INTERNATIONAL
CAMPUS OF
EXCELLENCE

UNIVERSIDAD POLITÉCNICA DE MADRID

**Escuela Técnica Superior de Ingeniería Agronómica,
Alimentaria y de Biosistemas**

***“Functional characterization of DEMETER-like DNA
demethylases during growth-dormancy cycles in poplar”***

Tesis Doctoral

Daniel Conde Rodríguez

Ingeniero de Montes

2017

Departamento de Biotecnología - Biología Vegetal
Escuela Técnica Superior de Ingeniería Agronómica,
Alimentaria y de Biosistemas
Universidad Politécnica de Madrid

Tesis Doctoral:

“Functional characterization of DEMETER-like DNA demethylases during growth-dormancy cycles in poplar”

Autor:

Daniel Conde Rodríguez

Ingeniero de Montes

Directores:

Isabel Allona Alberich (Doctora Ingeniera de Montes)

Pablo González-Melendi (Doctor Licenciado en Ciencias Biológicas)

Madrid, 2017

AGRADECIMIENTOS

Aunque creo que casi siempre es verdad eso de que todo acaba llegando, nunca estuve totalmente seguro de que llegaría el momento de escribir esta parte, así que no me queda otra (y además me apetece) que gritar “hallelujah”. Pero como dice la canción *“it's a cold and it's a broken Hallelujah”*.

Por encima de todo quiero dar las gracias a mi directora Isabel. Decidiste arriesgar y darme la oportunidad de realizar la tesis en tu laboratorio, a pesar de mis pocos conocimientos en biología molecular y celular. No tenías la obligación, y sin embargo siempre buscaste y encontraste la manera de que yo pudiera seguir adelante, lo que ha supuesto serias preocupaciones en más de un momento. He intentado corresponder a ese compromiso y apoyo con esfuerzo y dedicación. Gracias a Pablo. No se puede tener mejor codirector de tesis. Desde el principio has puesto a mi disposición eficaces protocolos de biología celular diseñados por ti, que se convertían en provechosos experimentos. Además durante estos años has ido convirtiendo los churros ilegibles que te he mandado en elegantes textos científicos. Ha sido un privilegio aprender de ti.

Gracias al Che Perales. Llegaste al laboratorio y me salvaste. Me salvaste literalmente cuando accediste a gastarte la plata en contratar a este que escribe sin conocerlo. No puedo expresar la gratitud que siento por haber podido trabajar con semejante talento para la ciencia. Ojalá se me haya pegado algo de ti. Gracias a Alicia, contigo empezó todo. Me has enseñado bien cómo hay que trabajar en un laboratorio. Sin tu ayuda no hubiera ni siquiera arrancado la tesis. Gracias a Jose, que durante estos años has sido para mí muchas cosas; un compañero, al que le ha faltado tiempo para ayudarme siempre que lo he necesitado; una motivación, por tus ganas de aprender, tu energía y tu inteligencia. Es un privilegio haber tenido un ejemplo de cómo hay que ser en el oficio tan cerca; y un amigo, con el que hablar y cantar. Gracias a Paolo, desde que llegaste al laboratorio los días han sido más felices, dentro y fuera de él, ojalá coincidamos en otra etapa más adelante. Gracias a Tamara, todo un ejemplo de trabajar mucho y muy bien. Gracias a Judith, siempre me ayudaste cuando te lo pedí. Gracias a Marco, da gusto tener a buenas personas como tú rondando en el trabajo. Merci beaucoup à Anne-Laure et Stéphane, Je n'aurais pas pu finir sans leur aide. Thank you very much to Matias and Chris for your huge contribution to this work. Gracias a todos los compañeros del CBGP que me han ayudado a lo largo de este periodo, en especial a Rosendo. Perdóname por la cantidad de trabajo que te he dado.

Gracias a mis padres, por la libertad para elegir el camino. Gracias a Laura, mi hermana, que siempre está orgullosa de mí. Sois la base sobre la que construyo todo. Gracias a mis

amigos de Ermua, Toribio, Asier, Augusto, Pinto, Rubén, Gallego, Cid, Dani, Serrano, Orujo, Bravo, Txinas, Prol, Javi, Abel, Campo, Oscar y Raúl. No os podéis imaginar la paz que da, y la presión que quita, saber que en el peor de los casos, y si todo va mal, me tengo que volver al agujero con vosotros. Gracias a Jose, aquí me quedo sin palabras. Gracias a los amigos de Cuba 8: David, Victor, Dominic, Yefrén, Rodri, Elena, Abraham y Reyes.

Gracias a la gente de Madrid por vuestro apoyo: Juan, Elena, Gonzalo, Luciano, Claire, Stefano, Noelia, Sonia, Dario, Christine, Okado y Zeus. En especial muchísimas gracias a dos pilares básicos: Ángel y Pita. Creo que vosotros dos habéis sido, fundamentalmente, los que habéis sufrido las consecuencias de todos los momentos estresantes y frustrantes por los que he atravesado durante la realización de la tesis. Muchas gracias por vuestro apoyo, comprensión y momentos terapias anti estrés en el museo del jamón.

Casi todo llega, y la hora de cerrar este ciclo también. Ha sido un viaje intenso, por un camino tortuoso de cuestas pronunciadas hacia arriba y hacia abajo. Por encima de todo ha sido un periodo feliz, y va a ser difícil dejar, a menos de momento, a mis amigos de Madrid de dentro y de fuera del laboratorio, por eso: *“it's a cold and it's a broken Hallelujah”*.

Este trabajo de investigación ha sido posible gracias a la financiación por medio de los siguientes proyectos de investigación: AGL2008-00168/FOR, AGL2011-22625, AGL2014-53352-R, KBBE PIM2010PKB-00702, COST Action FP0905 y PCIG13-GA-2013-631630.

INDEX

ABSTRACT	7
RESUMEN	8
ABBREVIATIONS.....	9
1. INTRODUCTION.....	11
1.1 WINTER DORMANCY	11
1.1.1 Winter dormancy entrance	12
1.1.2 Maintenance of dormancy	18
1.1.3 Winter dormancy exit	19
1.2 WINTER DORMANCY AND CHROMATIN REMODELING	19
1.3 DNA METHYLATION	21
1.3.1 DNA methylation and winter dormancy	21
1.3.2 Molecular mechanism of DNA methylation	23
1.3.3 DNA methylation and gene expression	24
1.3.4 Active DNA methylation.....	25
1.3.5 Active DNA demethylation by DMLs and gene expression.....	26
1.4 REFERENCES	29
2. OBJETIVES	42
3. POPLAR STEMS SHOW OPPOSITE EPIGENETIC PATTERNS DURING WINTER DORMANCY AND VEGETATIVE GROWTH	43
3.1 ABSTRACT	43
3.2 INTRODUCTION	44
3.3 MATERIAL AND METHODS.....	46
3.4 RESULTS	48
3.5 DISCUSSION	55
3.6 REFERENCES	57
4. A DEMETER-LIKE DNA DEMETHYLASE CONTROLS BUD BREAK IN POPLAR.....	61
4.1 ABSTRACT	61
4.2 MATERIAL AND METHODS.....	62
4.3 RESULTS AND DISCUSSION	67
4.4 CONCLUSION.....	73

4.5 REFERENCES.....	74
4.6 SUPPORTING INFORMATION FOR CHAPTER 4.....	79
5. A DNA DEMETHYLASE OVEREXPRESSION PROMOTES EARLY APICAL BUD MATURATION IN POPLAR THROUGH THE BIOSYNTHESIS AND ACCUMULATION OF FLAVONOID.....	82
5.1 ABSTRACT	82
5.2 INTRODUCTION	83
5.3 MATERIAL AND METHODS.....	84
5.4 RESULTS	90
5.5 DISCUSSION	101
5.6 CONCLUSION.....	104
5.7 REFERENCES.....	105
5.8 SUPPORTING INFORMATION FOR CHAPTER 5.....	113
6. GENERAL DISCUSSION	118
7. CONCLUSIONS.....	122

ABSTRACT

During their life cycle, plants are exposed to different environmental conditions that are often unfavorable. Their sessile condition has favored the acquisition of sophisticated molecular mechanisms that guarantee the plastic response to the different conditions faced during their development. Current advances indicate that changes in the functional state of chromatin by epigenetic modifications play important roles in the regulatory networks involved in plant responses to environmental cues. More specifically, several evidences in different plant species indicate that changes in the overall genomic DNA methylation promote tolerance to stressful environmental conditions, and also are associated with developmental programmed transition in response to the environment.

The main aim of this doctoral thesis was to evaluate the involvement of DNA methylation in the control of the annual growth-dormancy cycle of trees, and the molecular characterization of hybrid poplar *DEMETER like* genes to evaluate their regulatory role in these transitions through modifying the DNA methylation profile.

We performed an immunofluorescence-based protocol for the identification of epigenetic marks of closed and open chromatin states in hybrid poplar stems. The fluorescence signal of 5-methylcytosine was significantly higher in winter than in summer. Conversely, the fluorescence signal for acetylated Lys 8 of histone H4 was significantly higher in summer than in winter. Collectively, these results put forward an epigenetic control of winter dormancy in hybrid poplar stems.

The DNA methylation pattern observed in the SAM, from dormancy to the time point of bud break, indicates that the reactivation of the cell cycle occurs after genomic DNA demethylation in hybrid poplar. In addition, we demonstrated that the active DNA demethylation carried out by *DEMETER like 10* (PtaDML10) is required for bud break to allow the induction of cell metabolism genes needed for the reactivation of growth.

Finally, we provide evidence that the chestnut DML (CsDML), the homolog of hybrid poplar PtaDML6, induces the bud formation needed for the survival of the apical meristem under the harsh conditions of winter, by inducing flavonoids biosynthesis accumulated in the SAM and bud scales during winter dormancy entrance.

RESUMEN

A lo largo de su ciclo de vida, las plantas se ven frecuentemente sometidas a condiciones ambientales adversas. Su carácter sésil ha favorecido la adquisición de sofisticados mecanismos moleculares que garantizan una rápida y plástica respuesta a los cambios medioambientales. Avances científicos recientes señalan que los cambios en el estado funcional de la cromatina, a través de variaciones en las marcas epigenéticas, son un importante mecanismo de control de la respuesta de las plantas a los cambios ambientales. Más concretamente, varias evidencias indican que alteraciones en los niveles de metilación de ADN promueven la tolerancia a condiciones de estrés, estando también relacionados con transiciones hacia otros programas de desarrollo en respuesta al ambiente.

Con el fin de identificar marcas epigenéticas relacionadas con estados accesibles e inaccesibles de la cromatina en el tallo, pusimos a punto un protocolo basado en inmunofluorescencia. La fluorescencia que detecta los residuos de 5-metilcitosina fue significativamente mayor durante el invierno respecto al verano. Contrariamente, la fluorescencia que detecta la acetilación de la lisina 8 de la histona H4 fue significativamente mayor durante el verano respecto al invierno. Estos resultados sugieren la existencia de un control epigenético de la dormancia invernal en el tallo del chopo híbrido.

El perfil de metilación observado en el SAM, durante la dormancia hasta el momento de apertura de las yemas en primavera, mostró que la reactivación del ciclo celular ocurre después de un descenso del nivel de metilación del ADN genómico en el chopo híbrido. En este trabajo demostramos que la demetilación activa mediada por DEMETER like 10 (PtaDML10) es requerida para la apertura de la yema, ya que permite la inducción de genes relacionados con el metabolismo celular necesarios para la reactivación del crecimiento.

Finalmente, aportamos evidencias de una demetilasa de castaño (CsDML), y su homólogo en el chopo híbrido PtaDML6, que induce la formación de la yema apical necesaria para la supervivencia del meristemo bajo las condiciones adversas del invierno, a través de la biosíntesis de flavonoides que se acumulan en el SAM y en las escamas de la yema durante la entrada a la dormancia invernal.

ABBREVIATIONS

BSA: Bovine Serum Albumin

CDS: coding sequence

DEG: differentially expressed genes

DME: DEMETER

DML: demeter-like

DMR: differentially methylated regions

DNA: deoxyribonucleic acid

DPBA: diphenylboric acid-2-aminoethyl ester

FISH: fluorescence in situ hybridization

GAs: gibberellins

HA: hemagglutinin

HAT: histone acetyl transferase

HC: histoclear

HDAC: histone deacetylase

HhH: hairpin-helix-hairpin

HPCE: high performance capillary electrophoresis

HPLC: high performance liquid chromatography

KD: knock-down plants

LD: long day

OX: overexpressor plants

PBS: phosphate buffered saline: 137 mM NaCl; 0.27 mM KCl; 1 mM phosphate buffer, pH 7.4

PD: plasmodesmata

RdDM: RNA-directed DNA Methylation

RNA: ribonucleic acid

ROS1: REPRESSOR OF SILENCING 1

SAM: shoot apical meristem

SD: short day

TE: transposable elements

TF: transcription factor

TSS: transcriptional start site

UTR: untranslated terminal region

WGBS: whole genome bisulfite sequencing

WT: wild type

5mC: the addition of a methyl group to the carbon atom at the fifth position of a cytosine

1. INTRODUCTION

1.1 WINTER DORMANCY

Plant growth responds to cyclic developmental and environmental signals. Trees growing in boreal and temperate regions synchronize their growth with seasonal climatic changes in adaptive responses that are essential for their survival (Singh et al. 2016). In perennial plants, growth rate is markedly regulated by the establishment of periods of dormancy, which consist in the repression of cell division in meristem-containing structure. Temperate horticultural and forestry trees have developed three different types of vegetative dormancy: (i) paradormancy or apical dominance, which is the suppression of lateral bud growth by apical meristem. This is the strategy used by trees to achieve their typical architecture; (ii) ecodormancy, in which growth is arrested by environmental conditions but can resume when the conditions become again favorable; and (iii) endodormancy, which is caused by plant endogenous factors and requires a sustained exposure to low temperatures for regrowth to occur in spring (Lang et al. 1987). Endodormant plants are not able to grow under growth-promoting conditions, even in the absence of the external physiological signals that caused this state (Anderson et al. 2010). This dormancy strategy permits trees to survive through periods that are unfavorable for growth or are potentially hazardous, such as the winter season (**Figure 1.1**), to guarantee sexual reproduction and seed set in adult trees. In this case, growth inactivity is imposed on all meristems.

Figure 1.1. Seasonal growth-dormancy cycle in temperate perennial plants. The onset of winter dormancy coincides with changes in the environment. Decreasing day length promotes growth cessation and dormancy facilitated by low temperatures. Prolonged exposure to chilling temperatures is required for

dormancy release. Growth resumes when environmental conditions become favorable for growing (day length and temperature above critical thresholds). Modified from Shim et al. 2015.

1.1.1 *Winter dormancy entrance*

Photoperiod and temperature are the main cues controlling this process. Of these, photoperiod provides the most reliable information, in a variation predictable fashion (van der Schoot et al. 2014). Different plant systems depend on the photoperiod to program crucial developmental events in the apex, as the transition to flowering, and to terminal bud formation and dormancy in perennials (Vince-Prue 1994; Yanovsky & Kay 2002; Arora et al. 2003; Cooke et al. 2012). Low temperatures also trigger some developmental changes, but while photoperiod is perceived in the leaves, low temperatures are perceived directly in the apex.

The shoot apical meristem (SAM) of deciduous woody perennials shares common structural features with other angiosperms, but are unique in the seasonal regulation of vegetative and floral meristems. Throughout their life span, flowering occurs in the adult phase, restricted to some axillary meristems. In temperate and cold climates, photoperiodic timing and/or chilling sensing induce end-of-season growth arrest, dormancy cycling and flowering (Paul et al. 2014). Winter dormancy entry during autumn requires the concurrence of three developmental processes: apical bud development, acclimation to dehydration and cold and acquisition of dormancy. Apical bud development comprises growth cessation and bud formation. These processes are interconnected, but present differences in the molecular mechanisms of control. The dormant state becomes progressively established once growth ceases and the apical bud is formed (Ruttink et al. 2007).

