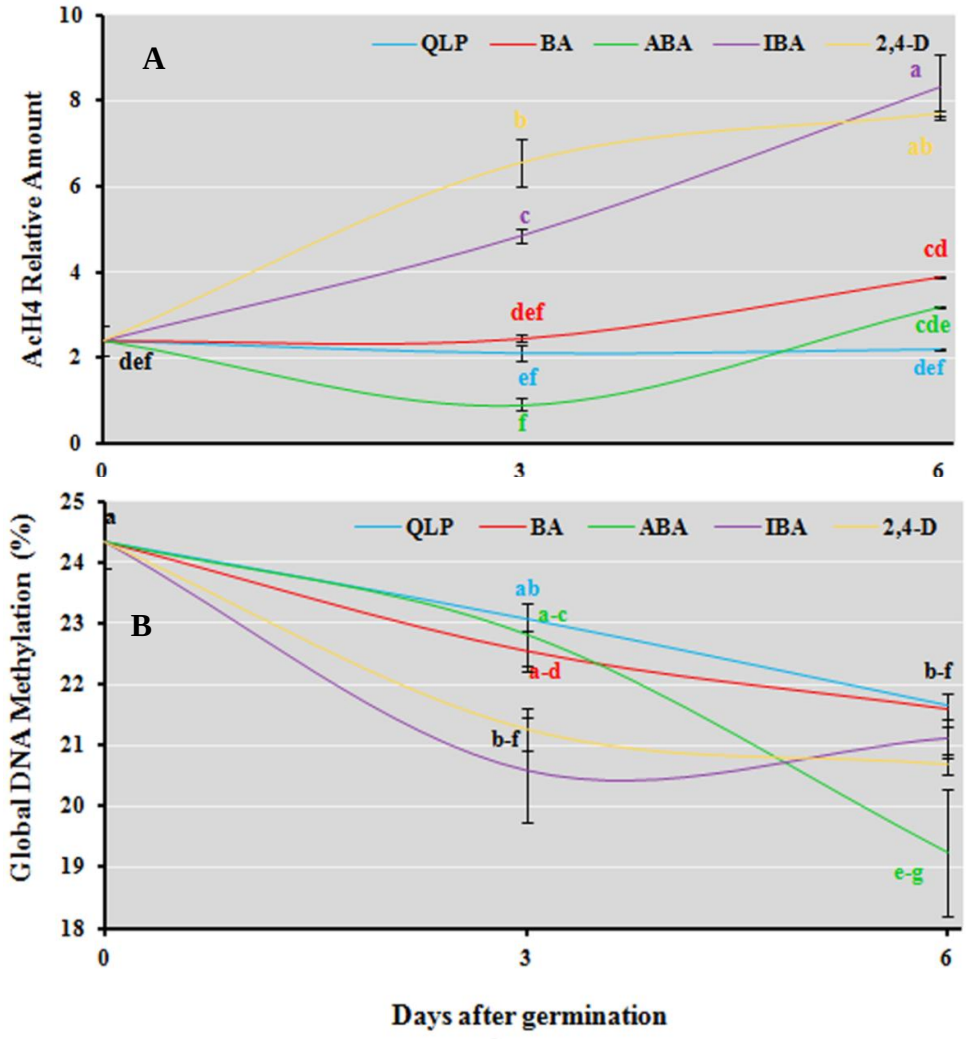


Figure 2.4: A) Acetylated H4 Histone content of *P. radiata* embryos cultured in QLP medium and supplemented with BA, ABA, IBA and 2, 4-D during 0, 3 and 6 days. Different letters indicate significant differences by Tuckey test after ANOVA D= 0.01%. B) 5-mdC content in *P. radiata* embryos cultured in QLP basal medium and QLP plus BA, ABA, IBA and 2, 4-D 22.19 µM.and for 3 and 6 days. Different letters indicate significant differences by Tuckey test after ANOVA D= 0.01%. Common statistic range share letters in black.

2.3.5. Immunohistochemistry

Immunolocalization analyses revealed the methylation and AcH4 landscape of each embryo in the different experimental situations. Whole embryos and a detail of both stem cell niches (SAM and RAM) are shown.

2.3.5.1. Mature zygotic embryo

Regarding to whole embryos (Fig 2.5), mature embryos showed two opposite situations with methylation along the tissue and a very low AcH4 signal. Both apical meristems followed the pattern of the whole embryos and presence of methylation and low signal of AcH4 were found.

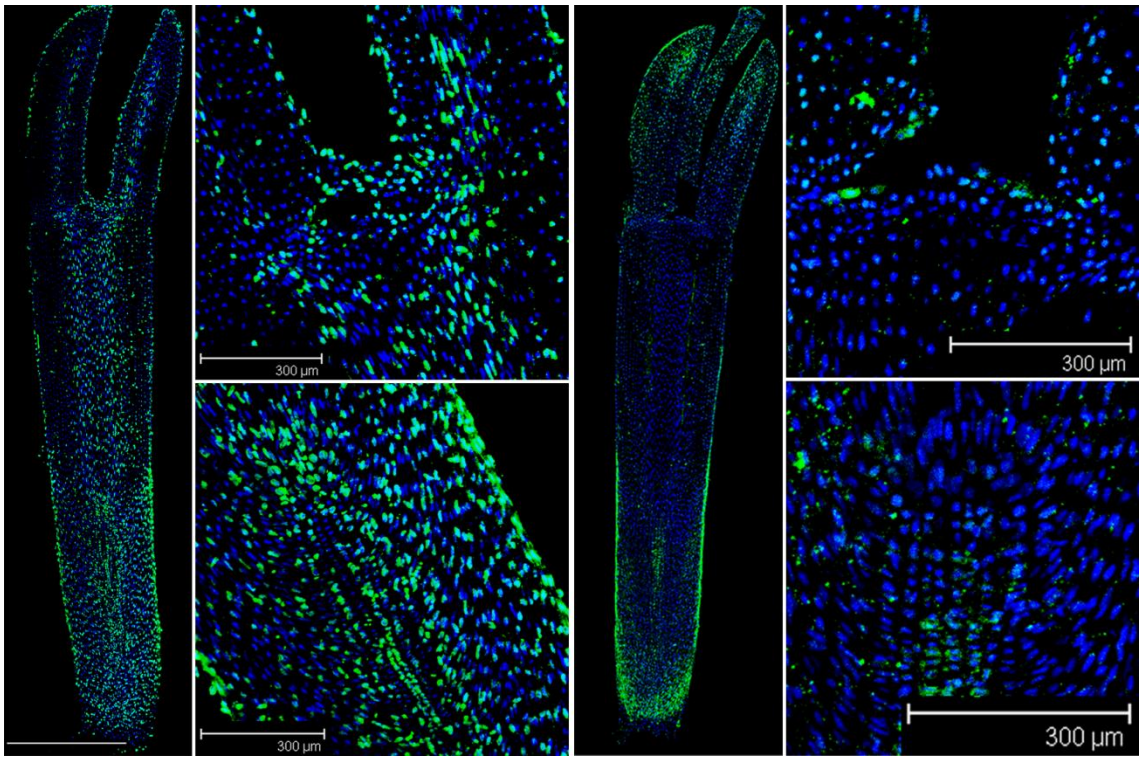


Figure 2.5: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* mature zygotic embryos. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

2.3.5.2. Embryos cultured in QLP

Embryos cultured in QLP for 3 days had the methylation signal antibody focused in the vascular cells of the hypocotyl and cotyledons. The whole embryo had methylation signal as well, but a lower number of labeled nuclei was present (Fig 2.6). Both RAM and SAM had a similar rate of methylation. Regarding to Ac H4, the RAM showed a high rate of labeled nuclei and the SAM only had several labeled nuclei. Embryos cultured for 6 days (Fig 2.7) had a high methylation signal in the mid bottom, and a low labeled nuclei in the SAM. Acetylated H4 signal was highly distributed along the tissue, except in the RAM where the columela area had no signal.

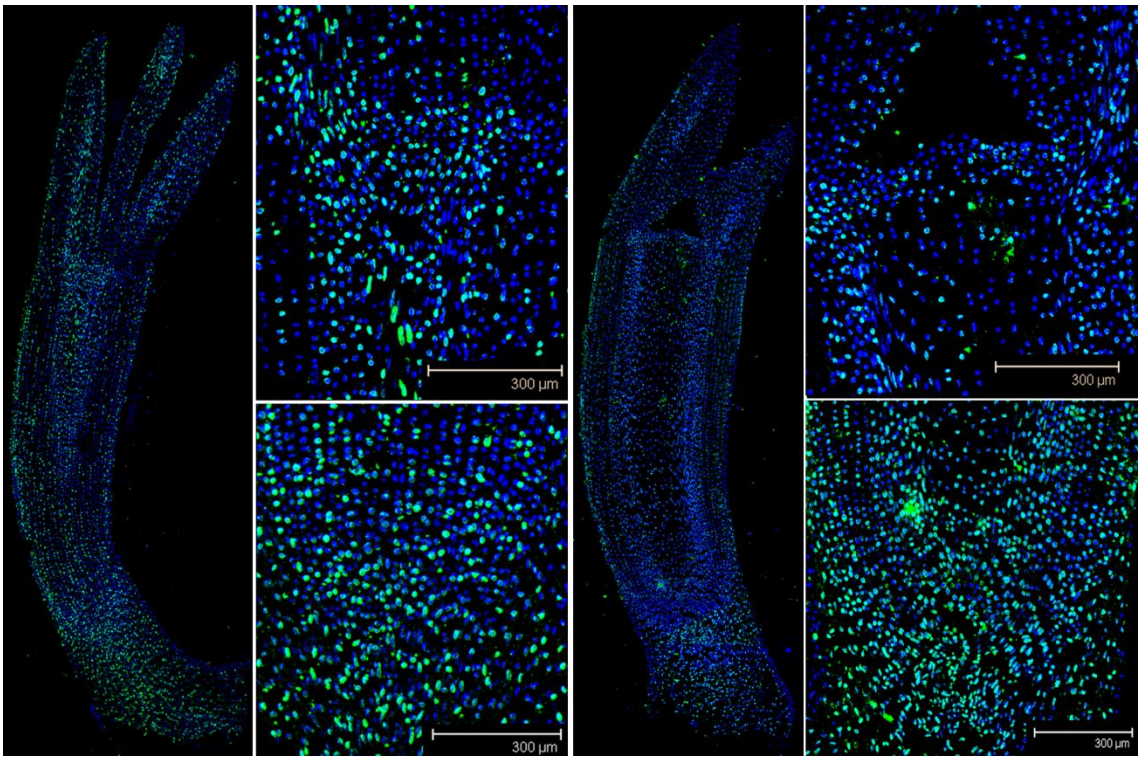


Figure 2.6: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 3 days in QLP basal medium. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

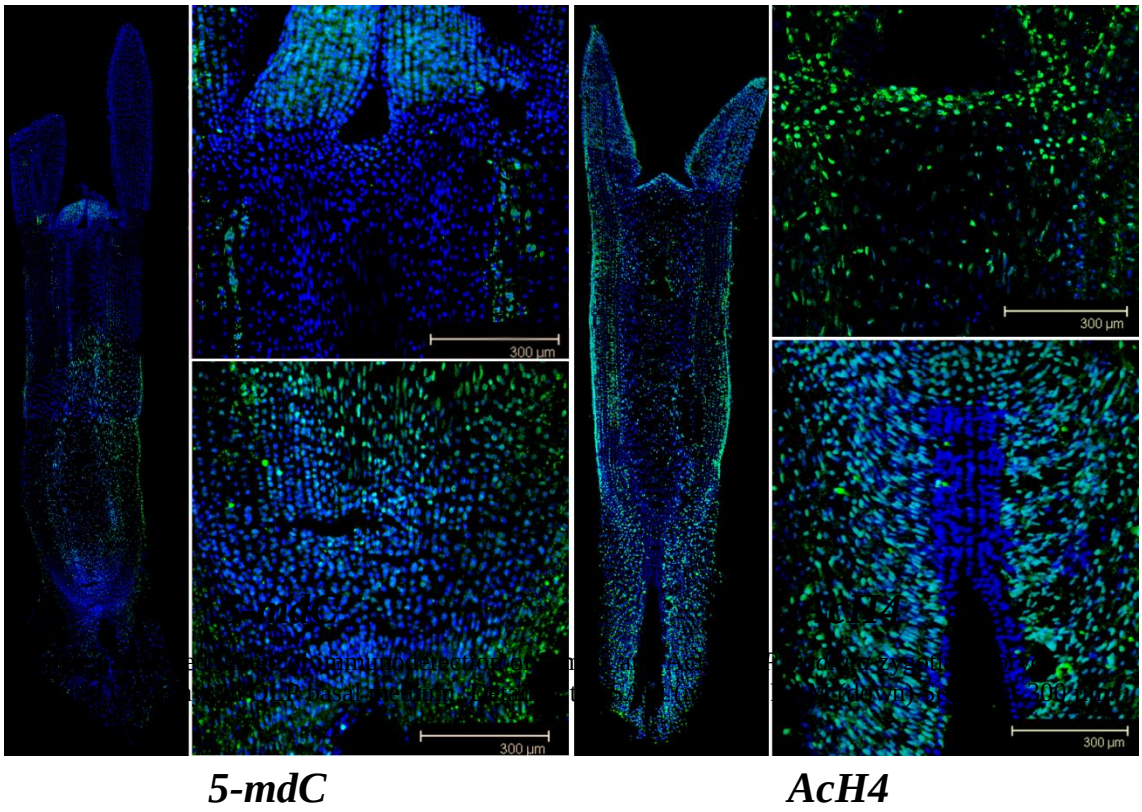


Figure 2.7 Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 6 days in QLP basal medium. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

2.3.5.3. Embryos cultured in QLP plus ABA

Methylation and AcH4 patterns at 3 days presented and even distribution along the tissue (Fig 2.8). The SAM in ABA was evenly methylated and a low density of AcH4 labeled nuclei was shown. In the RAM we could find a demethylated area covering the cells of the quiescent center (QC) and the columella, whereas AcH4 had a uniform nuclei labeling.

Methylation at day 6 showed the cotyledons with a low labeled nuclei density, and regarding both meristems, both signals showed a lightly high intensity, especially in the RAM (Fig 2.9).

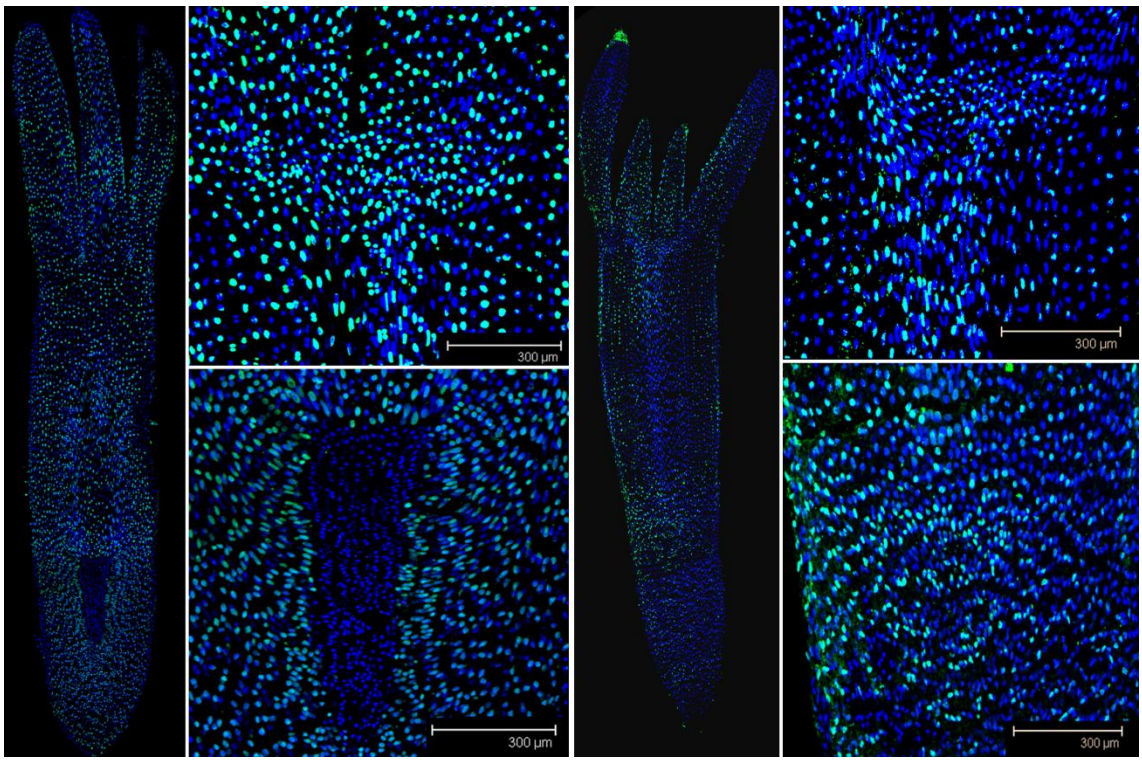


Figure 2.8: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 3 days in QLP basal medium plus ABA. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

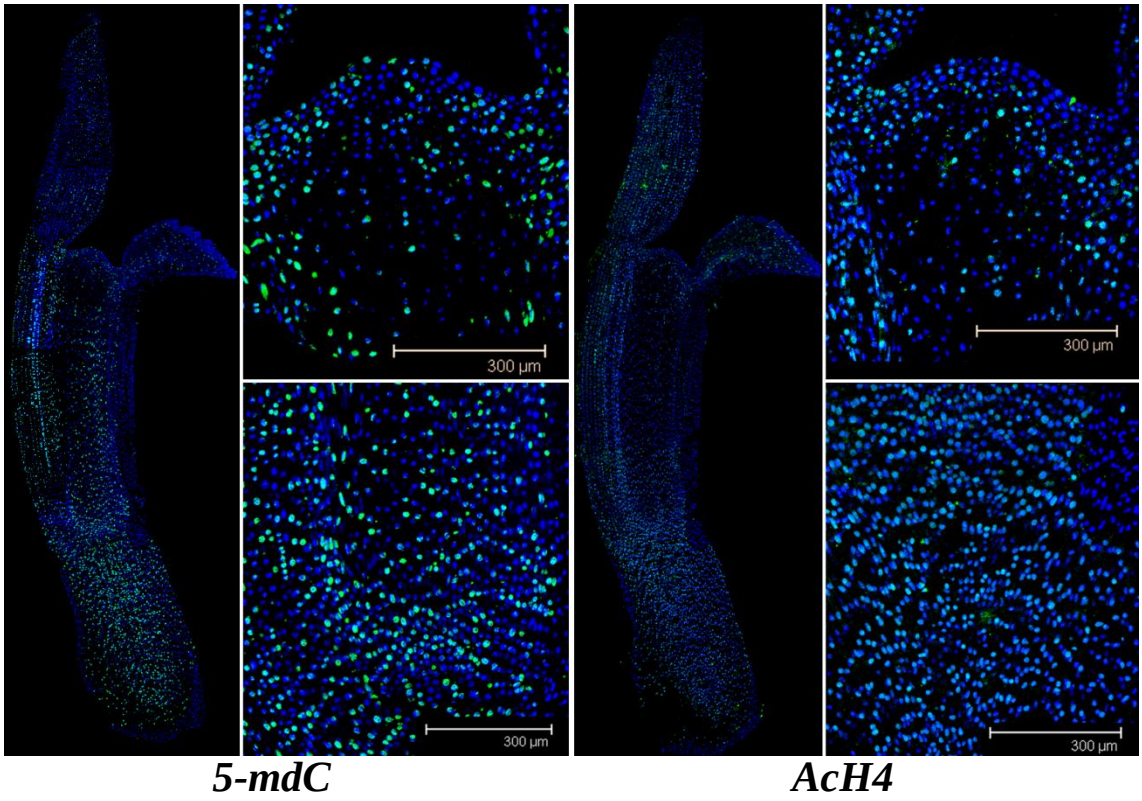


Figure 2.9: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 6 days in QLP basal medium plus ABA. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

2.3.5.4. Embryos cultured in QLP plus BA

Embryos cultured in QLP plus BA at day 3 (Fig 2.10) showed a high degree of methylation in the basal area of the embryo and a progressive demethylation as long as we go to the cotyledons. There was also a signal of AcH4 in the cotyledon area and a low signal in the rest of the embryo. Regarding SAM, it showed a high rate of methylation as well as RAM, but in the case of AcH4 there was a very low signal in SAM meristem.

Embryos cultured in BA kept the same pattern in methylation at day 6 and both meristems were highly methylated as well (Fig 2.11). On the other hand, there was a low signal of AcH4, in the whole embryo. A detail of the adventitious buds at 10 days (Fig 2.12) revealed an opposite pattern with no methylation and a high signal of AcH4.

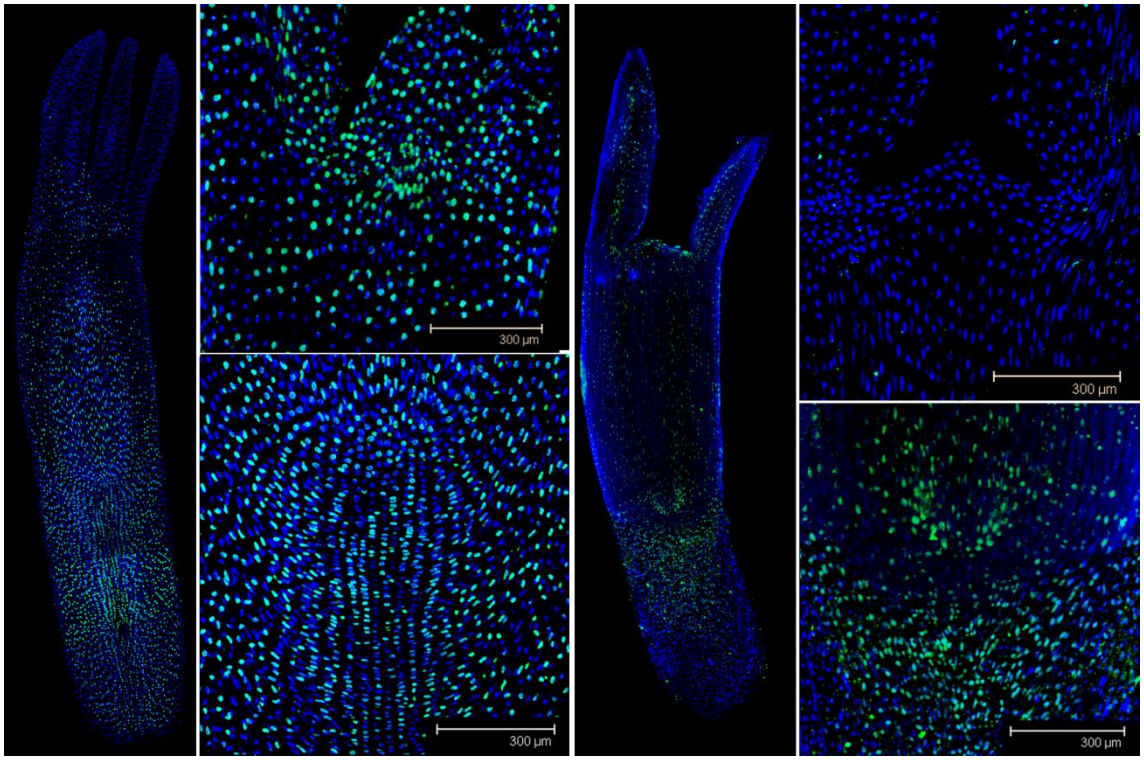
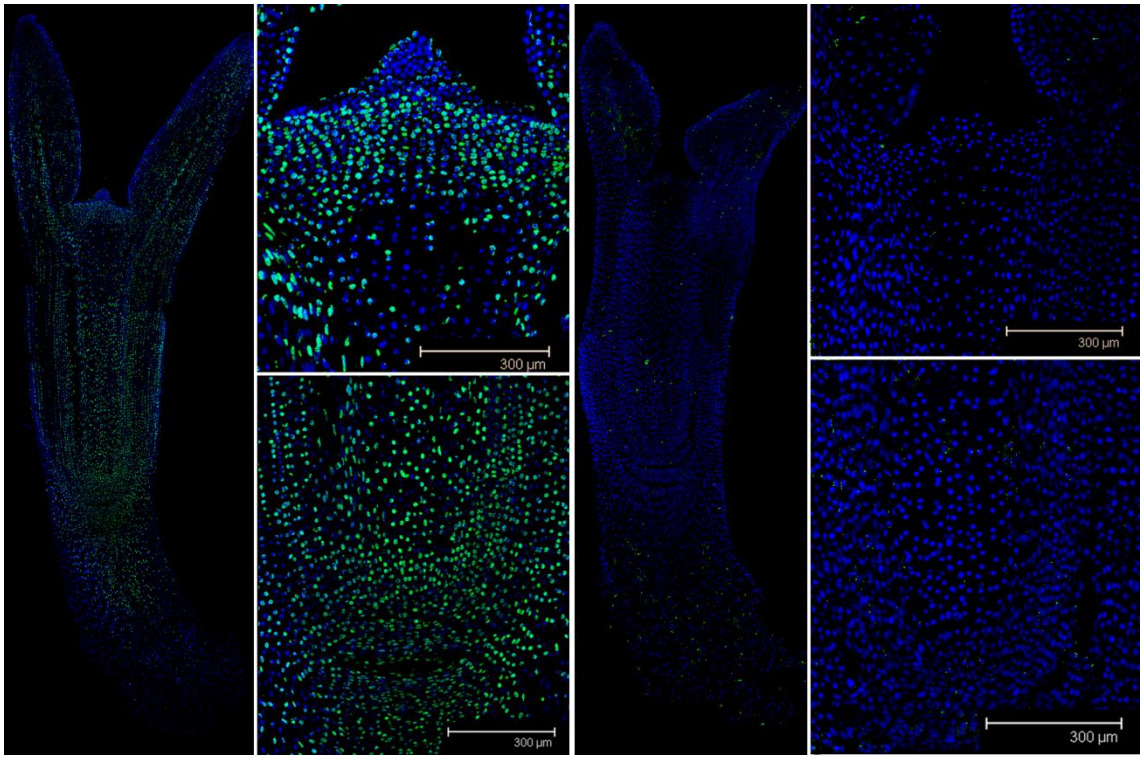
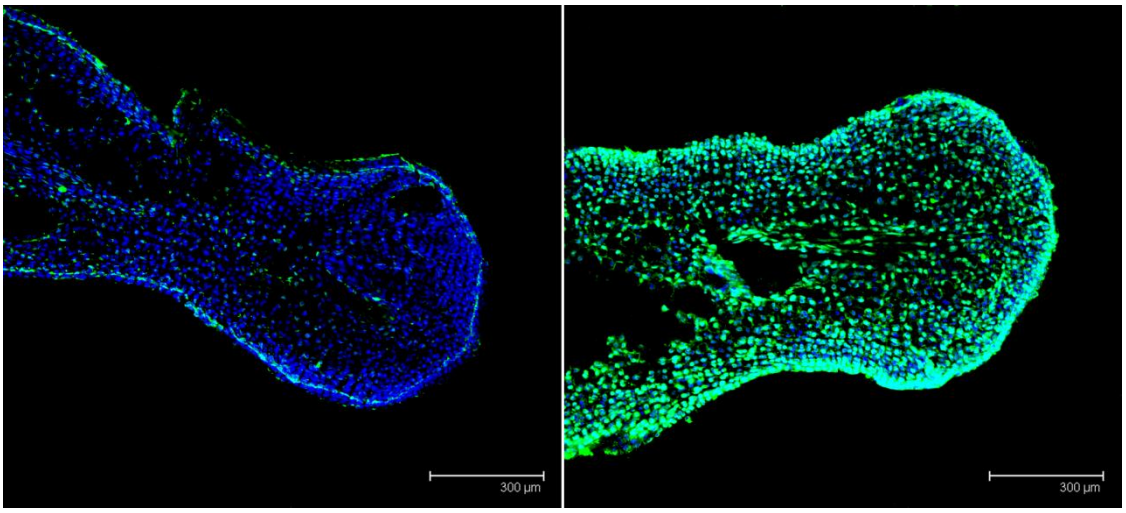


Figure 2.10: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 3 days in QLP basal medium plus BA. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.



5-mdC ***AcH4***

Figure 2.11: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 6 days in QLP basal medium plus BA. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.



5-mdC ***AcH4***

Figure 2.12: Detail of a cotyledon of a merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* adventitious buds of zygotic embryos cultured for 10 days in QLP basal medium plus BA. Scale bar 300 µm.

2.3.5.5. Embryos cultured in QLP plus IBA

IBA methylation and acetylation patterns at day 3 were present in the whole embryo (Fig 2.13). IBA presented a high methylation signal in RAM area, especially in the columella and we could also see an uneven demethylation in the apical dome of the SAM, and AcH4 signal was present in both meristems with a similar pattern than methylation pattern.

IBA methylation and acetylation followed an aberrant pattern at day 6 and although SAM kept its integrity, RAM began to disorganize (Fig 2.14). SAM meristems had both an uneven signal.

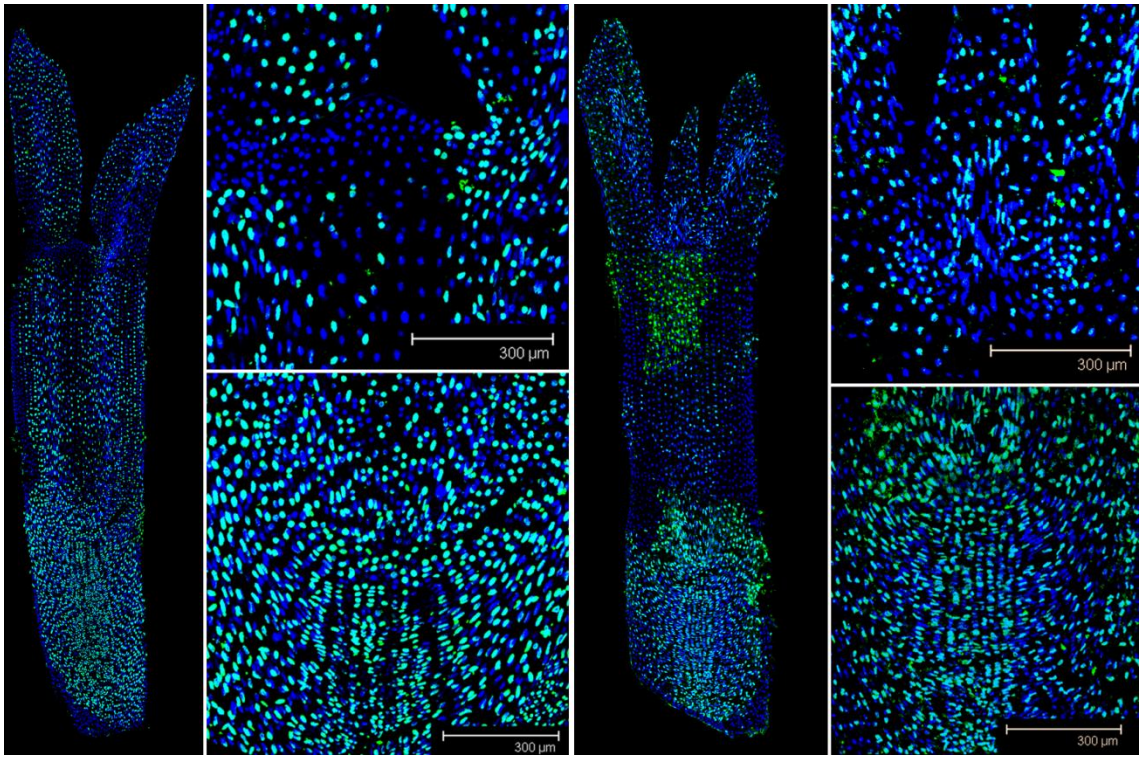
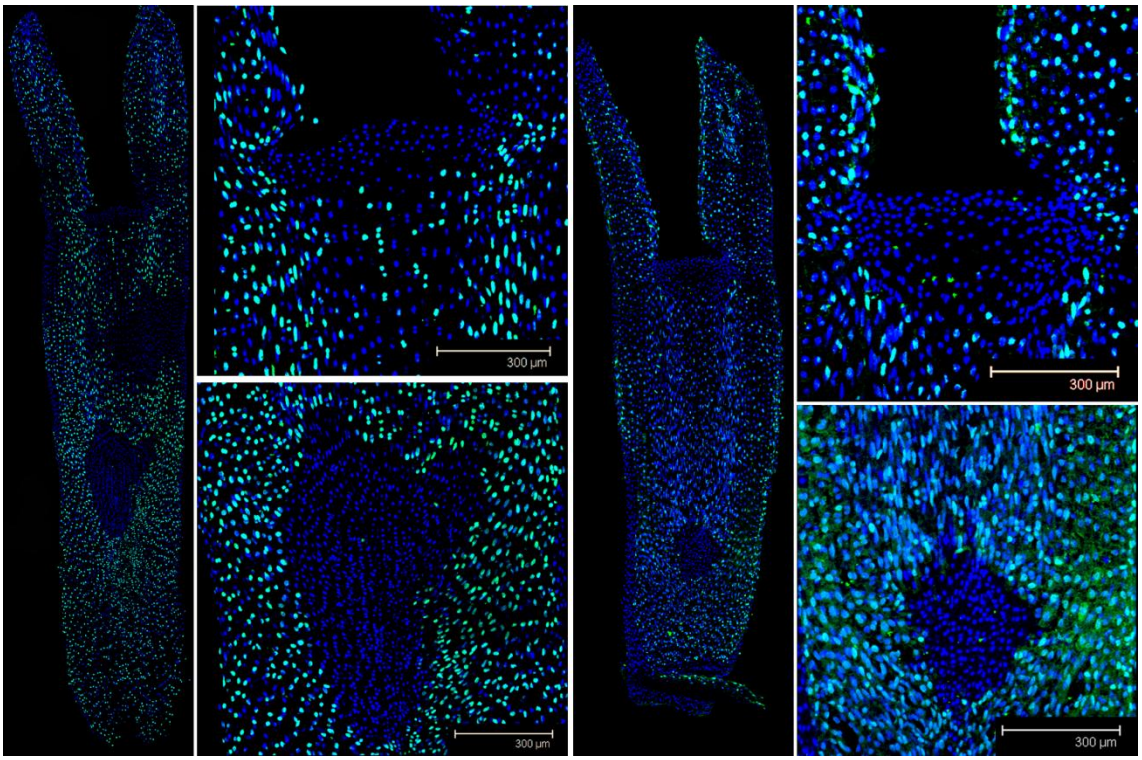


Figure 2.13: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 3 days in QLP basal medium plus IBA. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.