1.1.1.1 *Growth cessation*

Although photoperiodic responses are conserved (Vince-Prue, 1994), there might be considerable variation in the precise molecular mechanisms that operate among plants, or in the way they are implemented for a specific developmental process. Photoperiod-sensitive detection and transduction mechanisms in *Arabidopsis* and rice, might have been adopted by perennial species to regulate developmental transitions in different seasonal contexts (Horvath et al. 2003; Rinne & van der Schoot 2003; Böhlenius et al. 2006; Chao et al. 2007; Rohde & Bhalerao 2007; Ruonala et al. 2008; Horvath 2009; Hsu et al. 2011; Cooke et al. 2012; Mimida et al. 2013; Singh et al. 2016).

In many perennial plants, the shortening of the daylength at the end of the summer is the only environmental signal needed to induce growth cessation. Short Day (SD) conditions are then short enough to promote growth cessation. The critical daylength is longer in the north than

in the south latitudes. This adjustment is critical to ensure that the induction of dormancy and cold acclimation occur before winter.

In *Populus*, phytochromes act upstream of the regulatory pathway controlling SD-induced growth cessation. There have been found three phytochrome coding genes in poplar: *PHYA*, *PHYB1* and *PHYB2* (Howe et al. 1998). Ectopic expression of oat (*Avena sativa*) *PHYA* in *Populus* prevents SD-induced growth cessation (Olsen et al. 1997), and RNA interference (RNAi)-mediated downregulation of *PHYA* triggers earlier growth cessation and leads to phase changes of circadian clock genes (Kozarewa et al. 2010). Quantitative trait locus (QTL) and association mapping studies have identified *PHYB2* as one of the main loci controlling SD-induced growth arrest and bud formation (Frewen et al. 2000; Ingvarsson et al. 2006; McKnown et al. 2014). *Populus* transgenic trees with reduced expression of *LATE ELONGATED HYPOCOTYL (LHY)* and *TIMING OF CAB EXPRESSION 1 (TOC1)* led to a shortened internal period of clock-controlled gene expression rhythms and delayed SD-induced growth cessation (Ibáñez et al. 2010).

In *Populus*, the CO/FT module seems to control the extent of the growing period by sensing the day length. The overexpression of *FT (FLOWERING LOCUS T)* triggers flowering in juvenile poplars showing its conserved function in trees. It also controls SD-induced growth cessation and bud formation (Bohlenius et al. 2006) since downregulation of both *Populus FT* and *CO (CONSTANS)* brings about premature growth cessation and bud formation (Bohlenius et al. 2006). Moreover, *CO* and *FT* expression analysis of different *Populus* ecotypes from various latitudes, with different critical day length for growth cessation, showed that the phase of *CO* expression in relation with the light period highly correlated with *FT* capacity to be expressed. This supports the role of the CO/FT in photoperiod sensing, providing a possible mechanism for the different critical day lengths of trees originating from different latitudes (Bohlenius et al. 2006). In contrast, *CO* RNAi plants growth cessation does not occur as early as in *FT* RNAi lines, and transgenic poplars overexpressing *CO* display a normal growth cessation response (Hsu et al. 2012). These results suggest that, although CO has a role in activating *FT* in response to the photoperiod in *Populus*, other factors are likely to have even more important functions in daylength sensing.

In *Populus* there are two *FT* paralogues: *FT1* and *FT2*. Gene expression analyses showed that *FT2* is expressed during the vegetative growth in leaves, while *FT1* seems to be induced by chilling and it is expressed in dormant buds in late winter (Hsu et al. 2011). In *Arabidopsis*, FT acts in a complex with the transcription factor FD (Abe et al. 2005). Tylewicz et al. (2016) identified two *FD* homologs in *Populus*, *FD-like 1 (FDL1)* and *FD-like 2 (FDL2)*. Although both FDL1 and FDL2 can interact with FT1 and FT2, only FDL1 could be linked to

the control of growth cessation. Transgenic trees overexpressing *FDL1* form apical buds significantly later than the wild type (WT) under SD, while the opposite is true for *FDL1* RNAi trees. This suggests that the FT–FD complex is conserved in *Populus*, but acting in the maintenance of the growth in response to photoperiod. In *Arabidopsis*, *APETALA1* (*API*), the FT/FD target, is one of the first genes to be expressed in the developing flower meristem (Wigge et al. 2005; Kaufmann et al. 2010). *LAP1* (*LIKE API*) downregulation in *Populus* suppressed inhibition of growth cessation in *FTI*-overexpressing plants and led to slightly earlier growth cessation in a WT background (Azeez et al. 2014). This suggests a possible role for *LAP1* in the regulation of growth cessation. However, the very weak response of *LAP1* RNAi lines compared to *FT* RNAi suggests that other targets are important. *AINTEGUMENTA*-like 1 (*AIL1*), a direct target of *LAP1* (Azeez et al. 2014), activates the expression of cell cycle genes such as D-cyclins, and its downregulation under SD is needed for growth cessation (Karlberg et al. 2011). All these data were gathered in a model for a photoperiod control of growth cessation, comprising light perception by the phytochromes, circadian clock core associated genes, the CO/FT module, *LAP1* and the regulation of genes involved in the cell cycle control (Ding & Nilsson 2016) (**Figure 1.2**).

Figure 1.2. Simplified scheme showing similarities in the regulatory network controlling floral transition in *Arabidopsis* and vegetative growth in *Populus*. Arrows indicate activation, while flat-ended arrows indicate repression. Arrows do not necessarily indicate direct interactions. Modified from Ding & Nilsson 2016.

Gibberellins (GAs), which are involved in stem elongation and flowering time, are also implicated in growth cessation in trees (Eriksson et al. 2000). Both exogenous applications of bioactive GAs to *Populus* under SD conditions and plants overexpressing GA20 oxidase (GA20ox), leading to elevated levels of GA, delay growth cessation (Eriksson et al. 2000). However, overexpression of the GA receptor *PtGID1* does not affect the response to short days (Eriksson et al. 2000; Eriksson et al. 2015). These data suggest that level of bioactive GA rather than the availability of the GA receptor controls SD-induced growth cessation. Moreover, overexpression of GA20ox delays growth cessation under SD independently of *FT2* expression levels, implying that there are FT independent regulations of SD-induced growth cessation through GAs (Eriksson et al. 2015).

As autumn advances, the drops in temperature leads to a greater tolerance to cold and to leaf fall in deciduous trees (Welling & Palva 2006). Circadian clock genes, *CsLHY* and *CsTOC1*, expression in chestnut (*Castanea sativa*) are disrupted in response to cold, leading to constitutive and arrhythmic activation of these genes (Ramos et al. 2005). These changes are directly triggered by low temperature and are independent of the photoperiod. Similar changes are also observed in the expression of other genes of the central oscillator, including *CsPRR9*, *CsPRR7*, and *CsPRR5* (Ibáñez et al. 2008). Such disruption is also observed in *Populus sp.*, suggesting that cold alteration of the circadian clock is a common feature of woody plants (Ibáñez et al. 2010). Exposure of *C. sativa* seedlings to cold temperatures in the morning (when *CsLHY* expression is high and that of *CsTOC1* is low) and in the afternoon (when *CsLHY* expression is low and that of *CsTOC1* is high) resulted in both cases in upregulation of these genes (Ramos et al. 2005). Similar changes in the level of expression and rhythmicity have been found for some circadian clock output genes in *C. sativa* (Berrocal-Lobo et al. 2011; Moreno-Cortés et al. 2012). Taken together, these studies indicate that there is, in perennials, a widespread control of the disruption of the circadian clock caused by cold, possibly including layers of regulation other than the transcriptional ones (Allona et al. 2008)

Presumably, the disruption of the circadian clock has an important biological relevance in perennials. *Populus tremula x tremuloides* trees with reduced levels of *PttTOC1* show high and arrhythmic increases in the expression of *PttLHY1*, *PttLHY2* and *PttCBF1* in response to cold, and also have increased freezing tolerance (Ibáñez et al. 2010). Conversely, the loss of *PttLHY1* and *PttLHY2* expression leads to a loss of *PttCBF1* expression and reduced freezing

tolerance (Ibáñez et al. 2010), implying that the expression of *PttLHY1* and *PttLHY2* is necessary for the induction of *PttCBF1*.

Experimental observations have reinforced the hypothesis that the establishment of winter dormancy cannot be considered as strictly photoperiodic. In *Populus*, SD-induced growth cessation and bud formation processes are positively influenced by synergic crosstalk between SD and the lower temperatures of autumn (Rohde et al. 2010). Apple (*Malus pumila*), pear (*Pyrus communis*) and other species of the *Rosaceae* family are insensitive to photoperiod (Wareing 1956). In these species, low temperatures control growth cessation and dormancy induction independently of photoperiodic conditions (Cook et al. 2005; Heide & Prestrud 2005). Finally, SD and low temperatures have a synergistic effect in enhancing the freeze tolerance of buds and stem tissues of birch (*Betula pendula*) (Li et al. 2002).

1.1.1.2 Bud formation

Upon elongation arrest, the meristems cease growing and the apical bud is formed in response to SD conditions. Leaf primordia already formed under LD (long day) will develop mature leaves during growth cessation (Rohde et al. 2002). In poplar, only the new leaf primordia generated under SD will change their morphogenetic fate to generate the organs presented inside the apical bud. The structures forming the bud, in an inward order, are: (i) scales, originated from the first primordium initiated after SD, when its lamina senesces and abscises, and the stipular domain enlarges (Rohde et al. 2002); (ii) embryonic leaves, each with two stipules; (iii) and leaf primordia without stipules yet. Once these organs developed, the activity of the apical meristem ceases and the bud is formed (Rohde et al. 2002).

Using transcript and metabolite profiling, Ruttink et al. (2007) established a molecular timetable of events during bud development and dormancy induction in hybrid poplar (*Populus tremula* x *Populus alba*), which serves as a reference for functional studies (**Figure 1.3**). To date, few genes have been functionally characterized. Expression of a dominant-negative version of *ETHYLENE TRIPLE RESPONSE 1* (*ETR1*) or overexpression of *ABSCISIC ACID-INSENSITIVE 3* (*ABI3*) prevents the formation of closed apical buds upon SD induction, suggesting roles in bud development (Rohde et al. 2002; Ruonala et al. 2006). *FDL1* also has a role in bud development: its expression increases gradually during bud development and *FDL1* can interact with *ABI3*. Bud maturation marker genes in both *ABI3*-overexpressing and *FDL1*-overexpressing plants exhibit similar changes. These results suggest that *FDL1* and *ABI3* are components of a regulatory network controlling the bud maturation pathway (Tylewicz et al. 2016; Shing et al. 2016). The intimate connection between the regulation of growth cessation and bud formation makes its analysis difficult. Apical bud formation can be completed only

after growth cessation, which in turn is mainly triggered by the critical day length, although the time required for growth cessation and bud development can vary considerably with temperature (Rohde et al. 2011).

Some metabolic processes have been reported to change in the transition from the vegetative growth to the dormant state in trees: cease of cellulose synthesis, acceleration of lignin and starch synthesis, accumulation of anthocyanins, phenols and fats (Perry 1971). Bud scales accumulate phenolic components during maturation, leading to their final red-brown coloration (Rohde et al. 2002). Consistently, Ruttink et al. (2007) found that enzymes involved in flavonoid biosynthesis increase remarkably their expression at 3 to 4 weeks under SD conditions. The expression profiles of these genes are correlated with the time and extent of bud scale development.

Figure 1.3. Timetable of autumnal bud development. This process comprises bud formation (red), acclimation to dehydration and cold (blue), and dormancy acquisition (orange). Genes in each panel are highlighted with this colors according to the process they are involved in (Ruttink et al. 2007). Simultaneous with bud development, elongation growth (green) gradually ceases. Bud development is characterized by the sequential activation of SD perception, ethylene, and ABA signal transduction pathways. The two major phases of transcriptional and metabolic response are indicated by gray boxes. Arrows connect regulators and TFs to their putative downstream processes, without implying a genetic or

direct molecular interaction. Because of its putative nature, the link between low sugar and ethylene signal transduction is shown with a dashed arrow. Modified from Ruttink et al. 2007.

1.1.2 Maintenance of dormancy

The molecular control underlying dormancy regulation is still poorly understood. This can be explained, in part, by the difficulty of analyzing dormant meristematic tissues, the vascular cambium or the SAM, protected by the SD-induced apical bud. Apical bud formation and acquisition of the dormant state may be regulated by independent mechanisms. Trees overexpressing *ABI3* form aberrant apical buds, but acquired the dormant state (Rohde et al. 2002). The ectopic expression of a dominant-negative version of *ETR1* resulted in a delay or absence of apical bud, although acquiring dormancy state.

The role of plasmodesmata (PD) as putative regulators of dormancy has been investigated. PD, the conduits that connect cells symplastically, are closed during dormancy (Rinne & van der Schoot 1998; Rinne et al. 2001). Exposition to SD close PD gradually by deposition of callose (1,3 β -glucan) (Rinne & van der Schoot 1998). Based on these studies, dormancy establishment and maintenance could involve the symplastic isolation of SAM by blockage of PD. Overexpression of *PHYA* does not result in the closure of PD and dormancy cannot be induced (Ruonala et al. 2008). Nevertheless, more functional evidences are needed to understand whether the blockage of PD is the cause or the consequence of dormancy establishment and maintenance.

The ‘Evergreen’ (EVG) peach (*Prunus persica*) variety is insensitive to environmental signals and, consistently, there is not growth cessation and bud formation (Rodríguez et al. 1994). This phenotype is associated with a deletion in the *evg* locus containing a cluster of six repeated *DORMANCY ASSOCIATED MADS-BOX (DAM)* genes (Bielenberg et al. 2008). These *DAM* genes are homolog to the *SVP/AGL24* subfamily of *MADS-box* genes in *Arabidopsis*, which regulate flowering time via the CO/FT regulatory module (Wells et al. 2015). The *DAM* genes are expressed in buds and are clearly affected by photoperiod and chilling (Li et al. 2009). Overexpression of the *Prunus mume DAM6* gene in *Populus* triggered an early growth cessation and bud formation (Sasaki et al. 2011). *DAM-like* genes have also been identified from other perennials and their expression patterns suggest a possible role in the maintenance of dormancy (Jimenez et al. 2009; Ubi et al. 2010; Wu et al. 2012; Saito et al. 2013). EVG trees, like *FT* overexpressors, grow continuously during SD, suggesting that the *DAM* genes are involved in controlling growth cessation. Thus, it is possible that the *DAM* genes have direct roles in both the regulation of growth cessation and bud set, and not, or not exclusively, in the establishment

and maintenance of dormancy, but more functional characterization is needed to understand their function.

1.1.3 Winter dormancy exit

The exit from dormancy is often a temperature-dependent process. Once the chilling requirement is fulfilled, the trees are able to respond to the warmer temperatures of spring and promote bud break independently of the photoperiod (Ding & Nilsson 2016). The molecular mechanisms underlying bud break in spring are little understood. *Populus* trees overexpressing *CEN1*, a flowering repressor and homolog of *Arabidopsis* *TERMINAL FLOWER1* (*TFL1*), require longer chilling times than the wild-type, and RNAi transgenics shorter (Mohamed et al. 2010). Using activation tagging in *Populus*, Yordanov et al. (2014), identified a novel regulator named *EARLY BUD BREAK 1* (*EBB1*), which encodes a putative APETALA2/Ethylene responsive transcription factor. Ectopic expression of *EBB1* leads to precocious bud burst, while downregulation delays bud break. In Japanese pear (*Pyrus pyrifolia*), PpEBB1 can interact with the promoters of four *D-type cyclin* (*PpCYCD3*) genes in flower buds and induce their expression (Tuan et al. 2016). *EBB1* is predominantly expressed in the SAM, similar to the expression pattern of *CEN1* (Ruonala et al. 2008). However, it remains unclear whether they act together or via independent pathways.