5-mdC ***AcH4***

Figure 2.14: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 6 days in QLP basal medium plus IBA. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

2.3.5.6. Embryos cultured in QLP plus 2, 4-D

A complete demethylation and acetylation happened in both meristems at day 3 and a demethylation in other areas of the embryo also took place showing an aberrant pattern (Fig 2.15).

At day 6 there was still no organized pattern for methylation or acetylation and the RAM looked disorganized. The SAM had no signal of methylation or acetylation (Fig 2.16).

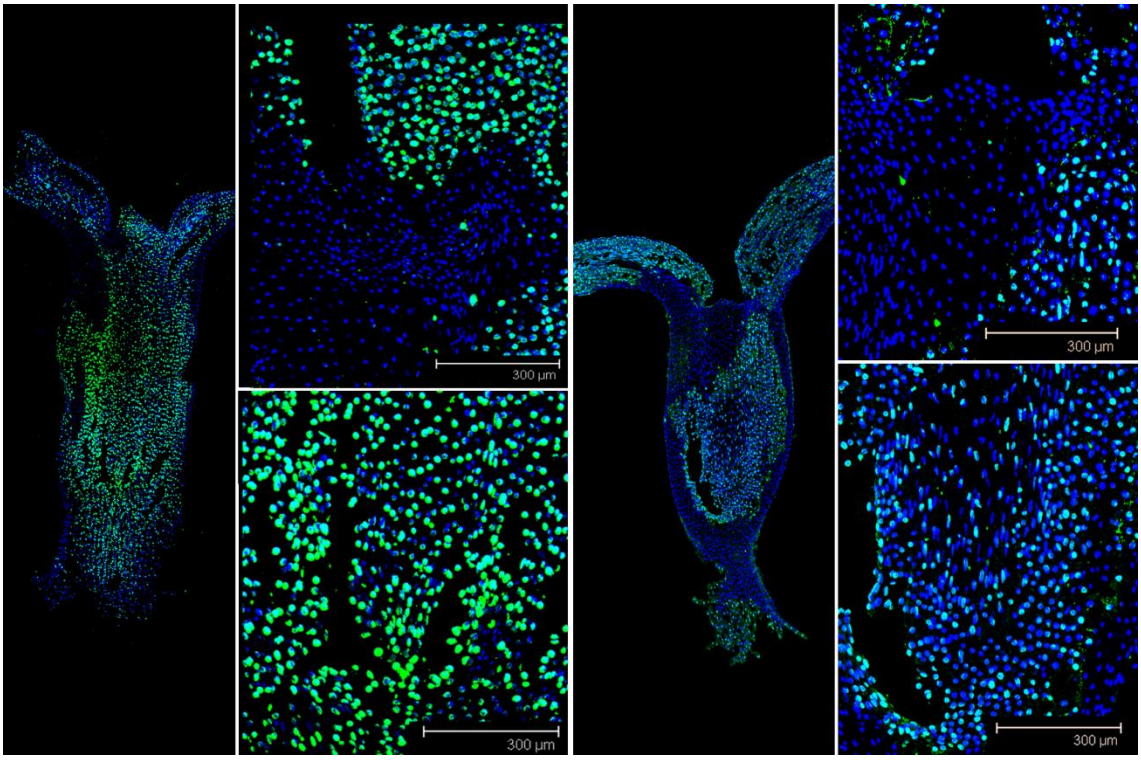


Figure 2.15: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 3 days in QLP basal medium plus 2, 4-D. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

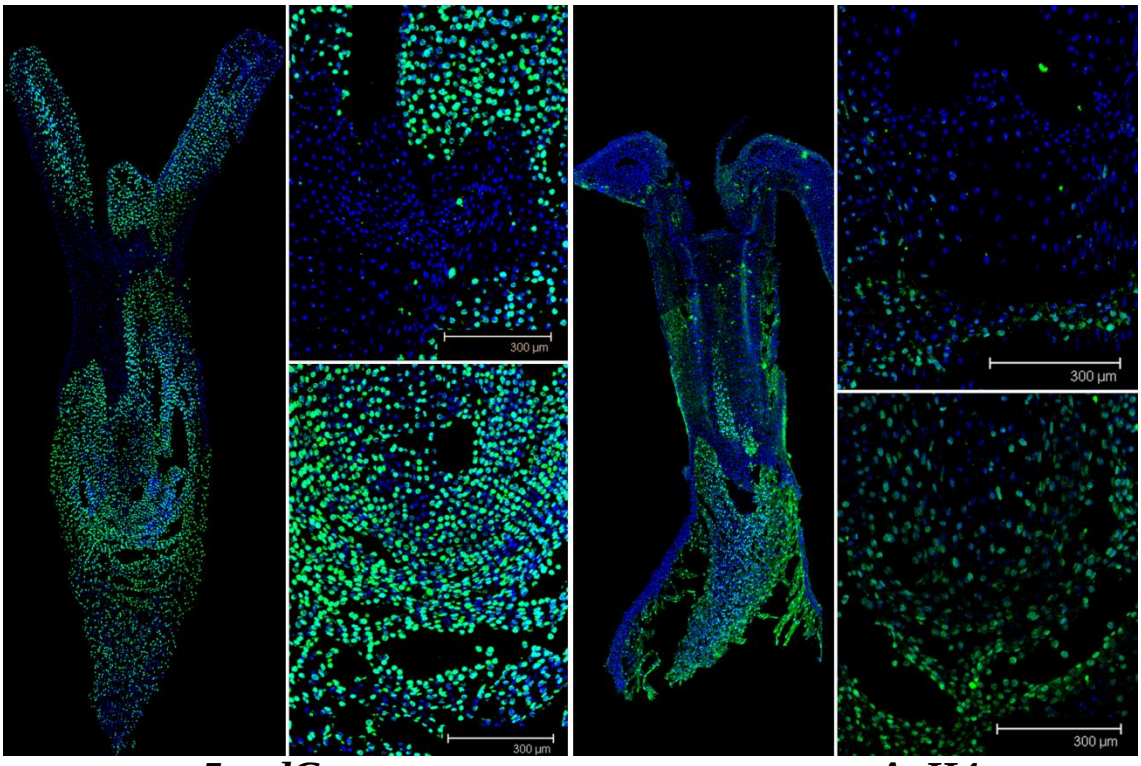


Figure 2.16: Merged image of immunodetection of 5-mdC and AcH4 in *P. radiata* zygotic embryos cultured for 6 days in QLP basal medium plus 2, 4-D. Detail of the SAM (up) and RAM (down) Scale bar 300 µm.

2.4. Discussion

2.4.1. Global methylation

2.4.1.1. Embryogenesis

Embryogenesis has been largely studied in conifers because up to date, the immature embryo is the only organ that respond to the induction of somatic embryogenesis (Klimaszewska et al. 2009; Marum et al. 2009) being a correct methylation pattern during embryogenesis essential for seed viability (Xiao et al. 2006).

2.4.1.2. Embryo cell reprogramming

DNA methylation is a dynamic mechanism defined as the methylation/demethylation changes which occur during plant growth and development. Global methylcytosine content in DNA varies widely across species, organs, and developmental states, and a demethylation happens before any differentiation program takes place (Ramchandani et al. 1999). Methylation in *in vitro* culture has been analyzed before in forest species (Fraga et al. 2002; Baurens et al. 2004; Klimaszewska et al. 2009), but there are no references regarding to adventitious organogenesis and other reprogramming responses or its distribution along the tissue.

The maximum methylation level was found in mature zygotic embryos, a characteristic behaviour of dormant tissues (Hasbún et al. 2007). After the beginning of the response in *in vitro* cultured embryos, the significant decrease in IBA and 2, 4-D points out the beginning of a dedifferentiation program, and could be due to an indirect root formation through a callus phase (Bergmann and Stomp 1994). Although ABA concentration did not avoid embryo germination, a delay regarding to QLP was observed. DNA methylation with a general decreasing tendency increases at day 10, which may be caused by ABA through DML3 transcript level reduction that increases DNA methylation

levels (Chinnusamy et al. 2008). In the case of embryos cultured in BA, it seemed strange that a decreasing level of methylation take place in embryos which did not vary their aspect except for a light swelling. Organs or tissues with low methylation levels have been described as transcriptionally active, but a methylation of genes homologues to cell cycle avoiding their expression and the inhibition of adventitious shoot induction when 5-aza-2–deoxycytidine was used (Prakash et al. 2003), indicates that active transcription during adventitious organogenesis is not focused in cell division, and that a specific methylation program is triggered.

2.4.2. AcH4 content

Following the pattern of methylation, auxins triggered a hyperacetylation process from day 3 with a bigger increase in the case of 2, 4-D but with a similar value at day 6. This fact is common during callus formation, given that it is a tissue that has a higher acetylation rate than differentiated tissues (Alatzas and Foundouli 2009). However this acetylation is regulated in those loci with hypermethylated promoters, involved in undifferentiated states of the cell. The effect of ABA in gene activation was evident with values at day 3 even lower than in mature embryos (Sokol et al. 2007; Chinnusamy et al. 2008). Cell cycle during adventitious shoot induction is inhibited in *Paulownia kawakamii* according to Prakash et al. (2003) and although histone deacetylation has been observed in tobacco protoplasts by Li et al. (2005) the cells of our embryos grown in BA with a reduced cell division and elongation kept their a low signal of AcH4.

2.4.3. Immunolocalization

In mature embryos, both methylation and acetylation patterns followed the same pattern than other dormant organs, where an important regulation mediated by DNA methylation takes place (Santamaría et al. 2009). Control embryos in QLP basal medium at day 3, kept whole embryo methylation pattern, although this tendency was followed at day

6 with a complete demethylation of RAM and cotyledons and an acetylation of the whole embryo except for the columela cells. This lack of acetylation precedes root elongation, and could be related to changes that take place during culture of zygotic embryos (Lin and Leung 2002a; Lin and Leung 2002b) indicating the importance of DNA methylation and chromatin remodeling regulating all developmental processes (Kornet and Scheres 2008; He et al. 2011).

In the case of embryos cultured in BA, the lightly swelling and the absence of elongation, make up the mirror of methylation and H4 acetylation of the embryo, and it is also known that bud induction inhibits growth not only in embryos but also in mature tissues (Zhang et al. 2010). Focusing in adventitious shoot induction, since day 6 methylation signal in the cotyledons decreased and at day 10 the two epigenetic marks changed their distribution in the adventitious bud due to the active growth that takes place (Zhang and Lemaux 2004). It is known that methylation has an influence in the shoot forming capacity, and the expression of the main genes involved in this process like *CUC1*, *CLV1*, *CLV3*, *ESR1*, and *WUS* depend on DNA methylation levels (Tokuji et al. 2011)

Regarding ABA embryos, a demethylation at day 3 of both apical meristems was the main difference regarding to control, and especially in the RAM where the quiescent center and the columela, of great importance in stem cell maintaining and growth (Vernoux and Benfey 2005; Durbak et al. 2012), were affected. Methylation and AcH4 levels at day 6 could be due to a delay in embryo germination according to Chinnusamy et al. (2008).

The similar pattern shown by the two auxins only differs in the modifications exerted in the apical meristems by 2, 4-D without methylation and acetylation signal at day 3 regarding to IBA. It is known that during normal development quiescent center and surrounding cells are highly methylated (Willemse et al. 2008). Auxins have a very important role in regulating this area (Vernoux et al. 2010) and it seems that in the case of 2, 4-D its activity in cell callus formation affects stem cells in a similar process than in

IBA. The lack of methylation of these areas could be related to cell division and callus formation that could be seen in the macromorphological images. Some genes like PIN family, whose expression is restricted to quiescent center or columela, could probably be regulated by methylation in this process as well (Xiao et al. 2006; Palovaara et al. 2010; Zhu 2010; Lafos et al. 2011).

2.5. Conclusions

The different responses induced by plant growth regulators BA, IAA, ABA y 2, 4-D showed a differential and coordinated dynamic of DNA methylation and H4 histone actylation in zygotic embryos of Pinus radiata.

Adventitious caulogenesis showed an even distribution of methylation signal decreasing at the same time acetylated H4 increased its signal, especially y new formed buds.

Auxins IBA and 2, 4-D, were the plant growth regulators with a biggest effect in DNA Methylation and H4 histone acetylation levels. Shoot and root meristems undergone drastic changes in both epigenetic marks after six days associated to undifferentiated cell formation.

2.6. References

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. 1997. Genes involved in organ separation in Arabidopsis: an analysis of the *cup-shaped cotyledon* mutant. *The Plant Cell Online* **9**: 841-841.

Aida M, Ishida T, Tasaka M. 1999. Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the *CUP-SHAPED COTYLEDON* and *SHOOT MERISTEMLESS* genes. *Development* **126**: 1563-1563.

Alatzas A, Foundouli A. 2009. Alterations in core histone variant ratios during maize root differentiation, callus formation and in response to plant hormone treatment. *Biol Res* **42**: 445-460.

Baurens FC, Nicolleau J, Legavre T, Verdeil JL, Monteuuis O. 2004. Genomic DNA methylation of juvenile and mature *Acacia mangium* micropropagated *in vitro* with reference to leaf morphology as a phase change marker. *Tree Physiol* **24**: 401-407.

Bergmann BA, Stomp AM. 1994. Effect of genotype on rooting of hypocotyls and *in vitro*-produced shoots of *Pinus radiata*. *Plant Cell Tissue Organ Cult* **39**: 195-202.

Bhalla P, Singh M. 2006. Molecular control of stem cell maintenance in shoot apical meristem. *Plant Cell Rep* **25**: 249-256.

Biondi S, Thorpe TA. 1982. Growth Regulator Effects, Metabolite Changes and Respiration during Shoot Initiation in Cultured Cotyledon Explants of *Pinus radiata*. *Bot Gaz* **143**: 20-25.

Brown MB, Forsythe AB. 1974. Robust tests for the equality of variances. *Journal of the American Statistical Association* **69**: 364-367.

Costa S, Shaw P. 2007. 'Open minded'cells: how cells can change fate. *Trends Cell Biol* **17**: 101-106.

Chinnusamy V, Gong Z, Zhu JK. 2008. Abscisic Acid mediated Epigenetic Processes in Plant Development and Stress Responses. *J Integr Plant Biol* **50**: 1187-1195.

Christianson ML, Warnick DA. 1985. Temporal requirement for phytohormone balance in the control of organogenesis *in vitro*. *Dev Biol* **112**: 494-497.

Durbak A, Yao H, McSteen P. 2012. Hormone signaling in plant development. *Curr Opin Plant Biol* **15**: 92-96

Fraga MF, Rodríguez R, Cañal MJ. 2002. Genomic DNA methylation-demethylation during ageing-reinvigoration of *Pinus radiata*. *Tree Physiol* **22**: 813-816.

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M. 2000. Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *The Plant Cell Online* **12**: 393-393.

Hasbún R, Valledor L, Berdasco M, Santamaría E, Cañal MJ, Rodríguez R. 2007. Dynamics of DNA methylation during chestnut trees development, Application to breeding programs. *Acta horticulturae* **760**: 563–566.

Hasbún R, Valledor L, Rodríguez JL, Santamaría E, Ríos D, Sánchez M, Cañal MJ, Rodríguez R. 2008. HPCE quantification of 5-methyl-2´-deoxycytidine in genomic DNA: Methodological optimization for chestnut and other woody species. *Plant Physiol Biochem* **46**: 815-822.

He G, Elling AA, Deng XW. 2011. The epigenome and plant development. *Annu Rev Plant Biol* **62**: 411-435.

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T. 2001. Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Natur* **409**: 1060-1063.

Klimaszewska K, Noceda C, Pelletier G, Label P, Rodriguez R, Lelu-Walter MA. 2009. Biological characterization of young and aged embryogenic cultures of *Pinus pinaster* (Ait.). *In Vitro Cell Dev Biol Plant* **45**: 20-33.

Kornet N, Scheres B. 2008. Stem Cell Factors in Plants: Chromatin Connections. in *Cold Spring Harbor Symp Quant Biol*, pp. 235-242. Cold Spring Harbor Laboratory Press.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Natur* **227**: 680-685.

Lafos M, Kroll P, Hohenstatt ML, Thorpe FL, Clarenz O, Schubert D. 2011. Dynamic regulation of H3K27 trimethylation during Arabidopsis differentiation. *PLoS Genetics* **7**: e1002040.

Li Y, Butenko Y, Grafi G. 2005. Histone deacetylation is required for progression through mitosis in tobacco cells. *Plant J* **41**: 346-352.

Lin X, Leung DWM. 2002a. Culture of isolated zygotic embryos of *Pinus radiata* D. Don. Part I: Factors influencing *in vitro* germination and growth of isolated embryos. *In Vitro Cell Dev Biol Plant* **38**: 191-197.

-. 2002b. Culture of isolated zygotic embryos of *Pinus radiata* D. Don. Part II: Biochemical changes associated with the conversion of isolated embryos. *In Vitro Cell Dev Biol Plant* **38**: 198-202.

Marum L, Loureiro J, Rodriguez E, Santos C, Oliveira MM, Miguel C. 2009. Flow cytometric and morphological analyses of *Pinus pinaster* somatic embryogenesis. *J Biotechnol* **143**: 288-295.

Meijón M, Valledor L, Santamaría E, Testillano PS, Risueño MC, Rodríguez R, Feito I, Cañal MJ. 2009. Epigenetic characterization of the vegetative and floral stages of azalea buds: Dynamics of DNA methylation and histone H4 acetylation. *J Plant Physiol* **166**: 1624-1636.

Palovaara J, Hallberg H, Stasolla C, Luit B, Hakman I. 2010. Expression of a gymnosperm PIN homologous gene correlates with auxin immunolocalization pattern at cotyledon formation and in demarcation of the procambium during *Picea abies* somatic embryo development and in seedling tissues. *Tree Physiol* **30**: 479-489.

Prakash AP, Kumar PP. 2002. PkMADS1 is a novel MADS box gene regulating adventitious shoot induction and vegetative shoot development in *Paulownia kawakamii*. *Plant J* **29**: 141-151.

Prakash AP, Kush A, Lakshmanan P, Kumar PP. 2003. Cytosine methylation occurs in a CDC48 homologue and a MADS-box gene during adventitious shoot induction in Petunia leaf explants. *J Exp Bot* **54**: 1361-1371.

Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M. 1999. DNA methylation is a reversible biological signal. *Proc Natl Acad Sci U S A* **96**: 6107-6112.

Santamaría ME, Hasbún R, Valera MJ, Meijón M, Valledor L, Rodríguez JL, Toorop PE, Cañal MJ, Rodríguez R. 2009. Acetylated H4 histone and genomic DNA methylation patterns during bud set and bud burst in *Castanea sativa*. *J Plant Physiol* **166**: 1360-1369.

Shapiro SS, Wilk MB. 1965. An analysis of variance test for normality (complete samples). *Biometrika* **52**: 591-611.

Sokol A, Kwiatkowska A, Jerzmanowski A, Prymakowska-Bosak M. 2007. Up-regulation of stress-inducible genes in tobacco and Arabidopsis cells in response to abiotic stresses and ABA treatment correlates with dynamic changes in histone H3 and H4 modifications. *Planta* **227**: 245-254.

Steel RGD, Torrie JH. 1980. *Principles and Procedures of Statistics. A Biometrical Approach. 2nd ed.* McGraw- Hill Book Co. Inc., New York.

Takada S, Hibara K, Ishida T, Tasaka M. 2001. The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. *Dev Biol* **128**: 1127-1135.

Tokuji Y, Takano S, Tonomura M, Tanaka S, Igari K, Watanabe T. 2011. Influence of 5′-azacitidine on promoting recovery of cell competence for shoot organogenesis in Arabidopsis. *Plant Cell, Tissue and Organ Culture* **106**: 289-297.

Valledor L, Jorrin-Novo JV, Rodríguez JL, Lenz C, Meijón M, Cañal MJ. 2010. Combined Proteomic and Transcriptomic analysis identifies differentially expressed pathways and stress responses associated to *Pinus radiata* needle maturation. *J Proteome Res* **9**: 3954-3979.

Vanyushin B, Ashapkin V, ed. 2006. *DNA methylation in plants*. Nova Biomedical Books, New York.

Vernoux T, Benfey PN. 2005. Signals that regulate stem cell activity during plant development. *Curr Opin Genet Dev* **15**: 388-394.

Vernoux T, Besnard F, Traas J. 2010. Auxin at the shoot apical meristem. *Cold Spring Harbor Perspectives in Biology* **2**: a001487.

Willemse J, Kulikova O, de Jong H, Bisseling T. 2008. A new whole-mount DNA quantification method and the analysis of nuclear DNA content in the stem-cell niche of Arabidopsis roots. *Plant J* **55**: 886-894.

Xiao W, Custard KD, Brown RC, Lemmon BE, Harada JJ, Goldberg RB, Fischer RL. 2006. DNA Methylation Is Critical for *Arabidopsis* Embryogenesis and Seed Viability. *Plant Cell* **18**: 805-814.

Zhang H, Horgan KJ, Reynolds PHS, Jameson PE. 2010. 6-Benzyladenine metabolism during reinvigoration of mature *Pinus radiata* buds *in vitro*. *Tree Physiol* **30**: 514-526.

Zhang S, Lemaux P. 2004. Molecular Analysis of *in vitro* Shoot Organogenesis. *Crit Rev Plant Sci* **23**: 325-335.
Zhu Y. 2010. The epigenetic involvement in plant hormone signaling. *Chin Sci Bull* **55**: 2198-2203.

CHAPTER 3. Differential Expression Analysis and Identification of Candidate Genes Involved in Adventitious Organogenesis

3.1. Introduction

Pinus radiata D. Don is a fast growth rate species with desirable timber quality being, together with Eucalyptus, the biggest forest plantations in mild climate countries like Chile, New Zealand, Australia, South Africa and Northern Spain (FAO 2011). To guarantee the sustainability of the plantations, production must still be improved by reforesting with clonal elite individuals (Walter et al. 2007). To achieve this goal, ongoing upgrading clonal multiplication programs based on classical breeding and biotechnological tools, must overcome the challenge that tree maturation involves, since the characteristic traits of an elite individual are only shown at a mature age.

Admitting that reinvigoration is a promising technology to propagate adult individuals, nowadays is limited to selected species and a reduced number of genotypes, being one of the most important barriers for clonal forestry (Montalbán et al. 2012). I*n vitro* clonal propagation from adult tissues has already been achieved (Prehn et al. 2003), but the response of few genotypes, like in somatic embryogenesis, and a difficult rooting later on, make of both techniques not a suitable solution at present (Montalbán et al. 2010).

Adventitious caulogenesis has been shown as a reliable instrument in *in vitro* mass multiplication although and a deeper understanding of the molecular basis of this process and the effect of the different growth regulators like cytokinins by a differential gene expression is determinant. Adventitious caulogenesis is a complex process which in conifers, contrary to angiosperms, remain partially unknown at gene expression profile (Christianson and Warnick 1985; Cary et al. 2002). Comparative genomics between angiosperms and gymnosperms at nucleotide level, and target gene identification and cloning may be a cumbersome process and the absence of a reference genome and the unknown sequences in other sequenced conifer

species make RNAseq technique not worthwhile. A suitable alternative is to identify these genes in each concrete species, adding new genes in public databases and clarifying their regulation in adventitious organogenesis processes, for a better understanding that guarantee the multiplication of elite individuals, and an approach able to identify sequences poorly represented in the mRNA population like Suppressive Subtractive Hybridization was used. For this aim, this work tries to identify genes involved in adventitious shoot organogenesis in zygotic embryos of *P. radiata* after induction by 6-Benzyladenine (BA). The organogenic response in the tissue is restricted to a small number of cells in epidermal and subepidermal cell layers (Yeung et al. 1981), therefore an additional experimental situation with embryos cultured in ABA inducing embryo arrest, was also used to identify sequences regarding BA that could not be shown differential between QLP control medium and BA experimental situation.

3.2. Methods

3.2.1. Plant Material.

P. radiata cones (Basque Country Coast ES06, batch 2614 producer 00949/05) were provided by the Breeding and Conservation Service of Forest Genetic Resources (Spanish Sea, Rural and Environment Ministry).

3.2.2. In vitro culture

Seed coats from *P. radiata* seeds were manually removed and after a sterilization with H_2O_2 (30 Vol) for 10 min and 3 rinses in sterile water, embryos were extracted and *in vitro* cultured for ten days at 25±2 ºC, 70–80 µmol m^{-2} s^{-1} light tensity and a 16:8 (day/light) photoperiod in three different media: QLP medium (Rodríguez et al. 2004), QLP plus ABA and QLP plus BA 22.14 µM as growth regulators under sterile conditions. For macroarray hybridization and quantitative PCR protocols, mature embryos and embryos cultured for 3 and 10 days in the aforementioned media were also used. Both plant growth regulators belong

to Duchefa Biochemie B.V. (Harleem, The Netherlands). Embryos for gene identification were cultured for 10 days, picked up, frozen in liquid nitrogen and stored at -80ºC until RNA extraction.

3.2.3. RNA extraction and amplification.

RNA was isolated from 75 mg of frozen tissue according to RNeasy Plant Mini Kit (Qiagen, Dusseldorf, Germany). RNA integrity was tested in agarose gel under denaturing conditions. For SSH, approximately 1 µg of total RNA from each of the embryos *in vitro* cultured were transcribed in to cDNA and amplified using the Super SMART cDNA synthesis kit (Clontech, Mountain View, CA, USA).

3.2.4. SSH library construction and sequencing.

cDNA Suppressive Subtractive Hybridization (SSH) was performed using PCR-Select Subtraction Kit (Clontech, Mountain View, CA, USA) according to manufacturer's instructions. The cDNA of embryos cultured in QLP was used as tester in both cases and the cDNA of embryos cultured in ABA or BA as driver for control libraries. For the adventitious organogenesis and embryo growth arrest libraries, cDNA of embryos cultured in BA or ABA was used as tester and cDNA of embryos cultured in QLP was used as driver. PCR products of subtracted cDNAs were directly inserted into pGEMT-Easy Vector (Promega, Madison, WI, USA) and transformed in competent *Escherichia coli* DH5α cells employing TransformAID Kit (Fermentas, Ontario, Canada). Clone selection was performed using LB medium (Bertani, 1951) plus Isopropyl β-D-1-thiogalactopyranoside (IPTG) (500 µM), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) (40 µg/mL) and ampicillin (100 µg/mL). Clones were picked up to establish each subtracted cDNA library constituted by 480 clones each. Selected clones were single-pass sequenced (Macrogen Inc., Seoul, Korea). Sequences were trimmed using SeqTrim v 0.098-w 0.1929 (Falgueras, J., Lara, A.J., et al. 2010) and Geneious 4.8 (Biomatters Inc. Auckland, New Zealand), and then assembled into 4

different batches: BA (expressed in BA), CBA (expressed in control of BA), ABA (expressed in ABA) and CABA (expressed in control of ABA) employing SeqManPro7.1 (Lasergene, DNASTAR Inc., Madison, WI, USA) to obtain sequence contigs. Contig sequence homology searches were performed using BLAST 2.2.18 software (http://www.ncbi.nlm.nih.gov/BLAST/) and custom local databases (described in Supporting Information). Homologies that showed e-values lower than 1×10^{-07} were considered significant. Functional classification was performed according to KEGG (Ogata, H., Goto, S., et al. 1999).

3.2.5. Dot Blot Macroarray.

Macroarrays were designed to test the differential expression of the identified contigs obtained after SSH. cDNA was PCR amplified directly from *E. coli* clones employing SP6 and T7 primers (PCR program: 94°C for 2 min, 36 x (94°C for 20 s; 50°C for 30 s); 72°C for 70 s and a final extension of 7 min at 72°C). Resultant PCR product was mixed with a denaturant solution of 1.5 M NaOH, 300mM Ethylenediaminetetraacetic acid (EDTA) pH 8.2 to reach a final concentration of 400 mM NaOH, 80 mM EDTA. A volume of 1 uL of the mixture was spotted in to Hybond N+ Membranes (GE Healthcare, Piscataway, NJ, USA). The membranes had 191 genes (95 and 96 probes each), corresponding to identified genes.

Reverse transcription from 1 µg of total RNA was performed by Superscript III first strand synthesis kit, (Invitrogen ©Life Technologies Corporation, Gaithersburg, MD, USA) and tail-labeled with DIG-UTP alkali-labile employing a terminal transferase (Roche, Basel, Switzerland). Membranes were prehybridized in Church's buffer (0.5 M phosphate buffer pH 7.2, 10 mM EDTA, 7% Sodium Dodecyl Sulfate (SDS), 1% Bovine serum albumin (BSA)) for 1 hour. Target cDNA was then added to fresh buffer at a final concentration of 50 ng mL^{-1} and hybridized overnight at 55°C. After two consecutive washings in 2x SSC-0.1% SDS and 1x SSC-0.1% SDS, the chemiluminiscence assay (CSPD ready to use, Roche, Basel, Switzerland) was done according to manufacturer´s instructions and autoradiography was

performed in a LAS-3000 minisystem (Fuji, Tokyo, Japan). Images were analyzed with Quantity One V.4.6.3 (Bio-Rad, Hercules, CA, USA). Three hybridization replicates were done for later statistical analysis.

3.2.6. Real Time Expression

Quantitative PCR was performed as follows: 10 ng of cDNA, 5µM of each primer and Perfecta SYBR Green (Quanta, Gaithersburg, MD, USA) were mixed and amplified using the ABI 7900HT system (Applied Biosystems, Foster City, CA, USA). Superscript III first strand synthesis kit, (Invitrogen, Gaithersburg, MD, USA) was used for cDNA synthesis. Three measurements for each transcript from a pool of individuals were analyzed using the LinReg software (Ruijter et al. 2009). Actin and Ubiquitin, previously selected by GeNorm software (Vandesompele et al. 2002), were used as endogenous controls to calculate relative expression. Primer sequences and Tm are indicated in Table S3.1.

3.2.7. Statistical Analysis

The univariate analysis of variance (ANOVA) was carried out in all EST data using *glm* procedure in SAS© software (SAS Institute Inc., Cary, USA) to determine the possible factor interactions. The multiple comparisons were performed using the post hoc Tukey´s HSD test after ANOVA.

The importance of multivariate analyses was also considered to analyze gene expression (Jacobsen et al. 2007; Grove et al. 2008; Valledor and Jorrín 2011). Principal component analysis (PCA) was performed on the Unscrambler 9.1 software (CAMO AS, Oslo, Norway). *P. radiata* mature embryos were used as control and embryos cultured in QLP, ABA and BA in 2 time points (3 and 10 days) were analyzed. Three membranes per situation were used and a total of 191 ESTs were evaluated. Macroarray data normalization was done dividing the intensity value of each spot by the intensity of all the spots of the membrane and multiplied by one thousand, and for PCA data, values were escalated.

Distribution of variance of PCAs was checked, and the most representative PCAs were selected with a higher percentage of variability explained. Confidence ellipses were calculated from loading matrix data.

Samples were evaluated by cluster analysis as well. Normalized data were clustered by Cluster 3.0 (de Hoon et al. 2004) employing Euclidean distance method over a complete linkage dissimilarity matrix and plotted employing Java Treeview 1.1.3 software (http://jtreeview.sourceforge.net).

After the selection of the genes which represented by PCA our model the best, statistical analysis of them in all experimental situations by a multivariate analysis of the variance (MANOVA) was done. MANOVA is usually used for expression analysis of two or more than two situations (Tsai and Chen 2009). It was performed by PROC GLM SAS© 9.3.1 software (SAS, Cary, USA), and multiple comparisons were done by Tukey-Kramer test.

3.3. Results

3.3.1. Characterization of suppressive subtractive hybridization libraries from P. radiata embryos

SSH applied to RNA from embryos cultured for 10 days in QLP medium and under ABA and BA treatments gave us as result 426 differential contigs after bioinformatic analysis. They were divided in four different libraries according to each experimental situation. Two libraries for the genes induced by BA and ABA growth regulators and two more libraries, acting as control, for the genes induced in QLP regarding BA or ABA; PRACBA (Control of BA) 77 contigs, PRABA (BA) 135 contigs, PRACABA (Control of ABA) 105 contigs and PRAABA (ABA) 109 contigs. Gene information of the four libraries is available in public repositories [GeneBank Accessions from GO448050 to GO448475]. Sequence homology searches were performed locally using BLASTx against a custom database from TAIR8pep,

Genebank, TGI/DFCI and Uniprot databases (updated 13/07/2011), identifying 53 genes from PRACBA, 110 genes from PRABA, 72 genes from PRACABA and 87 from PRAABA (Table S3.1). These genes were gathered into biological functions according to KEGG (Ogata et al. 1999) (Fig. 3.1). Different contigs shared homology with the same sequence being, probably, part of the same gene family.

The main differences between libraries were related to biological functions of Environmental Information Processing; Signal Transduction, Energy and Lipid Metabolism. Regarding Signal Transduction, both BA samples had a percentage of sequences (21 and 24 %) larger than ABA samples (8 and 9 %). Energy Metabolism sequences have their lowest value (3%) in ABA and both controls have the highest values (17 and 11%). Sequences in Lipid Metabolism showed a big difference with a very low value in BA samples (1 and 4% respectively) in regard with ABA samples (11 and 12%).

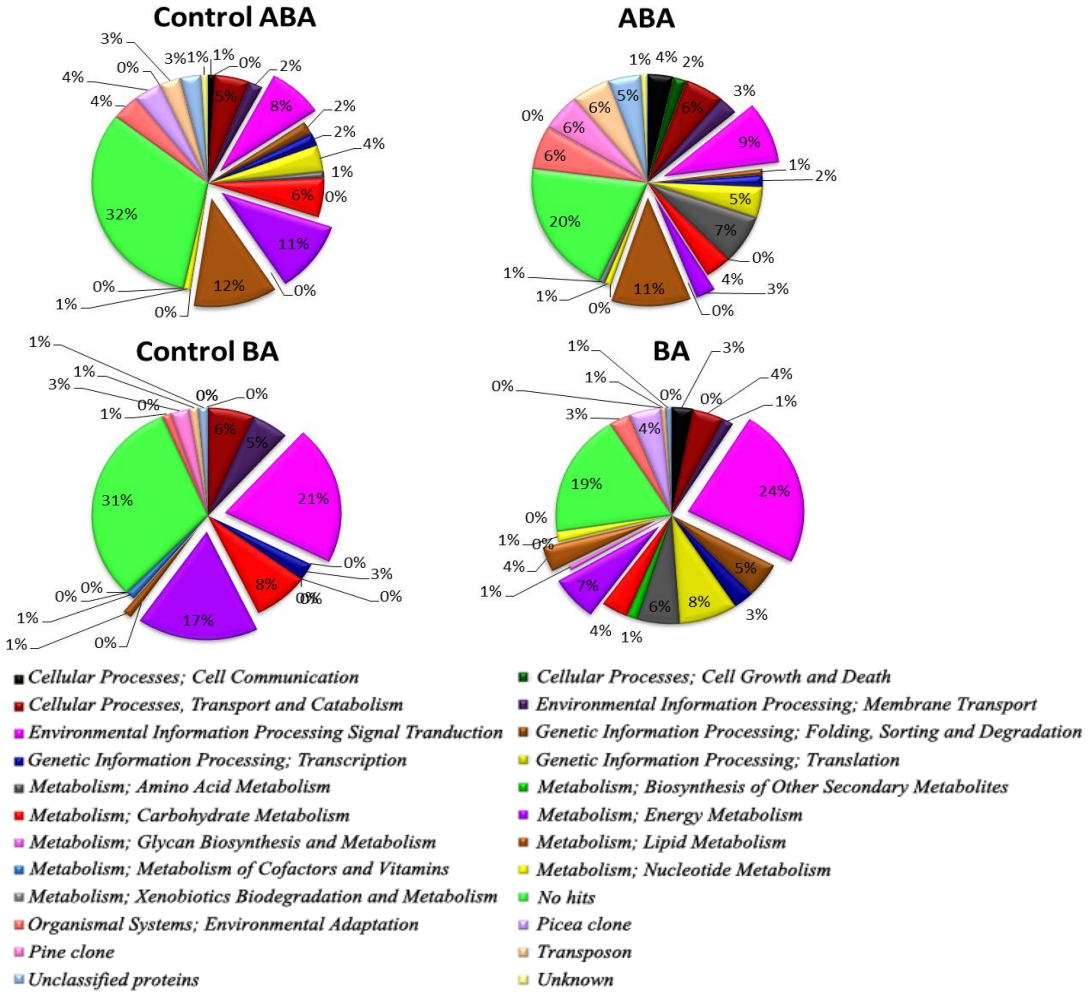


Figure 3.1: Biological function of 426 ESTs obtained from a SSH library in *P. radiata* embryos cultured for 10 days in basal media QLP and QLP plus growth regulators BA and ABA 22.14 µM.

3.3.2. Dot Blot Data Analysis

Dot Blot hybrydization allowed the quantifying of 191 genes in embryos from mature seeds, embryos cultured in basal medium (QLP) and QLP plus ABA and BA for 3 and 10 days (Table S3.2). All replicates showed a high correlation among them according to Pearson's correlation coefficient, as well as among treatments (Fig. S3.3). Data were analysed by Analysis of Variance (ANOVA) and by Principal Component Analysis (PCA).

A hierarchical clustering of the expression of the 191 ESTs tested along culture time using the Euclidean distance as a Heatmap image was done (Fig. 3.2). In this clustering, some groups which expression was associated to stress, signal transduction, or metabolism can be found indicating different pathways by the specific response patterns between the two growth regulators. Genes related to amino acid metabolism were overexpressed at 10 days, especially in QLP 10d and repressed in BA 3d and 10d, stress related genes were overexpressed mostly in ABA 10d, although there was a group of genes which expression was higher in QLP 3d.

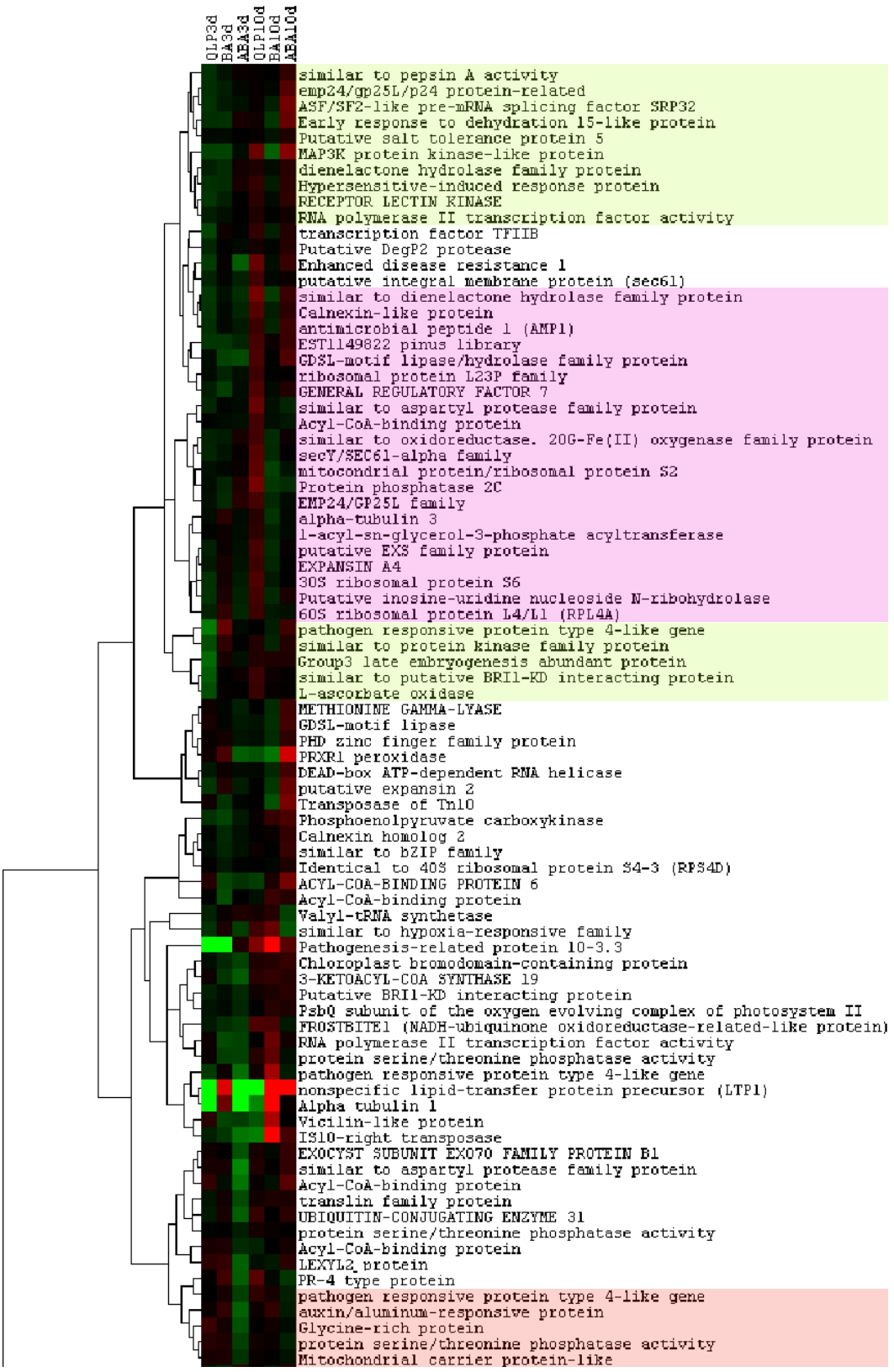


***Figure 3.2*:** Hierarchical clustering of *P. radiata* ESTs in zygotic embryos cultured under BA, ABA and without growth regulators along time (3 and 10 days) with regard to quiescent mature zygotic embryos. Red and green colors indicate an upregulation and downregulation respectively. Grouped sequences with similar function are highlighted (Green for stress pink for metabolism and red for signal transduction).

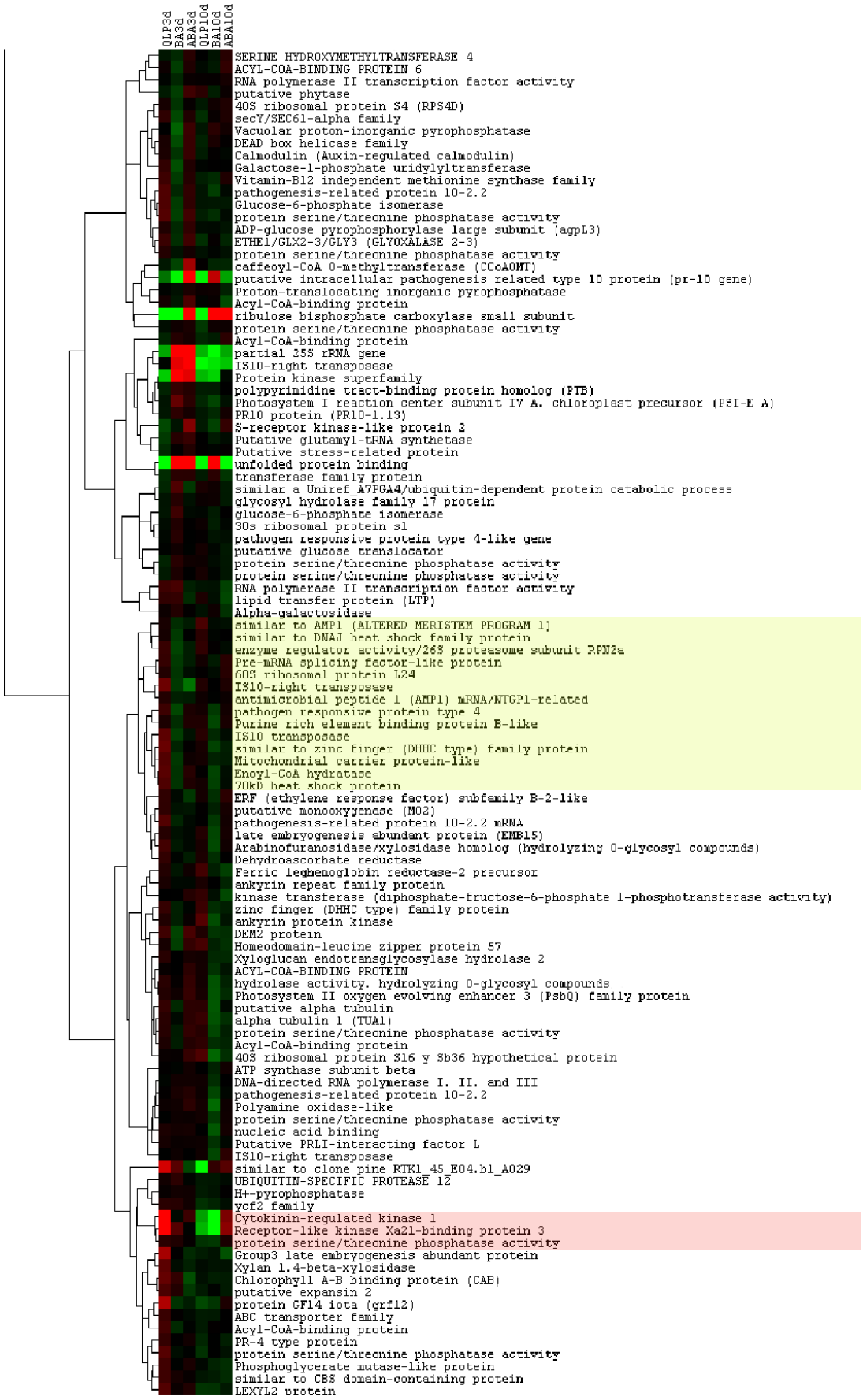


***Figure 3.2*:** Continued.

The analysis of the biological functions was determined by the significant differences using an ANOVA and expression data was normalized using mature embryos as a control expression. We focused on several functions induced by cytokinins like Energy and Lipid Metabolism and functions related to stress and signal transduction like Signal Transduction and Environmental Adaptation amongst others (Figure 3.3). These functions were represented by the variation of at least ten genes, rejecting those functional groups with a fewer number of genes, considering them not representative. The percentage of total genes analysed was an 82.19 %.

Embryos cultured in a media without any growth regulator showed a general repression of most of the analysed genes after 3 days of culture. However, after 10 days Folding, Sorting and Degradation, Translation and Amino Acid Metabolism were upregulated; meanwhile Energy Metabolism had no upregulated genes at day 10 and Lipid Metabolism decreased its percentage of genes. A different pattern was followed by the embryos cultured in ABA, with a decrease in the biological functions Transcription and Translation at day 10 and with no upregulated genes related to Transcription. On the other hand, Carbohydrate Metabolism increased their downregulated genes. Upregulated and downregulated BA genes from Folding, Sorting and Degradation, Transcription and Translation fell off their number at day 10. Amino Acid Metabolism decreased their downregulated genes and no upregulated genes were observed in the two time points. In the case of Energy Metabolism upregulated genes came up at day 10 but there was an increase of downregulated genes as well.

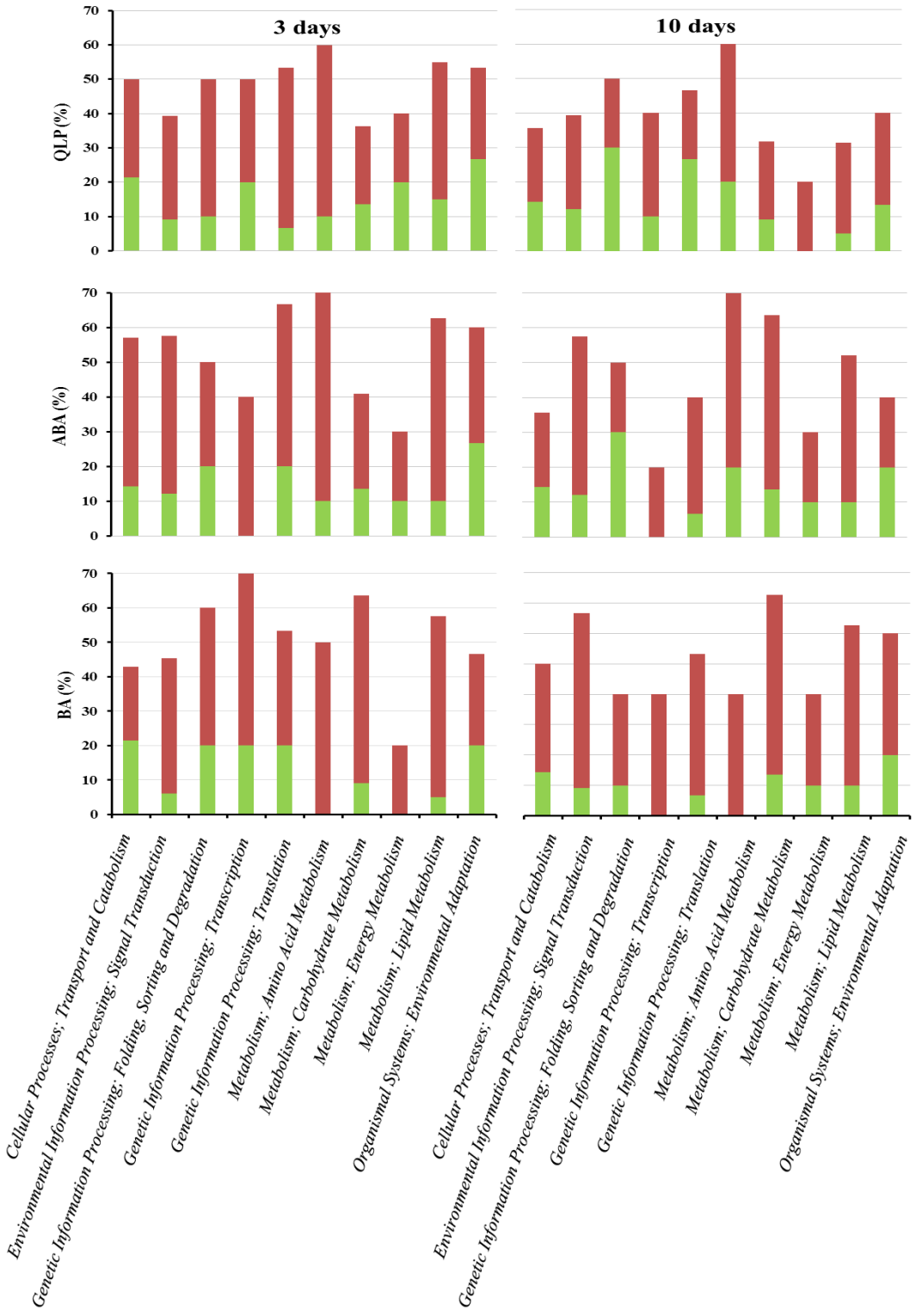


***Figure 3.3*:** Percentage of induced and repressed sequences of *P. radiata* gathered into the main biological functions in each different experimental situation (QLP, ABA and BA) and along culture time (3 and 10 days) regarding to mature embryos. Red/Green color represents repressed and induced ESTs respectively according to ANOVA.

Four main grouped situations were distinguished by Multivariate PC analysis: E0 Vs ABA3d in PC1, and in PC2 QLP 3d and 10d Vs BA10d, and several ESTs (Fig. 3.4; Fig. S3.4). The PC-1 and PC-2 analysis explained the 63% of the variance (37% and 26%,

respectively). Treatments formed by zygotic embryos of *P. radiata* cultured in QLP medium without plant growth regulators at 3 and 10 days (QLP3 and QLP 10) and embryos from matured seeds formed an association which, by PCA1 results, is negatively correlated with the embryos cultured in BA for 10 days. The unfolded protein binding (PRACABA03_F7, D3-2), RuBIsCO small subunit (PRACABA02_F3, C4-2), non specific lipid-transfer protein precursor (LTP1) (PRACABA05_G11, F1-2) and an Alpha tubulin 1 (PRACBA05_A9, H1-2) are positively correlated (upregulated) with embryos cultured in BA for 10 days and negatively correlated (downregulated) with mature embryos and embryos cultured in QLP for 3 and 10 days. On the other hand, by PCA 2, zygotic embryos cultured in QLP medium plus ABA for 3 days were negatively correlated with mature embryos. 25S rRNA gene (PRAABA04_D2, C12-1), a member of the protein kinase superfamily (PRABA01_H5, F7-1), a putative intracellular pathogenesis related type 10 protein (pr-10 gene) (PRABA02_G8, G4-1), a IS10-right transposase (PRABA02_H7, G6-1), a receptor-like kinase Xa21-binding protein 3 (XB3) (PRABA04_B6, H7-1) and a cytokinin-regulated kinase 1 (PRABA04_C3, H8-1) are positively correlated with ABA 3 days and negatively correlated with mature embryos.

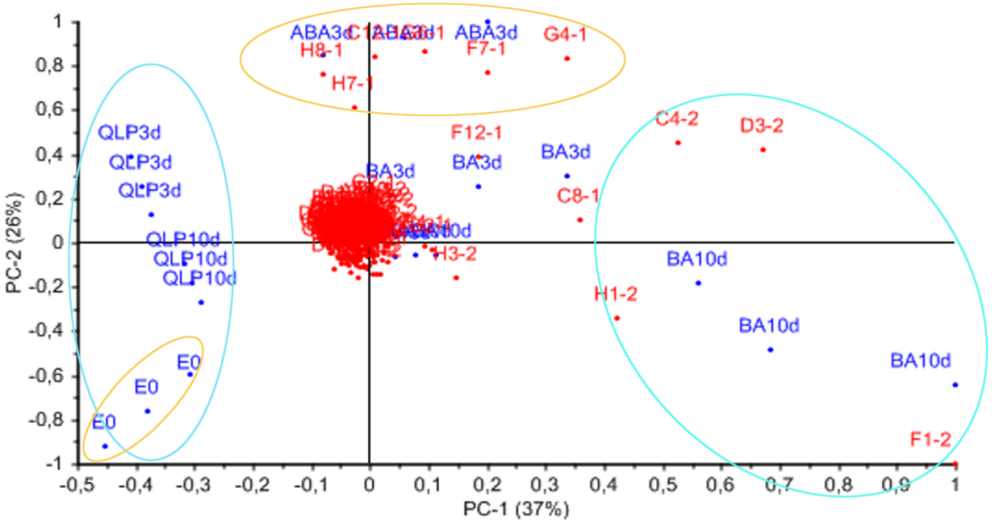


Figure 3.4: Principal Component Analysis of gene expression in *P. radiata* D. Don zygotic embryos in matured seeds and cultured in QLP basal medium and plus two different growth regulators (ABA and BA) cultured separately for 3 and 10 days. Unfolded protein binding (D3-2), RuBisCO small (C4-2), LTP1 (F1-2), Alpha tubulin 1 (H1-2), 25S rRNA (C12-1), protein kinase superfamily (F7-1), PR-10 (G4-1), IS10-right transposase (G6-1), XB3 (H7-1), CRK-1 (H8-1).

3.3.3. Validation of dot-blot quantitations by RT-qPCR.

qRT-PCR was performed on the genes associated to each experimental situation according to PC analysis. Their relative expression followed the same trend than in macroarray validating their expression, and it is shown in Fig. 3.6. Genes associated with ABA have an upregulation at day 3 in all experimental situations and a downregulation at day 10, except PR10 and IS10-right transposase which upregulation is mainly limited to ABA. RuBisCO small, unfolded protein binding, LTP1 and Alpha tubulin 1 showed the highest expression associated to BA at day 10, and LTP1 had a high expression in ABA at day 3 and 10 as well.

Multivariate Analysis of Variance (MANOVA) was performed on the expression data from the qRT-PCR to determine the significant positive and negative correlation among variables and their relationship along time. According to MANOVA correlation matrix expression, we showed that all genes had positive correlation among them (Table 3.1). Regarding statistical analysis performed, the expression of all genes was influenced not only by the treatment (medium where embryos were cultured) but also by the interaction of treatment (T) with time (D). In agreement with these results CRK1 showed significant differences with a *p*-value <0.01 meanwhile the rest of them showed significant differences with a *p*-value <0.001.

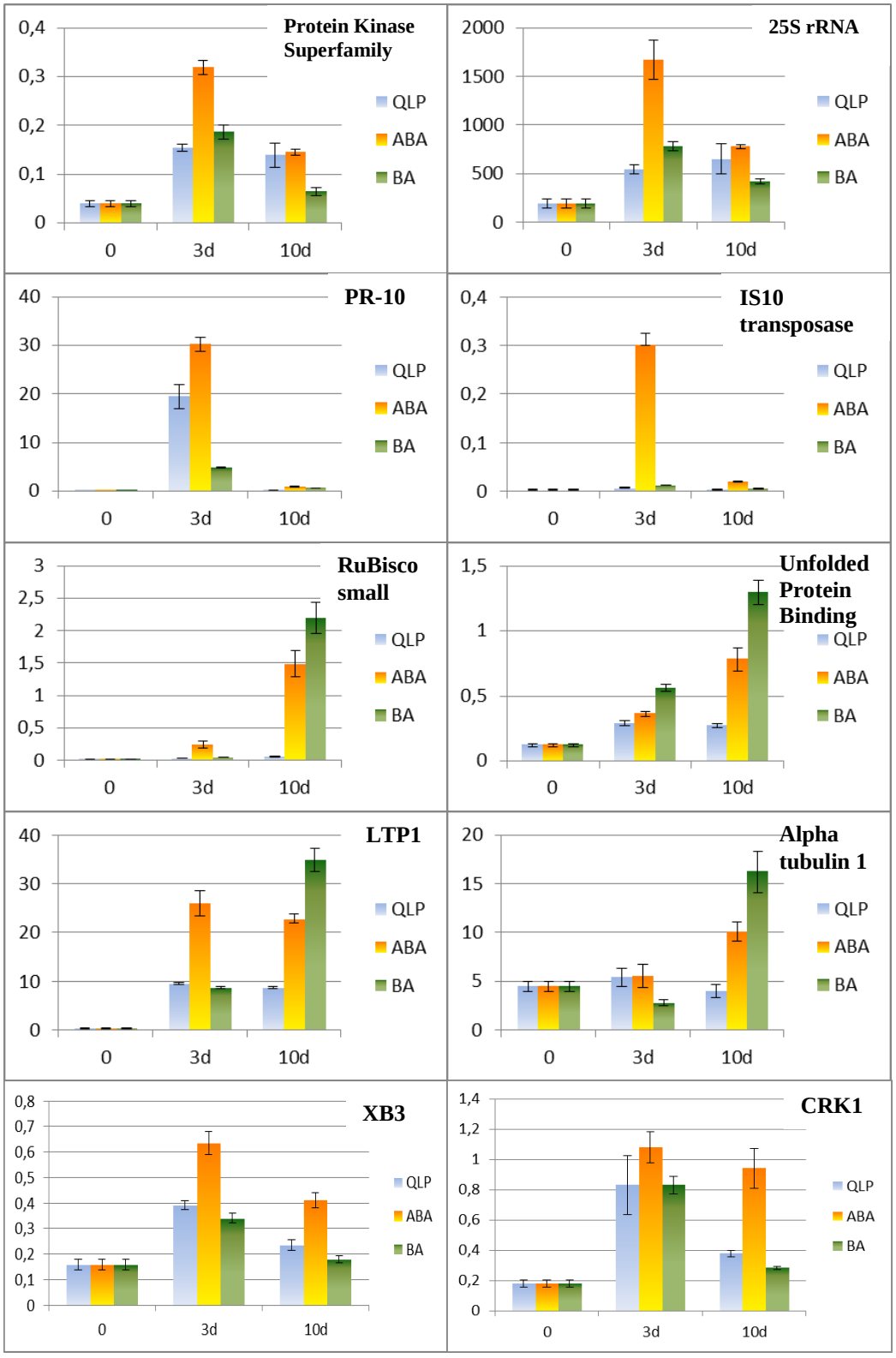


Figure 3.6: qRT-PCR gene expression along time (0, 3 and 10 days) and among treatment (QLP, ABA and BA) of the sequences selected from PC analysis. **PR-10**; Pathogenesis Related-10, **LTP1**; Lipid Transfer Protein 1, **XB3**; Xa21-binding protein 3, **CRK-1**; Cytokinin Regulated Kinase 1.

Table 3.1: Correlation matrix according to the MANOVA between EST qRT-PCR expression data and the associated probability with a linear relationship between them; **F7-1:** protein kinase superfamily, **C12-1:** 25S rRNA, **G4-1:** PR-10, **G6-1:** IS10-right transposase, **C4-2:** RuBisCO small, **D3-2:** unfolded protein binding, **F1-2:** LTP1, **H1-2:** Alpha tubulin 1, **H7-1:** XB3, **H8-1:** CRK-1.

	C12_1	**F7_1**	**G4_1**	**G6_1**	**H7_1**	**H8_1**	**C4_2**	**D3_2**	**F1_2**	**H1_2**
C12_1	1									
F7_1	0.30 ns	1								
G4_1	**0.45***	**0.60*****	1							
G6_1	**0.67*****	-0.004 ns	0.29 ns	1						
H7_1	0.17 ns	0.19 ns	**0.47***	0.14 ns	1					
H8_1	-0.04 ns	**0.43***	**0.46 ***	-0.17 ns	0.13 ns	1				
C4_2	-0.03 ns	-0.07 ns	-0.005 ns	-0.05 ns	-0.02 ns	-0.16 ns	1			
D3_2	0.17 ns	0.12 ns	0.26 ns	0.15 ns	0.31 ns	0.31 ns	0.37 ns	1		
F1_2	0.15 ns	0.23 ns	**0.43 ***	-0.14 ns	**0.58 ****	0.19 ns	0.29 ns	**0.52 ****	1	
H1_2	-0.19 ns	0.06 ns	0.02 ns	-0.34 ns	0.11 ns	0.15 ns	0.31 ns	0.35 ns	**0.42 ***	1

3.4. Discussion

A SSH strategy, that enriches sequences poorly represented in the mRNA population, was performed in 10 day-cultured *P. radiata* embryos in QLP and under the effect of BA and ABA. The dot blot array developed in this work has been effectively used to validate the SSH-based selection of genes, and its data analysis has showed us a high correlation and reproducibility also confirmed by RT-qPCR, and enabled us to associate different changes in gene expression to a specific experimental situation, a purpose for which dot-blotting is a current methodology and these kind of approaches are a source of new findings (Sugiyama et al. 2007; Valledor et al. 2010).

3.4.1. Gene annotation and biological function

Genes related to metabolism, that represented most of the genes in all of our libraries (Table S3.2), are the most abundant ones in all growth conditions showing an activation, as it was previously reported by (Lin and Leung 2002b), regardless of embryos developed isolated or together with the megagametophyte (Lin and Leung 2002a). In the case of BA situation, Signal Transduction genes had a high percentage probably due to a differential expression of genes encoding transcription factors in response to hormone treatment (Biondi and Thorpe 1982; Patel and Thorpe 1984; Cary et al. 2002).

3.4.2. Transcriptional profile induced by BA and ABA

To understand how the whole adventitious organogenesis works, the relevance of BA in this process and to establish a relationship between gene expression and macromorphological changes, two-way univariate analysis of variance, which let us associate gathered genes into different functional biological categories, was done.

Christianson and Warnick (1985) divided adventitious organogenesis in three different stages: acquisition of competence, induction and morphological differentiation. These stages

involve the establishment of new gene expression patterns and several research focused on adventitious caulogenesis has been done. In *Arabidopsis*, photosynthesis, transcription, metabolism and signalling related genes are upregulated due to photosynthetic activity caused by light exposure and treatment with Callus Induction Medium and Shoot Induction Medium (Che et al. 2002; Che et al. 2006). The absence of light during our experimental conditions could explain a downregulation in photosynthesis-related genes in the embryos cultured in QLP. However, exogenous cytokinins themselves increasing their active forms in the plant, have been shown to improve a recovery of photosynthesis (Hu et al. 2012). Contrarily, a downregulation in carbohydrate metabolic function takes place in ABA embryos regarding to those embryos in QLP which can affect seedling development as reported by Cutler et al. (2010) and Garciarrubio et al. (1997).

The small subunit of RuBIsCO small and the possible role of cytokinins in its regulation has been analysed in different situations finding evidences supporting this regulation in cytokinin-induced photomorphogenesis and chloroplast biogenesis in plants (Lochmanova et al. 2008). These evidences agree with our results in *in vitro* culture darkness conditions and the role of BA as inductor of photomorphogenesis in absence of light. An upregulation of photosynthesis mediated by cytokinins come along with an increase in metabolic functions (Lin and Leung 2002b), and new genes linked to metabolism like LPT1, Alpha-tubulin 1 and RuBisCO small were associated to adventitious organogénesis as well. The unfolded protein binding showed no known similarity in databases and no characteristic domains or profiles were found in the sequence against protein databases.

Non specific lipid-transfer protein precursor (LTP1) expression was increased in embryos supplemented with BA. LTP1 is mainly related to wax transport in cuticle synthesis (Costaglioli et al. 2005), and it has been already identified as differentially expressed in Arabidopsis during the end of the acquisition of competence stage and in the early stages of adventitious shoot induction in almond (Che et al. 2002; Santos et al. 2009), and also under

plant stress conditions. The role of exogenous application of cytokinins on wax biosynthesis has been proved in CER2, a gene involved in cuticular wax accumulation, showing that its expression is slowly induced by BA (Xia et al. 1997). Alpha-tubulin 1, together with Beta-tubulin, constitute microtubules which take part in mitosis, cytokinesis and cell differentiation in plants (Cai 2010). Several tubulins have been identified as differentially regulated by cytokinins in Arabidopsis light/dark experiments and α-tubulin 1 is accumulated in pea cotyledons after a treatment with BA (Lochmanova et al. 2008). This upregulation has been seen to be related to plant development and growth and nutrient assimilation (Rubio-Wilhelmi et al. 2011), as well as plant biomass productivity (Monteuuis et al. 2009). It has also been related to stress tolerance (Gordon et al. 2009) by scavenging reactive oxygen species amongst other responses (Hwang et al. 2012).