1.2 WINTER DORMANCY AND CHROMATIN REMODELING

The chilling requirement of perennial plants can be compared to the vernalization treatment required for flowering in *Arabidopsis* and other annual plants. In *Arabidopsis*, the FLOWERING LOCUS C (FLC) MADS-BOX protein is the central regulator of this process. FLC is a repressor of *FT* and, thus, of flowering. Upon vernalization the levels of FLC transcript and protein drop allowing flowering in late-flowering ecotypes (Sheldon et al. 2000). Two different *MADS-BOX* genes, *VERNALIZATION1* (*VRN1*) and *VRN2* control the flowering time in cereals. While *VRN1* is induced by vernalization and promotes flowering, *VRN2* represses *FT* until vernalization is completed (Reviewed in Trevaskis et al. 2007). Dormancy-associated *MADS-BOX* genes (*DAM*) seem to be regulators of bud dormancy in leafy spurge (*Euphorbia esula*) and peach (*Prunus persica*) perennial plants (Li et al. 2009; Horvath et al. 2010; Jiménez et al. 2010). Vernalization and bud dormancy are mediated by the regulation of *FLC*, *VRN1*, and *DAM* genes expression through common epigenetic mechanisms. Prolonged cold exposure promotes gene repression of leafy spurge *DAM1* and *Arabidopsis* *FLC* through the decrease of trimethylation of lysine 4 of histone H3 (H3K4me3) and increase of H3K27me3 in their loci (Bastow et al. 2004; Kim et al. 2005; Horvath et al. 2010). These epigenetic marks repress *VRN1* in barley (*Hordeum vulgare*) before vernalization (Oliver et al. 2009). In peach, *DAM6*

shows similar epigenetic changes associated with gene repression after dormancy release, in addition to a decrease of H3 acetylation in the chromatin around the ATG region (Leida et al. 2012). *PpEBBI* overexpression induces precocious bud break in Japanese pear, and its expression peaks shortly before bud enlargement, when levels of H3K4me3 increase in the 50 base pairs upstream and start codon regions in *PpEBBI* locus (Tuan et al. 2016).

In addition to histone modifications, in *Arabidopsis*, *FLC* expression is also regulated by higher order of chromatin structure, by a chromatin loop that involves a physical interaction of the 5' and 3' flanking regions of the *FLC* locus. This loop is disrupted in the early steps of vernalization, in the switch from the expression of *FLC* to a Polycomb-mediated repressive state (Crevillen et al. 2012). Moreover, the synthesis and action of long non-coding RNAs (lncRNAs) play a crucial role in the epigenetic regulation of *FLC* gene also in *Arabidopsis* (Turk & Coupland 2011). Both, a natural antisense transcript called COOLAIR, expressed from a promoter located in the 3' flanking region of *FLC*, and a sense intronic lncRNA named COLDAIR, participate in different phases of the cold-induced repression and the stable silencing of *FLC* by the Polycomb Repressive Complex 2 (PRC2) (Swiezewski et al. 2009; Heo & Sung 2011).

Several transcriptomic analyses in *Populus* have led to the identification of genes involved in the epigenetic regulation of gene expression, and of genes differentially expressed during active growth and winter dormancy. The entrance into dormancy of the vascular cambium involves a global change in the transcriptome of *Populus* (Schrader et al. 2004). The homologue of *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) was strongly upregulated during cambial dormancy in *Populus tremula*. *FIE* acts as part of PRC2, which is involved in the trimethylation of the lysine 27 of histone H3 (H3K27me3). This epigenetic mark is associated with stable gene silencing. *FIE* and *PICKLE* (*PKL*), which encodes an ATP-dependent chromatin remodeling protein associated with H3K27me3, are both rapidly upregulated following the perception of SD (Ruttink et al. 2007). In apical buds, Karlberg et al. (2010) reported that *Populus tremula x tremuloides* *GENERAL CONTROL NON-REPRESSIBLE 5 LIKE* (*GCN5-like*), a histone acetyl transferase (HAT), was upregulated in the apex after SD treatment. Also, after five weeks of SD, two putative histone deacetylases (HDAC), *HDA14* and *HDA08*, the histone lysine methyltransferase (HMT) *SET DOMAIN PROTEIN 20* (*SDG20*) and a gene involved in histone ubiquitination, *HISTONE MONO-UBIQUITINATION 2* (*HUB2*), were all up-regulated, while a putative *DEMETER-LIKE* (*DML*) gene was down-regulated over the same period. The authors proposed that the down-regulation of this latter gene could increase the level of DNA methylation and hence chromatin compaction and gene silencing, after SD perception. The analysis of dormant *Populus* stem tissues found

that many genes involved in chromatin remodeling were upregulated in winter compared to actively growing (summer) tissues (Ko et al. 2011). Moreover, transcriptomic analysis of *C. sativa* buds revealed higher expression of *CsHUB2* and *CsGCN5L* in dormant buds (Santamaría et al. 2011) as compared to growing ones. Further genetic approaches should be performed to identify the chromatin remodelers involved in the control of winter dormancy. Studies by Kumar & Wigge (2010) in *Arabidopsis* suggest that temperature-regulated chromatin remodeling could act as a “thermometer” and that this might be important in clock regulation of growth activation in perennials.

1.3 DNA METHYLATION

1.3.1 DNA methylation and winter dormancy

Genomic DNA methylation is an essential epigenetic mark in higher eukaryotes. This refers to the addition of a methyl group to the carbon atom at the fifth position of a cytosine (5mC). DNA methylation controls gene expression, genome protection and stability by transposon silencing, DNA recombination and other biological processes (Teixeira & Colot 2010; Saze et al. 2012; Mirouze et al. 2012). Dynamic DNA methylation/demethylation confers plasticity to plants in order to respond to environmental and/or developmental signals (Ramchandani et al. 1999). Reductions in the DNA methylation levels in a specific cell population or organ have been found prior the transition to a new developmental program. Zluvova et al. (2001) found that the central zone of the SAM in *Silene latifolia* remains highly methylated during the vegetative growth. However, upon the transition to the floral bud, the level of methylation decreases in the meristem, and is accompanied with higher cell division (Zluvova et al. 2001). In turn, cell differentiation mostly implies an increase of DNA methylation, as described during ageing of pine needles (Fraga et al. 2000). In *Azalea japonica*, floral buds have been shown to be hypermethylated compared with vegetative buds (Meijón et al. 2009).

During autumn, cell proliferation ceases and the quiescent state is established in the SAM, until the growth is resumed when the good environmental conditions return in spring. Changes in genomic DNA methylation levels have been observed in these periodic transitions in trees. In chestnut, apical bud maturation during autumn correlates with a progressive increment of the 5mC levels, while apical bud break is accompanied by a decrease of 5mC from the closed apical bud to the developmental state where the first unfolded leaves are visible (Santamaría et al. 2009). The opposite pattern was found for the acetylation of histone H4 (AcH4) during the same periods (Santamaría et al. 2009). Kumar et al. (2016) found in apple (*Malus x domestica*) a progressive decrease of DNA methylation in four developmental stages: dormant bud, silver tip,

green tip and initial fruit set. This dynamics in apple buds only occurred when the trees grew under environmental conditions that permitted the fulfillment of the chilling requirement for winter dormancy release (Kumar et al. 2006), highlighting the importance of environmental conditions in the developmental reprogramming by DNA methylation.

Transcriptomic analyses in different developmental states in the growth-dormancy transition of trees throughout the year identified putative enzymes involved in the DNA methylation dynamics. In 2010, Karlberg et al. found putative *DML* genes downregulated in growth-dormancy transition in poplar buds according to the increase of 5mC observed in other tree species during same period (Santamaría et al. 2009). Shim et al. (2014) identified 5 genes involved in DNA methylation, which were differentially expressed in different developmental stages of winter dormancy in poplar stems: two poplar homologs to *Arabidopsis METHYLTRANSFERASE 1 (MET1)*, a homolog to *CHROMOMETHYLASE 3 (CMT3)* and two homologs to *DEMETETER (DME)*. One poplar *DEMETETER like* gene (*DML*) was upregulated when the growth ceased during winter dormancy entrance, while the other *DML* was relatively higher expressed during dormancy exit after the chilling requirement was fulfilled. Howe et al. reported (2015) that this *DML* was downregulated during winter dormancy entrance and upregulated in the latest stage of winter dormancy also in lateral buds.

Acetylation/deacetylation dynamics by HATs and HDAC, respectively, regulates the accessibility of the transcription machinery to DNA and, thus, gene expression. Nuclear acetyl-CoA is the donor of acetyl groups for histone acetylation. This reaction is dependent on ATP-citrate lyase (ACL), which converts citrate and coenzyme A into acetyl-coA and oxaloacetate Wellen et al. (2009). Takahashi et al (2006) showed that Acetyl-CoA synthetase (ACS) also regulates global acetylation of histones in yeast. Deacetylation is carried out by sirtuins, the class III of HDAC. Rice sirtuin OsSRT1 directly targets H3K9ac and expression of many genes that are related to stress and metabolism (Zhong et al. 2013) (**Figure 1.4**).

Several methylation reactions are catalyzed by methyltransferases (MTases) that use S-adenosyl methionine (SAME) as donor of the methyl group to a variety of acceptor molecules, such as DNA, RNA or arginine and lysine residues. The end product of this reaction, S-adenosyl homocysteine (SAH), inhibits this enzymatic activity. SAH hydrolase (SAHH) breaks down SAH into adenosine and homocysteine, the precursor of methionine (Met), which in turn produces SAME (**Figure 1.4**).

SAHH and sirtuins are dependent on NAD^+ . Thus, the high NAD^+/NADH ratios during winter dormancy would promote DNA methylation and histone deacetylation reactions (Grillo & Colombatto 2008; Luo & Kuo 2009). These data indicate that energy fluxes through the TCA

cycle link the plant nutrient and metabolic status to the epigenetic regulation of gene expression (Figure 1.4).

Figure 1.4. NAD^+ , acetyl-CoA and SAMe perform a key role in the epigenetic control of transcription by methylation of DNA and post-translational modifications of histones. NAD^+ is required for the activity of sirtuins (HDAC). Acetyl-CoA, synthesized by acetyl-CoA synthetase (ACS) and ATP citrate lyase, provides the acetyl group for histone and protein acetyltransferases. SAMe is the methyl donor for DNA, RNA and histones methylation, releasing S-adenosyl homocysteine (SAH). NAD^+ is an essential coenzyme for SAHH, which breaks down SAH into adenosine and homocysteine. Homocysteine is the methionine precursor, recycling back SAMe. NAD^+ and citrate participate in the TCA and, therefore, are linked to the cell energy status. Modified from Luo & Kuo 2009.

1.3.2 Molecular mechanism of DNA methylation

Specific modifications of the histones, recruitment of protein complexes, and/or specific modification of the underlying DNA, regulate chromatin structural changes between two functional states, active euchromatin and inactive heterochromatin. Euchromatin is associated with histone hyperacetylation and cytosine hypomethylation while heterochromatin is related with histone hypoacetylation and cytosine hypermethylation (Richards & Elgin 2002).

Cytosine methylation in a symmetrical CG (mCG) context has been found across eukaryotes. In contrast to animals, where DNA methylation is mostly confined to this sequences, methylation in plants occur in three different contexts (CG, CHG and CHH, where H = A, C or T). CG and CHG are symmetrical contexts as there is a mirrored cytosine on the

opposing strand. This is essential for the recognition of the mechanism of maintenance of mCG. In plants, this is carried out by the METHYLTRANSFERASE 1 (MET1, ortholog of mammals Dnmt1). After replication VARIANT IN METHYLATION 1 (VIM1-5) (UHRF1 in mammals) recognizes the hemimethylated mCG sites and recruits MET1 (Dnmt1) to methylate the symmetrical sequences on the opposing strand. This pathway is essential to maintain mCG, since *met1* mutants lose the majority of mCG in their genome. Maintenance of mCHG is triggered by CHROMOMETHYLASE 3 (CMT3) and is based on a feedback loop involving the dimethylation of lysine 9 of histone H3 (H3K9me2) epigenetic mark (Du et al. 2012). The CHROMO and BROMO ADJACENT HOMOLOG (BAH) domains facilitate the binding of the CMT3 to H3K9me2, primarily present in heterochromatin. The histone methyltransferases KRYPTONITE (KYP), Su(var)3-9 homologue 5 (SUVH5), and SUVH6 contain the SRA domain that bind to methylated DNA, producing H3K9me2. This creates a cross-talk between mCHG and H3K9me2 that promotes chromatin packing and gene repression.

CHH is the asymmetrical context of DNA methylation, lacking the hemimethylated mirrored cytosine that guides methylation maintenance after replication. Hence, methylation needs to be re-established de novo by two different mechanisms. The first involves another CMT family member, CMT2. Like CMT3, also with CHROMO and BAH domains, it interacts with H3K9me2, causing methylation of CHH in these regions. The second mechanism, shared by the other two sequence contexts, is the RNA-directed DNA Methylation (RdDM). This pathway requires 24-nucleotide short-interfering RNAs (24-nt siRNAs) that are transcribed by the plant specific POLYMERASE IV (POL IV). These siRNAs guide DOMAINS REARRANGED 2 (DRM2) methyltransferase to target regions in all three contexts (Law et al. 2010; Matzke et al. 2014). RdDM is particularly associated with high levels of mCHH.

MET1, CMT2 and CMT3 methylation depend in part on the chromatin remodeller DECREASED DNA METHYLATION 1 (DDM1). Mutation of *DDM1* leads to a progressive loss of methylation in all three contexts over generations (Kakutani et al. 1995).

1.3.3 DNA methylation and gene expression

DNA methylation is mostly a repressive chromatin modification; however, this is not always the case (Niederhuth & Schmitz 2016). Methylation in the promoter affects transcription factor (TF) binding and, therefore, gene expression. A recent study in *Arabidopsis* revealed that 72% of the TF analyzed showed less affinity when their target sequences were methylated, 4% preferred methylated DNA and 24% did not show any differences (O'Malley et al. 2016). Whole genome methylomes performed in *Arabidopsis* and in *Populus trichocarpa* have unveiled that methylation in the promoter and/or upstream sites near (TSS) mainly represses

gene expression (Zhang et al. 2006; Vining et al. 2012; Lafon-Placette et al. 2013; Liang et al. 2014). The role of gene body methylation is still unclear. In plants and animals, mCG in gene bodies has been associated with higher gene expression, and more specifically, with constitutively expressed genes (Niederhuth & Schmitz 2016). These genes showed reduced mCG in and around the TSS. However, gene-body methylation in poplar has been correlated with tissue-specific expression and shows a more negative effect on transcription than promoter-gene methylation (Vining et al. 2012; Lafon-Placette et al. 2013). Generally methylation in CG of TSS is sufficient for transcriptional silencing (Niederhuth et al. 2016). These data suggest that the pattern of methylation and its context in gene body can be critical for down-regulate or up-regulate gene expression.

1.3.4 Active DNA methylation

A major feature of epigenetic variations is their reversibility and inheritability. Passive demethylation occurs when the machinery to copy methylated sequences to the newly replicated DNA strand is not recruited. Nevertheless, plants have evolved specific enzymes to remove 5mC residues from DNA, represented by the well-characterized *Arabidopsis* DNA glycosylases DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1) and DEMETER-LIKE 2 (DML2) and 3 (DML3). Similarly, all plant 5mC DNA glycosylases are named DEMETER-LIKE (DML) after the founding members of the *Arabidopsis* family. The DME/ROS1/DML enzymes contain a conserved DNA glycosylase domain belonging to the helix-hairpin-helix [4Fe-4S] iron-sulfur cluster superfamily (Choi et al. 2002). DMLs use their bifunctional glycosylase/lyase domain to catalyze both 5mC excision and the cleavage of a sugar-phosphate backbone via β - and δ -elimination reactions, creating a one-nucleotide gap in the DNA. Then, either the DNA phosphatase ZDP or the AP endonucleases APE1L and ARP processes the 3' end of the abasic site to generate a hydroxyl group (Lee et al. 2014; Li et al. 2015). Immediately following that, a yet-to-be identified DNA polymerase and DNA ligase I act sequentially to insert an unmethylated cytosine.

DML enzymes are much larger than other glycosylases, ranging from 1100 to over 1700 residues and contain two additional domains with no known homology to other proteins. The molecular mechanism of 5mC demethylation has been widespread investigated in *Arabidopsis* providing new and complete insight into the molecular underpinnings for 5mC specificity and excision mechanism by the DML family of enzymes, which include a complete characterization of their characteristic discontinuous glycosylase-lyase domain, which let the identification of essential amino acids needed for their activity. (Mok et al. 2010; Ponferrada-Marín et al. 2009; Ponferrada-Marín et al. 2010a,b; Parrilla-Doblas et al. 2013; Brooks et al. 2014).