This function of cytokinins is important in *in vitro* culture, given that it triggers a stress response, but genes related to stress like PR-10, protein kinase superfamily, XB3, CRK1, 25s rRNA and IS10-right transposase (Grandbastien 1998; Cao et al. 2005; Razem et al. 2006; Wang et al. 2006; Sels et al. 2008; Chae et al. 2010) are only found upregulated in those embryos cultured in ABA for 3 days, corroborating the role of cytokinins as phytohormones with an important role in stress (Zwack et al 2013).

It could be contradictory that 25S rRNA gene, which expression is linked to cell proliferation, was overexpressed in this situation, but a putative RNA binding domain, that has been found in the family of RNA binding proteins, named ribonucleoprotein consensus sequence (RNP-CS) (Bandziulis et al. 1989), is regulated by ABA, or by an ABA receptor (Li et al. 2002; Razem et al. 2006).

Regarding to expression of PR-10 gene (G4-1) associated to Receptor-like kinase XB3 (H7-1) and to LTP1 (F1-2), it has been shown that some of the LTP members have a similarity to plant allergens (Breiteneder and Ebner 2000; Salcedo et al. 2004) being likely a common regulatory pathway or a common localization in the same tissue. The same case

happens in LTP1 and XB3 with a common expression in the leaf epidermis (Bhat et al. 2005). Finally, significant and positive correlation between LTP1 (F1-2) and Alpha tubulin 1 (H1-2), indicates a co-expression already seen in cell wall formation, especially during early wood formation in *P. radiata* (Li et al. 2009), an upregulation of both genes by dehydration in *Sorghum bicolor* (Buchanan et al. 2005) and in this case induced by BA.

The remarkable differences in the expression of IS10-right transposase (G6-1) that, according to the MANOVA characteristic vector is the gene with most influence in our data analysis, could be explained because of *in vitro* culture procedures trigger a stress response *per se* (Hirochika 1993; Grandbastien 1998).

3.5. Conclusions

Caulogenic process requires the activation of metabolism and morphogenic competence acquisition with a differential expression of genes associated to these functions like RuBIsCO and LTP1. Contrary to the expected results, the ABA treatment did not significantly alter embryo inhibitory pathways. However, our data pointed out that the induction of adventitious caulogenesis by BA reasserted the role of cytokinins against stress repressing expression of genes induced by ABA and by *in vitro* culture in control situation. Thus this study represents a first step towards the understanding of adventitious organogenesis beyond the specific genes related to the process and a future analysis of their regulation.

3.6. Appendix

Supplemental Table S3.1: Primer sequence from constitutive genes and from ESTs were qRT-PCR was performed.

Gen	Secuence 5'->3'	Tm
PtActin F	CAC TGC ACT TGC TCC CAG TA	55
PtActin R	AAC CTC CGA TCC AAA CAC TG	55
PtTub F	GCA TTG CCC AGT CTG TTT CT	55
PtTub R	GAT GCA GAA TAA GGC CCA GA	55
PpUbiq F	GTC CCG CAC TGA CAA TCT CT	55
PpUbiq R	CTG AGT CCA TGC TCT TGC TG	55
Pw18S F	GCG AAA GCA TTT GCC AAG G	55
Pw18S R	ATT CCT GGT CGG CAT CGT TTA	55
GAPDH F	AAG ATC CTC GGG AAA GGA GA	55
GAPDH R	CTT CCT TGC TCA ATG CAA CA	55
PRACABA05_G11 F	GTG GGC TGG TGG TGT TTA TC	55
PRACABA05_G11 R	TTT CAC ATG GGC AAC TTC AA	55
PRACABA02_F3 F	TCC TCA AAA TTG TGG CTT CC	55
PRACABA02_F3 R	GCG TTG AGC CAG TGT GAG TA	55
PRABA04_B6F	TCC AAA ATG GAT CTG GAA GC	55
PRABA04_B6R	CAT GCG AGA AAC TCT GAC CA	55
PRABA04_C3F	ATC GCC AAT GTC AAG TGT GA	55
PRABA04_C3R	CCG ACG ATA TTT CGC ATT CT	55
PRAABA04_D2F	GGC TGA ATC TCA GTG GAT CG	55
PRAABA04_D2R	ACG GTC GTT GAG AAG AAT CC	55
PRABA01_H5F	AAC AAT ACT CCG CCT TCT GC	55
PRABA01_H5R	CAG CCT TCT CAA AAC CAT GC	55
PRABA02_H7F	TCC CAA CCT TGT TTC TGA GC	55
PRABA02_H7R	TTG AAA AGT CCT GCC TAC GG	55
PRABA02_G8F	GTG TGG TGA CTT GGA TCT GC	55
PRABA02_G8R	GCA GTA TAA GGT GGG GTT GG	55
PRACBA05_A9F	CGC CGT TTC ATT TAT TGA TCC	55
PRACBA05_A9R	TTG TGT TGT TCC CGT TAT TGC	55
PRACABA03_F7F	TTC CCC TTT TGC CCT ATT TT	55
PRACABA03_F7R	GGA AGG GAA GGA GAA AAA CG	55

Supplemental Table S3.2: List of the contigs isolated by SSH in which significant homologies with sequences available in public databases were found[a]. Normalized data expression regarding Mature embryos is shown.

ClonePRA[b]	Accession[c]	Bp	Homology accession[d]	E-value	Description	Three days			Ten days		
						QLP	BA	ABA	QLP	BA	ABA
					Cellular Processes; Cell Communication						
PRAABA03_B1	GO448327	829	TC99011[1]	$6x10^{-70}$	Calnexin homolog 2	0,9663	0,8342	0,9235	0,9982	10,247	11,752
PRABA02_B11	GO448145	174	TC99011[1]	$1x10^{-93}$	Calnexin-like protein	0,4622	0,6252	0,5214	0,9657	0,5435	0,8180
					Cellular Processes; Cell Growth and Death						
PRAABA01_B6	GO448330	490	TC88109[1]	0	Protein GF14 iota (grf12)	20,094	11,911	12,708	11,083	11,835	14,758
PRAABA03_E12	GO448275	767	TC81298[1]	0	Transcription factor TFIIB	0,4522	0,7545	0,6879	0,8871	0,5841	0,8292
PRACABA01_B5	GO448469	851	CT579120[2]	0	Ankyrin repeat family protein	10,189	0,8980	0,9016	10,192	0,9250	0,8003
PRACABA03_D8	GO448389	773	TC62907[1]	0	Ankyrin protein kinase	0,9133	0,7178	0,8426	10,769	0,5745	0,7527
					Cellular Processes; Transport and Catabolism						
PRAABA02_B3	GO448283	874	TC97357[1]	0	Exocyst subunit exo70 family protein B1	0,9474	0,8966	0,5769	0,9859	0,8601	10,876
PRAABA04_H11	GO448278	330	TC84602[1]	0	EMP24/GP25L/P24 PROTEIN-RELATED	0,4218	0,4754	0,5432	0,5996	0,5465	0,8492
PRAABA04_H5	GO448284	597	TC102151[1]	0	Alpha-tubulin 3	0,6517	0,8251	0,6559	0,8200	0,5213	0,6632
PRABA03_H1	GO448074	764	TC106352[1]	0	Putative glucose translocator	0,6750	0,7569	0,6786	0,7708	0,6649	0,5899
PRABA04_F6	GO448155	330	TC73550[1]	$6x10^{-70}$	Belongs to the EMP24/GP25L family	0,8618	0,7470	11,057	12,428	0,7527	0,8856
PRABA05_E3	GO448098	784	EX337073[2]	$2x10^{-40}$	DEM2 protein	15,592	0,8587	16,162	13,885	0,9426	0,9800
PRACABA04_C7	GO448379	397	AW011241[2]	$1x10^{-136}$	Putative alpha tubulin	0,9324	0,6098	0,9053	0,8995	0,4451	0,7202
PRACABA04_D8	GO448463	402	TC111024[1]	0	Alpha tubulin 1 (TUA1)	19,324	15,758	19,554	20,327	10,530	13,100
PRACABA05_F7	GO448418	520	CF391830[2]	0	Polyamine oxidase-like	11,916	12,103	13,357	12,220	0,6306	11,952
PRACBA05_D11	GO448239	512	TC88236[1]	0	Lipid transfer protein (LTP)	13,531	13,677	0,8989	12,536	10,389	0,7558

PRACBA03_G12	GO448190	798	TC54497[1]	0	Mitochondrial carrier protein-like	0,7985	0,8089	0,5659	0,7480	0,7303	0,6670
PRACBA04_H8	GO448259	839	TC54497[1]	0	Mitochondrial carrier protein-like	0,8823	0,4225	0,6546	0,6752	0,4952	0,5172
PRACBA05_A9	GO448221	201	TC111024[1]	$3x10^{-73}$	Alpha tubulin 1	10,155	22,446	0,8356	15,031	39,163	19,426
PRACBA05_D2	GO448237	856	TC45428[1]	0	CBS domain-containing protein	16,216	14,133	13,342	11,798	11,471	10,669
					Environmental Information Processing; Membrane Transport						
PRAABA01_D6	GO448304	858	TC97743[1]	0	Auxin/aluminum-responsive protein	0,9463	10,761	0,5634	0,9469	10,785	0,8112
PRACABA05_G11	GO448405	661	TC101508[1]	0	Nonspecific lipid-transfer protein precursor (LTP1)	0,4851	15,350	0,4776	0,7081	28,766	15,770
PRACBA01_C11	GO448194	772	TC92521[1]	0	Belongs to the ABC transporter family	0,9476	0,7454	0,6926	0,6336	0,6404	0,7392
					Environmental Information Processing; Signal Transduction						
PRAABA01_D4	GO448277	1039	TC82571[1]	0	Chloroplast bromodomain-containing protein	0,7770	0,6614	0,5401	0,9914	10,160	10,909
PRAABA01_H2	GO448343	779	TC102683[1]	0	Similar to zinc finger (DHHC type) family protein	15,890	0,9875	11,557	12,408	0,8557	10,065
PRAABA02_F4	GO448365	464	TC69156	$1x10^{-119}$	MAP3K protein kinase-like protein	0,9568	0,9511	11,591	16,006	0,8393	17,124
PRAABA03_G1	GO448336	670	TC91052[1]	0	Similar to hypoxia-responsive family	0,7550	10,343	0,7189	11,573	12,992	0,6312
PRAABA04_G1	GO448280	773	TC100219[1]	0	putative EXS family protein	0,5888	0,7365	0,6383	0,9374	0,5432	0,7156
PRAABA05_B11	GO448281	501	TC90217[1]	0	Hypersensitive-induced response protein	0,4411	0,4557	0,6881	0,7707	0,5115	0,7439
PRAABA05_B2	GO448323	759	TC73733[1]	0	Similar to protein kinase family protein	0,4813	0,9132	0,7322	0,8575	0,6199	0,8643
PRAABA05_G10	GO448369	723	TC87327[1]	$1x10^{-75}$	Early response to dehydration 15-like protein	0,4492	0,4436	0,6836	0,6559	0,4253	0,9516
PRAABA05_H3	GO448271	285	TC110337[1]	$9x10^{-98}$	Expansin A4	0,7973	0,9756	0,8150	11,106	0,7490	0,8763
PRABA01_H5	GO448109	734	TC57035[1]	0	Belongs to the protein kinase superfamily	32,075	58,608	51,597	32,903	31,758	40,878
PRABA02_B1	GO448184	451	TC80568[1]	0	Encodes a member of the ERF (ethylene response factor) subfamily B-2 of the plant specific ERF/AP2	0,9999	0,7874	0,6171	0,9016	0,6483	0,9874
PRABA02_G4	GO448067	374	TC90240[1]	0	General Regulatory Factor 7	0,5129	0,3878	0,5769	0,9308	0,5888	0,7555
PRABA02_H1	GO448078	602	TC93679[1]	0	Zinc finger (DHHC type) family protein	10,581	0,6823	0,8014	10,867	0,6213	0,6830

PRABA03_C9	GO448112	798	TC97469[1]	0	Antimicrobial peptide 1 (AMP1)	0,4514	0,5883	0,5219	0,8287	0,4849	0,8343
PRABA05_A7	GO448075	389	DR020528[2]	0	Calmodulin (Auxin-regulated calmodulin)	23,092	15,479	26,064	17,805	20,913	19,808
PRABA05_F2	GO448104	842	TC97231[1]	0	PHD zinc finger family protein	19,771	20,609	17,708	18,081	15,864	22,570
PRACABA01_C8	GO448425	463	TC96811[1]	$1x10^{-145}$	Protein phosphatase 2C	12,956	11,600	15,471	20,373	10,664	11,185
PRACABA03_H1	GO448392	525	TC97469[1]	0	Antimicrobial peptide 1 (AMP1) mRNA/NTGP1-related	0,8367	0,6005	0,6141	0,7651	0,7019	0,7538
PRACABA03_H10	GO448448	291	TC96811[1]	$1x10^{-160}$	Protein serine/threonine phosphatase activity	10,501	10,049	0,9058	0,6903	0,6678	0,9827
PRACABA04_H8	GO448382	199	TC93637[1]	$1x10^{-103}$	70kD heat shock protein	10,725	0,5965	0,8989	0,8566	0,5517	0,7430
PRACABA05_D7	GO448374	300	TC110337[1]	$1x10^{-141}$	Putative expansin 2	0,7001	11,145	0,8147	0,9333	0,6914	14,192
PRACBA01_A10	GO448200	271	TC110337[1]	$1x10^{-137}$	Putative expansin 2	0,9679	0,8287	0,5222	0,6245	0,6987	0,6262
PRACBA01_A6	GO448199	771	TC96811[1]	$6x10^{-81}$	Protein serine/threonine phosphatase activity	0,6399	0,6585	0,5526	0,7214	0,6850	0,6640
PRACBA01_C6	GO448203	761	TC96811[1]	$8x10^{-62}$	Protein serine/threonine phosphatase activity	0,7982	0,8505	0,8443	0,8689	0,6006	0,8582
PRACBA01_G10	GO448195	1152	TC96811[1]	$1x10^{-119}$	Protein serine/threonine phosphatase activity	0,9929	0,7312	10,408	0,9931	0,5513	0,7147
PRACBA03_B12	GO448193	1257	TC96811[1]	$1x10^{-162}$	Protein serine/threonine phosphatase activity	0,8199	0,5818	0,5744	0,9047	10,929	0,7335
PRACBA03_E2	GO448240	781	TC96811[1]	$1x10^{-72}$	Protein serine/threonine phosphatase activity	0,9586	0,9372	0,6334	0,8909	0,8718	0,7357
PRACBA04_B11	GO448186	1219	TC96811[1]	$1x10^{-152}$	Protein serine/threonine phosphatase activity	0,9368	0,7441	0,8831	0,7515	0,7930	0,7503
PRACBA05_A3	GO448218	884	TC96811[1]	$2x10^{-10}$	Protein serine/threonine phosphatase activity	10,232	0,4513	0,9526	0,5837	0,5190	0,6585
PRACBA05_D7	GO448238	727	TC96811[1]	$1x10^{-20}$	Protein serine/threonine phosphatase activity	0,7794	0,4729	0,5075	0,3643	0,5099	0,4612
PRACBA05_E10	GO448242	766	TC96811[1]	$1x10^{-29}$	Protein serine/threonine phosphatase activity	0,5512	0,6628	0,7070	0,3869	0,6597	0,6560
PRACBA05_F10	GO448247	743	TC96811[1]	$2x10^{-87}$	Protein serine/threonine phosphatase activity	0,6899	10,365	0,9227	0,9142	0,7986	0,7461
PRACBA05_G10	GO448196	1240	TC96811[1]	$1x10^{-114}$	Protein serine/threonine phosphatase activity	0,5550	0,7511	0,6531	0,7308	0,6335	0,5695

					Genetic Information Processing; Folding, Sorting and Degradation						
PRAABA04_F1	GO448290	419	ES869607[2]	$1x10^{-160}$	Enhanced disease resistance 1	0,6206	0,9112	0,4648	13,418	0,8161	11,307
PRABA01_A1	GO448083	628	TC8160[1]	$2x10^{-88}$	Ubiquitin-Specific Protease 12	0,7427	0,8394	0,6589	0,5857	0,5918	0,6353
PRABA02_A3	GO448079	478	CF390608[2]	$1x10^{-105}$	S-receptor kinase-like protein 2	0,8949	11,400	18,364	11,123	0,9291	14,683
PRABA03_D7	GO448093	766	TC89516[1]	$8x10^{-28}$	SECY/SEC61-ALPHA FAMILY	0,5044	0,5579	0,6537	0,9338	0,4814	0,6379
PRABA03_E8	GO448059	838	TC323115[1]	$9x10^{-68}$	Putative integral membrane protein (sec61)	0,5596	0,6800	0,5685	0,9839	0,6735	0,7458
PRABA04_B6	GO448125	816	TC112333[1]	0	Receptor-like kinase Xa21-binding protein 3	39,951	33,473	30,533	24,509	20,077	35,332
PRABA04_C3	GO448131	700	TC86842[1]	$1x10^{-143}$	Cytokinin-regulated kinase 1	43,366	31,409	33,563	26,746	19,608	35,508
PRABA05_H1	GO448177	804	TC89516[1]	0	Belongs to the secY/SEC61-alpha family	11,838	0,6927	11,274	0,7972	10,247	11,189
PRACABA02_G10	GO448458	335	TC71322[1]	$4x10^{-83}$	Enzyme regulator activity/26S proteasome subunit RPN2a	13,035	0,7143	0,8983	12,669	10,521	0,9827
PRACABA03_E7	GO448422	846	TC89540[1]	0	Ubiquitin-Conjugated Enzyme 31	11,112	13,231	0,8071	14,422	12,124	13,986
					Genetic Information Processing; Transcription						
PRAABA03_A6	GO448337	792	TC91729[1]	$1x10^{-113}$	Pre-mRNA splicing factor-like protein	10,501	0,6280	0,7403	0,9210	0,7493	10,095
PRAABA05_F3	GO448363	497	TC44867[1]	$1x10^{-130}$	ASF/SF2-like pre-mRNA splicing factor SRP32	0,4201	0,5076	0,5397	0,6281	0,4867	10,043
PRABA01_B2	GO448160	691	TC88388[1]	0	RNA polymerase II transcription factor activity	0,5492	0,4050	0,6095	0,6006	0,5934	0,6639
PRABA01_E10	GO448091	717	TC100241[1]	0	Similar to bZIP family	0,5833	0,4801	0,5635	0,6982	0,6272	0,8812
PRABA02_H9	GO448179	695	TC88729[1]	0	Purine rich element binding protein B-like	0,8592	0,5807	0,8756	0,8769	0,4814	0,8483
PRABA04_F2	GO448085	851	DR097404[2]	$1x10^{-131}$	Polypyrimidine tract-binding protein homolog (PTB)	0,8577	11,888	11,280	0,8813	0,8822	0,9900
PRACABA03_E5	GO448376	626	TC82884[1]	0	Homeodomain-leucine zipper protein 57	10,599	0,7050	11,805	12,592	0,8283	0,7606
PRACABA03_F3	GO448384	683	TC80804[1]	0	RNA polymerase II transcription factor activity	0,9696	10,256	10,887	12,116	10,710	11,557
PRACBA02_F12	GO448245	784	TC90936[1]	0	RNA polymerase II transcription factor activity	0,8266	0,5317	0,5602	0,7816	11,248	0,8367
PRACBA05_C8	GO448189	683	TC80804[1]	0	RNA polymerase II transcription factor activity	12,357	12,794	0,8123	0,8641	0,8739	0,6562
					Genetic Information Processing; Translation						
PRAABA02_C12	GO448310	777	TC97041[1]	0	Valyl-tRNA synthetase	0,5540	0,7292	0,8164	0,7724	0,8397	0,4173

PRAABA02_C8	GO448285	746	DR059452[2]	$2x10^{-13}$	Mitocondrial protein/ribosomal protein S2	0,9949	10,268	10,966	14,048	0,8100	0,9823
PRAABA02_E1	GO448301	691	TC89374[1]	0	Translin family protein	0,8306	0,9615	0,6473	0,9602	0,9968	0,9925
PRAABA04_D2	GO448265	770	TC113143[1]	0	Partial 25S rRNA gene	12,513	25,940	29,978	12,222	0,6347	12,517
PRABA01_A8	GO448077	543	TC83655[1]	$1x10^{-160}$	60S ribosomal protein L24	0,9869	0,5796	0,7671	0,8434	0,7739	10,145
PRABA01_D2	GO448068	933	TC44370[1]	$5x10^{-22}$	Ribosomal protein L23P family	0,8115	0,7098	0,7978	11,023	0,8404	0,8716
PRABA03_B10	GO448122	809	TC58034[1]	$1x10^{-117}$	DEAD-box ATP-dependent RNA helicase	0,5292	0,6627	0,5756	0,5807	0,4687	0,8586
PRABA03_F7	GO448102	792	TC106298[1]	$5x10^{-20}$	40S ribosomal protein S4 (RPS4D)	0,7145	0,4409	0,6579	0,5403	0,6922	0,7509
PRABA03_G10	GO448051	801	TC81777[1]	0	Identical to 40S ribosomal protein S4-3 (RPS4D)	0,7193	0,6140	0,6861	0,6985	0,6777	0,8188
PRABA04_A3	GO448146	864	TC97430[1]	0	30s ribosomal protein s1	0,9807	12,613	10,712	11,299	0,8635	10,093
PRABA04_E5	GO448143	630	TC95199[1]	$1x10^{-158}$	30S ribosomal protein S6	0,5263	0,7773	0,6054	0,9840	0,6149	0,7103
PRABA04_G7	GO448171	320	TC45796[1]	$1x10^{-46}$	Belongs to the DEAD box helicase family	29,816	16,869	33,989	22,322	29,998	27,091
PRACABA03_G1	GO448466	390	TC88726[1]	0	Putative glutamyl-tRNA synthetase	0,5703	0,9337	10,005	0,7955	0,6919	0,7455
PRACABA05_C3	GO448415	615	TC95578[1]	0	60S ribosomal protein L4/L1 (RPL4A)	0,8058	11,976	0,7851	12,515	0,8505	0,8911
PRACABA05_G10	GO448472	422	TC112765[1]	0	40S ribosomal protein S16 y Sb36 hypothetical protein	12,486	12,300	14,736	16,019	0,6768	0,9598
					Metabolism; Amino Acid Metabolism						
PRAABA01_H1	GO448342	506	TC89772[1]	0	ubiquitin-dependent protein catabolic process	0,6507	12,027	0,5657	0,9492	0,9045	0,7035
PRAABA02_B5	GO448331	820	TC45081[1]	$1x10^{-165}$	Similar to aspartyl protease family protein	11,129	11,006	0,4086	11,460	10,273	12,220
PRAABA02_D9	GO448315	598	TC111710[1]	0	Serine Hydroxymethyltransferase 4	0,9104	0,7895	13,088	10,452	0,9276	13,042
PRAABA02_F12	GO448357	749	TC91872[1]	$2x10^{-34}$	Similar to pepsin A activity	0,5409	0,6415	0,7533	0,7341	0,7112	0,8913
PRAABA02_G9	GO448338	808	TC86871[1]	0	Similar to aspartyl protease family protein	0,6164	0,7393	0,7184	11,499	0,7006	0,6242
PRAABA03_C9	GO448311	588	TC94215[1]	0	Similar to AMP1 (Altered Meristem Program 1)	11,062	0,7636	0,8523	13,454	0,9314	0,9055
PRAABA04_C5	GO448308	465	DR465947[2]	$2x10^{-39}$	Receptor Lectin Kinase	0,4626	0,5395	0,6610	0,8031	0,6124	0,8145

PRAABA04_G2	GO448279	669	TC96182[1]	0	Putative DegP2 protease	0,4587	0,5741	0,5477	0,6507	0,5635	0,7051
PRABA05_G7	GO448175	759	TC53750[1]	$3x10^{-12}$	Belongs to the vitamin-B12 independent methionine synthase family	13,738	0,7891	12,574	0,9257	0,8666	12,270
PRACABA05_C7	GO448393	609	TC82870[1]	0	PRXR1 peroxidase	0,8845	10,382	0,5985	0,6300	0,5352	13,950
					Metabolism; Biosynthesis of Other Secondary Metabolites						
PRABA02_G10	GO448105	679	TC109522[1]	0	Caffeoyl-CoA O-methyltransferase (CCoAOMT)	12,626	10,683	20,662	13,768	12,067	12,007
					Metabolism; Carbohydrate Metabolism						
PRAABA02_H7	GO448345	624	TC50829[1]	0	Similar to DNAJ heat shock family protein	0,7433	0,4460	0,6595	0,8246	0,6443	0,6618
PRAABA04_F6	GO448293	239	TC99823[1]	$1x10^{-99}$	Dihydrolipoyl dehydrogenase y Ferric leghemoglobin reductase-2 precursor hits muy parecidos	0,9865	0,7829	0,9172	11,044	0,7498	0,6272
PRAABA04_H1	GO448350	587	TC99394[1]	0	Putative phytase	0,7333	0,6110	10,331	0,9630	0,7210	0,8546
PRAABA05_D8	GO448296	590	TC63426[1]	$1x10^{-108}$	Vicilin-like protein metabolism	16,710	12,269	12,555	11,481	22,190	14,985
PRABA04_B4	GO448063	851	TC81691[1]	0	Glucose-6-phosphate isomerase	0,5302	0,8326	0,5712	0,5834	0,5096	0,6139
PRABA04_E7	GO448090	827	AW011453[2]	$2x10^{-41}$	Glycosyl hydrolase family 17 protein	0,8236	10,054	0,8342	0,9187	0,9294	0,8064
PRABA05_C5	GO448053	133	Q45NN8[2]	$3x10^{-15}$	ETHE1/GLX2-3/GLY3 (Glyoxalase 2-3)	32,469	19,557	33,376	19,332	25,925	20,691
PRABA05_D4	GO448141	846	TC81691[1]	0	Glucose-6-phosphate isomerase	30,223	16,404	27,069	20,357	20,113	20,057
PRABA05_H8	GO448181	772	TC98466[1]	0	Galactose-1-phosphate uridylyltransferase	11,889	0,6226	11,161	0,9526	0,9782	0,9636
PRACABA01_C2	GO448434	249	TC109639[1]	$1x10^{-124}$	Phosphoglycerate mutase-like protein	11,694	0,8884	10,078	0,8680	0,7569	0,7105
PRACABA02_F3	GO448428	460	TC92228[1]	0	RuBIsCO small subunit	0,6401	0,8148	30,173	0,6470	22,195	24,808
PRACABA02_G1	GO448467	617	TC81818[1]	0	ADP-glucose pyrophosphorylase large subunit (agpL3)	0,9865	0,6952	10,544	0,8133	0,8178	0,7806
PRACABA03_D11	GO448407	472	TC86604[1]	0	Belongs to the enoyl-CoA hydratase	11,260	0,5986	0,9398	0,8004	0,5317	0,7384
PRACABA03_E2	GO448406	591	AM171731[2]	$1x10^{-69}$	Xyloglucan endotransglycosylase hydrolase 2	10,467	0,8902	0,9810	0,9658	0,7379	0,7329
PRACABA04_A9	GO448455	648	TC86984[1]	0	Arabinofuranosidase/xylosidase homolog (hydrolyzing O-glycosyl compounds)	0,9095	0,5994	0,6534	0,7649	0,4281	0,7374
PRACBA01_B8	GO448201	774	TC268643[1]	$3x10^{-12}$	Xylan 1,4-beta-xylosidase	11,297	0,7150	0,6363	0,7169	0,6909	0,6963

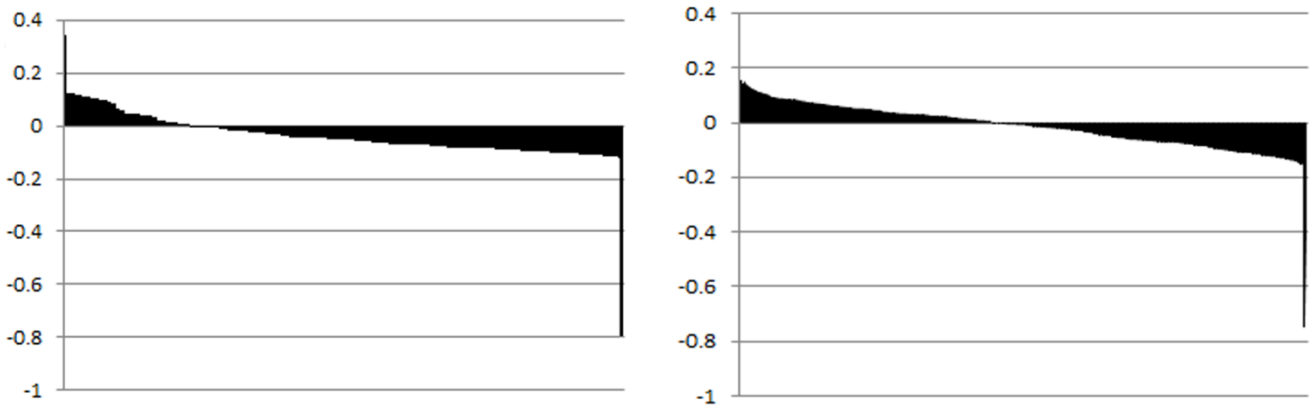
PRACBA01_D5	GO448204	736	CO224045[2]	$1x10^{-100}$	L-ascorbate oxidase	0,5652	0,8587	0,8257	10,337	0,8633	0,8464
PRACBA01_E1	GO448206	619	TC91676[1]	$8x10^{-09}$	Dehydroascorbate reductase	0,9140	0,7129	0,6240	0,8308	0,5612	0,7359
PRACABA05_G8	GO448383	756	TC86984[1]	0	LEXYL2 protein	0,9341	0,9594	0,4638	0,7430	0,7077	0,9307
PRACBA04_A8	GO448220	726	TC86984[1]	0	LEXYL2 protein	10,631	0,8071	0,8816	0,5806	0,5866	0,6761
PRACBA05_B4	GO448224	799	TC93186[1]	$2x10^{-07}$	Kinase transferase (diphosphate-fructose-6-phosphate 1-phosphotransferase activity)	0,9921	0,9848	11,303	11,816	0,9353	0,7119
PRACBA05_D3	GO448197	374	TC88113[1]	0	Alpha-galactosidase	10,462	11,337	10,151	0,9352	10,575	0,8107
					Metabolism; Energy Metabolism						
PRABA03_A4	GO448144	807	TC86741[1]	0	Putative monooxygenase (MO2)	0,7435	0,5105	0,4994	0,5853	0,4641	0,6584
PRABA04_F1	GO448151	834	TC76694[1]	$1x10^{-102}$	Photosystem I reaction center subunit IV A, chloroplast precursor (PSI-E A)	0,8225	12,198	10,282	0,7599	0,6602	0,8308
PRACABA01_H10	GO448442	706	TC104195[1]	$1x10^{-23}$	PsbQ subunit of the oxygen evolving complex of photosystem II	11,126	11,488	10,295	11,748	13,499	13,146
PRACABA02_C5	GO448414	860	TC82426[1]	0	ATP synthase subunit beta	0,8465	0,8242	0,9004	0,8426	0,7300	0,8959
PRACABA03_B8	GO448438	783	TC6000[1]	$5x10^{-23}$	Frostbite1 (NADH-ubiquinone oxidoreductase-related-like protein)	11,627	10,728	10,155	15,905	15,715	11,648
PRACABA05_H12	GO448385	396	TC98525[1]	0	Chlorophyll A-B binding protein (CAB)	15,091	12,705	0,6750	0,9674	0,9034	0,9177
PRACBA02_A11	GO448214	286	TC81556[1]	$1x10^{-148}$	Oxygen evolving enhancer 3 (PsbQ) family protein energy	11,358	10,467	12,294	10,807	0,5910	0,7801
PRABA02_F4	GO448056	720	TC109304[1]	0	Proton-translocating inorganic pyrophosphatase	0,5890	0,5363	0,7157	0,6522	0,6628	0,4661
PRABA03_G8	GO448172	776	BQ699593[2]	$2x10^{-43}$	H+-pyrophosphatase	0,7631	0,8016	0,7929	0,6443	0,6682	0,7452
PRABA04_F8	GO448158	763	TC62274[1]	$1x10^{-160}$	Vacuolar proton-inorganic pyrophosphatase	21,074	11,469	26,832	18,992	25,062	22,827
					Metabolism; Lipid Metabolism						
PRAABA03_F12	GO448349	774	TC108403[1]	0	Group3 late embryogenesis abundant protein	0,4571	0,8907	0,7554	0,9228	0,9112	0,9075
PRAABA01_D12	GO448294	505	TC108403[1]	0	Group3 late embryogenesis abundant protein	15,687	0,9544	0,7895	0,8434	0,8775	0,6398
PRAABA01_E10	GO448263	484	TC97307[1]	0	Acyl-CoA-binding protein	11,505	11,168	12,973	11,972	0,9107	10,349
PRAABA01_F5	GO448282	864	DV974041[2]	0	3-Ketoacyl-CoA Synthase 19	0,6887	0,4257	0,2850	0,7908	0,8228	0,7772