Although the biochemical process of the base excision needed for the active DNA demethylation has been well characterized, very little is known about the locus-specific targeting of DMLs. Previous studies suggested that the RNA-binding protein ROS3 is required for the recruitment of ROS1 to its target loci (Zheng et al. 2008). Interestingly, a HAT, INCREASED DNA METHYLATION 1 (IDM1), was identified as another important regulator of DNA demethylation that function upstream of ROS1 with the assistance of IDM2, an alpha-crystalline domain protein. IDM1 recognizes chromatin regions that are methylated in CG context with low levels of histone 3 lysine 4 and arginine 2 (H3K4; H3R2) methylation, and catalyzes H3K18 and H3K23 acetylation, which triggers ROS1-mediated DNA demethylation (Qian et al. 2012, 2014). In 2014, Li et al. found that, IDM1 and IDM2 interact with MBD7 and IDL1. MBD7 is a member of the methyl-CpG-binding domain (MBD) family. MBD7 directly binds to the target loci, enabling the H3K18 and H3K23 acetylation by IDM1, opening the chromatin in that loci, that are subsequently demethylated by ROS1 (Li et al. 2015) (**Figure 1.5**).

Figure 1.5. Working model for the IDM1-IDM2-IDL1-MBD7 complex functioning in DML mediated active DNA demethylation. Modified from Li et al. 2015.

1.3.5 Active DNA demethylation by DMLs and gene expression

As DNA methylation is a key epigenetic mark involved in many aspects of plant development and stress response (Kumar et al. 2013), it was supposed that active DNA

demethylation could play a significant role in several plant processes. The first approaches to identify the targets of DMLs were carried out in *Arabidopsis thaliana*. Peterman et al. (2007) used a 5'-methylcytosine antibody for DNA precipitation and genome-tilling microarray to identify genomic regions hyper and hypo methylated in *ros1*, *dml2* and *dml3* triple mutant in comparison to WT. *ROS1*, *DML2* and *DML3* are expressed in a wide range of plant organs. They found that the overall methylation levels of WT and the triple mutant were very similar. Despite this, they found 179 discrete loci hypermethylated in the triple mutant in comparison to the WT interspersed among the five chromosomes. Of these loci, over 80% were within or near genes. More specifically, they showed that DMLs primarily demethylate at 5' and 3' of the transcribed region of these genes. Also, DMLs control the dynamic methylated/demethylated status of transposons indicating the putative role of DMLs in the maintenance of the epigenome plasticity (Zhu et al. 2007). In contrast to *ROS1*, *DML2* and *DML3*, DME is preferentially expressed in the central cell of the female gametophyte before fertilization. DME prevents CG hypermethylation of its target genes, thus activating their gene expression. This process is necessary for the activation of the expression of the imprinted genes like *MEDEA* (*MEA*) (Hsieh et al. 2009; Gehring et al. 2009). Gene imprinting refers to the differential allele expression of paternal and maternal alleles depending on parental origin. The loss-of-function mutation of DME results in aberrant endosperm and embryo development because of DNA hypermethylation and down-regulation of the maternal alleles of imprinted genes (Choi et al. 2002). In 2007, Ohr et al. 2007, reported on *CaMV:DME Arabidopsis* plants ectopically expressing DME in pollen and stamens, which after Affymetrix GeneChip arrays analysis, allowed the identification of target genes normally silenced in the male reproductive organs. They found 94 activated genes in both stamen and pollen. The first work showed that the main role of DML is to remove 5mC from genes and the second showed that this activity is important for the transcriptional activation of their targets.

Apart from the more documented gene imprinting mechanism, the biological implication of gene activation carried out by DMLs is now beginning to be understood, and it seems to be very specific in each plant species. Yu et al. (2012) showed that some transposable elements (TE) and repeats are demethylated by *ROS1*, activating the transcription of some immune-response genes, containing TE or repeats in their promoter regions, impairing the multiplication and vascular propagation of the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis* leaves. Also in *Arabidopsis*, Le et al. in 2014, showed that the *ros1*, *dml2* and *dml3* triple mutant is more susceptible to the fungal pathogen *Fusarium oxysporum*. They reported that the downregulated stress response genes were enriched for short transposable elements in their promoter. In the triple mutant, these genes showed an increment in mCG level and reduced mCHH, suggesting that RdDM, necessary for mCHH, may depend on the active DNA

demethylation necessary for the activation of stress response genes. Activation of genes by DMLs is necessary also for abiotic stress responses. In 2015, Bharti et al. showed that *AtROS1* overexpression in tobacco showed tolerance to salt stress, and that the tolerance was correlated with higher expression levels of genes encoding enzymes of the flavonoid biosynthetic and antioxidant pathways. Interestingly, they showed that the differences of methylation levels in their promoter, between *AtROS1* overexpressing plants and WT, are increased under salt stress, indicating that the demethylation activity of *AtROS1*, is increased under stress conditions.

From the point of view of plant development, we have mentioned that *Arabidopsis* DME is necessary for seed viability. Nevertheless, *Arabidopsis ros1*, *dml2*, and *dml3* single, double, or triple mutants do not show any developmental alterations (Gong et al. 2002; Penterman et al. 2007). Recent studies have revealed that DMLs are important regulators of postembryonic developmental programs in other plant species. Several studies suggested the importance of active DNA demethylation in controlling gene expression in tomato. Previous studies indicated that genome cytosine methylation levels decrease by 30% in pericarp of fruits during ripening, although DNA replication is very limited at this stage (Teyssier et al. 2008). Moreover, methylome dynamics in tomato fruit pericarp revealed substantial changes in the distribution of DNA methylation over the tomato genome during fruit development, including demethylation during ripening at specific promoters such as the *NON RIPENING* (*NOR*) and *COLORLESS NON RIPENING* (*CNR*) promoters (Zhong et al. 2013). This observation motivated the study of tomato DMLs and in 2016, Liu et al. found that *SIDML2*, a tomato DML, control the ripening process. *SIDML2* activates the expression of genes necessary of this process, like *RIN* (*RIPENING INHIBITOR*), *NOR* and *CNR* by a direct demethylation in their promoter. In *Medicago truncatula*, the legume–Rhizobium symbiosis leads to the formation of a new organ, the root nodule, involving coordinated and massive induction of specific genes. A demethylases, *MtDME*, is essential for nodule development and regulates the expression of 1,425 genes, some of which are critical for plant and bacterial cell differentiation (Satgé et al. 2016).

1.4 REFERENCES

- Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T. 2005. FD, a bZIP Protein Mediating Signals from the Floral Pathway Integrator FT at the Shoot Apex. *Science* 309: 1052–1056.
- Anderson J V., Horvath DP, Chao WS, Foley ME. 2010. Bud dormancy in perennial plants: A mechanism for survival. *Topics in Current Genetics* 21: 69–90.
- Allona I, Ramos A, Ibañez C, Contreras A, Casado R, Aragoncillo C. 2008. Review. Molecular control of winter dormancy establishment in trees. *Spanish Journal of Agricultural Research* 6: 201–210.
- Azeez A, Miskolczi P, Tylewicz S, Bhalerao RP. 2014. A tree ortholog of APETALA1 mediates photoperiodic control of seasonal growth. *Current Biology* 24: 717–724.
- Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen R a, Dean C. 2004. Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature* 427: 164–167.
- Berrocal-Lobo M, Ibañez C, Acebo P, Ramos A, Perez-Solis E, Collada C, Casado R, Aragoncillo C, Allona I. 2011. Identification of a homolog of Arabidopsis DSP4 (SEX4) in chestnut: Its induction and accumulation in stem amyloplasts during winter or in response to the cold. *Plant, Cell and Environment* 34: 1693–1704.
- Bharti P, Mahajan M, Vishwakarma AK, Bhardwaj J, Yadav SK. 2015. AtROS1 overexpression provides evidence for epigenetic regulation of genes encoding enzymes of flavonoid biosynthesis and antioxidant pathways during salt stress in transgenic tobacco. *Journal of Experimental Botany* 66: 5959–5969.
- Bielenberg DG, Wang Y, Li Z, Zhebentyayeva T, Fan S, Reighard GL, Scorza R, Abbott AG. 2008. Sequencing and annotation of the evergrowing locus in peach [*Prunus persica* (L.) Batsch] reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. *Tree Genetics and Genomes* 4: 495–507.
- Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O. 2006. CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science (New York, N.Y.)* 312: 1040–3.
- Brooks SC, Fischer RL, Huh JH, Eichman BF. 2014. 5-methylcytosine recognition by

Arabidopsis thaliana DNA glycosylases DEMETER and DML3. *Biochemistry* 53: 2525–2532.

Chao WS, Foley ME, Horvath DP, Anderson J V. 2007. Signals regulating dormancy in vegetative buds. *International Journal of Plant Developmental Biology* 1: 49–56.

Choi Y, Gehring M, Johnson L, Hannon M, Harada JJ, Goldberg RB, Jacobsen SE, Fischer RL. 2002. DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in *Arabidopsis*. *Cell* 110: 33–42.

Cooke JEK, Eriksson ME, Junttila O. 2012. The dynamic nature of bud dormancy in trees: Environmental control and molecular mechanisms. *Plant, Cell and Environment* 35: 1707–1728.

Crevillén P, Sonmez C, Wu Z, Dean C. 2013. A gene loop containing the floral repressor FLC is disrupted in the early phase of vernalization. *The EMBO journal* 32: 140–8.

Ding J, Nilsson O. 2016. Molecular regulation of phenology in trees-because the seasons they are a-changin'. *Current Opinion in Plant Biology* 29: 73–79.

Du J, Zhong X, Bernatavichute Y V., Stroud H, Feng S, Caro E, Vashisht AA, Terragni J, Chin HG, Tu A, *et al.* 2012. Dual binding of chromomethylase domains to H3K9me2-containing nucleosomes directs DNA methylation in plants. *Cell* 151: 167–180.

Eriksson ME, Hoffman D, Kaduk M, Mauriat M, Moritz T. 2015. Transgenic hybrid aspen trees with increased gibberellin (GA) concentrations suggest that GA acts in parallel with FLOWERING LOCUS T2 to control shoot elongation. *New Phytologist* 205: 1288–1295.

Eriksson ME, Israelsson M, Olsson O, Moritz T. 2000. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology* 18: 784–788.

Fraga MF, Rodriguez R, Canal MJ. 2002. Genomic DNA methylation-demethylation during aging and reinvigoration of *Pinus radiata*. *Tree Physiology* 22: 813–816.

Frewen BE, Chen THH, Howe GT, Davis J, Antje Rohde, Boerjan W, Bradshaw HD. 2000. Quantitative trait loci and candidate gene mapping of bud set and bud flush in populus. *Genetics* 154: 837–845.

Gehring M, Huh JH, Hsieh TF, Penterman J, Choi Y, Harada JJ, Goldberg RB, Fischer RL. 2006. DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. *Cell* 124: 495–506.

Gong Z, Morales-Ruiz T, Ariza RR, Roldán-Arjona T, David L, Zhu JK. 2002. ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. *Cell* 111: 803–814.

Grillo MA, Colombatto S. 2008. S-adenosylmethionine and its products. *Amino Acids* 34: 187–193.

Heide OM, Prestrud a K. 2005. Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. *Tree physiology* 25: 109–114.

Heo JB, Sung S, Kim DH, Doyle MR, Sung S, Amasino RM, Sung S, Amasino RM, Wood CC, Lucia F De, *et al.* 2011. Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. *Science (New York, N.Y.)* 331: 76–9.

Horvath DP, Anderson J V., Chao WS, Foley ME. 2003. Knowing when to grow: Signals regulating bud dormancy. *Trends in Plant Science* 8: 534–540.

Horvath DP, Sung S, Kim D, Chao W, Anderson J. 2010. Characterization, expression and function of DORMANCY ASSOCIATED MADS-BOX genes from leafy spurge. *Plant Molecular Biology* 73: 169–179.

Howe GT, Bucciaglia PA, Hackett WP, Furnier GR, Cordonnier-Pratt MM, Gardner G. 1998. Evidence that the phytochrome gene family in black cottonwood has one PHYA locus and two PHYB loci but lacks members of the PHYC/F and PHYE subfamilies. *Molecular Biology and Evolution* 15: 160–175.

Howe GT, Horvath DP, Dharmawardhana P, Priest HD, Mockler TC, Strauss SH. 2015. Extensive Transcriptome Changes During Natural Onset and Release of Vegetative Bud Dormancy in Populus. *Frontiers in plant science* 6: 1–28.

Hsieh T-F, Ibarra CA, Silva P, Zemach A, Eshed-Williams L, Fischer RL, Zilberman D. 2009. Genome-wide demethylation of Arabidopsis endosperm. *Science (New York, N.Y.)* 324: 1451–4.

Hsu C-Y, Adams JP, Kim H, No K, Ma C, Strauss SH, Drnevich J, Vandervelde L, Ellis JD, Rice BM, *et al.* 2011. FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. *Proceedings of the National Academy of Sciences of the United States of America* 108: 10756–61.

Ibañez C, Ramos A, Acebo P, Contreras A, Casado R, Allona I, Aragoncillo C. 2008. Overall alteration of circadian clock gene expression in the chestnut cold response. *PLoS ONE*

3.

Ibáñez C, Kozarewa I, Johansson M, Ogren E, Rohde A, Eriksson ME. 2010. Circadian clock components regulate entry and affect exit of seasonal dormancy as well as winter hardiness in *Populus* trees. *Plant physiology* 153: 1823–33.

Ingvarsson PK, García MV, Hall D, Luquez V, Jansson S. 2006. Clinal variation in phyB2, a candidate gene for day-length-induced growth cessation and bud set, across a latitudinal gradient in European aspen (*Populus tremula*). *Genetics* 172: 1845–1853.

Jiménez S, Lawton-Rauh AL, Reighard GL, Abbott AG, Bielenberg DG. 2009. Phylogenetic analysis and molecular evolution of the dormancy associated MADS-box genes from peach. *BMC Plant Biology* 9: 81.

Jiménez S, Reighard GL, Bielenberg DG. 2010. Gene expression of DAM5 and DAM6 is suppressed by chilling temperatures and inversely correlated with bud break rate. *Plant Molecular Biology* 73: 157–167.

Kakutani T, Jeddeloh J a, Richards EJ. 1995. Characterization of an *Arabidopsis thaliana* DNA hypomethylation mutant. *Nucleic acids research* 23: 130–137.

Karlberg A, Bako L, Bhalerao RP. 2011. Short day-mediated cessation of growth requires the downregulation of AINTEGUMENTALIKE1 transcription factor in hybrid aspen. *PLoS Genetics* 7.

Karlberg A, Englund M, Petterle A, Molnar G, Sjödin A, Bako L, Bhalerao RP. 2010. Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology* 27: 1–16.

Kaufmann K, Wellmer F, Muiño JM, Ferrier T, Wuest SE, Kumar V, Serrano-Mislata A, Madueño F, Krajewski P, Meyerowitz EM, *et al.* 2010. Orchestration of floral initiation by APETALA1. *Science (New York, N.Y.)* 328: 85–9.

Kim SY, He Y, Jacob Y, Noh Y-S, Michaels S, Amasino R. 2005. Establishment of the vernalization-responsive, winter-annual habit in *Arabidopsis* requires a putative histone H3 methyl transferase. *The Plant cell* 17: 3301–10.

Ko JH, Prassinis C, Keathley D, Han KH. 2011. Novel aspects of transcriptional regulation in the winter survival and maintenance mechanism of poplar. *Tree Physiology* 31: 208–225.

Kozarewa I, Ibáñez C, Johansson M, Ögren E, Mozley D, Nylander E, Chono M, Moritz T, Eriksson ME. 2010. Alteration of PHYA expression change circadian rhythms and timing of bud set in *Populus*. *Plant Molecular Biology* 73: 143–156.

Kumar S, Kumari R, Sharma V, Sharma V. 2013. Roles, and establishment, maintenance and erasing of the epigenetic cytosine methylation marks in plants. *Journal of Genetics* 92: 629–666.