PRAABA02_H9	GO448347	815	TC97307[1]	$3x10^{-18}$	Acyl-CoA-binding protein 6	10,621	0,7440	11,487	0,9665	0,9018	12,262
PRAABA05_C7	GO448273	862	TC90825[1]	$1x10^{-35}$	GDSL-motif lipase/hydrolase family protein	0,6653	0,4705	0,4370	0,9755	0,6967	0,9892
PRAABA05_E2	GO448361	619	TC86471[1]	0	Phosphoenolpyruvate carboxykinase	0,4203	0,3539	0,5008	0,6161	0,7741	0,8499
PRAABA05_H11	GO448292	581	TC86504[1]	0	Late embryogenesis abundant protein (EMB15)	0,8274	0,6458	0,6753	0,8195	0,4626	0,7748
PRAABA05_H6	GO448270	628	CO224077[2]	$6x10^{-44}$	1-acyl-sn-glycerol-3-phosphate acyltransferase	0,6376	0,7012	0,6153	0,8066	0,5388	0,6217
PRABA01_E6	GO448094	520	DV974282[2]	$2x10^{-70}$	GDSL-motif lipase	0,8016	0,6794	0,6083	0,6335	0,5180	0,9698
PRABA04_E11	GO448147	631	TC83589[1]	$5x10^{-07}$	Acyl-CoA-binding protein	0,5760	0,8416	0,7870	0,5604	0,6006	0,8674
PRABA04_E6	GO448054	405	TC97307[1]	0	Acyl-CoA-binding protein	0,6927	0,6683	0,6541	0,9561	0,6600	0,6281
PRABA04_F7	GO448157	780	TC97307[1]	$2x10^{-93}$	Acyl-CoA-binding protein	10,256	0,7329	10,330	0,9695	12,666	11,880
PRABA05_F6	GO448163	749	TC97307[1]	$1x10^{-108}$	Acyl-CoA-binding protein	15,047	15,265	11,458	11,926	13,659	14,859
PRACABA01_B10	GO448465	750	TC97307[1]	$6x10^{-47}$	Acyl-CoA-binding protein 6	11,360	0,7673	0,8328	0,7942	11,039	14,191
PRACABA01_C6	GO448433	778	TC97307[1]	$2x10^{-25}$	Acyl-CoA-binding protein	11,046	0,9273	13,253	0,9999	10,538	0,7202
PRACABA01_D11	GO448411	750	TC97307[1]	$1x10^{-11}$	Acyl-CoA-binding protein	13,545	10,934	0,6792	11,736	10,869	15,065
PRACABA04_D5	GO448462	270	TC84520[1]	$1x10^{-148}$	Hydrolase activity, hydrolyzing O-glycosyl compounds/T17H3.3	0,9986	0,8650	10,387	0,9130	0,5359	0,7188
PRACABA05_A10	GO448456	699	TC97307[1]	$9x10^{-83}$	Acyl-CoA-binding protein	10,450	0,6334	0,7781	0,6953	0,8023	0,7895
PRACBA04_G11	GO448255	484	TC97307[1]	0	Acyl-CoA-binding protein	0,8202	0,6326	0,8459	0,8793	0,5777	0,6106
					Metabolism; Metabolism of Cofactors and Vitamins						
PRACBA04_A1	GO448217	447	TC85738[1]	0	Putative PRLI-interacting factor L	0,9080	0,8644	0,8728	0,8798	0,6438	0,8002
					Metabolism; Nucleotide Metabolism						
PRAABA05_G2	GO448267	427	TC102993[1]	0	Methionine Gamma-Lyase	0,6142	0,5383	0,4569	0,5561	0,4161	0,7616
PRABA02_E2	GO448095	739	TC55194[1]	0	Putative inosine-uridine nucleoside N-ribohydrolase	0,5228	0,8153	0,5846	0,9544	0,5562	0,8011
PRABA04_F5	GO448058	496	DR746302[2]	0	DNA-directed RNA polymerase I, II, and III	0,9046	0,9838	0,9834	0,9773	0,7743	0,9245

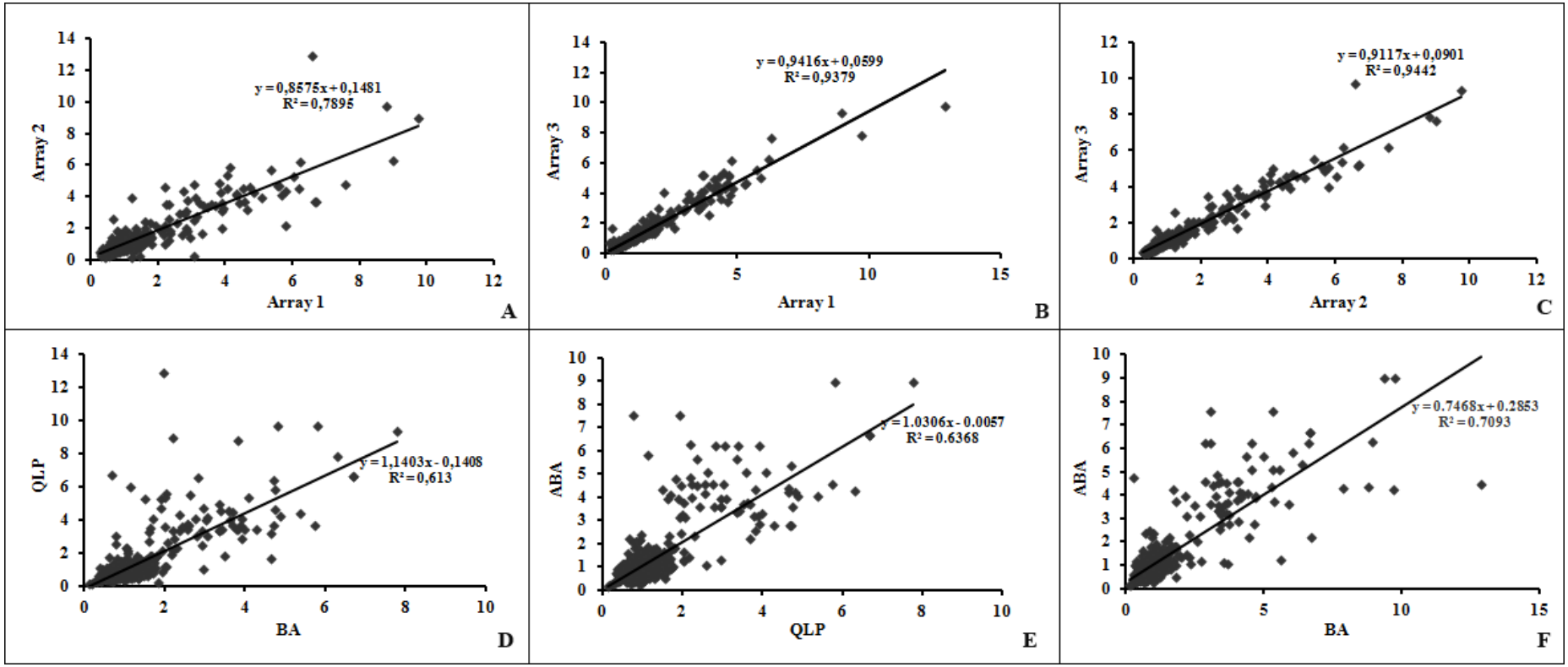
					Metabolism; Xenobiotics Biodegradation and Metabolism						
PRAABA03_D12	GO448302	805	TC89504[1]	$1x10^{-88}$	Dienelactone hydrolase family protein	0,8068	0,7471	10,824	11,433	0,9130	12,006
PRAABA05_A12	GO448264	295	TC89504[1]	$1x10^{-165}$	Similar to dienelactone hydrolase family protein	0,4301	0,5526	0,4577	0,9873	0,4134	0,7975
					Organismal Systems; Environmental Adaptation						
PRAABA02_D10	GO448266	448	TC91330[1]	0	Pathogen responsive protein type 4-like gene	15,315	18,652	12,801	18,816	25,673	16,670
PRAABA03_B6	GO448324	736	TC91330[1]	$6x10^{-13}$	PR-4 type protein	0,9001	11,214	0,5248	12,196	0,8515	0,6770
PRAABA03_E6	GO448268	784	TC89631[1]	0	Pathogen responsive protein type 4-like gene	0,6285	14,329	10,311	10,686	0,9621	12,998
PRAABA03_G10	GO448333	806	TC103006[1]	$1x10^{-08}$	Pathogenesis-related protein 10-3.3	23,935	25,133	40,753	45,099	57,814	43,332
PRAABA03_G7	GO448335	708	TC89631[1]	$1x10^{-103}$	Pathogen responsive protein type 4	10,139	0,5489	0,8952	0,8670	0,5991	0,8631
PRAABA05_B9	GO448354	835	TC109793[1]	$1x10^{-119}$	Pathogen responsive protein type 4-like gene	10,577	11,351	0,8143	12,267	11,360	0,9386
PRABA01_A3	GO448080	599	TC100346[1]	$2x10^{-12}$	Putative salt tolerance protein 5	10,138	10,041	10,645	11,200	0,9423	12,685
PRABA02_G8	GO448082	733	TC103006[1]	0	Putative intracellular pathogenesis related type 10 protein (pr-10 gene)	51,009	43,391	90,942	43,421	68,727	48,238
PRABA04_C7	GO448072	769	TC87701[1]	0	PR10 protein (PR10-1.13)	0,5278	0,7497	0,6731	0,5214	0,4655	0,6678
PRABA05_H2	GO448070	375	DR095252[2]	0	Pathogenesis-related protein 10-2.2	16,811	10,020	15,075	11,040	0,8682	11,715
PRACABA01_B11	GO448457	726	TC89631[1]	$1x10^{-48}$	PR-4 type protein	18,373	15,131	16,195	12,649	14,943	13,056
PRACABA03_G6	GO448381	375	DR095252[2]	0	Pathogenesis-related protein 10-2.2	0,8317	0,9096	0,9525	0,8687	0,5626	0,7976
PRACABA04_A4	GO448452	631	DR095252[2]	$1x10^{-123}$	Pathogenesis-related protein 10-2.2 mRNA	10,506	0,5941	0,6956	0,7398	0,5186	0,8037
PRACABA05_A12	GO448394	545	TC89631[1]	0	Pathogen responsive protein type 4-like gene	0,7383	0,9068	0,7607	0,7822	0,6945	0,7130
PRACBA04_F7	GO448185	548	Q8H469[2]	$4x10^{-24}$	Putative stress-related protein	0,5607	0,6957	0,7766	0,6870	0,6655	0,6907
					Pine clone						
PRAABA04_D10	GO448303	795	TC101505[1]	0	EST1149822 pinus library	0,5980	0,5929	0,6581	10,482	0,8464	10,092
PRABA02_E8	GO448092	735	TC104063[1]	$2x10^{-37}$	Similar to clone pine RTK1_45_E04.b1_A029	39,520	34,785	28,782	17,545	33,346	34,756

					Transposon						
PRAABA01_C2	GO448318	825	CB670399[2]	$4x10^{-11}$	IS10 transposase	12,135	0,6643	0,8266	10,264	0,7455	0,8444
PRAABA03_B7	GO448322	821	TC388561[1]	$3x10^{-58}$	IS10-right transposase	14,533	0,7289	0,4678	11,201	0,9755	11,038
PRABA02_H7	GO448110	688	TC388561[1]	0	IS10-right transposase	36,853	50,641	67,399	18,725	23,618	25,462
PRACABA03_B9	GO448437	837	Q0KII0[2]	$7x10^{-16}$	Transposase of Tn10	0,9992	0,7900	0,9326	0,9554	0,6840	13,463
PRACABA05_D9	GO448464	693	TC388561[1]	0	IS10-right transposase	0,8529	0,9003	0,8575	0,9052	0,6323	0,9931
PRACBA05_C1	GO448228	828	TC388561[1]	0	IS10-right transposase	11,305	10,471	0,8253	0,7998	21,214	14,680
					Unclassified proteins						
PRAABA05_A9	GO448295	509	TC96107[1]	0	Similar to oxidoreductase, 2OG-Fe(II) oxygenase family protein	0,4628	0,4888	0,6566	0,8056	0,4287	0,6196
PRAABA05_C10	GO448309	799	TC97328[1]	$1x10^{-79}$	Similar to putative BRI1-KD interacting protein	0,7160	10,562	11,036	12,793	11,111	10,784
PRAABA05_C9	GO448316	737	TC97328[1]	0	Putative BRI1-KD interacting protein 118	0,5295	0,5915	0,4914	0,7005	0,7441	0,7867
PRACABA04_F12	GO448396	605	TC97052[1]	0	Transferase family protein	0,9081	11,931	11,893	0,9650	11,936	0,8764
PRACABA04_G4	GO448395	354	TC112949[1]	$1x10^{-177}$	Belongs to the ycf2 family	10,486	10,378	0,9619	0,7162	0,7653	0,8463
PRACABA05_F2	GO448390	596	TC86817[1]	0	Nucleic acid binding	0,9098	0,8799	0,8875	0,8065	0,4946	0,8807
PRACBA03_C9	GO448226	845	TC25382[1]	$7x10^{-07}$	Glycine-rich protein	13,153	11,720	0,7410	10,747	11,551	10,334
					Unknown						
PRACABA03_F7	GO448430	703	TC99011[1]	$5x10^{-35}$	Unfolded protein binding	0,7739	18,018	14,744	0,8340	15,191	0,8265

[a]Functional classification was based according to KEGG (Ruepp et al., 2004). [b] Clone reference in PRA library. [c]dbEST accession number. [d] Homology accession number according to public databases: (1) TGI, (2) Genbank.



Supplemental Figure S3.3:Loading plot of PCA 1 and 2 from expression data of *Pinus radiata* zygotic embryos cultured on QLP and on QLP supplemented with BA and with ABA at 3 and 10 days. Data has been normalized with expression values of mature embryos



Supplemental Figure S3.4: Correlation plots of macroarray data expression analysis among replicates (A, B and C) and among treatments (D, E and F) from *P. radiata* embryos *in vitro* cultured in the presence of BA, ABA and control media.

3.7. References

Bandziulis RJ, Swanson MS, Dreyfuss G. 1989. RNA-binding proteins as developmental regulators. *Genes Dev* **3**: 431-437.

Bertani G. 1951. Studies on lysogenesis I.: The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**: 293-300.

Bhat RA, Miklis M, Schmelzer E, Schulze-Lefert P, Panstruga R. 2005. Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. *Proc Natl Acad Sci U S A* **102**: 3135–3140.

Biondi S, Thorpe TA. 1982. Growth Regulator Effects, Metabolite Changes and Respiration during Shoot Initiation in Cultured Cotyledon Explants of *Pinus radiata*. *Bot Gaz* **143**: 20-25.

Breiteneder H, Ebner C. 2000. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* **106**: 27-36.

Buchanan CD, Lim S, Salzman RA, Kagiampakis I, Morishige DT, Weers BD, Klein RR, Pratt LH, Cordonnier-Pratt MM, Klein PE. 2005. Sorghum bicolor's transcriptome response to dehydration, high salinity and ABA. *Plant Mol Biol* **58**: 699-720.

Cai G. 2010. Assembly and disassembly of plant microtubules: tubulin modifications and binding to MAPs. *J Exp Bot* **61**: 623-626.

Cao X, Li K, Suh SG, Guo T, Becraft PW. 2005. Molecular analysis of the *CRINKLY4* gene family in *Arabidopsis thaliana*. *Planta* **220**: 645-657.

Cary AJ, Che P, Howell SH. 2002. Developmental events and shoot apical meristem gene expression patterns during shoot development in *Arabidopsis thaliana*. *Plant J* **32**: 867-877.

Costaglioli P, Joub s J, Garcia C, Stef M, Arveiler B, Lessire R, Garbay B. 2005. Profiling candidate genes involved in wax biosynthesis in *Arabidopsis thaliana* by microarray analysis. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1734**: 247-258.

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. 2010. Abscisic Acid: Emergence of a Core Signaling Network. *Annu Rev Plant Biol* **61**: 651-679.

Chae L, Pandey GK, Luan S, Cheong YH, Kim KN. 2010. Protein Kinases and Phosphatases for Stress Signal Transduction in Plants. in *Abiotic Stress Adaptation in Plants*, pp. 123-163.

Che P, Gingerich DJ, Lall S, Howell SH. 2002. Global and Hormone-Induced Gene Expression Changes during Shoot Development in Arabidopsis. *Plant Cell* **14**: 2771-2785.

Che P, Lall S, Nettleton D, Howell SH. 2006. Gene Expression Programs during Shoot, Root, and Callus Development in Arabidopsis Tissue Culture. *Plant Physiol* **141**: 620-637.

Christianson ML, Warnick DA. 1985. Temporal requirement for phytohormone balance in the control of organogenesis *in vitro*. *Dev Biol* **112**: 494-497.

de Hoon MJL, Imoto S, Nolan J, Miyano S. 2004. Open source clustering software. *Bioinformatics* **20**: 1453-1454

Falgueras J, Lara AJ, Fernández-Pozo N, Cantón FR, Pérez-Trabado G, Claros MG. 2010. SeqTrim: a high-throughput pipeline for pre-processing any type of sequence read. *BMC Bioinformatics* **11**: 38.

FAO. 2011. *State of the World's Forests*. Food and Agriculture Organization of the United Nations, Rome, Italy.

Garciarrubio A, Legaria JP, Covarrubias AA. 1997. Abscisic acid inhibits germination of mature Arabidopsis seeds by limiting the availability of energy and nutrients. *Planta* **203**: 182-187.

Gordon SP, Chickarmane VS, Ohno C, Meyerowitz EM. 2009. Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. *Proc Natl Acad Sci U S A* **106**: 16529-16534.

Grandbastien M. 1998. Activation of plant retrotransposons under stress conditions. *Trends Plant Sci* **3**: 181-187.

Grove H, Jørgensen BM, Jessen F, Søndergaard I, Jacobsen S, Hollung K, Indahl U, Færgestad EM. 2008. Combination of statistical approaches for analysis of 2-DE data gives complementary results. *J Proteome Res* **7**: 5119-5124.

Hirochika H. 1993. Activation of tobacco retrotransposons during tissue culture. *The EMBO Journal* **12**: 2521-2528.

Hu L, Wang Z, Huang B. 2012. Growth and Physiological Recovery of Kentucky Bluegrass from Drought Stress as Affected by a Synthetic Cytokinin 6-Benzylaminopurine. *Crop Sci* **52**: 2332-2340.

Hwang I, Sheen J, Müller B. 2012. Cytokinin signaling networks. *Annu Rev Plant Biol* **63**: 353-380.

Jacobsen S, Grove H, Jensen K, Sørensen H, Jessen F, Hollung K, Uhlen A, Jørgensen B, Faergestad E, Søndergaard I. 2007. Multivariate analysis of 2-DE protein patterns--practical approaches. *Electrophoresis* **28**: 1289-1299.

Li J, Kinoshita T, Pandey S, Ng CKY, Gygi SP, Shimazaki K, Assmann SM. 2002. Modulation of an RNA-binding protein by abscisic-acid-activated protein kinase. *Natur* **418**: 793-797.

Li X, Wu HX, Dillon SK, Southerton SG. 2009. Generation and analysis of expressed sequence tags from six developing xylem libraries in *Pinus radiata* D. Don. *BMC Genomics* **10**: 41.

Lin X, Leung DWM. 2002a. Culture of isolated zygotic embryos of *Pinus radiata* D. Don. Part I: Factors influencing *in vitro* germination and growth of isolated embryos. *In Vitro Cell Dev Biol Plant* **38**: 191-197.

Lin X, Leung DWM. 2002b. Culture of isolated zygotic embryos of *Pinus radiata* D. Don. Part II: Biochemical changes associated with the conversion of isolated embryos. *In Vitro Cell Dev Biol Plant* **38**: 198-202.

Lochmanova G, Zdrahal Z, Konecna H, Koukalova S, Malbeck J, Soucek P, Valkova M, Kiran NS, Brzobohaty B. 2008. Cytokinin-induced photomorphogenesis in dark-grown Arabidopsis: a proteomic analysis. *J Exp Bot* **59**: 3705–3719.

Montalbán IA, De Diego N, Moncaleán P. 2010. Bottlenecks in *Pinus radiata* somatic embryogenesis: improving maturation and germination. *Trees-Structure and Function* **24**: 1061-1071

Montalbán IA, Novák O, Rolčik J, Strnad M, Moncaleán P. 2012. Endogenous cytokinin and auxin profiles during in vitro organogenesis from vegetative buds of *Pinus radiata* adult trees. *Physiol Plant*.

Monteuuis O, Baurens FC, Goh DKS, Quimado M, Doulbeau S, Verdeil JL. 2009. DNA Methylation in Acacia mangium In vitro and Ex-vitro Buds, in relation to their Within-shoot Position, Age and Leaf Morphology of the Shoots. *Silvae Genet* **58**: 287-292.

Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. 1999. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **27**: 29-34.

Patel KR, Thorpe TA. 1984. *In vitro* differentiation of plantlets from embryonic explants of lodgepole pine (*Pinus contorta* Dougl. ex Loud). *Plant Cell Tissue Organ Cult* **3**: 131-142.

Prehn D, Serrano C, Mercado A, Stange C, Barrales L, Arce-Johnson P. 2003. Regeneration of whole plants from apical meristems of *Pinus radiata*. *Plant Cell Tissue Organ Cult* **73**: 91-94.

Razem FA, El-Kereamy A, Abrams SR, Hill RD. 2006. The RNA-binding protein FCA is an abscisic acid receptor. *Natur* **439**: 290-294.

Rodríguez R, Fraga MF, Berdasco M, Diego LB, Valledor L, Hasbun R, Rodríguez A, Valdés AE, Ritter E, Espinel S et al. 2004. Applied and basic studies on *Pinus radiata* D. Don. *Curr Top Plant Biol* **5**: 143-162.

Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Begoña B, Rios JJ, Romero L, Blumwald E, Ruiz JM. 2011. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. *Environ Exp Bot* **72**: 167-173.

Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, Van Den Hoff MJB, Moorman AFM. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* **37**: e45.

Salcedo G, Sanchez-Monge R, Diaz-Perales A, Garcia-Casado G, Barber D. 2004. Plant non-specific lipid transfer proteins as food and pollen allergens. *Clinical & Experimental Allergy* **34**: 1336-1341.

Santos AM, Oliver MJ, Sánchez AM, Payton PR, Gomes JP, Miguel C, Oliveira MM. 2009. An integrated strategy to identify key genes in almond adventitious shoot regeneration. *J Exp Bot* **60**: 4159-4173.

Sels J, Mathys J, De Coninck B, Cammue B, De Bolle M. 2008. Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiol Biochem* **46**: 941–950.

Sugiyama S, Yamamoto K, Nishimura N, Nakagawa M, Maruta Y, Ando J. 2007. Adequate design of customized cDNA macroarray for convenient multiple gene expression analysis. *J Biosci Bioeng* **103**: 74-81.

Tsai CA, Chen JJ. 2009. Multivariate analysis of variance test for gene set analysis. *Bioinformatics* **25**: 897-897.

Valledor L, Jorrin-Novo JV, Rodríguez JL, Lenz C, Meijón M, Cañal MJ. 2010. Combined Proteomic and Transcriptomic analysis identifies differentially expressed pathways and stress responses associated to *Pinus radiata* needle maturation. *J Proteome Res* **9**: 3954-3979.

Valledor L, Jorrín J. 2011. Back to the Basics: Maximizing the information obtained by quantitative two dimensional gel electrophoresis analyses by an appropriate experimental design and statistical analyses. *Journal of proteomics* **74**: 1-18.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *genome biology* **3**: 0034.0031–0034.0011.

Walter C, Carson M, Carson S. 2007. Conifers In: Biotechnology in Agriculture and Forestry. in *Transgenic Crops V* (ed. EPaM Davey), pp. 447-471. Springer-Verlag, Berlin Heidelberg.

Wang YS, Pi LY, Chen X, Chakrabarty PK, Jiang J, De Leon AL, Liu GZ, Li L, Benny U, Oard J. 2006. Rice XA21 binding protein 3 is a ubiquitin ligase required for full Xa21-mediated disease resistance. *The Plant Cell Online* **18**: 3635–3646.

Xia Y, Nikolau BJ, Schnable PS. 1997. Developmental and hormonal regulation of the Arabidopsis CER2 gene that codes for a nuclear-localized protein required for the normal accumulation of cuticular waxes. *Plant Physiol* **115**: 925-937.

Yeung EC, Aitken-Christe J, Biondi S, Thorpe TA. 1981. Shoot histogenesis in cotyledon explants of radiata pine. *Bot Gaz* **142**: 494-501.

Zwack PJ, Robinson BR, Risley MG, Rashotte AM. 2013. Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses. *Plant Cell Physiol*. **In press**

Chapter 4. Epigenetic regulation of candidate genes by DNA methylation and chemical unmasking of other candidate genes.

4.1. Introduction

All *in vitro* culture techniques like adventitious organogenesis or somatic embryogenesis, involve processes of cell dedifferentiation/redifferentiation in a greater or lesser extent due to the acquisition of different reprogramming patterns (Zhao et al. 2008). Cell dedifferentiation during *in vitro* propagation implies changes in regulation of specific genes by epigenetic marks, including DNA methylation in several species (Chan et al. 2005; Li et al. 2011; De-la-Pena et al. 2012).

5-Aza-2-deoxycytidine is a hypomethylating nucleoside analog that is incorporated during DNA replication and was synthetised as a chemotherapeutic agent against cancer (Čihák 1974). The use of 5-Aza-2-deoxycytidine in mouse cell cultures has been shown to activate genes or repetitive sequences in a concentration dependent manner by demethylating their promoter regions. In Arabidopsis, using the demthylating drug 5-Aza-2-deoxycytidine, cell dedifferentiation has been associated with promoter hypermethylation dependent downregulation of TTG1, GSTF5, SUVH8, CCD7 and fimbrin genes (Berdasco et al. 2008), and in potato 5-Aza-2-deoxycytidine has been used in *in vitro* culture in cell suspensions to identify different patterns of DNA methylation and its relationship with H3 and H4 acetylation patterns (Law and Suttle 2005). Cells acquiring pluripotency have been described as exhibiting hypomethylation-dependent upregulation of several members of the NAC (NAM/ATAF1/CUC2) domain family (Avivi et al. 2004), and demethylating treatments using 5-Aza-2-deoxycytidine has been found to improve somatic embryogenesis by recovering of cell competence (Tokuji et al. 2011; Fraga et al. 2012).

Previous research in adventitious organogenesis gene analysis in *Pinus radiata* let us identify several sequences like RuBIsCO and LTP1 related to metabolism, an IS10 transposase and stress related proteins. Cell cultures represent a simple, highly reproducible system to determine the effects in gene expression by demethylating agent 5-Aza-2-deoxycytidine and to identify genes regulated by DNA methylation (Berdasco et al. 2008). Therefore, a methylated DNA immunoprecipitation between *Pinus radiata* control cells and cells cultured with 5-Aza-2-deoxycytidine in the adventitious organogenesis-related genes has been performed. An identification of candidate genes sensitive to be regulated by DNA methylation has also been performed in the same experimental situation by the hybridization of a dot blot developed in the caulogenic process.

4.2. Methods

4.2.1. Plant Material.

P. radiata cones (Basque Country Coast ES06, batch 2614 producer 00949/05) were provided by the Breeding and Conservation Service of Forest Genetic Resources (Spanish Sea, Rural and Environment Ministry).

4.2.2. In vitro culture and DNA methylation settings

4.2.2.1. Suspension culture settings

Suspension culture was obtained according to Azevedo et al. (2008) with several modifications. Seeds were surface-sterilised by immersion in 35% H_2O_2 for 20 min, followed by thorough rinsing using sterile H_2O and germinated in culture flasks containing QLP medium. Eight root sections from one month old seedlings were transferred to a Petri dish containing 20 ml of 0.8 (w/v) agarised MS medium pH 6, supplemented with 3 % glucose, 5 mg L^{-1} dithiothreitol, 0.1 g L^{-1} myoinositol, 1 mg L^{-1}

BA and 2 mg L^{-1} 2,4-D. Calli were allowed to develop for 6 weeks and were subcultured every 2 weeks. Suspension cultures were initiated from 8-week old callus by transferring 1 g to 250 ml Erlenmeyer flasks containing 10 mL of MS liquid medium and incubating in the dark, at 25 ºC on an orbital shaker at 100 rpm. After 3 days, when volume of culture medium was reduced, 10 mL of fresh medium were added. After 1 week of culture a volume of 25 mL was added and 25 mL of fresh medium were added every 12–14 days. After one month of growing in the Erlenmeyer flask with a final volume of 100 mL, 10 mL of the culture were transferred into 15 ml of fresh medium to restart the cycle. The steps to get a sustainable culture must be flexible because a high density of cells in the culture determines a proper growth.

4.2.2.2. Cell viability and 5-Aza-2-deoxycytidine treatment

A volume of 25 mL of the cell suspension was cultured together with two different concentrations (0.5 and 5 µM) of the demethylating agent 5-Aza-2-deoxycytidine (Sigma-Aldrich St. Louis, MO, USA) for 10 days. To establish the appropriate concentration of demethylating agent a cell viability test using 0.5 % (w/v) FDA (Fluorescein diacetate 6-isothiocyanate) (Sigma-Aldrich St. Louis, MO, USA) in acetone in a 1:50 dilution in the same MS culture medium was performed. Percentage of viability was evaluated during culture period in control suspension and in the two cell suspensions cultured with the demethylating agent in a fluorescence microscope Eclipse E600 (Nikon, Tokyo, Japan) with an objective Nikon Plan Fluor 20X (Nikon, Tokyo, Japan). Under UV light, fluorescein production from FDA of living cells can be seen regarding to dead cells that had no fluorescence. Using 3 different fields of vision for each treatment a counting of the living and dead cells was done.

4.2.2.3.- Quantification of global DNA methylation

4.2.2.3.1.- DNA extraction

A fresh weight of 150 mg of cell suspension was used for DNA extraction with DNeasy Plant Mini Kit (QIAGEN N.V. Venlo, The Netherlands) according to manufacturer´s instructions. DNA was concentrated with a DyNA Vap (Labnet Edison, NJ, USA) connected to a vacuum pump (Heto Guang Dong, China) and resuspended in 10 µL of ddH_2O (0.75 µg μL^{-1}).

4.2.2.3.2.- DNA hydrolysis

DNA samples, with at least 5 µg of genomic DNA, were denatured for 5 min at 95 ºC and then chilled on ice. Enzymatic hydrolisis was carried out by adding 1.25 µL of 10 mM $ZnSO_4$ and 2.5 µL of 200 U mL^{-1} Nuclease P1 in 30 mM $C_2H_3O_2Na$ (Sigma-Aldrich St. Louis, MO, USA) mixture. Samples were incubated for at least 16 h at 37 ºC, under moist conditions to avoid desiccation. A volume of 2.5 µL 0.5 M, pH 8.3 Tris-HCl (Tris(hydroxymethyl)aminomethane hydrochloride) (Sigma-Aldrich St. Louis, MO, USA) and 0.5 µL 50 U mL^{-1} in 2.5 M $(NH_4)_2SO_4$ alkaline phosphatase (Sigma-Aldrich St. Louis, MO, USA) were added later on, and samples were incubated another 2 h at 37 ºC to remove phosphate group. After centrifugation at 14 000 rpm for 20 min, supernatant was recovered and stored at -20 ºC until analysis.

4.2.2.3.3.- Capillary electrophoresis procedure

Global methylation quantification was carried out by High Performance Capillary Electrophoresis technique as previously described by (Hasbún et al. 2008) with several modifications. An eCAP capillary tubing (Beckman, Palo Alto, CA, USA) 75 µm ID, 375 µm OD and 60 cm long in an Agilent CE system device was used. Samples were run at 23 ºC and under a voltage of 7 Kv and a negative pressure of - 4

mbar. Data were obtained with Agilent 3D-CE Chemstation software (Agilent Technologies Santa Clara, CA, USA). Five analytical samples were measured in control and 5-Aza-2-deoxycytidine treatment and time of induction. The percentage of DNA methylation was calculated as follows: mdC peak area x 100/ (dC peak area + mdC peak area).

4.2.3. Methylated DNA Immunoprecipitation

4.2.3.1. DNA isolation

A fresh weight of 300 mg of *in vitro* cultured cell suspensions was used for DNA extraction by nuclei purification as follows:

Frozen tissue was grinded and powder was transferred to a 10 mL tube containing 8 mL of Buffer A (0.44 M sucrose, 10 mM Tris-HCl; pH 8.0, 5 mM β-ME and 0.15 mM PMFS). Samples were kept in ice from 20 to 30 min with a gently shaking every 5 min. A mousseline was used to filter the samples and the filtered solution was centrifuged at 3000 g for 15 min at 4 ºC.

Pellet was resuspended in 5 mL of buffer B (0.25 M sucrose, 10 mM Tris-HCl; pH 8.0, 10 mM MgCl2, 1% Triton X-100, 5 mM β-ME and 0.15 mM PMSF) and kept in ice for 10 min with gently shaking. Samples were centrifuged at 3000 g for 10 min at 4 ºC to pellet nuclei. Several additional extractions in buffer B were performed until pellet was mostly white. Pellet was resuspended in 8 mL of buffer C (0.25 M sucrose, 10 mM Tris-HCl; pH 8.0, 10 mM MgCl2, 5 mM β-ME and 0.15 mM PMSF) and centrifuged at 3000 g for 10 min at 4 ºC. Nuclei were resuspended in 1 mL of nuclei lysis buffer (50 mM Tris-HCl; pH 8, 10 mM EDTA, 1 % SDS and 15 µM PMSF). Samples were phenol/chloroform purified and DNA was ethanol precipitated and quantified.

4.2.3.2. DNA sonication

An amount of 20 µg of genomic DNA was diluted in 300 µL of IP buffer (100 mM Na-phosphate; pH 7.0, 1.4 M NaCl and 0.5 % Triton X-100) in a 1.5 mL tube. A total of 7 pulses of 90 % intensity for 15 seconds to each sample at 4 ºC were performed with an ultrasonic processor UP200S (Hielscher Ultrasonics GmbH, Teltow, Germany). A verification of the size of the fragment of sonicated DNA on a 2% agarose gel was performed with an average size of 400 bp and a range from 200 to 800 bp. A total of 14 µg of DNA was placed in two 1.5 mL tubes (7 µg of DNA each).

4.2.3.3. Immunoprecipitation of methylated DNA

Samples were heat-denatured and immediately placed in ice for 10 min. One tube of each situation was stored at −20°C as an input control. An amount of 10 µg of antibody (monoclonal mouse anti-5-methylcytidine. Ref. BI-MECY-0100; Eurogentec Liège, Belgium) was added to each sample and the mixture was incubated on a rotating platform at 4°C. A volume of 50 µl of magnetic Dynabeads Protein G (Invitrogen ©Life Technologies Corporation, Gaithersburg, MD, USA) was added to the DNA-antibody mixture and it was incubated for 2 h on a rotating platform at 4°C. The washing steps were simplified by the use of a magnetic rack (Invitrogen ©Life Technologies Corporation, Gaithersburg, MD, USA) and removing the unbound DNA-antibody mixture. Magnetic beads were washed 3 times and resuspended in 250 µl of digestion buffer (50 mM Tris; pH 8.0, 10 mM EDTA and 0.5 % SDS) with 100 µg of Proteinase K (Sigma Chemical Co., St. Louis, MO, USA) and incubated overnight on a rotating platform at 50 °C.

4.2.3.4. Purification of methylated DNA

Samples were purified with phenol:chloroform:isoamyl alcohol (v/v 25:24:1) and for separation of aqueous phase, a 2 ml heavy 5 prime phaselock tube (5 Prime→3

Prime Inc, Boulder, CO, USA) was used according to manufacturer´s instructions. For DNA precipitation 1/10 of the total volume of 5 M NaCl, 2 volumes of 100 % ethanol and 1.5 µl of glycogen (20 mg/ml stock) were added. Samples were precipitated at −80°C overnight and centrifuged at 20 000 g for 10 min at 4°C. Pellet was resuspended in TE buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA) and DNA was quantified.

4.2.4. Quantitative PCR.

Quantitative PCR was performed as follows: 10 ng of immunoprecipitated DNA, 5µM of each primer and Brilliant III Ultra-Fast SYBR® Green (Agilent Technologies, Palo Alto, CA, USA) were mixed according to manufacturer´s instructions and amplified using the ABI 7900HT system (Applied Biosystems, ©Life Technologies Corporation, Foster City, CA, USA). Four replicates for each transcript from a pool of individuals were analyzed using the LinReg software (Ruijter et al. 2009). Primer sequences and Tm are indicated in Appendix (Table S4.1).

4.2.5. Dot blot macroarray.

RNA was isolated from 75 mg of frozen tissue according to RNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands). RNA integrity was tested in agarose gel under denaturing conditions.

cDNA was PCR amplified directly from *E. coli* clones Employing SP6 and T7 primers (PCR program: 94°C for 2 min, 36 x (94°C for 20 s; 50°C for 30 s); 72°C for 70 s and a final extension of 7 min at 72°C). Resultant PCR product was mixed with 1.5 M NaOH, 300mM Ethylenediaminetetraacetic acid (EDTA) pH 8.2 to reach a final concentration of 400 mM NaOH, 80 mM EDTA. From the mixture, 1 uL was spotted into two Hybond N+ Membranes (GE Healthcare, Piscataway, NJ, USA). The membranes had 191 probes (96 and 95 probes each), corresponding to identified genes. Reverse transcription from 1 µg of total RNA was performed by Superscript III first

strand synthesis kit, (Invitrogen ©Life Technologies Corporation, Gaithersburg, MD, USA) and tail-labeled with DIG-UTP alkali-labile employing a terminal transferase (Roche, Basel, Switzerland). Membranes were prehybridized in Church's buffer (0.5 M phosphate buffer pH 7.2, 10 mM EDTA, 7 % Sodium Dodecyl Sulfate (SDS), 1 % Bovine serum albumin (BSA)) for 1 hour. Target cDNA was then added to fresh buffer at a final concentration of 50 ng $mL^{-1}$and hybridized overnight at 55°C. After two consecutive washings in 2x SSC-0.1 % SDS and 1x SSC-0.1 % SDS the chemiluminiscence assay (CSPD ready to use, Roche, Basel, Switzerland) was done according to manufacturer´s instructions and autoradiography was performed in a LAS-3000 minisystem (Fuji, Tokyo, Japan). Images were analysed with Quantity One V.4.6.3 (Bio-Rad, Hercules, CA, USA). Three hybridization replicates were done for later statistical analysis.

4.2.6. Statistical analysis

The univariate analysis of variance (ANOVA) was carried out in all EST data using *glm* procedure in SAS© software (SAS Institute Inc., Cary, NC, USA) to determine the possible factor interactions. The multiple comparisons were performed using the post hoc Tukey´s HSD test after ANOVA.

4.3. Results

4.3.1. Cell suspension and DNA methylation settings

The effect of two concentrations of 5-Aza-2-deoxycytidine in the culture was assessed to select the most appropriate concentration of the demethylating drug. Cell viability was monitored in treated and control suspensions for 8 days. Cell culture grown with 5 µM showed significant differences regarding to cell survival of control culture from day 5 (Figure 4.1), decreasing strongly cell viability from this time point.

The cell culture grown with 0.5 µM had no differences in cell viability regarding control culture along the experiment. This concentration of 5-Aza-2-deoxycytidine was selected as the optimal concentration, and it was used in the following steps.

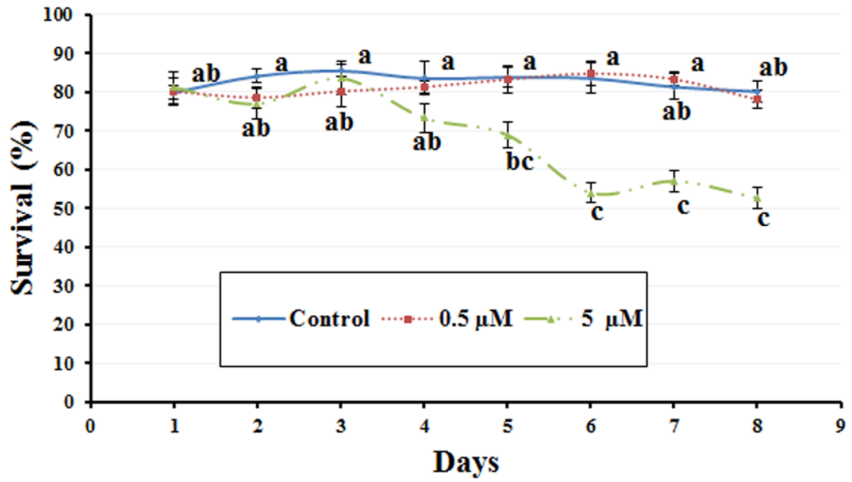


Figure 4.1: Percentage of survival in cultured cells of *P. radiata* for 8 days with and without 0.5 and 5 µM 5-Aza-2-deoxycytidine. Different letters indicate significant differences by Tuckey test after ANOVA D= 0.01%.

The optimal exposition time of cell suspensions to 5-Aza-2-deoxycytidine to induce changes in gene methylation was determined using global DNA methylation values as the unique indicator. Global DNA methylation pattern along time in the presence of 5-Aza-2-deoxycytidine was similar to control culture until day 6, in which statistical differences regarding to control were observed as is shown in Figure 4.2. From these results, 6 days of culture were chosen for the macroarray differential expression analysis.

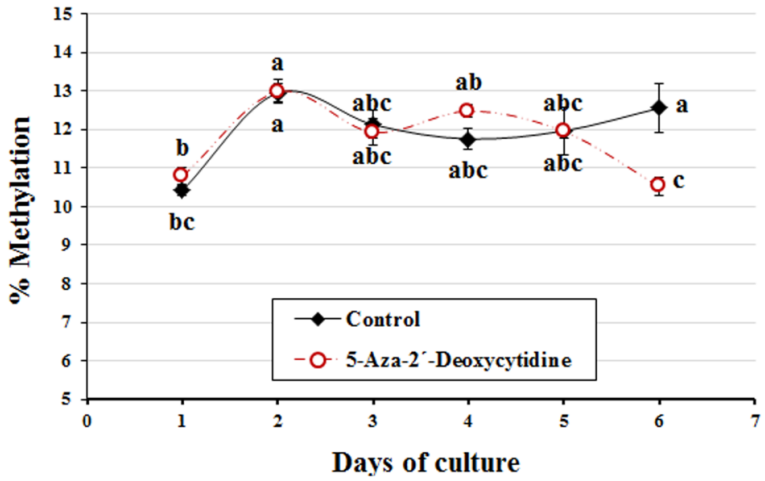


Figure 4.2: Percentage of global DNA methylation in cultured cells of *P. radiata* for 6 days with and without 0.5 µM 5-Aza-2-deoxycytidine. Different letters indicate significant differences by Tuckey test after ANOVA D= 0.01%.

4.3.2. Methylation analysis by DNA immunoprecipitation

A Methylated DNA Immuno Precipitation (MeDIP) was performed in the DNA extracted from the cell suspension cultures at day 6 with and without 0.5 µM 5-Aza-2-deoxycytidine.

The relative amount of DNA in input (IN) and immunoprecipitated (IP) samples of the previously identified genes upregulated in adventitious organogenesis RuBIsCO small, Alpha tubulin 1 and LTP1, and downregulated genes 25S rRNA, PR-10, IS10-right transposase, XB3 and CRK1 (Rodriguez et al., under review) was tested to measure the enrichment of DNA between IP regarding to IN. The differential immunoprecipitation induces an enrichment in IP samples of specific genes compared with IN samples. Starting with the same amount of DNA, by quantitative PCR the differences between IP/IN in a specific gene can be measured, and the enrichment in IP samples is statistically analyzed to find significant differences.

In control samples (Fig. 4.3) LTP1, PR-10, IS10-right transposase and Xa21BP3 were statistically different ($p < 0.01$) and were considered as methylated in their sequence. 25S rRNA was the only gene statistically not methylated ($p < 0.001$).

In 5-Aza-2-deoxycytidine cell cultures (Fig. 4.4), all sequences except IS10 right transposase and α-Tubulin 1, showed statistical differences, indicating that methylation in all of them has been decreased by the action of the drug.

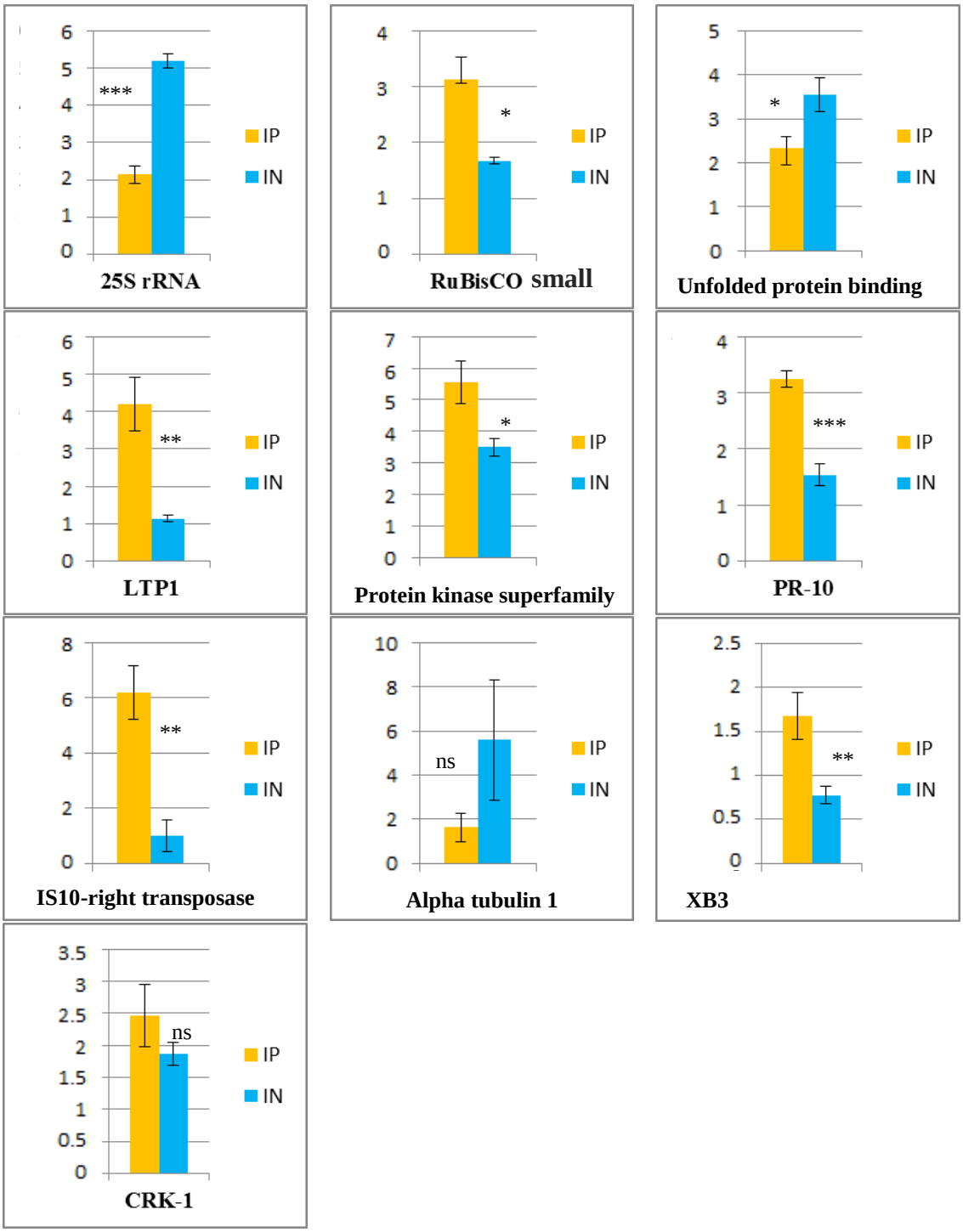


Figure 4.3: Relative amount of DNA in input (IN) and in immunoprecipitated (IP) samples of different genes related to adventitious organogenesis in *P. radiata* control suspension culture at 6 days without 5-Aza-2-Deoxycytidine. ns: non-significant; *<0.05; **<0.01; ***<0.001

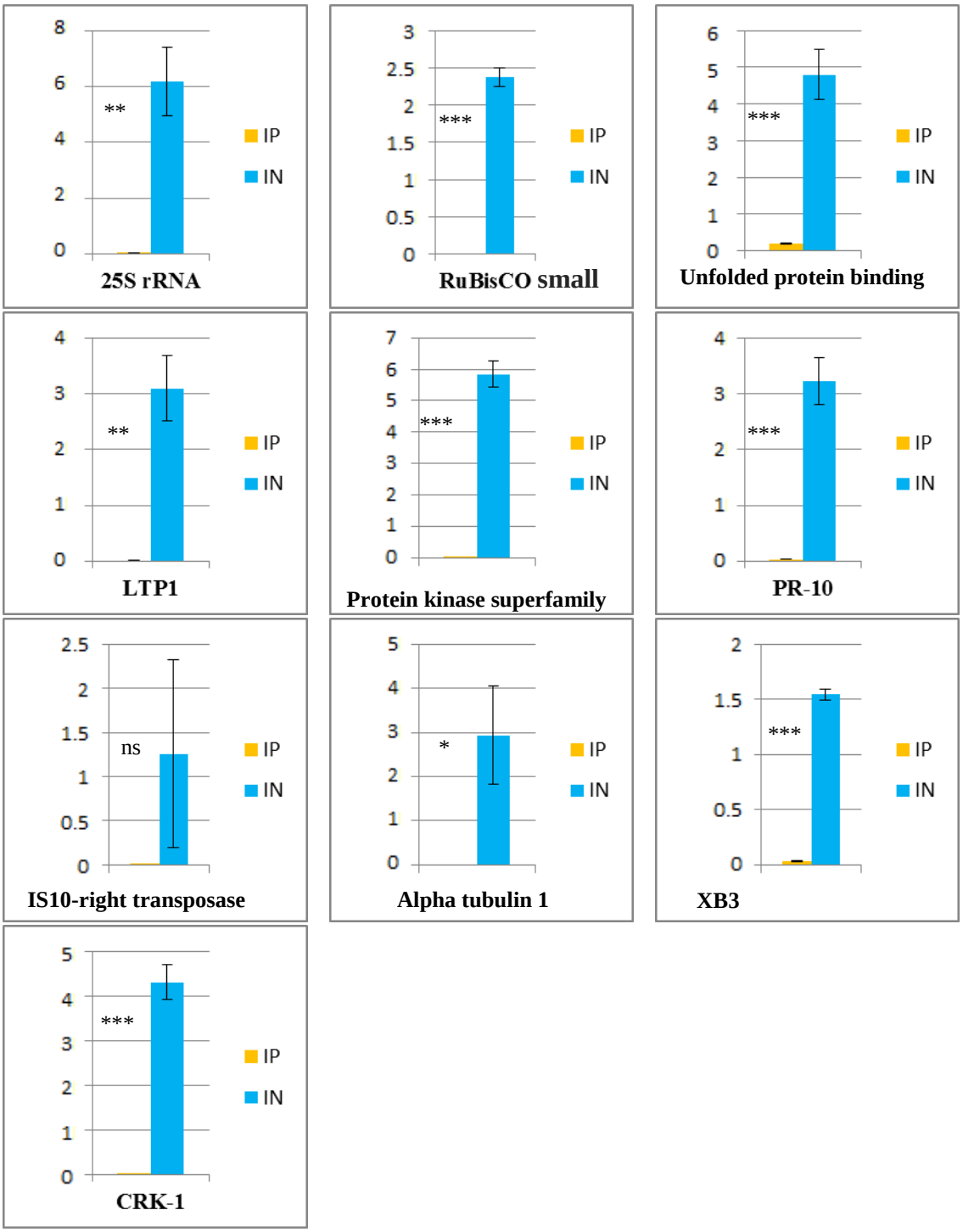


Figure 4.4: Relative amount of DNA in input (IN) and immunoprecipitated (IP) samples of different genes related to adventitious organogenesis in *P. radiata* suspension culture at 6 days with 5-Aza-2-Deoxycytidine. ns: non-significant; *<0.05; **<0.01; ***<0.001

DNA methylation enrichment was compared in all samples between control and 5-Aza-2-Deoxycytidine and cells from control showed a higher general enrichment regarding to 5-Aza-2-Deoxycytidine (Fig. 4.5). LTP1, PR-10, IS10-right transposase and XB3 with a fold enrichment of (3.7, 2.1, 6.1 and 2.1) respectively in control, were the statistically methylated genes.

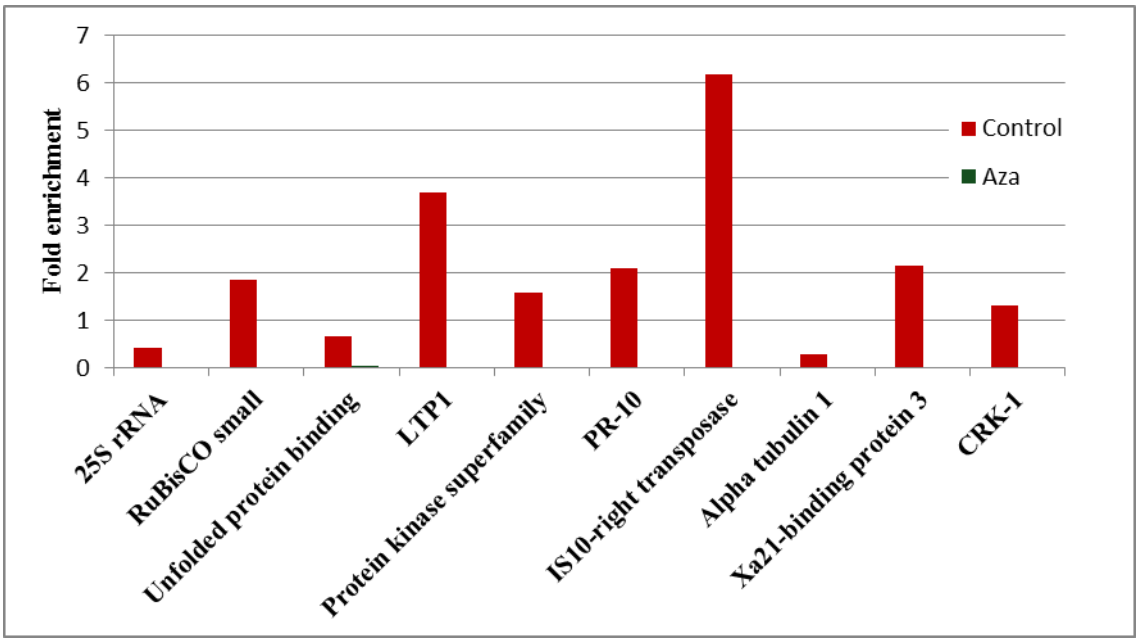


Figure 4.5: Enrichment of DNA methylation relative to input genomic DNA of sequences differentially expressed during adventitious organogenesis in *P. radiata* suspension culture at 6 days with and without 5-Aza-2-Deoxycytidine.

4.3.3. Dot blot data analysis

The Expression of 191 sequences (Table S4.2) representing each of the different biological functions was measured by southern dot blotting in 6 days cultured cells in the presence or absence of 5-Aza-2-deoxycytidine 0.5 µM. All the analysed sequences showed changes in their expression ratio: the 49 % of the sequences had more expression in the presence of the demethylating agent (Fig. 4.6). In macroarray data an analysis of variance was performed to identify sequences differentially expressed between 5-Aza-2-deoxycytidine cultured cells and control. Genes analysed by MeDIP found out a significant increase of expression in sequence 25S rRNA, although it was

statistically unmethylated in control. Statistically significant sequences upregulated in 5-Aza-2-deoxycytidine were 25S rRNA and Early response to dehydration 15-like protein, whereas in control the overexpressed sequences were *AMP1 (ALTERED MERISTEM PROGRAM 1)*, Pathogen responsive protein type 4-like gene, Calnexin-like protein, Putative glucose translocator, EMP24/GP25L family, Acyl-CoA-binding protein, PsbQ subunit of the oxygen evolving complex ofphotosystem II, mitocondrial ATP synthase subunit beta, *UBIQUITIN-CONJUGATING ENZYME 31*, 70kD heat shock protein, Photosystem II oxygen evolving, enhancer 3 (PsbQ) family protein and Protein serine/threonine phosphatase activity (Table S4.3). Expression levels of these statistically significant sequences in 5-Aza-2-deoxycytidine and in control cultures are shown in Fig. 4.7.

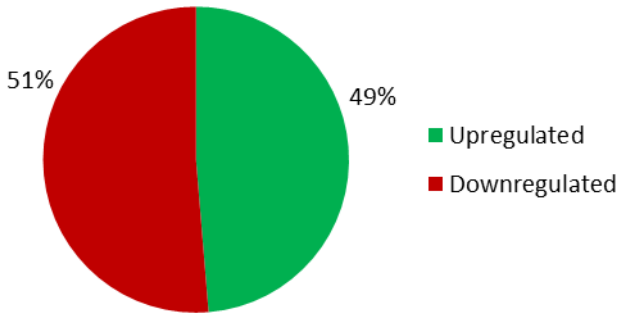


Figure 4.6: Expression ratio from sequences of *P. radiata* cell suspension cultured for 6 days with 0.5 µM 5-Aza-2-deoxycytidine regarding to control cell culture.

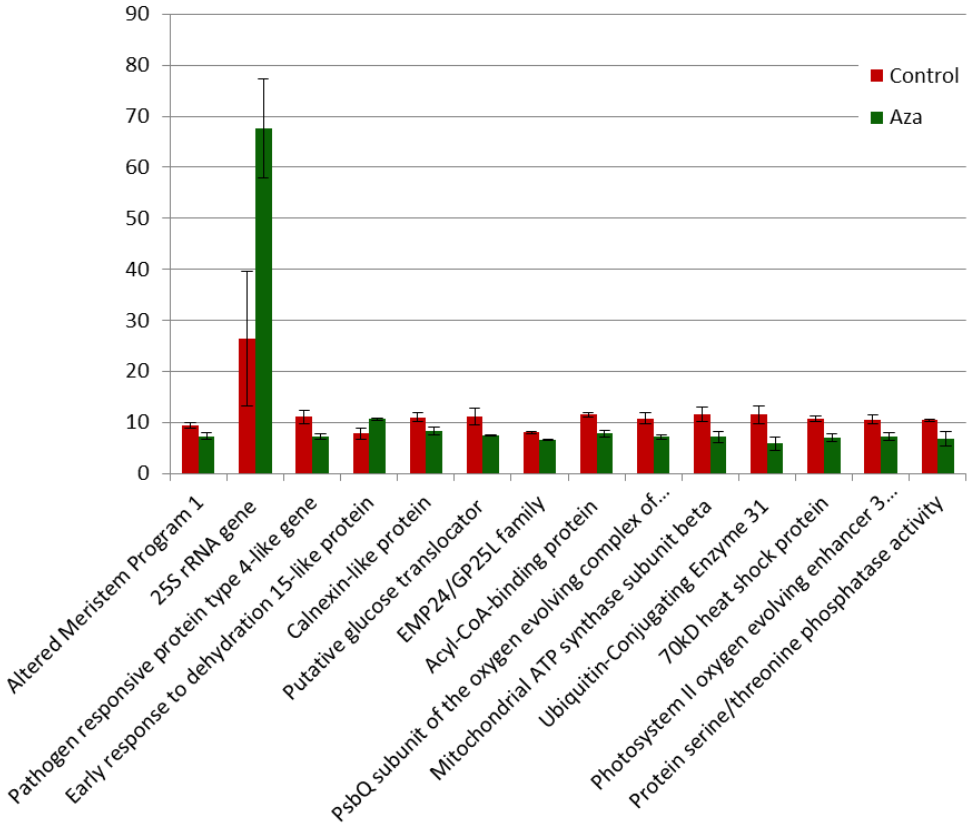


Figure 4.7. Relative expression of sequences of *P. radiata* cell suspension cultured for 6 days with and without 5-Aza-2-deoxycytidine found statistically different according to ANOVA.

4.4. Discussion

4.4.1. Cell suspension culture and global DNA demethylation

It is known that the toxicity of 5-Aza-2-deoxycytidine is concentration-dependent and it is related to the cell cycle, leading cell to apoptosis and variations in DNA methylation (Murakami et al. 1995; Meng et al. 2011). When using the demethylating agent 5-Aza-2-deoxycytidine, it is essential to be sure that the cell viability does not jeopardise the response of the suspension cell culture to different concentrations of the drug. In mouse, it has also been reported a concentration-dependent demethylation of genic regions with low concentrations (0.1 µM) of 5-Aza-2-deoxycytidine by (Lim et al. 2009) and our results partially agree, being genes all the sequences upregulated, and having gene sequences and the IS10 transposase demethylated in 0.5 µM 5-Aza-2-deoxycytidine cell culture.

Global methylation decreased in response to 5-Aza-2-deoxycytidine by inhibiting the DNA methyltransferase activity. The compound is included in the DNA mainly during replication, and according to the results from Aquea et al. (2008), DNA content in *P. radiata* cultures doubled after 4 days of culture and in *Pinus sylvestris* cell mass growth doubled after 6 of culture (Vuosku et al. 2012). These data indicate that after 6 days of culture our cells underwent no more than one cell division, being sufficient to find differential results in global DNA methylation and in gene sequence methylation as well in our cell cultures.

4.4.2. Demethylated and upregulated genes by 5-Aza-2-deoxycytidine

The identification of sequences differentially methylated in the genome can be tackled by using MeDIP technique as a first and efficient approximation. The influence of being methylated in promoter or gene body have a different role in gene expression (Vining et al. 2012; Lafon-Placette et al. 2013) and CG islands involved in this regulation are assessed by bisulfite sequencing (Krueger et al. 2012; Su et al. 2013).

All sequences analysed showed a reduction in DNA methylation in cell suspension cultures in 5-Aza-2-deoxycytidine regarding to control, in accordance with results using the same technique in Arabidopsis (Berdasco et al. 2008; Hudson et al. 2011). 5-Aza-2-deoxycytidine greatly induced the overexpression of 25S rRNA in *P. radiata* cell suspension. In this regard, the ribosomal RNA (rRNA) has been reviewed to be in tandem arrays containing hundreds of copies that are stochastically silenced, and this silencing is due to methylation in its promoter sequence (Chan et al. 2005; Neves et al. 2005). This methylation varies because of environmental conditions, and under stress or a 5-Aza-2-deoxycytidine treatment there is an extensive demethylation and chromatin decondensation (Santos et al. 2011). Regarding to *in vitro* culture, a demethylation in rDNA is necessary to cell dedifferentiation in tobacco (Koukalova et al. 2005) and in humans CpG island hypermethylation-associated silencing of small

nucleolar RNAs is related to cancer (Ferreira et al. 2012). This relationship between dedifferentiation and DNA methylation rate in rDNA shows the importance in development processes (Koukalova et al. 2005) and how a regulation in the tandem rRNA genes could be dependent on each external stimulus or growth regulator. For these reasons, 25S rRNA sequence should be analysed with particular attention because small variations in this sequence enrichment suggest the regulation of an important number of copies.

Early Response to Dehydration 15-like protein (ERD15) expression was also increased in 5-Aza-2-deoxycytidine cell suspension. ERD15 has been reported to regulate cell defense and to modify hormone signalling under stress (Alves et al. 2011a). Due to the presence of 5-Aza-2-deoxycytidine in cell suspension induce a strong stress effect, the overexpression of ERD15 gene expression could indicate a change in signaling pathways which regulated the control cell suspension response, resulting in the activation of new pathway that may transduce a programmed cell death signal (Alves et al. 2011b).

Other genes related to stress, such as LTP-1, PR-10, XB3 also presented a significant enrichment. LTP1 has been proposed to belong PR-14 pathogenesis-related protein family with the particularity that possess a signal peptide that target them to the cell secretory pathway and can be localised at the cell wall, being this extra cellular location not a general rule (Sels et al. 2008). In the same way PR-10 sequence has been associated with plant defence in both biotic and abiotic stress (Liu and Ekramoddoullah 2006) and has a Bet v I allergen domain being part of the food and pollen allergens member of PR-10 proteins (Fernandes et al. 2013).

Finally, XB3 is an ubiquitin ligase able to autophosphorylate Serine and Threonine residues to improve protein stability (Deng and Gmitter 2003; Wang et al. 2006; Xu et al. 2006), and it is involved in pathogen recognition triggering a really fast

response in plant initial defense by several post-translational modifications of the already present proteins (Stulemeijer and Joosten 2008; Trujillo and Shirasu 2010); and finally IS10-right transposase sequence (IS10R) a kind of sequences induced mainly by stress conditions (Grandbastien 1998).

This research reports the first results that indicate a regulation of these genes by DNA methylation in their sequence, except in the case of IS10R where (Roberts et al. 1985) found a regulation in the adenine residues in *Escherichia coli*, and a regulation by methylation of cytosine residues has been proposed by (Hudson et al. 2011).

4.4.3. Downregulated genes by 5-Aza-2-deoxycytidine

Macroarray expression analysis revealed different sequences upregulated in control and related to stress response in endoplasmic reticulum like calnexin, HSP70 *UBIQUITIN-CONJUGATING ENZYME 31* and EMP24/GP25L (Williams 2006; Tuteja 2009; Miura and Hasegawa 2010), and related to several stress responses like ACBP2 (Xiao and Chye 2011; Du et al. 2013). Desjardins et al. (2007) found out that *in vitro* culture affects cells provoking a stress situation triggering systemic defense mechanisms and developmental responses, that could be already activated before 5-Aza-2-deoxycytidine experiment. The methylation of the different sequences in control related to stress diverge from already expressing sequences in the same experimental situation, which may indicate a differential regulation by DNA methylation of sequences in specific stress responses as it has been proposed by Dowen et al. (2012). The upregulation of ERD15 in Aza-2-deoxycytidine could have a relationship in the stress caused in the endoplasmic reticulum related genes downregulating their expression, because of changes in specific stress pathways (Alves et al. 2011b).