Kumar G, Rattan UK, Singh AK. 2016. Chilling-Mediated DNA Methylation Changes during Dormancy and Its Release Reveal the Importance of Epigenetic Regulation during Winter Dormancy in Apple (*Malus x domestica* Borkh.). *Plos One* 11: e0149934.

Kumar SV, Wigge PA. 2010. H2A.Z-Containing Nucleosomes Mediate the Thermosensory Response in *Arabidopsis*. *Cell* 140: 136–147.

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 Phytologist* 197: 416–430.

Lang G, Early J, Martin G, Darnell R. 1987. Endo-, para-, and ecodormancy: physiological terminology and classification for dormancy research. *HortScience* 22: 371–377.

Law JA, Jacobsen SE. 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nature Reviews. Genetics* 11: 204–220.

Le T-N, Schumann U, Smith NA, Tiwari S, Au P, Zhu Q-H, Taylor JM, Kazan K, Llewellyn DJ, Zhang R, *et al.* 2014. DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in *Arabidopsis*. *Genome Biology* 15: 458.

Lee J, Jang H, Shin H, Choi WL, Mok YG, Huh JH. 2014. AP endonucleases process 5-methylcytosine excision intermediates during active DNA demethylation in *Arabidopsis*. *Nucleic Acids Research* 42: 11408–11418.

Leida C, Conesa A, Llácer G, Badenes ML, Ríos G. 2012. Histone modifications and expression of DAM6 gene in peach are modulated during bud dormancy release in a cultivar-dependent manner. *New Phytologist* 193: 67–80.

Li Y, Córdoba-Cañero D, Qian W, Zhu X, Tang K, Zhang H, Ariza RR, Roldán-Arjona T, Zhu JK. 2015a. An AP Endonuclease Functions in Active DNA Dimethylation and Gene

Imprinting in Arabidopsis. *PLoS Genetics* 11.

Li C, Puhakainen T, Welling A, Viherä-Aarnio A, Ernstsén A, Junttila O, Heino P, Palva ET. 2002. Cold acclimation in silver birch (*Betula pendula*). Development of freezing tolerance in different tissues and climatic ecotypes. *Physiologia Plantarum* 116: 478–488.

Li Z, Reighard GL, Abbott AG, Bielenberg DG. 2009. Dormancy-associated MADS genes from the EVG locus of peach [*Prunus persica* (L.) Batsch] have distinct seasonal and photoperiodic expression patterns. *Journal of Experimental Botany* 60: 3521–3530.

Li Q, Wang X, Sun H, Zeng J, Cao Z, Li Y, Qian W. 2015b. Regulation of Active DNA Demethylation by a Methyl-CpG-Binding Domain Protein in *Arabidopsis thaliana*. *PLoS genetics* 11: e1005210.

Liang D, Zhang Z, Wu H, Huang C, Shuai P, Ye C-Y, Tang S, Wang Y, Yang L, Wang J, *et al.* 2014. Single-base-resolution methylomes of *Populus trichocarpa* reveal the association between DNA methylation and drought stress. *BMC genetics* 15 Suppl 1: S9.

Liu R, How-Kit A, Stammiti L, Teyssier E, Rolin D, Mortain-Bertrand A, Halle S, Liu M, Kong J, Wu C, *et al.* 2015. A DEMETER-like DNA demethylase governs tomato fruit ripening. *Proceedings of the National Academy of Sciences of the United States of America* 112: 10804–9.

Luo, J and Kuo M-H. 2009. Linking nutrient metabolism to epigenetics. *Cell Science*.

Matzke MA, Mosher RA. 2014. RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nature Reviews Genetics* 15: 394–408.

Mckown AD, Klápště J, Guy RD, Geraldés A, Porth I, Hannemann J, Friedmann M, Muchero W, Tuskan GA, Ehlting J, *et al.* 2014. Genome-wide association implicates numerous genes underlying ecological trait variation in natural populations of *Populus trichocarpa*. *New Phytologist* 203: 535–553.

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. *Journal of Plant Physiology* 166: 1624–1636.

Mimida N, Komori S, Suzuki A, Wada M. 2013. Functions of the apple TFL1/FT orthologs in phase transition. *Scientia Horticulturae* 156: 106–112.

Mirouze M, Lieberman-Lazarovich M, Aversano R, Bucher E, Nicolet J, Reinders J, Paszkowski J. 2012. Loss of DNA methylation affects the recombination landscape in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* 109: 5880–5.

Mohamed R, Wang CT, Ma C, Shevchenko O, Dye SJ, Puzey JR, Etherington E, Sheng X, Meilan R, Strauss SH, *et al.* 2010. *Populus* CEN/TFL1 regulates first onset of flowering, axillary meristem identity and dormancy release in *Populus*. *Plant Journal* 62: 674–688.

Mok YG, Uzawa R, Lee J, Weiner GM, Eichman BF, Fischer RL, Huh JH. 2010. Domain structure of the DEMETER 5-methylcytosine DNA glycosylase. *Proceedings of the National Academy of Sciences of the United States of America* 107: 19225–30.

Moreno-Cortés A, Hernández-Verdeja T, Sánchez-Jiménez P, González-Melendi P, Aragoncillo C, Allona I. 2012. CsRAV1 induces sylleptic branching in hybrid poplar. *New Phytologist* 194: 83–90.

Niederhuth CE, Schmitz RJ. 2016. Putting DNA methylation in context: from genomes to gene expression in plants. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*.

O'Malley RC, Huang SC, Song L, Lewsey MG, Bartlett A, Nery JR, Galli M, Gallavotti A, Ecker JR. 2016. Cistrome and Epicistrome Features Shape the Regulatory DNA Landscape. *Cell* 165: 1280–1292.

Ohr H, Bui AQ, Le BH, Fischer RL, Choi Y. 2007. Identification of putative *Arabidopsis* DEMETER target genes by GeneChip analysis. *Biochemical and Biophysical Research Communications* 364: 856–860.

Oliver SN, Finnegan EJ, Dennis ES, Peacock WJ, Trevaskis B. 2009. Vernalization-induced flowering in cereals is associated with changes in histone methylation at the VERNALIZATION1 gene. *Proceedings of the National Academy of Sciences of the United States of America* 106: 8386–91.

Olsen JE, Junttila O, Nilsen J, Eriksson ME, Martinussen I, Olsson O, Sandberg G, Moritz T. 1997. Ectopic expression of oat phytochrome A in hybrid aspen changes critical daylength for growth and prevents cold acclimatization. *Plant Journal* 12: 1339–1350.

Parrilla-Doblas JT, Ponferrada-Marín MI, Roldán-Arjona T, Ariza RR. 2013. Early steps of active DNA demethylation initiated by ROS1 glycosylase require three putative helix-

invading residues. *Nucleic Acids Research* 41: 8654–8664.

Paul LK, Rinne PLH, Van der Schoot C. 2014. Shoot meristems of deciduous woody perennials: Self-organization and morphogenetic transitions. *Current Opinion in Plant Biology* 17: 86–95.

Penterman J, Zilberman D, Huh JH, Ballinger T, Henikoff S, Fischer RL. 2007. DNA demethylation in the Arabidopsis genome. *Proceedings of the National Academy of Sciences of the United States of America* 104: 6752–6757.

Perry TO. 1971. Dormancy of trees in winter. *Science (New York, N.Y.)* 171: 29–36.

Ponferrada-Marín MI, Martínez-Macías MI, Morales-Ruiz T, Roldán-Arjona T, Ariza RR. 2010. Methylation-independent DNA binding modulates specificity of repressor of silencing 1 (ROS1) and facilitates demethylation in long substrates. *Journal of Biological Chemistry* 285: 23032–23039.

Ponferrada-Marín MI, Parrilla-Doblas JT, Roldán-Arjona T, Ariza RR. 2011. A discontinuous DNA glycosylase domain in a family of enzymes that excise 5-methylcytosine. *Nucleic Acids Research* 39: 1473–1484.

Ponferrada-Marín MI, Roldán-Arjona T, Ariza RR. 2009. ROS1 5-methylcytosine DNA glycosylase is a slow-turnover catalyst that initiates DNA demethylation in a distributive fashion. *Nucleic Acids Research* 37: 4264–4274.

Qian W, Miki D, Lei M, Zhu X, Zhang H, Liu Y, Li Y, Lang Z, Wang J, Tang K, *et al.* 2014. Regulation of active DNA Demethylation by an α -crystallin domain protein in Arabidopsis. *Molecular Cell* 55: 361–371.

Qian W, Miki D, Zhang H, Liu Y, Zhang X, Tang K, Kan Y, La H, Li X, Li S, *et al.* 2012. A histone acetyltransferase regulates active DNA demethylation in Arabidopsis. *Science (New York, N.Y.)* 336: 1445–8.

Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M. 1999. DNA methylation is a reversible biological signal. *Proceedings of the National Academy of Sciences of the United States of America* 96: 6107–6112.

Ramos A, Pérez-Solís E, Ibáñez C, Casado R, Collada C, Gómez L, Aragoncillo C, Allona I. 2005. Winter disruption of the circadian clock in chestnut. *Proceedings of the National Academy of Sciences of the United States of America* 102: 7037–42.

Richards EJ, Elgin SCR. 2002. Epigenetic codes for heterochromatin formation and silencing: Rounding up the usual suspects. *Cell* 108: 489–500.

Rinne PLH, Kaikuranta PM, Van Schoot C Der. 2001. The shoot apical meristem restores its symplasmic organization during chilling-induced release from dormancy. *Plant Journal* 26: 249–264.

Rinne PL, Schoot C Van Der. 2003. Plasmodesmata at the crossroads between development, dormancy, and defense. *Canadian Journal of Botany* 81: 1182–1197.

Rinne PL, van der Schoot C. 1998. Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenetic events. *Development (Cambridge, England)* 125: 1477–1485.

Rodriguez-A J, Sherman WB, Scorza R, Wisniewski M, Okie WR. 1994. ‘Evergreen’ peach, its inheritance and dormant behavior. *Journal of the American Society for Horticultural Science* 119: 789–792.

Rohde A, Bastien C, Boerjan W. 2011. Temperature signals contribute to the timing of photoperiodic growth cessation and bud set in poplar. *Tree Physiology* 31: 472–482.

Rohde A, Bhalerao RP. 2007. Plant dormancy in the perennial context. *Trends in Plant Science* 12: 217–223.

Rohde A, Prinsen E, De Rycke R, Engler G, Van Montagu M, Boerjan W. 2002. PtABI3 impinges on the growth and differentiation of embryonic leaves during bud set in poplar. *The Plant cell* 14: 1885–901.

Ruonala R, Rinne PLH, Baghour M, Moritz T, Tuominen H, Kangasjärvi J. 2006. Transitions in the functioning of the shoot apical meristem in birch (*Betula pendula*) involve ethylene. *Plant Journal* 46: 628–640.

Ruonala R, Rinne PLH, Kangasjärvi J, van der Schoot C. 2008. CENL1 expression in the rib meristem affects stem elongation and the transition to dormancy in *Populus*. *The Plant cell* 20: 59–74.

Ruttink T, Arend M, Morreel K, Storme V, Rombauts S, Fromm J, Bhalerao RP, Boerjan W, Rohde A. 2007. A molecular timetable for apical bud formation and dormancy induction in poplar. *The Plant cell* 19: 2370–90.

Saito T, Bai S, Ito A, Sakamoto D, Saito T, Ubi BE, Imai T, Moriguchi T. 2013.

Expression and genomic structure of the dormancy-associated MADS box genes MADS13 in Japanese pears (*Pyrus pyrifolia* Nakai) that differ in their chilling requirement for endodormancy release. *Tree Physiology* 33: 654–667.

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

Santamaría ME, Rodríguez R, Cañal MJ, Toorop PE. 2011. Transcriptome analysis of chestnut (*Castanea sativa*) tree buds suggests a putative role for epigenetic control of bud dormancy. *Annals of Botany* 108: 485–498.

Sasaki R, Yamane H, Ooka T, Jotatsu H, Kitamura Y, Akagi T, Tao R. 2011. Functional and Expressional Analyses of PmDAM Genes Associated with Endodormancy in Japanese Apricot. *Plant physiology* 157: 485–497.

Satgé C, Moreau S, Sallet E, Lefort G, Auriac M-C, Remblière C, Cottret L, Gallardo K, Noirot C, Jardinaud M-F, *et al.* 2016. Reprogramming of DNA methylation is critical for nodule development in *Medicago truncatula*. *Nature Plants* 2: 16166.

Saze H, Tsugane K, Kanno T, Nishimura T. 2012. DNA methylation in plants: Relationship to small rnas and histone modifications, and functions in transposon inactivation. *Plant and Cell Physiology* 53: 766–784.

Singh RK, Svystun T, Aldahmash B, Maria A, Bhalerao RP. 2016. Photoperiod- and temperature-mediated control of phenology in trees – a molecular perspective. *New Phytologist*: doi: 10.1111/nph.14346.

Schrader J, Moyle R, Bhalerao R, Hertzberg M, Lundeberg J, Nilsson P, Bhalerao RP. 2004. Cambial meristem dormancy in trees involves extensive remodelling of the transcriptome. *Plant Journal* 40: 173–187.

Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ, Dennis ES. 2000. The molecular basis of vernalization: the central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences of the United States of America* 97: 3753–3758.

Shim D, Ko J-H, Kim W-C, Wang Q, Keathley DE, Han K-H. 2014. A molecular framework for seasonal growth-dormancy regulation in perennial plants. *Horticulture Research* 1: 14059.

Swiezewski S, Liu F, Magusin A, Dean C. 2009. Cold-induced silencing by long antisense transcripts of an Arabidopsis Polycomb target. *Nature* 462: 799–802.

Takahashi H, McCaffery JM, Irizarry RA, Boeke JD. 2006. Nucleocytosolic Acetyl-Coenzyme A Synthetase Is Required for Histone Acetylation and Global Transcription. *Molecular Cell* 23: 207–217.

Teixeira FK, Colot V. 2010. Repeat elements and the Arabidopsis DNA methylation landscape. *Heredity* 105: 14–23.

Teyssier E, Bernacchia G, Maury S, How Kit A, Stammitti-Bert L, Rolin D, Gallusci P. 2008. Tissue dependent variations of DNA methylation and endoreduplication levels during tomato fruit development and ripening. *Planta* 228: 391–399.

Trevaskis B, Tadege M, Hemming MN, Peacock WJ, Dennis ES, Sheldon C. 2007. Short Vegetative Phase-like MADS-box genes inhibit floral meristem identity in barley. *Plant Physiology* 143: 225–235.

Tuan PA, Bai S, Saito T, Imai T, Ito A, Moriguchi T. 2016. Involvement of EARLY BUD-BREAK, an AP2/ERF transcription factor gene, in bud break in Japanese pear (*Pyrus pyrifolia* Nakai) lateral flower buds: expression, histone modifications and possible target genes. *Plant Cell Physiology* 57: 1038–1047.

Turck F, Coupland G. 2011. When vernalization makes sense. *Science* 331: 36–37.

Tylewicz S, Tsuji H, Miskolczi P, Petterle A, Azeez A, Jonsson K, Shimamoto K, Bhalerao RP. 2015. Dual role of tree florigen activation complex component FD in photoperiodic growth control and adaptive response pathways. *Proceedings of the National Academy of Sciences of the United States of America* 112: 3140–5.

Ubi BE, Sakamoto D, Ban Y, Shimada T, Ito A, Nakajima I, Takemura Y, Tamura F, Saito T, Moriguchi T. 2010. Molecular Cloning of Dormancy-associated MADS-box Gene Homologs and Their Characterization during Seasonal Endodormancy Transitional Phases of Japanese Pear. *Journal of the American Society for Horticultural Science* 135: 174–182.

Van Der Schoot C, Paul LK, Rinne PLH. 2014. The embryonic shoot: A lifeline through winter. *Journal of Experimental Botany* 65: 1699–1712. New Phytologist: doi: 10.1111/nph.14346

Vince-Prue D. 1986. The duration of light and photoperiodic responses.

Photomorphogenesis in plants.269–305.

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.

Wareing PF. 1956. Photoperiodism in Woody Plants. *Annual Review of Plant Physiology* 7: 191–214.