A meristem development and pattern formation gene *ALTERED MERISTEM PROGRAM 1 (AMP1)*, a hypothetical glutamate carboxypeptidase, has been found as differential in control cells. Redifferentiation/differentiation genes have already been

identified as hypermethylated genes in 5-Aza-2-deoxycytidine cultured cells of Arabidopsis (Berdasco et al. 2008) and the activation of genes involved in development pathways could have an effect in its expression (Lohe and Chaudhury 2002).

Sequences related to photosynthesis and electron transport have also been found. PsbQ protein is part of the lumenal complex of photosystem II (PS II), known as oxygen-evolving complex (OEC). As well as PsbQ, two more extrinsic subunits PsbO and PsbP are present in the OEC (Roose et al. 2007; Bricker and Frankel 2011). ATP synthase subunit beta is part of the F1 complex, where is found the catalytic core that synthetises/hydrolyses ATP. ATP synthases are membrane-bound enzyme complexes/ion transporters that combine ATP synthesis and/or hydrolysis with the transport of protons across a membrane. ATPases can harness the energy from a proton gradient, using the flux of ions across the membrane via the ATP synthase proton channel to drive the synthesis of ATP (Jonckheere et al. 2012). It could be surprising to see the case of RuBIsCO small, a gene connected to photosynthesis which is regulated by DNA methylation ($p<0.05$), with a clear demethylation in 5-Aza-2-deoxycytidine cell cultures but with no upregulation of its expression maybe caused by 5-Aza-2-deoxycytidine or by culture conditions in a dark chamber. It has been reported by Hudson et al. (2011) a downregulation of photosynthetic genes in Arabidopsis cells cultured with 5-Aza-2-deoxycytidine because of the effect of the drug in nuclear encoded plastid proteins. Although recent research indicates a relationship between net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration, and DNA methylation candidate markers (Ma et al. 2012).

4.5. Conclusions

The use of 5-Aza-2-deoxycytidine as demethylating agent has been shown very effective causing differences in global methylation regarding to control at day 6 as well as significant differences in immunoprecipitated DNA enrichment in most of the analyzed sequences. These results point out that its application in different developmental situations could be an appropriate method to study different culture conditions, so it will permit us asses the importance of methylation processes regulating those genes involved in plant development, especially in other micropropagation processes such as somatic embryogenesis where the genotype is determinant in whole plant regeneration.

The differential enrichment of sequences related to stress and downregulated in adventitious organogenesis, reasserts the importance of DNA methylation in the regulation and specificity of this response, and four new sequences regulated by DNA methylation in their nucleotidic sequence have been found. Furthermore, new possible candidate genes regulated by DNA methylation and connected to plant morphogenesis and photosynthesis have been identified.

Finally, the differential expression of 25S rRNA EST and the previous information about its regulation has revealed this sequence as an excellent candidate for being regulated by DNA methylation in adventitious organogenesis.

4.6. Appendix

Supplemental Table S4.1: Primer sequence from ESTs for MeDIP-qRT-PCR.

Gen	Secuencia 5'->3'	Tm
PRACABA05_G11 F	GTG GGC TGG TGG TGT TTA TC	55
PRACABA05_G11 R	TTT CAC ATG GGC AAC TTC AA	55
PRACABA02_F3 F	TCC TCA AAA TTG TGG CTT CC	55
PRACABA02_F3 R	GCG TTG AGC CAG TGT GAG TA	55
PRABA04_B6F	TCC AAA ATG GAT CTG GAA GC	55
PRABA04_B6R	CAT GCG AGA AAC TCT GAC CA	55
PRABA04_C3F	ATC GCC AAT GTC AAG TGT GA	55
PRABA04_C3R	CCG ACG ATA TTT CGC ATT CT	55
PRAABA04_D2F	GGC TGA ATC TCA GTG GAT CG	55
PRAABA04_D2R	ACG GTC GTT GAG AAG AAT CC	55
PRABA01_H5F	AAC AAT ACT CCG CCT TCT GC	55
PRABA01_H5R	CAG CCT TCT CAA AAC CAT GC	55
PRABA02_H7F	ACC AGT ACC AAC CCA GCT TC	55
PRABA02_H7R	GAC CTT GCG AGC ATT CTA CC	55
PRABA02_G8F	GTG TGG TGA CTT GGA TCT GC	55
PRABA02_G8R	GCA GTA TAA GGT GGG GTT GG	55
PRACBA05_A9F	TCT ATA ATG CGA GGG GAA AAT C	55
PRACBA05_A9R	TGT GTG GTT TTG GAC TTG TTG	55
RACABA03_F7F	TCC ACC TTC ATC CCT CTA CC	55
PRACABA03_F7R	GCA AGA AGC AAA AGA TGA CG	55

Supplemental Table S4.2: Data expression in control and 5-Aza-2-deoxycytidine of the list of the contigs isolated by SSH in Chapter 3.

Accession	Bp	Homology accession	E-value	Description	EST mean Expression	
					5-Aza-2-deoxycytidine	Control
				Cellular Processes; Cell Communication		
GO448327	829	TC99011[1]	$6x10^{-70}$	Calnexin homolog 2	112,004	91,566
GO448145	174	TC99011	$1x10^{-93}$	Calnexin-like protein	83,161	110,006
				Cellular Processes; Cell Growth and Death		
GO448330	490	TC88109	0	Protein GF14 iota (grf12)	63,583	61,860
GO448275	767	TC81298	0	Transcription factor TFIIB	92,255	98,856
GO448469	851	CT579120	0	Ankyrin repeat family protein	103,361	95,824
GO448389	773	TC62907	0	Ankyrin protein kinase	95,862	106,018
				Cellular Processes; Transport and Catabolism		
GO448283	874	TC97357	0	Exocyst subunit exo70 family protein B1	81,037	61,848
GO448278	330	TC84602	0	EMP24/GP25L/P24 PROTEIN-RELATED	97,660	99,928
GO448284	597	TC102151	0	Alpha-tubulin 3	95,729	101,475
GO448074	764	TC106352	0	Putative glucose translocator	74,805	110,705
GO448155	330	TC73550	$6x10^{-70}$	Belongs to the EMP24/GP25L family	65,345	79,972
GO448098	784	EX337073	$2x10^{-40}$	DEM2 protein	44,874	87,470
GO448379	397	AW011241	$1x10^{-136}$	Putative alpha tubulin	103,823	97,593
GO448463	402	TC111024	0	Alpha tubulin 1 (TUA1)	101,538	97,036
GO448418	520	CF391830	0	Polyamine oxidase-like	120,282	104,532
GO448405	661	TC101508	0	Lipid transfer protein (LTP)	164,622	158,237
GO448190	798	TC54497	0	Mitochondrial carrier protein-like	83,861	102,832
GO448259	839	TC54497	0	Mitochondrial carrier protein-like	104,082	107,262
GO448221	201	TC111024	$3x10^{-73}$	Alpha tubulin 1	87,261	92,557
GO448237	856	TC45428	0	CBS domain-containing protein	101,843	112,507
				Environmental Information Processing; Membrane Transport		

GO448304	858	TC97743	0	Auxin/aluminum-responsive protein	103,436	67,294
GO448194	772	TC92521	0	Belongs to the ABC transporter family	155,416	96,616
GO448239	512	TC88236	0	lipid transfer protein (LTP)	128,571	100,382
				Environmental Information Processing; Signal Transduction		
GO448277	1039	TC82571	0	Chloroplast bromodomain-containing protein	85,075	100,419
GO448343	779	TC102683	0	Similar to zinc finger (DHHC type) family protein	83,794	76,674
GO448365	464	TC69156	$1x10^{-119}$	MAP3K protein kinase-like protein	110,223	81,444
GO448336	670	TC91052	0	Similar to hypoxia-responsive family	95,291	134,766
GO448280	773	TC100219	0	putative EXS family protein	75,566	84,094
GO448281	501	TC90217	0	Hypersensitive-induced response protein	93,160	111,486
GO448323	759	TC73733	0	Similar to protein kinase family protein	89,820	108,758
GO448369	723	TC87327	$1x10^{-75}$	Early response to dehydration 15-like protein	105,962	77,762
GO448271	285	TC110337	$9x10^{-98}$	Expansin A4	92,333	113,752
GO448109	734	TC57035	0	Belongs to the protein kinase superfamily	116,926	94,574
GO448184	451	TC80568	0	Encodes a member of the ERF (ethylene response factor) subfamily B-2 of the plant specific ERF/AP2	91,905	77,996
GO448067	374	TC90240	0	General Regulatory Factor 7	159,997	99,226
GO448078	602	TC93679	0	Zinc finger (DHHC type) family protein	100,062	91,960
GO448112	798	TC97469	0	Antimicrobial peptide 1 (AMP1)	88,249	96,408
GO448075	389	DR020528	0	Calmodulin (Auxin-regulated calmodulin)	58,951	77,153
GO448104	842	TC97231	0	PHD zinc finger family protein	121,433	146,860
GO448425	463	TC96811	$1x10^{-145}$	Protein phosphatase 2C	74,009	97,463
GO448392	525	TC97469	0	Antimicrobial peptide 1 (AMP1) mRNA/NTGP1-related	133,726	131,301
GO448448	291	TC96811	$1x10^{-160}$	Protein serine/threonine phosphatase activity	130,964	110,714
GO448382	199	TC93637	$1x10^{-103}$	70kD heat shock protein	69,312	107,365
GO448374	300	TC110337	$1x10^{-141}$	Putative expansin 2	94,626	106,025
GO448200	271	TC110337	$1x10^{-137}$	Putative expansin 2	104,593	109,167
GO448199	771	TC96811	$6x10^{-81}$	Protein serine/threonine phosphatase activity	89,352	96,438
GO448203	761	TC96811	$8x10^{-62}$	Protein serine/threonine phosphatase activity	159,675	101,174
GO448195	1152	TC96811	$1x10^{-119}$	Protein serine/threonine phosphatase activity	108,116	113,258

GO448193	1257	TC96811	$1x10^{-162}$	Protein serine/threonine phosphatase activity	109,287	107,744
GO448240	781	TC96811	$1x10^{-72}$	Protein serine/threonine phosphatase activity	106,135	105,979
GO448186	1219	TC96811	$1x10^{-152}$	Protein serine/threonine phosphatase activity	115,689	99,455
GO448218	884	TC96811	$2x10^{-10}$	Protein serine/threonine phosphatase activity	85,810	95,396
GO448238	727	TC96811	$1x10^{-20}$	Protein serine/threonine phosphatase activity	68,067	103,758
GO448242	766	TC96811	$1x10^{-29}$	Protein serine/threonine phosphatase activity	99,223	95,371
GO448247	743	TC96811	$2x10^{-87}$	Protein serine/threonine phosphatase activity	122,981	97,762
GO448196	1240	TC96811	$1x10^{-114}$	Protein serine/threonine phosphatase activity	86,368	111,023
				Genetic Information Processing; Folding, Sorting and Degradation		
GO448290	419	ES869607	$1x10^{-160}$	Enhanced disease resistance 1	75,624	139,307
GO448083	628	TC8160	$2x10^{-88}$	Ubiquitin-Specific Protease 12	70,485	138,052
GO448079	478	CF390608	$1x10^{-105}$	S-receptor kinase-like protein 2	97,525	76,308
GO448093	766	TC89516	$8x10^{-28}$	SECY/SEC61-ALPHA FAMILY	70,413	78,566
GO448059	838	TC323115	$9x10^{-68}$	Putative integral membrane protein (sec61)	60,531	76,624
GO448125	816	TC112333	0	Receptor-like kinase Xa21-binding protein 3	111,840	126,272
GO448131	700	TC86842	$1x10^{-143}$	Cytokinin-regulated kinase 1	135,319	117,002
GO448177	804	TC89516	0	Belongs to the secY/SEC61-alpha family	176,678	99,638
GO448458	335	TC71322	$4x10^{-83}$	Enzyme regulator activity/26S proteasome subunit RPN2a	96,223	111,820
GO448422	846	TC89540	0	Ubiquitin-Conjugated Enzyme 31	58,264	114,876
				Genetic Information Processing; Transcription		
GO448337	792	TC91729	$1x10^{-113}$	Pre-mRNA splicing factor-like protein	95,680	94,098
GO448363	497	TC44867	$1x10^{-130}$	ASF/SF2-like pre-mRNA splicing factor SRP32	82,033	103,307
GO448160	691	TC88388	0	RNA polymerase II transcription factor activity	138,257	105,524
GO448091	717	TC100241	0	Similar to bZIP family	142,782	96,090
GO448179	695	TC88729	0	Purine rich element binding protein B-like	97,401	72,089
GO448085	851	DR097404	$1x10^{-131}$	Polypyrimidine tract-binding protein homolog (PTB)	114,705	75,038
GO448376	626	TC82884	0	Homeodomain-leucine zipper protein 57	120,356	100,347
GO448384	683	TC80804	0	RNA polymerase II transcription factor activity	67,877	103,382
GO448245	784	TC90936	0	RNA polymerase II transcription factor activity	67,177	81,707

GO448189	683	TC80804	0	RNA polymerase II transcription factor activity	75,119	91,621
				Genetic Information Processing; Translation		
GO448310	777	TC97041	0	Valyl-tRNA synthetase	76,448	71,833
GO448285	746	DR059452	$2x10^{-13}$	Mitocondrial protein/ribosomal protein S2	124,839	75,821
GO448301	691	TC89374	0	Translin family protein	147,336	88,168
GO448265	770	TC113143	0	Partial 25S rRNA gene	264,296	676,080
GO448077	543	TC83655	$1x10^{-160}$	60S ribosomal protein L24	126,649	99,628
GO448068	933	TC44370	$5x10^{-22}$	Ribosomal protein L23P family	182,700	100,396
GO448122	809	TC58034	$1x10^{-117}$	DEAD-box ATP-dependent RNA helicase	97,951	71,928
GO448102	792	TC106298	$5x10^{-20}$	40S ribosomal protein S4 (RPS4D)	104,619	80,861
GO448051	801	TC81777	0	Identical to 40S ribosomal protein S4-3 (RPS4D)	127,306	82,647
GO448146	864	TC97430	0	30s ribosomal protein s1	77,450	125,659
GO448143	630	TC95199	$1x10^{-158}$	30S ribosomal protein S6	89,018	91,756
GO448171	320	TC45796	$1x10^{-46}$	Belongs to the DEAD box helicase family	63,032	81,378
GO448466	390	TC88726	0	Putative glutamyl-tRNA synthetase	91,490	102,008
GO448415	615	TC95578	0	60S ribosomal protein L4/L1 (RPL4A)	123,118	121,318
GO448472	422	TC112765	0	40S ribosomal protein S16 y Sb36 hypothetical protein	89,326	101,557
				Metabolism; Amino Acid Metabolism		
GO448342	506	TC89772	0	ubiquitin-dependent protein catabolic process	87,865	78,399
GO448331	820	TC45081	$1x10^{-165}$	Similar to aspartyl protease family protein	106,649	68,634
GO448315	598	TC111710	0	Serine Hydroxymethyltransferase 4	129,656	95,942
GO448357	749	TC91872	$2x10^{-34}$	Similar to pepsin A activity	108,339	72,058
GO448338	808	TC86871	0	Similar to aspartyl protease family protein	111,243	111,088
GO448311	588	TC94215	0	Similar to AMP1 (Altered Meristem Program 1)	72,832	93,959
GO448308	465	DR465947	$2x10^{-39}$	Receptor Lectin Kinase	99,009	102,469
GO448279	669	TC96182	0	Putative DegP2 protease	92,746	124,825
GO448175	759	TC53750	$3x10^{-12}$	Belongs to the vitamin-B12 independent methionine synthase family	107,092	130,183
GO448393	609	TC82870	0	PRXR1 peroxidase	138,772	136,247

				Metabolism; Biosynthesis of Other Secondary Metabolites		
GO448105	679	TC109522	0	Caffeoyl-CoA O-methyltransferase (CCoAOMT)	162,560	101,800
				Metabolism; Carbohydrate Metabolism		
GO448345	624	TC50829	0	Similar to DNAJ heat shock family protein	90,797	100,669
GO448293	239	TC99823	$1x10^{-99}$	Dihydrolipoyl dehydrogenase y Ferric leghemoglobin reductase-2 precursor hits muy parecidos	74,499	131,621
GO448350	587	TC99394	0	Putative phytase	113,486	100,423
GO448296	590	TC63426	$1x10^{-108}$	Vicilin-like protein metabolism	141,663	143,608
GO448063	851	TC81691	0	Glucose-6-phosphate isomerase	77,649	105,396
GO448090	827	AW011453	$2x10^{-41}$	Glycosyl hydrolase family 17 protein	146,243	124,412
GO448053	133	Q45NN8	$3x10^{-15}$	ETHE1/GLX2-3/GLY3 (Glyoxalase 2-3)	70,066	82,536
GO448141	846	TC81691	0	Glucose-6-phosphate isomerase	55,406	77,328
GO448181	772	TC98466	0	Galactose-1-phosphate uridylyltransferase	87,749	94,760
GO448434	249	TC109639	$1x10^{-124}$	Phosphoglycerate mutase-like protein	89,849	88,000
GO448428	460	TC92228	0	RuBIsCO small subunit	144,271	149,684
GO448467	617	TC81818	0	ADP-glucose pyrophosphorylase large subunit (agpL3)	97,395	111,499
GO448407	472	TC86604	0	Belongs to the enoyl-CoA hydratase	142,770	123,603
GO448406	591	AM171731	$1,00E^{-69}$	Xyloglucan endotransglycosylase hydrolase 2	89,340	93,918
GO448455	648	TC86984	0	Arabinofuranosidase/xylosidase homolog (hydrolyzing O-glycosyl compounds)	106,401	115,131
GO448201	774	TC268643	$3,00E^{-12}$	Xylan 1,4-beta-xylosidase	108,308	98,159
GO448204	736	CO224045	e^{-100}	L-ascorbate oxidase	192,059	146,871
GO448206	619	TC91676	$8,00E^{-09}$	Dehydroascorbate reductase	180,935	96,254
GO448220	726	TC86984	0	LEXYL2 protein	112,377	122,614
GO448224	799	TC93186	$2,00E^{-07}$	kinase transferase (diphosphate-fructose-6-phosphate 1-phosphotransferase activity)	84,851	81,486
GO448197	374	TC88113	0	Alpha-galactosidase	72,318	119,514
				Metabolism; Energy Metabolism		
GO448144	807	TC86741	0	putative monooxygenase (MO2)	212,617	106,189
GO448151	834	TC76694	$1x10^{-102}$	Photosystem I reaction center subunit IV A, chloroplast precursor (PSI-E A)	106,010	83,062
GO448442	706	TC104195	$1,00E^{-23}$	PsbQ subunit of the oxygen evolving complex of photosystem II	86,176	81,438
GO448414	860	TC82426	0	ATP synthase subunit beta	135,031	84,858

GO448438	783	TC6000	$5{,}00E^{-23}$	FROSTBITE1 (NADH-ubiquinone oxidoreductase-related-like protein)	57,518	80,503
GO448385	396	TC98525	0	Chlorophyll A-B binding protein (CAB)	71,624	107,832
GO448214	286	TC81556	e^{-148}	oxygen evolving enhancer 3 (PsbQ) family protein energy	71,396	115,362
GO448056	720	TC109304	0	Proton-translocating inorganic pyrophosphatase	138,042	105,458
GO448172	776	BQ699593	$2x10^{-43}$	H+-pyrophosphatase	91,551	104,642
GO448158	763	TC62274	$1x10^{-160}$	Vacuolar proton-inorganic pyrophosphatase	72,345	105,488
				Metabolism; Lipid Metabolism		
GO448294	505	TC108403	0	Group3 late embryogenesis abundant protein	79,548	75,890
GO448263	484	TC97307	0	ACYL-COA-BINDING PROTEIN	112,168	74,420
GO448282	864	DV974041	0	3-KETOACYL-COA SYNTHASE 19	96,292	90,618
GO448347	815	TC97307	$3{,}00E^{-18}$	ACYL-COA-BINDING PROTEIN 6	90,442	91,033
GO448273	862	TC90825	$1{,}00E^{-35}$	GDSL-motif lipase/hydrolase family protein	77,929	104,450
GO448361	619	TC86471	0	Phosphoenolpyruvate carboxykinase	93,351	87,576
GO448292	581	TC86504	0	late embryogenesis abundant protein (EMB15)	114,398	80,895
GO448270	628	CO224077	$6{,}00E^{-44}$	1-acyl-sn-glycerol-3-phosphate acyltransferase	84,865	142,409
GO448094	520	DV974282	$2x10^{-70}$	GDSL-motif lipase	93,476	106,997
GO448147	631	TC83589	$5x10^{-07}$	Acyl-CoA-binding protein	74,240	67,203
GO448054	405	TC97307	0	Acyl-CoA-binding protein	90,251	67,132
GO448157	780	TC97307	$2x10^{-93}$	Acyl-CoA-binding protein	72,970	82,193
GO448163	749	TC97307	$1x10^{-108}$	Acyl-CoA-binding protein	218,301	120,189
GO448465	750	TC97307	$6{,}00E^{-47}$	ACYL-COA-BINDING PROTEIN 6	138,700	95,047
GO448433	778	TC97307	$2{,}00E^{-25}$	Acyl-CoA-binding protein	86,970	99,207
GO448411	750	TC97307	$1{,}00E^{-11}$	Acyl-CoA-binding protein	77,790	114,635
GO448462	270	TC84520	e^{-148}	hydrolase activity, hydrolyzing O-glycosyl compounds/T17H3.3	120,002	108,957
GO448456	699	TC97307	$9{,}00E^{-83}$	Acyl-CoA-binding protein	72,260	95,562
GO448255	484	TC97307	0	Acyl-CoA-binding protein	114,441	115,358
				Metabolism; Metabolism of Cofactors and Vitamins		
GO448217	447	TC85738	0	Putative PRLI-interacting factor L	104,405	111,893

				Metabolism; Nucleotide Metabolism		
GO448267	427	TC102993	0	METHIONINE GAMMA-LYASE	101,527	66,892
GO448095	739	TC55194	0	Putative inosine-uridine nucleoside N-ribohydrolase	72,343	85,001
GO448058	496	DR746302	0	DNA-directed RNA polymerase I, II, and III	79,651	72,229
GO448383	756	TC86984	0	LEXYL2 protein	85,571	88,100
				Metabolism; Xenobiotics Biodegradation and Metabolism		
GO448302	805	TC89504	$1{,}00E^{-88}$	dienelactone hydrolase family protein	73,539	88,229
				Organismal Systems; Environmental Adaptation		
GO448266	448	TC91330	0	pathogen responsive protein type 4-like gene	141,391	108,685
GO448324	736	TC91330	$6{,}00E^{-13}$	PR-4 type protein	114,080	103,768
GO448268	784	TC89631	0	pathogen responsive protein type 4-like gene	107,072	94,626
GO448333	806	TC103006	$1{,}00E^{-08}$	Pathogenesis-related protein 10-3.3	129,539	166,939
GO448335	708	TC89631	e^{-103}	pathogen responsive protein type 4	108,843	125,061
GO448354	835	TC109793	e^{-119}	pathogen responsive protein type 4-like gene	72,483	110,786
GO448080	599	TC100346	$2x10^{-12}$	Putative salt tolerance protein 5	126,078	100,198
GO448082	733	TC103006	0	putative intracellular pathogenesis related type 10 protein (pr-10 gene)	217,518	166,460
GO448072	769	TC87701	0	PR10 protein (PR10-1.13)	84,753	79,538
GO448070	375	DR095252	0	pathogenesis-related protein 10-2.2	87,760	102,222
GO448457	726	TC89631	$1{,}00E^{-48}$	PR-4 type protein	184,394	109,964
GO448381	375	DR095252	0	pathogenesis-related protein 10-2.2	127,055	117,494
GO448452	631	DR095252	e^{-123}	pathogenesis-related protein 10-2.2 mRNA	116,415	106,659
GO448394	545	TC89631	0	pathogen responsive protein type 4-like gene	95,855	107,179
GO448185	548	Q8H469	$4{,}00E^{-24}$	Putative stress-related protein	120,423	117,150
				Pine clone		
GO448303	795	TC101505	0	EST1149822 pinus library	103,708	106,546
GO448092	735	TC104063	$2x10^{-37}$	similar to clone pine RTK1_45_E04.b1_A029	79,802	121,129
				Transposon		
GO448318	825	CB670399	$4{,}00E^{-11}$	IS10 transposase	97,337	83,075
GO448322	821	TC388561	$3{,}00E^{-58}$	IS10-right transposase	72,270	144,554

GO448110	688	TC388561	0	IS10-right transposase	104,227	95,060
GO448437	837	Q0KII0	$7{,}00E^{-16}$	Transposase of Tn10	172,177	101,662
GO448464	693	TC388561	0	IS10-right transposase	96,835	110,666
GO448228	828	TC388561	0	IS10-right transposase	71,362	98,433
				unclassified proteins		
GO448264	295	TC89504	e^{-165}	similar to dienelactone hydrolase family protein	109,918	120,644
GO448295	509	TC96107	0	similar to oxidoreductase, 2OG-Fe(II) oxygenase family protein	93,003	85,888
GO448309	799	TC97328	$1{,}00E^{-79}$	similar to putative BRI1-KD interacting protein	78,377	94,592
GO448316	737	TC97328	0	Putative BRI1-KD interacting protein 118	87,159	131,759
GO448396	605	TC97052	0	transferase family protein	81,170	101,231
GO448395	354	TC112949	e^{-177}	Belongs to the ycf2 family	75,809	103,704
GO448390	596	TC86817	0	nucleic acid binding	140,854	123,066
GO448226	845	TC25382	$7{,}00E^{-07}$	Glycine-rich protein	101,286	100,936
				unknown		
GO448349	774	TC108403	0	TC108403/podría ser Group3 late embryogenesis abundant protein	155,741	89,486
GO448430	703	TC99011	$5{,}00E^{-35}$	unfolded protein binding	151,415	162,988

[a]Functional classification was based according to KEGG (Ruepp et al., 2004). [b] Clone reference in PRA library. [c]dbEST accession number. [d] Homology accession number according to public databases: (1) TGI, (2) Genbank.

Supplemental Table S4.3. Analysis of variance ANOVA for differentially expressed sequences of *Pinus radiata* cell culture. for 6 days with and without 0.5 µM 5-Aza-2-deoxycytidine

Variable	Factors	Df	Sum Sq	Mean Sq	F value	Pr(>F)
AMP1 (ALTERED MERISTEM PROGRAM 1)	Treat	1	6.695	6.695	6.074	0.0694
	Residuals	4	4.409	1.102		
25S rRNA gene	Treat	1	2544	2543.5	6.324	0.0657
	Residuals	4	1609	402.2		
Pathogen responsive protein type 4-like gene	Treat	1	22.01	22.007	7.165	0.0554
	Residuals	4	12.29	3.072		
Early response to dehydration 15-like protein	Treat	1	11.928	11.928	6.193	0.0676
	Residuals	4	7.704	1.926		
Calnexin-like protein	Treat	1	10.81	10.81	5.567	0.0777
	Residuals	4	7.768	1.942		
Putative glucose translocator	Treat	1	19.33	19.333	4.579	0.0991
	Residuals	4	16.89	4.222		
EMP24/GP25L family	Treat	1	3.209	3.209	26.29	0.00685
	Residuals	4	0.488	0.122		
Acyl-CoA-binding protein	Treat	1	20.363	20.363	28.46	0.00595
	Residuals	4	2.862	0.715		
PsbQ subunit of the oxygen evolving complex of photosystem II	Treat	1	19.66	19.665	9.2	0.0387
	Residuals	4	8.55	2.138		
Mitocondrial ATP synthase subunit beta	Treat	1	29	29	5.776	0.0741
	Residuals	4	20.08	5.02		
UBIQUITIN-CONJUGATING ENZYME 31	Treat	1	48.07	48.07	6.521	0.0631
	Residuals	4	29.49	7.37		
70kD heat shock protein	Treat	1	21.721	21.721	15.18	0.0176
	Residuals	4	5.723	1.431		
Photosystem II oxygen evolving enhancer 3 (PsbQ) family protein	Treat	1	16.477	16.477	9.223	0.0385
	Residuals	4	7.146	1.787		
Protein serine/threonine phosphatase activity	Treat	1	19.11	19.108	6.744	0.0602
	Residuals	4	11.33	2.833		

4.7. References

Alves MS, Fontes EPB, Fietto LG. 2011a. EARLY RESPONSIVE to DEHYDRATION 15, a new transcription factor that integrates stress signaling pathways. *plant signaling & behavior* **6**: 1993-1996.

Alves MS, Reis PA, Dadalto SP, Faria JA, Fontes EP, Fietto LG. 2011b. A novel transcription factor, ERD15 (Early Responsive to Dehydration 15), connects endoplasmic reticulum stress with an osmotic stress-induced cell death signal. *J Biol Chem* **286**: 20020-20030.

Aquea F, Poupin MJ, Matus JT, Gebauer M, Medina C, Arce-Johnson P. 2008. Synthetic seed production from somatic embryos of *Pinus radiata*. *Biotechnol Lett* **30**: 1847-1852.

Avivi Y, Morad V, Ben-Meir H, Zhao J, Kashkush K, Tzfira T, Citovsky V, Grafi G. 2004. Reorganization of specific chromosomal domains and activation of silent genes in plant cells acquiring pluripotentiality. *Dev Dyn* **230**: 12-22.

Azevedo H, Dias A, Tavares RM. 2008. Establishment and characterization of *Pinus pinaster* suspension cell cultures. *Plant Cell Tissue Organ Cult* **93**: 115-121.

Berdasco M, Alcázar R, García-Ortiz MV, Ballestar E, Fernández AF, Roldán-Arjona T, Tiburcio AF, Altabella T, Buisine N, Quesneville H. 2008. Promoter DNA hypermethylation and gene repression in undifferentiated Arabidopsis cells. *PLoS One* **3**: e3306.

Bricker TM, Frankel LK. 2011. Auxiliary functions of the PsbO, PsbP and PsbQ proteins of higher plant Photosystem II: a critical analysis. *Journal of Photochemistry and Photobiology B: Biology* **104**: 165-178.

Čihák A. 1974. Biological effects of 5-azacytidine in eukaryotes. *Oncology* **30**: 405-422.

Chan SWL, Henderson IR, Jacobsen SE. 2005. Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat Rev Genet* **6**: 351-360.

De-la-Pena C, Nic-Can G, Ojeda G, Herrera-Herrera JL, Lopez-Torres A, Wrobel K, Robert-Diaz ML. 2012. KNOX1 is expressed and epigenetically regulated during in vitro conditions in Agave spp. *BMC Plant Biol* **12**: 203.

Deng Z, Gmitter FG. 2003. Cloning and characterization of receptor kinase class disease resistance gene candidates in Citrus. *Theor Appl Genet* **108**: 53-61.

Desjardins Y, Dubuc JF, Badr A. 2009. In vitro culture of plants: a stressful activity! (ed. (ISHS)), pp. 29-50.

Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR. 2012. Widespread dynamic DNA methylation in response to biotic stress. *Proc Natl Acad Sci U S A* **109**: E2183-2191.

Du ZY, Chen MX, Chen QF, Xiao S, Chye ML. 2013. Overexpression of Arabidopsis acyl-CoA-binding protein ACBP2 enhances drought tolerance. *Plant Cell Environ* **36**: 300-314.

Fernandes H, Michalska K, Sikorski M, Jaskolski M. 2013. Structural and functional aspects of PR-10 proteins. *FEBS J* **280**: 1169-1199.

Ferreira HJ, Heyn H, Moutinho C, Esteller M. 2012. CpG island hypermethylation-associated silencing of small nucleolar RNAs in human cancer. *RNA biology* **9**: 881 - 890

Fraga HPF, Vieira LN, Caprestano CA, Steinmacher DA, Micke GA, Spudeit DA, Pescador R, Guerra MP. 2012. 5-Azacytidine combined with 2, 4-D improves somatic embryogenesis of Acca sellowiana (O. Berg) Burret by means of changes in global DNA methylation levels. *Plant Cell Rep*: 1327-1328.

Grandbastien M. 1998. Activation of plant retrotransposons under stress conditions. *Trends Plant Sci* **3**: 181-187.

Hasbún R, Valledor L, Rodríguez JL, Santamaría E, Ríos D, Sánchez M, Cañal MJ, Rodríguez R. 2008. HPCE quantification of 5-methyl-2´-deoxycytidine in genomic DNA: Methodological optimization for chestnut and other woody species. *Plant Physiol Biochem* **46**: 815-822.

Hudson K, Luo S, Hagemann N, Preuss D. 2011. Changes in global gene expression in response to chemical and genetic perturbation of chromatin structure. *PLoS One* **6**.

Jonckheere AI, Smeitink JA, Rodenburg RJ. 2012. Mitochondrial ATP synthase: architecture, function and pathology. *J Inherit Metab Dis* **35**: 211-225.

Koukalova B, Fojtova M, Lim KY, Fulnecek J, Leitch AR, Kovarik A. 2005. Dedifferentiation of tobacco cells is associated with ribosomal RNA gene hypomethylation, increased transcription, and chromatin alterations. *Plant Physiol* **139**: 275-286.

Krueger F, Kreck B, Franke A, Andrews SR. 2012. DNA methylome analysis using short bisulfite sequencing data. *Nat Meth* **9**: 145-151.