Welling A, Palva ET. 2006. Molecular control of cold acclimation in trees. *Physiologia Plantarum* 127: 167–181.

Wells CE, Vendramin E, Tarodo SJ, Verde I, Bielenberg DG. 2015. A genome-wide analysis of MADS-box genes in peach [*Prunus persica* (L.) Batsch]. *BMC Plant Biology* 15: 41.

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D. 2005. Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science (New York, N.Y.)* 309: 1056–9.

Wu RM, Walton EF, Richardson AC, Wood M, Hellens RP, Varkonyi-Gasic E. 2012. Conservation and divergence of four kiwifruit SVP-like MADS-box genes suggest distinct roles in kiwifruit bud dormancy and flowering. *Journal of Experimental Botany* 63: 797–807.

Yanovsky MJ, Kay S a. 2003. Living by the calendar: how plants know when to flower. *Nature reviews. Molecular cell biology* 4: 265–275.

Yordanov YS, Ma C, Strauss SH, Busov VB. 2014. EARLY BUD-BREAK 1 (EBB1) is a regulator of release from seasonal dormancy in poplar trees. *Proceedings of the National Academy of Sciences of the United States of America* 111: 10001–10006.

Yu A, Lepère G, Jay F, Wang J, Bapaume L, Wang Y, Abraham A-L, Penterman J, Fischer RL, Voinnet O, *et al.* 2013. Dynamics and biological relevance of DNA demethylation in *Arabidopsis* antibacterial defense. *Proceedings of the National Academy of Sciences of the United States of America* 110: 2389–94.

Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SWL, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, *et al.* 2006. Genome-wide High-Resolution Mapping and Functional Analysis of DNA Methylation in *Arabidopsis*. *Cell* 126: 1189–1201.

Zheng X, Pontes O, Zhu J, Miki D, Zhang F, Li W-X, Iida K, Kapoor A, Pikaard CS, Zhu J-K. 2008. ROS3 is an RNA-binding protein required for DNA demethylation in

Arabidopsis. *Nature* 455: 1259–1262.

Zhong S, Fei Z, Chen Y-R, Zheng Y, Huang M, Vrebalov J, McQuinn R, Gapper N, Liu B, Xiang J, *et al.* 2013a. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nature biotechnology* 31: 154–9.

Zhong X, Zhang H, Zhao Y, Sun Q, Hu Y, Peng H, Zhou DX. 2013b. The Rice NAD⁺-Dependent Histone Deacetylase OsSRT1 Targets Preferentially to Stress- and Metabolism-Related Genes and Transposable Elements. *PLoS ONE* 8.

Zhu J, Kapoor A, Sridhar V V., Agius F, Zhu JK. 2007. The DNA Glycosylase/Lyase ROS1 Functions in Pruning DNA Methylation Patterns in Arabidopsis. *Current Biology* 17: 54–59.

Zluvova J, Janousek B, Vyskot B. 2001. Immunohistochemical study of DNA methylation dynamics during plant development. *Journal of experimental botany* 52: 2265–2273.

2. OBJECTIVES

The principal aim of this PhD project was to understand the involvement of DNA methylation in the control of the annual cycle in trees, and the molecular characterization of hybrid poplar *DEMETER like* genes to evaluate their role in tree development. With this purpose, the following objectives were established:

1. To assess a putative epigenetic control of winter-dormancy transitions by a fluorescence-based method in vibratome-thick sections from hybrid poplar stems followed by the quantification of the signal in a representative population of individual nuclei on a confocal microscope, to detect epigenetic marks of closed and open chromatin states.
2. To determine the global DNA methylation profile in the hybrid poplar SAM from the dormant state to the time point of bud break in trees growing under natural conditions, to relate the dynamics of DNA methylation with the control of dormancy-active growth transitions in the apical meristem.
3. To identify and characterize hybrid poplar *DEMETER*-like proteins to explore their role during the annual cycle.
4. To characterize a chestnut *DEMETER*-like protein, which had been previously found in our laboratory in a winter subtractive hybridization, to determine its role during the annual cycle.

3. POPLAR STEMS SHOW OPPOSITE EPIGENETIC PATTERNS DURING WINTER DORMANCY AND VEGETATIVE GROWTH¹

3.1 ABSTRACT

In this study the identification of epigenetic marks of closed and open chromatin states has been performed by immunofluorescence in vibratome-thick sections from poplar stems, followed by quantification of the signal in a representative population of individual nuclei on a confocal microscopy. Unlike other methods often used for detection of global DNA methylation levels, the procedure hereby proposed can be applied to a wide range of specimens regardless of the purity and yield of genomic DNA extraction and in a tissue-dependent manner. Using antibodies to 5-methylcytidine and acetylated lysine 8 of histone H4, as reliable indicators of transcription incompatible and compatible chromatin states respectively, the influence of epigenetic regulation on differential gene expression during the growth and arrest periods in xylem and phloem tissues was assessed. The fluorescence signal of 5-methylcytosine was significantly higher in winter as compared to summer. Conversely, the fluorescence signal for acetylated Lys 8 of histone H4 was significantly higher in summer than in winter in both tissues examined. Collectively, these results put forward an epigenetic control of winter dormancy in poplar stems.

Keywords: epigenetics, immunofluorescence, DNA methylation, acetylated histone H4, poplar, winter dormancy

¹ This chapter reproduces the text of the paper: Conde D, González-Melendi P, Allona I. 2013. Poplar stems show opposite epigenetic patterns during winter dormancy and vegetative growth. *Trees - Structure and Function* 27: 311–320.

3.2 INTRODUCTION

Plant development is extremely adaptable, reacting to changes in environmental conditions through a complex network of response mechanisms to abiotic and biotic stresses. Winter dormancy is the strategy used by perennial plants to survive the harsh conditions of winter in temperate and cold regions and is a major determinant of the ecological adaptability, distribution and reproductive success (Chuine & Beaubien 2001; Horvath et al. 2003; Allona et al. 2008). In long-lived forest species, the length of the dormancy period limits the growing season, affecting wood production and quality. The complex mechanism of dormancy commences with an initial stage called ecodormancy, when the growth of buds and cambium meristems is arrested under given environmental factors induced by short days (SD). In this period, the plant forms autumn buds and, simultaneously, develops moderate cold tolerance. However, in some species of the Rosaceae family, the establishment of winter dormancy cannot be considered strictly photoperiodic and low temperatures, with or without short day lengths, may also initiate the dormancy process (Wareing 1956; Battey 2000; Heide & Prestrud 2005). The second stage, brought about by endogenous plant factors within the dormant tissue, is called endodormancy. During this time, leaf senescence and abscission occur in deciduous trees and the plants show their maximum adaptation to the cold. After this period, a chilling requirement needs to be fulfilled for growth to resume. These two states, ecodormancy and endodormancy, were described by Lang in 1987.

The CONSTANS/FLOWERING LOCUS T (*CO/FT*) regulatory module, which controls flowering in *Arabidopsis* in response to day-length variations (Hayama & Coupland 2003), besides playing the same role in aspen trees also controls growth cessation (Böhlenius et al. 2006). *CO* transcription, which is regulated by the circadian clock, peaks at day light in long days (LD) and at night in short days (SD). Since the CO protein is markedly labile in darkness, its accumulation is prevented during the night. For this reason, CO induces the expression of FT only in LD conditions and, consequently, flowering is not induced in SD conditions (Suárez-López et al. 2001; Yanovsky & Kay 2002; Sawa et al. 2007). However, the role played by low temperatures in the establishment of winter dormancy may differ in trees compared to *Arabidopsis*, since disruption of the circadian clock has been reported when chestnut seedlings are exposed to a period (1 week) of cold treatment (4°C) (Bieniawska et al. 2008; Ibáñez et al. 2008; Ramos et al. 2005). In addition, Ibáñez et al. (2010) recently reported that freezing tolerance and the expression of *Populus CBF1* are compromised by the down-regulation of *Populus* LHY genes. Temperature is also known to affect bud formation in poplar trees (Penfield 2008; Rohde et al. 2011).

Gene expression can also be controlled epigenetically by dynamic switches between transcriptionally compatible and incompatible chromatin states. Methylation of DNA cytosine residues and lysine acetylation of histones constitute reliable epigenetic marks of different functional chromatin forms (Finnegan et al. 1998; Wagner 2003; Tariq & Paszkowski 2004; Berdasco et al. 2008; Feil & Fraga 2012). Methylation of cytosine in the 5' position of the pyrimidine ring, which requires the activity of MET1, DRM2 or CMT3 methyltransferases, depending on the targets, promotes closed chromatin states and therefore prevents the binding of transcription factors and other proteins to DNA (Finnegan et al. 2000). Plant DNA methylation can occur in different cytosine contexts (CG, CHG and CHH, where H can be A, C or T) (Vining et al. 2012). Methylation/demethylation of DNA is a dynamic and reversible process, which responds to exogenous and endogenous factors (Ramchandani et al. 1999). Interactions between different types of histone post-translational modifications, the so-called histone code, also affect gene expression. Among these, the acetylation of Lys 8 of histone H4 is a hallmark of euchromatin (potentially active) (Jenuwein & Allis 2001; Richards & Elgin 2002). In this context, the vernalization-mediated epigenetic silencing of FLC is required to trigger flowering in *Arabidopsis* (Amasino 2004) and DNA demethylation and histone acetylation precede dormancy exit in potato tubers (Law & Suttle 2003).

An epigenetic regulation of the transitions into and out of dormancy has been proposed (reviewed by Horvath et al. 2003; Rohde & Bhalerao 2007). The expression of the DORMANCY ASSOCIATED MADS-BOX (DAM) genes during endodormancy is associated to trimethylation of Lys 4 of histone H3, an epigenetic mark of transcribed genes, whereas its repression involves trimethylation of Lys 27 of histone H3 (Horvath et al. 2010). Global DNA methylation changes have been determined and quantified by high performance capillary electrophoresis (HPCE) after genomic DNA extraction in different plant materials: chesnut bud set and burst (Santamaría et al. 2009), flowering in azalea (Meijón et al. 2009), aging in pine trees (Valledor et al. 2007) and undifferentiated *Arabidopsis* cells (Berdasco et al. 2008). Recently, a thorough survey of DNA methylation profiles by MeDIP-seq followed by bisulfite sequencing in poplar vegetative and reproductive tissues has revealed a lower gene expression when methylation occurred in the body of the gene than in the promoter (Vining et al. 2012).

Extraction of genomic DNA of sufficient yield and purity for HPCE analysis is highly depending on the starting material. In this work, we propose an immunofluorescence-based method for the detection of epigenetic marks using specific antibodies followed by the systematic quantification of the fluorescence signal from a representative sample size of single nuclei in confocal microscopy images. This procedure hereby proposed can be applied to a wide range of specimens and in a tissue-dependent manner. This method appears as reliable and

reproducible to examine the influence of epigenetic modifications in controlling winter dormancy in poplar tree stems.

3.3 MATERIAL AND METHODS

Plant material

The plant material for the study of epigenetic marks was obtained from 2-year old branches of four-year-old hybrid poplar tremula x alba INRA 717 1B4 trees planted in the Centre for Plant Biotechnology and Genomics (CBGP) in Pozuelo de Alarcón, Madrid (3°49'W, 40°24'N). Average temperatures during harvesting were 3.6°C (20 December 2010) and 22.5°C (21 June 2011). Plant material for the detection of pectins was obtained from 2-year-old branches of thirty-year-old *Populus alba* trees planted in the Forestry Engineering School of Madrid (3°43'W, 40°26'N). Average temperatures during harvesting were 7.2°C (20 December 2009) and 21.2°C (26 June 2010).

Fixation and sectioning

Straight after their collection in a water-containing Erlenmeyer flask, the branches were chopped off in 0.5-cm long portions and immediately immersed in a freshly prepared solution of 4% formaldehyde in PBS (phosphate buffered saline: 137 mM NaCl; 0.27 mM KCl; 1 mM phosphate buffer, pH 7.4). The formaldehyde solution was made up from paraformaldehyde powder by continuous heating and stirring until a clear solution was obtained. This solution can be stored in aliquots at -20°C avoiding repeated freezing. Fixation was performed under vacuum followed by o/n at 4°C. The samples were kept at 4°C in a 0.1% solution of formaldehyde in PBS until further use.

Formaldehyde-fixed 0.5-cm long portions of poplar branches were sectioned (40-50 µm thick) under water using a vibratome (1000 plus). The sections were then dried down onto 24-well plates and stored at -20°C.

Structural analysis

To characterize the structure of the plant material and identify the different stem tissues, 40-50 µm-thick sections were stained either with a 1% solution of toluidine blue O for the general structure (Panreac 253535.1210), 2.5% phloroglucinol-HCl for lignin (Sigma P3502) or calcofluor white for cellulose (Sigma 18909). Toluidine blue and phloroglucinol-stained sections were observed on a Zeiss Axiophot microscope under bright field and photographed with a Leica DFC 300FX CCD colour camera. Stacks of calcofluor-stained sections were collected on a Zeiss 710 confocal microscope under the laser excitation line of 405 nm. A

second approach was the detection of natural autofluorescence to highlight structural references and in this way identify the locations of the different stem tissues in the immunofluorescence assays. For this, the stacks of confocal sections were obtained under the laser excitation lines of 405, 488 and 543 nm.

Immunofluorescence

Frozen 40-50 μm thick sections stored at -20°C were thawed at room temperature and submerged into PBS in 24-well plates. The sections were permeabilized by dehydration/rehydration steps in the graded methanol series: 30, 50, 70, 90 and 100%, for 5 minutes each and then in PBS for 5 minutes. The cell walls were partially digested with 2% cellulase (Onozuka-R10) in PBS for 2 h at room temperature, followed by two washes in PBS for 10 minutes each. When detecting methyl-esterified pectins, this step was substituted by treatment with 0.1% tween 20 in PBS for 15 minutes to preserve the integrity of the cell wall. After cellulase digestion, sections to be incubated with the antibody to detect DNA methylation sites were subjected to an additional treatment with 2N HCl for 30 minutes, to expose the epitopes, followed by three washes in PBS for 10 minutes each. For all three primary antibodies used, unspecific binding was blocked with a 5% BSA (Bovine Serum Albumin, Sigma A2153) solution in PBS for 20 minutes. The sections were then transferred to 10-well slides (Fisher Scientific Inc., Pittsburgh, USA) and incubated with either the mouse monoclonal antibody against 5-methylcytidine (BI-MECY-0100, EUROGENTEC), the rabbit anti-acetylated lysine 8 of histone H4 antibody (Abcam, 15823) or the rat JIM7 antibody to methyl-esterified pectins (Knox et al. 1990). The antibodies used to detect methylated DNA and the acetylated lysine8 of H4 were applied at a 1/50 dilution and the samples incubated overnight at 4°C in a humid chamber. The JIM7 antibody was used undiluted and applied for 1 hour at room temperature. The corresponding secondary antibodies (anti-rat 488, anti-rabbit 488 and anti-mouse 546, Alexa, Molecular Probes) were added at a dilution 1/25 in 3% BSA in PBS for 1 hour in darkness. After three washes in PBS for 10 minutes each, the samples were mounted on slides with VectaShield (Vector laboratories, Inc, Burlingame, CA 94010) to preserve the fluorescence. Next, the preparations were observed under the fluorescence microscope (Zeiss AxioPhot) under the blue (ex 450-490, FT 510, em LP 520) light filter set (for the visualization of pectins) and the confocal microscope (Zeiss 710) under the wavelength excitation lines of 488 nm and 543 nm, for the visualization of acetylated lysine 8 of histone H4 and 5-methylcytidine coupled to Alexa 488 and Alexa 546, respectively. A PL APO 63x/1.4 objective was used. The fluorescence microscopy images were captured using a Leica CCD colour camera DFC 300FX, through the programme Leica Application Suite 2.8.1 build 1554. For confocal series capture, the same settings (pinhole size, gain, offset and laser intensity) were

used in all the experiments performed using the same primary antibody (anti-5 methylcytidine or anti-acetylated Lys 8 of histone H4).

Immunofluorescence quantification

Vibratome sections of plant tissues can show overlapping nuclei from different cell layers. This produces blurred images when observed under a fluorescence microscope. Thus, to isolate and accurately quantify the fluorescence emitted by individual nuclei, the immunolabelled sections were examined by confocal microscopy. This eliminates the out-of-focus signals and permits the identification of complete nuclei by examining consecutive images of the Z-series obtained.

Using the ZEN 2009 Light Edition package (Carl Zeiss MicroImaging GmbH 2006-2009), projections of maximum fluorescence images from each Z-series were obtained and used to quantify fluorescence. These images were analysed using ImageJ 1.43 software (Wayne Rashand, National Institutes of Health, USA, <http://imagej.nih.gov/ij>). The contour of each nucleus was manually outlined and the average fluorescence intensity per area was obtained. This is the sum of the fluorescence on each pixel within the outlined area (a number from 0 to 255 per pixel) divided by the total number of pixels of this area. The data collected were exported to Microsoft Excel for statistical analysis. The total fluorescence intensity was represented in histograms.

Statistical analysis

DNA methylation and histone acetylation levels were compared by two-way repeated measures ANOVA followed by the Holm-Sidak test using season and tissue examined (xylem or phloem) as factors. All statistical tests were performed using Sigma Stat 3.0.1 software.

3.4 RESULTS

Structural analysis

As a control of tissue fixation and preservation, some histochemical analyses were performed on the sections. The general anatomy of the sections was examined by toluidine blue staining (**Fig. 3.1a**). Xylem parenchymal radial cells were identified among the xylem vessels as dark blue rows. The purple-stained phloem area was flanked by the inwards vascular cambial meristem and the outwards pale-bluish sclerenchyma. In the outermost regions of the stem, collenchymal and epidermal tissues were clearly identified. Specific dyes were also used to detect the presence of lignin and cellulose in the sections. Phloroglucinol stained the lignified xylem vessels and the sclerenchyma fibres (**Fig. 3.1b**). Calcofluor white fluorescence detected

cellulose mainly in the non-lignified areas (**Fig. 3.1c**). Lignin-containing structures appeared dark under 405 nm light excitation due the quenching of autofluorescence by the presence of Evan's blue in the commercial staining solution. These observations indicated the good preservation of the tissues used in the immunofluorescence experiments conducted on specimens obtained in winter (**Fig. 3.1**) and summer (data not shown). Although xylem and phloem tissues were clearly recognized on both sides of the vascular cambium in the stained stem sections, these observations cannot be used to locate these tissues in the confocal micrographs.

Figure 3.1. Histochemical examination of vibratome cross-sections of two-year-old poplar branches. **(a)** The different tissues can be identified through Toluidine Blue O staining: phloem (phl), xylem (xy), vascular cambium (ca), sclerenchyma fibres (sc) and collenchyma (col). Xylem radial cell are indicated with arrows. **(b)** Phloroglucinol stains lignified xylem vessels (xy) and sclerenchyma fibres (sc). **(c)** Calcofluor white reveals the presence of cellulose in the non-lignified areas of phloem (phl) and collenchyma (col).

The pattern of natural autofluorescence emission by the different cell components was determined under the laser excitation lines of 405, 488 and 543 nm (**Fig. 3.2**). Lignified structures exhibited intense autofluorescence at 405 (**Fig. 3.2a**) and 488 nm (Fig. 2b), whereas they appeared dark under the 543 nm laser (**Fig. 3.2c**). Some autofluorescence was observed in the radial parenchymal cells, mainly under the 543 nm laser line. Thus, autofluorescence provides hallmarks for the unequivocal identification of the xylem and phloem areas in the stem sections to be used for the determination of DNA-methylation and histone acetylation levels.

Figure 3.2. Autofluorescence patterns produced in the same poplar stem cross-section under different excitation wavelengths. **(a)** Emission profile after excitation under the 405 nm laser line revealing the lignified structures of xylem (xy) and sclerenchyma (sc). **(b)** Emission profile after excitation under the 488 nm laser line showing the lignified structures of xylem (xy) and sclerenchyma (sc). **(c)** Excitation under the 543 nm laser line highlighting radial parenchyma cells of the xylem (arrows), phloem (phl) and collenchyma (col). (*) xylem vessels; (ca) vascular cambium.

Positive control for the immunofluorescence experiments

As a control of adequate permeabilization through the thick-vibratome sections of stems in the immunofluorescence assays, we used an antibody against a marker expected to provide a differential expression pattern in the growing season versus the dormancy stage. The relationship between methyl-esterified and non-esterified pectins plays an important role in the mechanical properties of plant cell walls throughout development (Ryden et al. 2003). Accordingly, we were able to observe a different pattern of labelling with the antibody JIM 7, specific to methyl-esterified pectins in the summer and winter specimens. This fluorescence signal was detected in the vascular cambial cells in the summer specimens (**Fig. 3.3a**) and was very weak in winter specimens (**Fig. 3.3b**).

Figure 3.3. Immunofluorescence detection of methyl-esterified pectins using the JIM7 antibody in vibratome sections of two-year-old poplar branches. **(a)** High levels of esterified pectins were detected in the cambium cells (ca) during proliferation in summer, **(b)** while the fluorescence signal in the same tissue was low in winter. Phloem (phl); xylem (xy).

Immunolocalization of epigenetic marks

To assess the influence of epigenetic modifications in the control of winter dormancy, immunofluorescence experiments were performed with antibodies against well-known markers of different functional states of chromatin. DNA methylation and acetylation of lysine 8 of histone H4 are considered reliable indicators of transcriptionally incompatible and compatible chromatin states, respectively. The antibody to 5-methylcytidine (5-mC) was able to label the nuclei with high specificity. Different global DNA methylation levels were detected in the growing season versus winter dormancy in both tissues analysed (phloem and xylem). Thus, summer specimens examined under the confocal microscope showed a low intensity of fluorescence in nuclei of the xylem radial cells and in the phloem cells (**Fig. 3.4a,b**), while the fluorescence signal was much higher for both tissues in winter specimens (**Fig. 3.4c,d**). When fluorescence intensity was compared, a 2-fold higher intensity was found in winter compared to summer (**Fig. 3.4e,f**) (**Table 3.1**). These differences were statistically significant ($P < 0.001$). In sections collected in summer the fluorescence signal was mainly located in the outer nuclear regions, where telomeric and centromeric heterochromatin attaches to the nuclear envelope, in both the phloem and xylem areas. In winter specimens, the intensity of labelling was distributed to wider areas of the nucleus in both tissues.

Using an antibody to the acetylated lysine 8 of histone H4, a higher intensity of fluorescence in summer tissues was observed, as a sign of increased gene activity. Although the measure of total fluorescence signal did not show such striking differences as for 5-mC, the intensity of fluorescence in summer tissues was significantly higher (1.3 to 1.6-fold) (**Fig. 3.5a,b**) than in winter (**Fig. 3.5c,d**), particularly in the xylem parenchyma cells. Quantification in xylem and phloem tissues (**Table 2**) and its representation in histograms revealed a significantly higher signal in summer than in winter ($P < 0.001$) (**Fig. 3.5e,f**). No differences were observed in the subnuclear distribution of fluorescence.

5-Methylcytosine

Figure 3.4. Immunofluorescence detection of 5-methylcytidine in vibratome sections of two-year-old poplar branches. **(a,b)** In the summer time, the fluorescence signal is low in both the phloem (a) and xylem (b). **(c,d)** In contrast, fluorescence is high in winter in both tissues –phloem (c) and xylem (d). The highest intensity of labelling may be observed at the nuclear periphery. **(e,f)** Histograms comparing fluorescence signals emitted by the nuclei in phloem (e) and xylem (f) tissue in summer versus winter.

Fluorescence intensity was around two-fold higher in winter than in summer specimens. Raw data can be found in Table 3.1.

Table 3.1. Total fluorescence intensity/area after 5mC immunodetection.

	SUMMER		WINTER	
	Phloem	Xylem	Phloem	Xylem
Total fluorescence intensity/area	46.50	39.88	85.22	99.25
N	112	47	55	69
SE	2.30	2.55	4.71	5.01

Table 3.2. Total fluorescence intensity/area after acetylated K8 of histone H4 immunodetection.

	SUMMER		WINTER	
	Phloem	Xylem	phloem	Xylem
Total fluorescence intensity/area	138.27	120.22	102.00	75.85
N	83	44	214	118
SE	5.23	7.42	2.73	3.51

Figure 3.5. Immunofluorescence detection of the acetylated lysine 8 of histone H4 in vibratome sections of two-year-old poplar branches. **(a,b)** In summer time, the fluorescence signal is high in both the phloem (a) and xylem (b). **(c,d)** In contrast, fluorescence is low in winter in both tissues – phloem (c) and xylem (d). **(e,f)** Histograms comparing fluorescence signals emitted by nuclei in phloem (e) and xylem (f) tissues in summer and winter. Fluorescence intensity was higher (1.3-fold in phloem, 1.6-fold in xylem) in specimens collected in summer compared to winter. Raw data can be found in Table 2.

3.5 DISCUSSION

An efficient fixation protocol was applied to preserve the specimens of poplar two-year old branches. This is indicated by histochemical staining of vibratome sections, which served to identify the distribution of the stem tissues and some structural components of the cells, such as lignin and cellulose. In effect, both these techniques have been successfully used to detail the anatomy of over-expressing and knock-down transgenic poplar in relation to wild type plants (Moreno-Cortés et al. 2012).

To optimize the immunofluorescence procedure in thick vibratome stem sections, the JIM 7 antibody against methyl-esterified pectins was used as a positive control of permeabilization. The fluorescence signal emitted was localized in the cell walls of the vascular cambial region during the growing season. This observation is consistent with reports that have related the presence of esterified pectins to proliferation states (Barány et al. 2010) and suggests that these forms of cell wall pectins are compatible with cell growth. In addition, low levels of esterified pectins were detected during winter dormancy, indicating the cessation of the vascular cambial activity. These findings also revealed the sensitivity of this method to distinguish between winter and summer specimens.

The role of global epigenetic control in differential gene expression under diverse physiological conditions has traditionally been addressed by calculating the percentage of 5-methyldeoxycytidine in relation to total deoxycytidines after genomic DNA extraction and HPCE analysis (Fraga et al. 2002). This methodology often requires the optimization of existing protocols to different plant sources to achieve a sufficient quantity of DNA and of a high purity. Genomic DNA extraction procedures produce irregular results when applied to poplar branches and stems, the only aerial plant materials present throughout the year. Thus, our alternative approach was the immunofluorescence detection of 5-methylcytidine in thick vibratome sections using specific antibodies followed by quantification of the fluorescent signal emitted. To this aim, a confocal microscopy analysis was used since it not only removes the out of focus signal, providing clearer images than a conventional fluorescence microscope, but also eliminates the autofluorescence that could interfere with the quantification results. Complete confocal Z series across the whole thickness of the vibratome cuts were obtained and sections spanning a given nucleus were selected using free software. Single measures of total fluorescence on maximum projections were taken in a representative sample size such that comparisons between samples can be made with sufficient statistical significance to provide meaningful results. However, the quantification of fluorescence on sum projections of confocal stacks is advisable when the estimate the amount of a cellular target bound to the fluorochrome,

such as the ploidy level by DAPI staining (González-Melendi et al. 2005) is required. This was not absolutely needed in our study since we were looking for seasonal changes.

Approximately a 2-fold higher intensity of fluorescence was measured in winter than in summer specimens after immunolabelling with the antibody to 5mC. The wider distribution of the signal in winter nuclei was consistent with a general down regulation of gene activity in the dormancy period. These findings are consistent with those of studies in other tissues and organs, which have related increased DNA methylation to aging and differentiation (Fraga et al. 2005; Santamaría et al. 2009; Valledor et al. 2007) and DNA demethylation to transcription resuming after dormancy release in potato tubers (Law & Suttle 2003). However, the identification of epigenetic marks does not allow correlations between chromatin state and gene expression. Recently a thorough analysis of DNA methylation by MeDIP-seq followed by bisulfite sequencing in different tissues and at different developmental stages in *Populus trichocarpa* has permitted to relate gene expression with the methylation status of different regions of the genes (Vining et al. 2012).

The reliability of the method here proposed was tested using antibodies to another epigenetic mark. The acetylation of histone H4 makes chromatin less compact and therefore more accessible to transcription factors by altering the conformation of the nucleosomes (Jenuwein & Allis 2001). Thus, we would expect an antibody to the acetylated Lys 8 of H4 to give rise to an opposite pattern of labelling to the one described above. Previous studies have shown converse labelling patterns of DNA methylation and acetylation of histone H4 in apical bud dormancy in chestnut (Santamaría et al. 2009). As expected, our immunofluorescence data for the acetylated Lys 8 of histone H4 revealed an opposite seasonal pattern to that described for methylated DNA, supporting the consistency of the quantification method.

This procedure of fluorescence quantification in confocal microscopy images after 5-methylcytidine immunofluorescence emerges as a reliable and reproducible method of determining global DNA methylation as a previous study to the application of the state of the art technologies that provide sequence information. Since the antigens to detect the epigenetic marks are highly conserved among eukaryotes, we demonstrated the consistency of the method by the detection of acetylated lysine 8 for H4. Besides providing global data for the correlation of gene activity to developmental processes, this approach also permits a detailed analysis in a tissue-dependent manner. Epigenetic marks of low transcriptional activity i.e. DNA methylation and histone H4 hypoacetylation are detected during the winter, and chromatin modifications compatible with gene expression i.e. DNA hypomethylation and histone H4 acetylation are found when growth resumes. Thus, our findings suggest an epigenetic control of winter dormancy in poplar stems.

3.6 REFERENCES

- Allona I, Ramos A, Ibañez C, Contreras A, Casado R, Aragoncillo C (2008) Review. Molecular control of winter dormancy establishment in trees. Spanish Journal of Agricultural Research 6 (special issue): 201-210
- Amasino R (2004) Vernalization, competence, and the epigenetic memory of winter. The Plant Cell 16: 2553-2559
- Barány I, Fadón B, Risueño MC, Testillano PS (2010) Cell Wall Components and Pectin Esterification Levels as Markers of Proliferation and Differentiation Events During Pollen Development and Pollen Embryogenesis in *Capsicum annuum* L. J Exp Bot 61(4):1159-1175.
- Batley NH (2000) Aspects of seasonality. Journal of Experimental Botany 51:1769-1780
- Berdasco M, Alcázar R, García-Ortiz MV, Ballestar E, Fernández AF, Roldán-Arjona T, Tiburcio AF, Altabella T, Buisine N, Quesneville H, Baudry A, Lepiniec L, Alaminos M, Rodríguez R, Lloyd A, Colot V, Bender J, Canal MJ, Esteller M, Fraga MF (2008) Promoter DNA hypermethylation and gene repression in undifferentiated Arabidopsis cells. PloS one 3:e3306
- Bieniaszewska Z, Espinoza C, Schlereth A, Sulpice R, Hinch DK, Hannah MA (2008) Disruption of the Arabidopsis circadian clock is responsible for extensive variation in the cold-responsive transcriptome. Plant Physiology 147:263-279
- Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O (2006) CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. Science 312:1040-1043
- Chuine I, Beaubien EG (2001) Phenology is a major determinant of tree species range. Ecology Letters 4: 500-510
- Feil R, Fraga MF (2012) Epigenetics and the environment: emerging patterns and implications. Nature Reviews 13:97-109
- Finnegan EJ, Genger RK, Peacock WJ, Dennis ES (1998) DNA Methylation in Plants. Annual Review of Plant Physiology and Plant Molecular Biology 49:223-247

Finnegan EJ, Peacock WJ, Dennis ES (2000) DNA methylation, a key regulator of plant development and other processes. *Current Opinion in Genetics & Development* 10:217-223

Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M (2005) Epigenetic differences arise during the lifetime of monozygotic twins. *Proceedings of the National Academy of Sciences of the United States of America* 102:10604-10609

Fraga MF, Uriol E, Borja Diego L, Berdasco M, Esteller M, Canal MJ, Rodriguez R (2002) High-performance capillary electrophoretic method for the quantification of 5-methyl 2'-deoxycytidine in genomic DNA: application to plant, animal and human cancer tissues. *Electrophoresis* 23:1677-1681

González-Melendi P, Ramírez C, Testillano PS, Kumlehn J, Risueño MC (2005) Three dimensional confocal and electron microscopy imaging define the dynamics and mechanisms of diploidisation at early stages of barley microspore-derived embryogenesis. *Planta* 222:47-57

Hayama R, Coupland G (2003) Shedding light on the circadian clock and the photoperiodic control of flowering. *Current Opinion in Plant Biology* 6:13-19

Heide OM, Prestrud AK (2005) Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. *Tree Physiology* 25:109-114

Horvath DP, Anderson JV, Chao WS, Foley ME (2003) Knowing when to grow: signals regulating bud dormancy. *Trends in Plant Science* 8:534-540

Horvath DP, Sung S, Kim D, Chao W, Anderson J (2010) Characterization, expression and function of DORMANCY ASSOCIATED MADS-BOX genes from leafy spurge. *Plant Molecular Biology* 73:169-179

Ibáñez C, Ramos A, Acebo P, Contreras A, Casado R, Allona I, Aragoncillo C (2008) Overall alteration of circadian clock gene expression in the chestnut cold response. *PloS one* 3:e3567

Ibáñez C, Kozarewa I, Johansson M, Ogren E, Rodhe A, Eriksson ME (2010). Circadian clock components regulate entry and affect exit of seasonal dormancy as well as winter hardiness in *Populus* trees. *Plant Physiology* 153(4):1823-1833

Jenuwein T, Allis CD (2001) Translating the histone code. *Science* 293:1074-1080

Lang GA, Early JD, Martin GC, Darnell RL (1987) Endo-, para-, and ecodormancy: physiological terminology and classification for dormancy research. *Hort Science* 22:371-377.