Lafon-Placette C, Faivre-Rampant P, Delaunay A, Street N, Brignolas F, Maury S. 2013. Methylome of DNase I sensitive chromatin in *Populus trichocarpa* shoot apical meristematic cells: a simplified approach revealing characteristics of gene-body DNA methylation in open chromatin state. *New Phytol* **197**: 416-430.

Law RD, Suttle JC. 2005. Chromatin remodeling in plant cell culture: patterns of DNA methylation and histone H3 and H4 acetylation vary during growth of asynchronous potato cell suspensions. *Plant Physiol Biochem* **43**: 527-534.

Li W, Liu H, Cheng ZJ, Su YH, Han HN, Zhang Y, Zhang XS. 2011. DNA methylation and histone modifications regulate de novo shoot regeneration in Arabidopsis by modulating WUSCHEL expression and auxin signaling. *PLoS Genetics* **7**.

Lim HW, Iwatani M, Hattori N, Tanaka S, Yagi S, Shiota K. 2009. Resistance to 5-aza-2'-deoxycytidine in Genic Regions Compared to Non-genic Repetitive Sequences. *J Reprod Dev* **56**: 86-93.

Liu JJ, Ekramoddoullah AKM. 2006. The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses. *Physiol Mol Plant Pathol* **68**: 3-13.

Lohe AR, Chaudhury A. 2002. Genetic and epigenetic processes in seed development. *Curr Opin Plant Biol* **5**: 19-25.

Ma K, Song Y, Jiang X, Zhang Z, Li B, Zhang D. 2012. Photosynthetic response to genome methylation affects the growth of Chinese white poplar. *Tree Genetics & Genomes* **8**: 1407-1421.

Meng HX, Hackett JA, Nestor C, Dunican DS, Madej M, Reddington JP, Pennings S, Harrison DJ, Meehan RR. 2011. Apoptosis and DNA Methylation. *Cancers* **3**: 1798-1820.

Miura K, Hasegawa PM. 2010. Sumoylation and other ubiquitin-like post-translational modifications in plants. *Trends Cell Biol* **20**: 223-232.

Murakami T, Li X, Gong J, Bhatia U, Traganos F, Darzynkiewicz Z. 1995. Induction of apoptosis by 5-azacytidine: drug concentration-dependent differences in cell cycle specificity. *Cancer Res* **55**: 3093-3098.

Neves N, Delgado M, Silva M, Caperta A, Morais-Cecilio L, Viegas W. 2005. Ribosomal DNA heterochromatin in plants. *Cytogenet Genome Res* **109**: 104-111.

Roberts D, Hoopes BC, McClure WR, Kleckner N. 1985. IS10 transposition is regulated by DNA adenine methylation. *Cell* **43**: 117-130.

Roose JL, Wegener KM, Pakrasi HB. 2007. The extrinsic proteins of Photosystem II. *Photosynth Res* **92**: 369-387.

Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, Van Den Hoff MJB, Moorman AFM. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* **37**: e45.

Santos AP, Ferreira L, Maroco J, Oliveira MM. 2011. Abiotic stress and induced DNA hypomethylation cause interphase chromatin structural changes in rice rDNA loci. *Cytogenet Genome Res* **132**: 297-303.

Sels J, Mathys J, De Coninck B, Cammue B, De Bolle M. 2008. Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiol Biochem* **46**: 941–950.

Stulemeijer IJE, Joosten MHAJ. 2008. Post translational modification of host proteins in pathogen triggered defence signalling in plants. *Mol Plant Pathol* **9**: 545-560.

Su J, Yan H, Wei Y, Liu H, Liu H, Wang F, Lv J, Wu Q, Zhang Y. 2013. CpG_MPs: identification of CpG methylation patterns of genomic regions from high-throughput bisulfite sequencing data. *Nucleic Acids Res* **41**: e4.

Tokuji Y, Takano S, Tonomura M, Tanaka S, Igari K, Watanabe T. 2011. Influence of 5′-azacitidine on promoting recovery of cell competence for shoot organogenesis in Arabidopsis. *Plant Cell, Tissue and Organ Culture* **106**: 289-297.

Trujillo M, Shirasu K. 2010. Ubiquitination in plant immunity. *Curr Opin Plant Biol* **13**: 402-408.

Tuteja N. 2009. Integrated Calcium Signaling in Plants. in *Signaling in Plants* (ed. NM Springer), pp. 29-49.

Vining KJ, Pomraning KR, Wilhelm LJ, Priest HD, Pellegrini M, Mockler TC, Freitag M, Strauss SH. 2012. Dynamic DNA cytosine methylation in the Populus trichocarpa genome: tissue-level variation and relationship to gene expression. *BMC Genomics* **13**: 27.

Vuosku J, Suorsa M, Ruottinen M, Sutela S, Muilu-Mäkelä R, Julkunen-Tiitto R, Sarjala T, Neubauer P, Häggman H. 2012. Polyamine metabolism during exponential growth transition in Scots pine embryogenic cell culture. *Tree Physiol* **32**: 1274-1287.

Wang YS, Pi LY, Chen X, Chakrabarty PK, Jiang J, De Leon AL, Liu GZ, Li L, Benny U, Oard J. 2006. Rice XA21 binding protein 3 is a ubiquitin ligase required for full Xa21-mediated disease resistance. *The Plant Cell Online* **18**: 3635–3646.

Williams DB. 2006. Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. *J Cell Sci* **119**: 615-623.

Xiao S, Chye ML. 2011. New roles for acyl-CoA-binding proteins (ACBPs) in plant development, stress responses and lipid metabolism. *Prog Lipid Res* **50**: 141-151.

Xu WH, Wang YS, Liu GZ, Chen X, Tinjuangjun P, Pi LY, Song WY. 2006. The autophosphorylated Ser^{686}, Thr^{688}, and Ser^{689} residues in the intracellular juxtamembrane domain of XA21 are implicated in stability control of rice receptor like kinase. *Plant J* **45**: 740-751.

Zhao XY, Su YH, Cheng ZJ, Zhang XS. 2008. Cell fate switch during *in vitro* plant organogenesis. *J Integr Plant Biol* **50**: 816-824.

5. General discussion

Methylation in *in vitro* culture has been analysed before in forest species, and it has been reported that has a direct relationship with juvenility and phase change affecting different tissues regarding type of explant and plant age (Fraga et al. 2002a; Fraga et al. 2002b; Read and Bavougian 2012). In this research, adventitious organogenesis induced by the cytokinin benzyl adenine BA in zygotic embryos of *Pinus radiata* was evaluated from an epigenetic point of view. A general overview of the role of DNA methylation and histone H4 acetylation during this process was compared with other developmental situations like adventitious rooting by the action of auxins indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic_acid (2,4-D), and germination arrest provoked by abscisic acid (ABA). Several genes involved in the organogenic process were identified by subtractive suppressive hybridization, and their biological function and expression were analysed by dot blot array hybridization. Furthermore, a regulation of the identified genes by DNA methylation was assessed in cell cultures in presence of the demethylating agent 5-Aza-2-deoxycytidine by methylated DNA immunoprecipitation (MeDIP) and new candidate genes were identified with the hybridization of the array in the same experimental situation.

Mature zygotic embryos showed the highest methylation level, being in agreement with previous reports in dormant buds (Hasbún et al. 2007; Santamaría et al. 2009). The specific pattern of demethylation followed by embryos cultured in auxins and BA differed from the distribution in control embryos, probably due to the differential developmental pathway that take place during adventitious organogenesis according to Christianson and Warnick (1985) These suggested stages involve the establishment of new gene expression patterns that showed a similar demethylation dynamic, although adventitious shoot induction occurred in a direct way with an

inhibition of elongation of embryos, whereas auxin root induction happened through a callus stage, indicating the activation of different gene pathways (Overvoorde et al. 2010). Embryos cultured in ABA also followed a differential demethylation pattern regarding to control, triggering a stress response that is known to provoke a DNA demethylation (Kim et al. 2012). The pattern of acetylated histone H4 followed an opposite tendency than DNA methylation, confirming the role these epigenetic marks have in gene regulation (Meijón et al. 2010; Valledor et al. 2010).

Global methylation and H4 acetylation levels along the embryo were also studied, and the results showed a differential pattern of acetylation-methylation in embryos cultured with the different growth regulators. In ornamental *Azalea japonica*, a specific cell and tissue pattern was detected during floral bud induction, transition and vegetative development (Meijón et al. 2009). As expected by macromorphological aspect of embryos and in spite of the fact that auxins and BA had a similar global methylation results, the distribution of both epigenetic marks in the tissue was different. The epigenetic regulation of embryos cultured with auxins is mainly focused in the shoot and root apical meristems (SAM and RAM), regions whose cells are highly methylated in normal development that are sensitive to be regulated by these growth regulators (Vernoux and Benfey 2005; Willemse et al. 2008). Aberrant patterns in the studied epigenetic marks were also identified according to callus formation that it is not limited to both apical meristems. In BA embryos, there was an even methylation in the whole embryo that could be related to growth inhibition, a situation that takes place during bud induction by BA in *P. radiata* mature tissues (Zhang et al. 2010). On the other hand, after 10 days of culture cotyledons changed the distribution of the signals, and methylation decreased meanwhile acetylation showed up especially in new created buds, given that in these regions an active growth and a high metabolism rate take place (Zhang and Lemaux 2004). RAM was also demethylated in embryos cultured in ABA

after 3 days of culture, and according to Chinnusamy et al. (2008) a delay in embryo germination and changes in DNA methylation and H4 acetylation affecting the apical meristem could be happening.

The identification of candidate genes related to the adventitious shoot induction and an analysis of their expression and biological function, could help us to identify the differential pathways involved. In this sense, the hybridization of a dot blot array with 191 genes from the 426 sequences identified and a later on statistical analysis, let us observe an upregulation of metabolic biological functions as previously reported by Che et al. (2002); (2006). Several genes related to metabolism were connected to this experimental situation, having RuBIsCO, non-specific lipid-transfer protein precursor (LTP1) and Alpha-tubulin 1. These genes have already been identified as differentially expressed under cytokinin treatment in Arabidopsis during the end of the acquisition of competence stage and during light/dark experiments (Che et al. 2002; Lochmanova et al. 2008). We have to highlight that these results pointed to photosynthesis activation, although there was an absence of light in our experimental conditions. In this regard, a cytokinin-induced photomorphogenesis and chloroplast biogenesis has been reported by Lochmanova et al. (2008), and cytokinins can also improve a recovery of photosynthesis after a stress situation (Hu et al. 2013).

Cytokinins not only promote photosynthesis after a stress situation but have a relevant role in stress response delaying senescence (Hu et al. 2013; Zwack et al. 2013). As a consequence, several genes related to stress like PR-10, protein kinase superfamily, XB3, CRK1, 25s rRNA and IS10-right transposase, have been found to be upregulated in embryos cultured in ABA, but they were downregulated in BA agreeing with this mitigation of stress by cytokinins (Hwang et al. 2012; Vining et al. 2012; Giron et al. 2013).

Global DNA methylation and the identification and biological function analysis of genes involved in adventitious organogenesis, led us to design an experimental system in which we could easily identify new candidate genes related to dedifferentiation/redifferentiation stages, and find out a regulation by DNA methylation of those genes involved in adventitious organogenesis. A chemical unmasking by 5-Aza-2-deoxycytidine in undifferentiated cell cultures was the best option according to previous results in Arabidopsis (Berdasco et al. 2008; Hudson et al. 2011). The hybridization of the dot blot array in this situation and MeDIP gave us as result a differential upregulation of 25S rRNA and ERD15. Both sequences are related to stress situations and their functions has been proposed to interconnect hormone signalling to external stimulus, and cell stress and undifferentiation (Koukalova et al. 2005; Alves et al. 2011a; Alves et al. 2011b). The non significant methylation of 25S rRNA according to MeDIP results could be related to its expression upregulation because from the hundreds of copies in the genome, it has been reported that each of them is differentially regulated by methylation (Chan et al. 2005; Neves et al. 2005), so it must be taken into account that small variations in DNA methylation in this sequence , even if there is no significant difference, could imply big changes in gene expression.

The differential methylation obtained by MeDIP also revealed a transposon and several genes related to stress like LTP-1, PR-10, XB3. These results provided us the first reports of DNA methylation in these sequences associated to stress induced by *in vitro* culture and drug application, corroborating the direct relationship between several stress situations and a regulation by DNA methylation as stress response (Cassells and Curry 2001; Boyko and Kovalchuk 2008).

Two main groups of genes were upregulated in control cells. One of them was related to stress, and more specifically to stress response in endoplasmic reticulum. Their upregulation could be induced by *in vitro* culture itself (Smulders and de Klerk

2011; Deng et al. 2013) before the experiment with 5-Aza-2-deoxycytidine, and differential methylation of genes identified by MeDIP in control could suggest a differential control of genes involved in specific stress responses (Dowen et al. 2012). The upregulation of ERD15 in Aza-2-deoxycytidine could explain parts of these differential response, so it has a relationship with the stress caused in the endoplasmic reticulum related genes, downregulating their expression and inducing the expression of other genes involved in specific stress pathways (Alves et al. 2011b). The second group is based in genes related to photosynthesis and electron transport, maybe because of the effect of 5-Aza-2-deoxycytidine with a possible role in nuclear genes of the chloroplast, as has been reported by Hudson et al. (2011). Finally, it has special importance meristem development and pattern formation in cell reprogramming processes, and *ALTERED MERISTEM PROGRAM 1* regulates embryo formation in Arabidopsis (Vidaurre et al. 2007). Berdasco et al. (2008) identified several genes involved in keeping an undifferentiated state in Arabidopsis cell cultures, and the regulation of these kind of genes between them (Lohe and Chaudhury 2002), could indicate a demethylation in genes with a repressive effect in *AMP1*.

5.1. References

Alves MS, Fontes EPB, Fietto LG. 2011a. EARLY RESPONSIVE to DEHYDRATION 15, a new transcription factor that integrates stress signaling pathways. *plant signaling & behavior* **6**: 1993-1996.

Alves MS, Reis PA, Dadalto SP, Faria JA, Fontes EP, Fietto LG. 2011b. A novel transcription factor, ERD15 (Early Responsive to Dehydration 15), connects endoplasmic reticulum stress with an osmotic stress-induced cell death signal. *J Biol Chem* **286**: 20020-20030.

Berdasco M, Alcázar R, García-Ortiz MV, Ballestar E, Fernández AF, Roldán-Arjona T, Tiburcio AF, Altabella T, Buisine N, Quesneville H. 2008. Promoter DNA hypermethylation and gene repression in undifferentiated Arabidopsis cells. *PLoS One* **3**: e3306.

Boyko A, Kovalchuk I. 2008. Epigenetic control of plant stress response. *Environ Mol Mutagen* **49**: 61-72.

Cassells A, Curry R. 2001. Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. *Plant Cell Tissue Organ Cult* **64**: 145-157.

Chan SWL, Henderson IR, Jacobsen SE. 2005. Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nat Rev Genet* **6**: 351-360.

Che P, Gingerich DJ, Lall S, Howell SH. 2002. Global and Hormone-Induced Gene Expression Changes during Shoot Development in Arabidopsis. *Plant Cell* **14**: 2771-2785.

Che P, Lall S, Nettleton D, Howell SH. 2006. Gene Expression Programs during Shoot, Root, and Callus Development in Arabidopsis Tissue Culture. *Plant Physiol* **141**: 620-637.

Chinnusamy V, Gong Z, Zhu JK. 2008. Abscisic Acid mediated Epigenetic Processes in Plant Development and Stress Responses. *J Integr Plant Biol* **50**: 1187-1195.

Christianson ML, Warnick DA. 1985. Temporal requirement for phytohormone balance in the control of organogenesis *in vitro*. *Dev Biol* **112**: 494-497.

Deng Y, Srivastava R, Howell S. 2013. Endoplasmic Reticulum (ER) Stress Response and Its Physiological Roles in Plants. *International Journal of Molecular Sciences* **14**: 8188-8212.

Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR. 2012. Widespread dynamic DNA methylation in response to biotic stress. *Proc Natl Acad Sci U S A* **109**: E2183-2191.

Fraga MF, Cañal MJ, Rodríguez R. 2002a. *In vitro* morphogenic potential of differently aged *Pinus radiata* trees correlates with polyamines and DNA methylation levels. *Plant Cell Tissue Organ Cult* **70**: 139-145.

Fraga MF, Rodríguez R, Cañal MJ. 2002b. Genomic DNA methylation-demethylation during ageing-reinvigoration of *Pinus radiata*. *Tree Physiol* **22**: 813-816.

Giron D, Frago E, Glevarec G, Pieterse CMJ, Dicke M, Biere A. 2013. Cytokinins as key regulators in plant-microbe-insect interactions: connecting plant growth and defence. *Funct Ecol*: n/a-n/a.

Hasbún R, Valledor L, Berdasco M, Santamaría E, Cañal MJ, Rodríguez R. 2007. Dynamics of DNA methylation during chestnut trees development, Application to breeding programs. *Acta horticulturae* **760**: 563–566.

Hu L, Wang Z, Huang B. 2013. Effects of Cytokinin and Potassium on Stomatal and Photosynthetic Recovery of Kentucky Bluegrass from Drought Stress. *Crop Sci* **53**: 221-231.

Hudson K, Luo S, Hagemann N, Preuss D. 2011. Changes in global gene expression in response to chemical and genetic perturbation of chromatin structure. *PLoS One* **6**.

Hwang I, Sheen J, Müller B. 2012. Cytokinin signaling networks. *Annu Rev Plant Biol* **63**: 353-380.

Kim JM, To TK, Seki M. 2012. An epigenetic integrator: new insights into genome regulation, environmental stress responses and developmental controls by histone deacetylase 6. *Plant Cell Physiol* **53**: 794-800.

Koukalova B, Fojtova M, Lim KY, Fulnecek J, Leitch AR, Kovarik A. 2005. Dedifferentiation of tobacco cells is associated with ribosomal RNA gene hypomethylation, increased transcription, and chromatin alterations. *Plant Physiol* **139**: 275-286.

Lochmanova G, Zdrahal Z, Konecna H, Koukalova S, Malbeck J, Soucek P, Valkova M, Kiran NS, Brzobohaty B. 2008. Cytokinin-induced photomorphogenesis in dark-grown Arabidopsis: a proteomic analysis. *J Exp Bot* **59**: 3705–3719.

Lohe AR, Chaudhury A. 2002. Genetic and epigenetic processes in seed development. *Curr Opin Plant Biol* **5**: 19-25.

Meijón M, Feito I, Valledor L, Rodriguez R, Canal MJ. 2010. Dynamics of DNA methylation and Histone H 4 acetylation during floral bud differentiation in azalea. *BMC Plant Biol* **10**: 10.

Meijón M, Valledor L, Santamaría E, Testillano PS, Risueño MC, Rodríguez R, Feito I, Cañal MJ. 2009. Epigenetic characterization of the vegetative and floral stages of azalea buds: Dynamics of DNA methylation and histone H4 acetylation. *J Plant Physiol* **166**: 1624-1636.

Neves N, Delgado M, Silva M, Caperta A, Morais-Cecilio L, Viegas W. 2005. Ribosomal DNA heterochromatin in plants. *Cytogenet Genome Res* **109**: 104-111.

Overvoorde P, Fukaki H, Beeckman T. 2010. Auxin control of root development. *Cold Spring Harbor Perspectives in Biology* **2**.

Read PE, Bavougian CM. 2012. In vitro rejuvenation of woody species. *Methods in molecular biology (Clifton, NJ)* **11013**: 383-395.

Santamaría ME, Hasbún R, Valera MJ, Meijón M, Valledor L, Rodríguez JL, Toorop PE, Cañal MJ, Rodríguez R. 2009. Acetylated H4 histone and genomic DNA methylation patterns during bud set and bud burst in *Castanea sativa*. *J Plant Physiol* **166**: 1360-1369.

Smulders MJM, de Klerk GJ. 2011. Epigenetics in plant tissue culture. *Plant Growth Regul* **63**: 137-146.

Valledor L, Meijón M, Hasbún R, Jesús Cañal M, Rodríguez R. 2010. Variations in DNA methylation, acetylated histone H4, and methylated histone H3 during *Pinus radiata* needle maturation in relation to the loss of *in vitro* organogenic capability. *J Plant Physiol* **167**: 351-357.

Vernoux T, Benfey PN. 2005. Signals that regulate stem cell activity during plant development. *Curr Opin Genet Dev* **15**: 388-394.

Vidaurre DP, Ploense S, Krogan NT, Berleth T. 2007. AMP1 and MP antagonistically regulate embryo and meristem development in Arabidopsis. *Development* **134**: 2561-2567.

Vining KJ, Pomraning KR, Wilhelm LJ, Priest HD, Pellegrini M, Mockler TC, Freitag M, Strauss SH. 2012. Dynamic DNA cytosine methylation in the Populus trichocarpa genome: tissue-level variation and relationship to gene expression. *BMC Genomics* **13**: 27.

Willemse J, Kulikova O, de Jong H, Bisseling T. 2008. A new whole-mount DNA quantification method and the analysis of nuclear DNA content in the stem-cell niche of Arabidopsis roots. *Plant J* **55**: 886-894.

Zhang H, Horgan KJ, Reynolds PHS, Jameson PE. 2010. 6-Benzyladenine metabolism during reinvigoration of mature *Pinus radiata* buds *in vitro*. *Tree Physiol* **30**: 514-526.

Zhang S, Lemaux P. 2004. Molecular Analysis of *in vitro* Shoot Organogenesis. *Crit Rev Plant Sci* **23**: 325-335.

Zwack PJ, Robinson BR, Risley MG, Rashotte AM. 2013. Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses. *Plant Cell Physiol*.

6. Conclusions

1. The different responses induced by plant growth regulators BA, IAA, ABA y 2, 4-D showed a differential and opposed dynamic of DNA methylation and H4 histone actylation in zygotic embryos of *Pinus radiata*.
2. Adventitious caulogenesis is characterized by an even distribution of methylation signal decreasing at the same time acetylated H4 increased its signal, especially y new formed buds.
3. Auxins IBA and 2, 4-D, were the plant growth regulators with a biggest effect in DNA Methylation and H4 histone acetylation levels. Shoot and root meristems undergone drastic changes in both epigenetic marks after six days associated to undifferentiated cell formation.
4. Caulogenic process requires the activation of metabolism and morphogenic competence acquisition with a differential expression of genes associated to these functions like RuBIsCO and LTP1.
5. Induction of adventitious caulogenesis by BA reasserted the role of cytokinins against stress repressing expression of genes induced by ABA and by *in vitro* culture in control situation.
6. Demethylant agent Aza-2-deoxycytidine at a concentration of 0.5 µM provoked differences in global methylation of somatic cells of *Pinus radiata* after six days, inducing significant changes in expression of cellular stress, morphogenesis and photosynthesis related genes.
7. Methylated DNA Immuno Precipitation (MeDIP) confirmed the importance of DNA methylation in regulation of gene expression pointing out a regulation by methylation of new genes involved in adventitious organogenesis and response to stress unmasked by the treatment with Aza-2-deoxycytidine.

8. Induction of expression of 25S rRNA caused by the demethylating drug and the differential regulation by DNA methylation of the different copies present in the genome, make of this sequence a relevant candidate to study during adventitious organogenesis.

7. Resumen

Las coníferas representan más del 50 % de la masa forestal del planeta y en particular en España, el 94,4 % de este área pertenece al género *Pinus* teniendo a *Pinus radiata* D. Don como la tercera especie más importante. Debido a que los bosques juegan un papel crucial en el cambio climático, han sido incluidos en el protocolo de Kyoto por su capacidad de acumulación de CO_2, considerada incluso mayor que la de la atmósfera. Para incrementar la producción, la multiplicación de genotipos élite a través de la caulogénesis adventicia *in vitro* a partir de embriones zigóticos es la técnica más eficaz. Estas técnicas se consideran una posible fuente de variación epigenética que provocan la activación de transposones y modificaciones de genes específicos. La epigenética puede ser entendida como un sistema para regular selectivamente la activación o inactivación de la expresión génica y a nivel molecular la metilación del ADN y las modificaciones post-traduccionales de histonas entre otros factores, cooperan en su conjunto.

Teniendo en cuenta estos antecedentes, en este estudio se aborda la regulación epigenética de la organogénesis adventicia inducida por la citoquinina bencil adenina (BA) en embriones zigóticos de *Pinus radiata*. El papel de la metilación del ADN y la acetilación de la histona H4 como reguladores de la organogénesis adventicia se comparó con otros programas de desarrollo como la rizogénesis adventicia inducida por la acción de la auxina ácido indol-3-butírico (IBA), la formación de tejido indiferenciado (callo) inducida por el ácido 2,4-diclorofenoxiacético (2,4-D) y la inhibición de la germinación por el ácido abscísico (ABA). Mediante una hibridación sustractiva supresora se identificaron diferentes genes relacionados con la caulogénesis adventicia, y se analizó su función biológica y su expresión mediante la hibridación de un dot blot. También se constató mediante una inmunoprecipitación de ADN metilado (MeDIP) una regulación mediante metilación del ADN de los genes identificados

asociados a calulogénesis y los inducidos en cultivos celulares en presencia del agente demetilante 5-Aza-2-deoxicitidina. Asimismo se identificaron nuevos genes candidatos mediante la hibridación de un dot blot en esta misma situación experimental.

Para abordar el estudio de la dinámica global y espacio-temporal de la metilación del ADN en la caulogénesis adventicia y otras respuestas organogénicas que implican reprogramación celular, se evaluaron los niveles de 5-mC global y su distribución espacio temporal mediante inmunolocalización. El mayor grado de metilación se observó en los embriones zigóticos maduros en estado quiescente coincidiendo con resultados previos en órganos en dormición. En los procesos organogénicos, incluida la germinación como situación control, se observó una dinámica diferencial en patrones específicos de metilación o demetilación. En los embriones cultivados con auxinas y BA el patrón difiere del que siguen los embriones control a partir del sexto día de cultivo, ya que se produce un descenso debido, probablemente, a un desarrollo diferencial con cambios de expresión génica que acontecen durante la organogénesis adventicia. Los embriones cultivados en ABA también siguieron un patrón diferencial en relación con el control, con una demetilación posiblemente debida al desencadenamiento de una respuesta de estrés. El patrón de la histona H4 acetilada siguió una tendencia opuesta al de la metilación del ADN, confirmando el papel antagonista y la interconexión de estas marcas epigenéticas en la regulación génica.

Como era de esperar, de acuerdo con su aspecto morfológico y a pesar de que las auxinas y el BA tenían un valor de metilación global similar, la distribución de ambas marcas epigenéticas en el tejido fue diferente. En los embriones cultivados en BA hay una metilación uniforme en todo el embrión que puede estar relacionada con una inhibición del crecimiento. Durante la caulogénesis la metilación disminuye, incrementándose paralelamente la acetilación de H4, especialmente en las nuevas

yemas en formación, zonas de crecimiento muy activo. En los embriones cultivados con auxinas se observan cambios muy drásticos en las marcas epigenéticas de los meristemos radicular y caulinar, regiones muy sensibles a la señalización por reguladores del crecimiento. En el meristemo radicular de los embriones cultivados en ABA también se produce una demetilación a los tres días, debido probablemente al efecto inductor del desencadenamiento de la respuesta al estrés de esta hormona.

El análisis de expresión diferencial e identificación de genes candidatos implicados en la organogénesis adventicia, fue abordado mediante una hibridación supresora sustractiva y la hibridación de un dot blot con 191 genes seleccionados de un total de 426 secuencias identificadas. Mediante un análisis estadístico de los resultados se constató una inducción de las funciones relacionadas con el metabolismo, destacando el incremento en la expresión de algunos genes relacionados con estas funciones como: *RuBIsCO*, *PRECURSOR PROTEICO DE TRANSPORTADOR NO ESPECÍFICO DE LÍPIDOS 1* (LTP1) y *α-tubulina 1*. Estos genes han sido identificados como diferenciales en respuesta a BA durante la fase de adquisición de competencia y fotomorfogénesis inducida por este regulador del crecimiento.

Las citoquininas no sólo mejoran la fotosíntesis después de un periodo de estrés, sino que tienen también un papel importante en la transmisión de señal en mecanismos de defensa y senescencia asociados al estrés. Como consecuencia, varios genes relacionados con estrés como son: *RELACIONADO CON PATOGÉNESIS 10 (PR-10)*, un gen perteneciente a la superfamilia de las proteina quinasas, *PROTEÍNA 3 DE UNIÓN A Xa21 (XB3)*, *QUINASA REGULADA POR CITOQUININAS 1 (CRK1)*, *25s ARNr* y *transposasa IS10-right*, que muestran un incremento en su expresión en ABA, están reprimidos en BA de acuerdo con el papel en la mitigación del estrés que tienen las citoquininas.

La metilación global y la identificación y el análisis de la función biológica de los genes relacionados con la organogénesis adventicia, nos condujeron a investigar la regulación epigenética mediante metilación en el ADN de genes candidatos, y el desenmascaramiento químico de otros posibles genes candidatos. Para ello se planteó un diseño experimental mediante el cual se pudieran identificar nuevos genes candidatos relacionados con procesos de desdiferenciación/rediferenciación de una forma fácil. Un desenmascaramiento químico mediante un tratamiento con 5-Aza-2-deoxicitidina en cultivos celulares indiferenciados fue considerada la mejor opción según los resultados previos obtenidos por otros grupos de investigación en *Arabidopsis thaliana*.

La hibridación de un dot blot en esta situación y una inmunoprecipitación del ADN metilado nos proporcionaron unos resultados diferenciales en la inducción de 25S ARNr y *RÁPIDA RESPUESTA A DESHIDRATACIÓN 15 (ERD15)* frente al tratamiento con el agente demetilante. Ambas secuencias, relacionadas con estrés y con la transducción de señal hormonal frente a estímulos externos como funciones principales, resultan inducidas tras el tratamiento con la droga. La metilación no significativa de 25S rRNA, de acuerdo a los datos proporcionados por el MeDIP, puede ser relacionada con una inducción de su expresión, ya que existen cientos de copias en el genoma que pueden tener una regulación diferencial mediante metilación, por lo que pequeños cambios de metilación pueden implicar grandes cambios de expresión génica. El enriquecimiento diferencial de las secuencias metiladas obtenido a través de MeDIP nos permitió también identificar otras secuencias, como un transposón y varios genes relacionados con estrés como LTP-1, PR-10 y XB3. Estos resultados suponen la primera reseña de la metilación en estos genes asociados a estrés inducido por el cultivo *in vitro* y la aplicación de la droga demetilante, corroborando la relación directa entre

las diferentes situaciones de estrés y una regulación mediante la metilación del ADN como respuesta a estrés.

En los cultivos celulares utilizados como control se identificaron dos grupos principales de genes de acuerdo a su función biológica y un gen relacionado con desarrollo del meristemo y patrones de formación de órganos como es *PROGRAMA DEL MERISTEMO ALTERADO 1 (AMP1)* que regula la formación del embrión en *Arabidopsis*. El primero de los grupos está relacionado con estrés, y más concretamente con estrés en el retículo endoplasmático, cuya represión podría tener su significado con la inducción de ERD15 en el tratamiento con 5-Aza-2-deoxicitidina mitigando esta situación de estrés. El segundo grupo se compone de genes relacionados con fotosíntesis y transporte de electrones los cuales son reprimidos por 5-Aza-2-deoxicitidina probablemente debido a su posible acción en los genes nucleares del cloroplasto.

8. Conclusiones

1. Las diferentes respuestas inducidas por los reguladores del crecimiento BA, IAA, ABA y 2, 4-D mostraron una dinámica diferencial y opuesta de la metilación del ADN y la acetilación de la histona H4 en embriones zigóticos de *Pinus radiata*.
2. La caulogénesis adventicia se caracteriza por una distribución uniforme de la metilación que disminuye en los cotiledones incrementándose paralelamente la acetilación de H4 especialmente en nuevas yemas en formación.
3. Las auxinas, IBA y 2, 4-D, fueron los reguladores del crecimiento con mayor efecto tanto en los niveles de metilación del ADN como en la acetilación de la histona H4. Los meristemos caulinar y radicular sufrieron cambios drásticos en ambas marcas epigenéticas a los seis días asociados a la formación de masas celulares indiferenciadas.
4. El proceso caulogénico requiere la activación del metabolismo y de la adquisición de competencia reflejado en una expresión diferencial de genes asociados a estas funciones como son RuBIsCO y LTP1.
5. El efecto de BA en la inducción de la caulogénesis adventicia confirmó el papel atenuante del estrés de las citoquininas reprimiendo la expresión de genes inducidos con ABA y de genes relacionados con estrés inducidos por el cultivo *in vitro* en la situación control.
6. El uso de 5-Aza-2-deoxicitidina a una concentración 0.5 µM provocó diferencias en la metilación global de células somáticas de *Pinus radiata* a los seis días de cultivo, induciendo cambios significativos en genes relacionados con estrés celular, morfogénesis y fotosíntesis.
7. El uso de la inmunoprecipitación de ADN metilado (MeDIP) ratificó la importancia de la metilación del ADN en la regulación de expresión génica, indicando una regulación por metilación de algunos genes candidatos y de nuevos genes asociados

a la organogénesis adventicia y a respuesta a estrés desenmascarados por el tratamiento con 5-Aza-2-deoxicitidina.

8. En base a la inducción de la expresión de 25S rARN provocada por la droga demetilante y la regulación diferencial de las múltiples copias presentes en el genoma por metilación, se considera una secuencia candidata relevante para el estudio de su regulación por metilación en procesos de organogénesis adventicia.