Law RD, Suttle JC (2003) Transient decreases in methylation at 5'-CCGG-3' sequences in potato (*Solanum tuberosum* L.) meristem DNA during progression of tubers through dormancy precede the resumption of sprout growth. *Plant Molecular Biology* 51:437-447

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

Moreno-Cortés A, Hernández-Verdeja T, Sánchez-Jiménez P, González-Melendi P, Aragoncillo C, Allona I (2012) CsRAV1 induces sylleptic branching in hybrid poplar. *New Phytologist* 194:83-90

Penfield S (2008) Temperature perception and signal transduction in plants. *The New Phytologist* 179: 615-628

Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M (1999) DNA methylation is a reversible biological signal. *Proceedings of the National Academy of Sciences of the United States of America* 96:6107-6112

Ramos A, Pérez-Solís E, Ibáñez C, Casado R, Collada C, Gómez L, Aragoncillo C, Allona I (2005) Winter disruption of the circadian clock in chestnut. *Proceedings of the National Academy of Sciences of the United States of America* 102:7037-7042

Richards EJ, Elgin SC (2002) Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* 108:489-500

Rohde A, Bastien C, Boerjan W (2011) Temperature signals contribute to the timing of photoperiodic growth cessation and bud set in poplar. *Tree Physiology* 31:472-482

Rohde A, Bhalerao RP (2007) Plant dormancy in the perennial context. *Trends in Plant Science* 12:217-223

Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter WD, McCann MC (2003) Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. *Plant Physiology* 132:1033-1040

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

Sawa M, Nusinow DA, Kay SA, Imaizumi T (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in *Arabidopsis*. *Science* 318:261-265

Suarez-Lopez P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G (2001) CONSTANS mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* 410:1116-1120

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

Valledor L, Hasbún R, Meijón M, Rodríguez JL, Santamaría ME, Viejo M, Berdasco M, Feito I, Fraga MF, Cañal MJ, Rodríguez R (2007) Involvement of DNA methylation in tree development and micropropagation. *Plant Cell Tiss Organ Cult* 91:75-86

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

Wagner D (2003) Chromatin regulation of plant development. *Current Opinion in Plant Biology* 6:20-28

Wareing PF (1956) Photoperiodism in woody plants. *Annu Rev Plant Physiol* 7:191-214

Yanovsky MJ, Kay SA (2002) Molecular basis of seasonal time measurement in *Arabidopsis*. *Nature* 419: 308-312

4. A DEMETER-LIKE DNA DEMETHYLASE CONTROLS BUD BREAK IN POPLAR

4.1 ABSTRACT

Dormancy-growth cycle is a pivotal developmental and physiological process essential for the survival of perennial temperate and boreal forests (Penuelas & Filella 2001). Dormancy control is being targeted to climate adaptation in a global warming environment. The main molecular players already unravelled in dormancy regulation include: During entrance, FLOWERING LOCUS T (FT) acting as photoperiodic signal for growth cessation (Böhlenius et al. 2006; Ding & Nilsson 2016; Singh et al. 2016) and FD-LIKE1 (FDL1) and ABA INSENSITIVE 3 (ABI3) controlling bud maturation and cold acclimation in poplar (Tylewicz et al. 2015). Throughout dormancy release, CENTRORADIALIS-LIKE1 (CENL1) as a negative regulator in poplar (Mohamed et al. 2010) and changes of the functional state of chromatin as histone modifications at DAM genes in peach (Leida et al. 2012; Rios et al. 2014). At the exit, EARLY BUD-BREAK1 (EBB1) involved in bud burst in *Populus* (Yordanov et al. 2014; Busov et al. 2016). Here, we address the contribution of genomic DNA methylation in dormancy-growth cycle's regulation. The active removing of DNA methylation by DEMETER-like 5 methyl-cytosine demethylases (DMLs) plays a key role in plant development (Zhu 2009; Liu et al. 2015; Satgé et al. 2016). Through an integrative approach, we show that bud break is preceded by a reduction of genomic DNA methylation in poplar apex while PtaDML10 is induced after chilling fulfilment during ecodormancy. During dormancy-growth transition in poplar, PtaDML10 is a key regulator required for reactivation of shoot apical meristem (SAM) and growth in spring through active DNA demethylation. In summary, PtaDML10 enable the induction of cell metabolism genes and key regulators of meristem development, and the downregulation of bud dormancy genes. These data implicate DEMETER mediated DNA demethylation in the control of environmentally induced developmental stage transitions in plants.

Keywords: Bud break, DEMETER, DNA demethylases, poplar, winter dormancy, DNA methylation

4.2 MATERIAL AND METHODS

Phylogenetic analyses to identify poplar DML proteins

Arabidopsis protein sequences of DMLs were retrieved using the TAIR10 website (www.arabidopsis.org). These sequences were used as queries to BLAST the *Populus trichocarpa* proteome (Phytozome v11, <https://phytozome.jgi.doe.gov/pz/portal.html>) and identify homolog proteins in this organism. These protein were aligned using MAFFT (E-INS-i algorithm) (Katoh & Standley 2013) and edited in Jalview 2.8 (Waterhouse et al. 2009). The best evolutionary model of the alignment was highlighted using ProtTest 2.4 (Abascal et al. 2005). The maximum likelihood tree was inferred with the RAxML tool (Stamatakis 2006) with 100 bootstrap replicates and visualized in the iTOL website <http://itol.embl.de> (Letunic & Bork 2007). Bootstrap values were generated with 100 replicates.

The conserved regions of Arabidopsis and poplar DMLs were assembled in the PROSITE website (<http://prosite.expasy.org/mydomains/>),

Plant material and growth conditions

To explore the 5mC profile from winter dormancy to bud break, 28 apical buds were collected weekly from 7 hybrid poplar trees (*Populus tremula* x *alba* INRA clone 717 1B4) at the Centre for Plant Biotechnology and Genomics (CBGP) in Pozuelo de Alarcón, Madrid (3°49'W, 40°24'N) over the period January 13 to April 14, 2015. Each week, genomic DNA was extracted from the 28 dissected apices for HPLC.

Annual expression patterns for poplar *PaDML8* and *PaDML10* were measured in 2-year-old poplar branches (*Populus alba*) growing under natural conditions in Madrid (Spain). The expression profiles of *PtaDML8* and *PtaDML10* throughout winter were measured in 2-year-old poplar branches and apical buds of hybrid poplar (*Populus tremula* x *alba* INRA clone 717 1B4) at CBGP.

To examine *PtaDML10* induction in pre-chilling and post-chilling conditions, cuttings were collected from five 6-year-old hybrid poplars growing under natural conditions. In a first experiment, cuttings were harvested on December 11, when the buds were in a dormant state and the chilling requirement had not been fulfilled (pre-chilling cuttings). In a second experiment, cuttings were collected on March 7, when the chilling requirement had been fulfilled but buds were still in an ecodormant state. Thirty-six cuttings (approximately 12 cm long) per tree were collected for each experiment. 120 cuttings were placed vertically in empty blue pipette tip racks filled with water to cover the bottom 5 cm of the cuttings (Figure 3). Each

box contained 15 cuttings (three cuttings of each selected tree). Cuttings in racks were transferred to the growth chamber under LD conditions (16 h light/) at 22°C. Apical buds were scored as six developmental stages of bud burst (stage 0 to stage 5) according to UPOV (1981).

For phenological assays, plantlets of *in vitro*-cultivated wild type and two *PtaDML8-10* RNAi lines, KD2 and KD5, were transferred to 3.5 L pots containing blond peat, pH 4.5. Before any photoperiodic treatment, the plants were grown in a chamber under controlled LD conditions (16h light, 21°C, 65% relative humidity and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity). After 4 weeks the plants were transferred to SD conditions (8h light, 19°C, 65% relative humidity and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity) for 12 weeks to induce apical bud formation. Bud formation progression was scored as stage 3 (fully growing apex) to 0 (fully formed apical bud) according to (Rohde et al. 2011). Dormant plants were kept under the conditions SD, 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and 4°C for 0, 4 and 8 weeks. Monitoring of bud burst after dormancy induction and chilling was conducted in LD conditions at 22°C. Regrowth was scored for the six developmental stages of bud burst (stage 0 to stage 5) according to UPOV (1981). Significant differences in apex state were identified through pairwise comparisons between KD and WT plants using the Wilcoxon Rank Sum test after Shapiro-Wilk confirmation of the non-normal distribution of data. All statistical tests were run in R (www.r-project.org).

We repeated the phenological experiment to induce winter dormancy induction and release (with 4 weeks of chilling fulfillment) in WT, KD2 and KD5 plants. When growth resumed, the topmost (apical) bud of each plant was collected when the first visual changes in bud development were evident. Buds with morphological changes indicating initial stages of bud break were removed, so all apical buds were collected at stage 0. We collected 30 apical buds from 30 plants for each of the WT, KD2 and KD5 genotypes; 15 were used for RNA extractions and sequencing, and 15 for genomic DNA extractions for HPLC and whole genome bisulfite sequencing analyses.

RT-PCR expression analyses

Total RNA extraction, single-stranded cDNA synthesis, primer design, real-time PCRs and data analysis were performed as we described previously (Ibañez et al. 2008). The gene-specific primers used are described in **Fig. S4.4a**.

Probe synthesis and fluorescence in situ hybridization (FISH)

Apical buds for this experiment were harvested on April 14, 2015. A 271 base pair (bp) fragment of the 5' portion of the untranslated terminal region (UTR) of *PtaDML10* DNA was cloned into pGem-T (Promega, Madison, WI, USA). The probe was *in vitro* transcribed to

obtain digoxigenin-UTP (DIG) labeled RNA probes using T7 and Sp6 RNA polymerases for the sense and the anti-sense mRNA probes, respectively (Roche Applied Science, Mannheim, Germany).

Apices were fixed and preserved as previously described in (Conde et al. 2013) before embedding in paraffin wax. The specimens were washed twice in PBS (30 min each) and dehydrated in a series of 30%, 50%, 70% (1 h each) and 95% ethanol overnight. Specimens were then washed in 100% ethanol for 12 h. Ethanol was then replaced with a mixture 50% histoclear (HC)-50% ethanol overnight and 100% HC for 12 h (twice). Vials containing the apices were filled up to a quarter of their volume with paraplast and incubated at 42°C for 12 h. Next, another quarter volume was added, followed by incubation at 60°C overnight. Paraplast in HC was replaced with melted paraffin wax (60°C), and this was replenished every 12 h for 5 days. Finally, the apices were placed in molds and, after solidification at room temperature, they were kept at 4°C. Sections of 15-20 µm were obtained in a microtome Leica 2055 (Leica Microsystems, Wetzlar, Germany) and the paraffin was removed in HC for 15 min and then in 100% ethanol for 15 min. Finally, the sections were rehydrated in a series of 96% and 70% ethanol for 10 min each and, finally, in PBS for 15 min.

After deparaffination, dehydration and rehydration, as previously described, bud sections were treated with 2% cellulase and proteinase K (1 µg ml⁻¹) at 37°C, for 1 hour each. Afterwards, 30 ng µl⁻¹ of either the sense or the antisense probe diluted in the hybridization mixture (50% formamide(v/v), 10% dextrane sulfate (w/v), 10 mM Pipes, 1 mM EDTA, 300 mM NaCl, 1000 ng µl⁻¹ tRNA) was applied to the sections and incubated at 50°C overnight. After hybridization the slides were washed in 1X Saline Sodium Citrate buffer (SSC) for 30 minutes at 50°C. DIG detection and fluorescence visualization were carried out with a primary anti-DIG (Sigma) antibody, diluted 1/3000 in 3% BSA in PBS for 1 hour at room temperature, and a secondary anti mouse 546 (ALEXA, Molecular Probes) antibody, applied 1/25 in 3% BSA in PBS for 1 hour at room temperature in the dark. Images were acquired using a Leica TCS-SP8 confocal microscope under the laser excitation line of 561 nm. Gain and offset conditions were maintained in all captures for comparison. Images were acquired using a Leica TCS-SP8 confocal microscope.

Generating binary vector constructs and plant transformation

A 419 bp fragment of Poplar *DML10* was amplified from *Populus alba* winter stem cDNA using gene specific primers with attB sites (**Fig. S4.4b**). We followed the protocol described in (Moreno-Cortés et al. 2012) for RNAi fragment insertion in the hybrid poplar genome. Plantlets were generated in kanamycin-containing selection medium and independently

transformed individuals (lines) were screened by reverse transcription (RT)-PCR to down-regulate *PtaDME10* expression.

RNA sequencing in shoot apices

Expression profiles were examined by RNA sequencing (RNA-Seq) in dormant apical bud, at the same time point, just before growth resume after winter dormancy induction and release of *PtaDML10* KD2, *PtaDML10* KD5 and wild-type hybrid poplars. Previous to RNA extraction, apices were dissected under a stereomicroscope to remove the leaflets surrounding the shoot apical meristem. RNA extraction was performed following earlier published protocol (Ibáñez et al. 2008). Libraries were prepared using 1 µg of RNA in the NEBNext® Ultra™ Directional RNA Library Prep Kit following the supplier's instructions (New England Biolabs, Ipswich, MA, USA). Sequencing was done with an Illumina NextSeq500 in high-throughput mode (2×150 cycles) at the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL, USA).

Pre-processing and assembly of sequence data were conducted using the web-based platform Galaxy at <https://usegalaxy.org/> (Giardine et al. 2005; Blankenberg et al. 2010; Goecks et al. 2010), which includes tools to remove reads containing N blurs, adapter sequences, and sequences shorter than 15 nucleotides. Quality control of reads was carried out with the FastQC Galaxy tool. Removal of rRNA, if needed, was performed locally with the SortMeRNA tool. The TopHat Galaxy tool was used to map clean reads to the *Populus tremula* x *P. alba* INRA clone 717-1B4 genome using the default settings. We used the *summarizeOverlaps* function (packages Genomic Alignments of R) to generate the matrix of read count for each gene from BAM files. Differentially expressed genes (DEG) lists were obtained using the function *exactTest* (edgeR package of R). For DEG analyses, *PtaDML10* KD2 and *PtaDML10* KD5 transcriptomes were considered as biological replicates when comparing to WT, using a *FDR* < 0.05. Using this strategy we tried to identify only genes that are consistently differentially regulated between *PtaDML10* KD transgenic lines, detecting the biological impact of the transgene in down-stream regulation. Raw data for the RNAseq were deposited in Gene Expression Omnibus at NCBI under GEO accession number GSE87445.

DNA methylation analysis

Genomic DNA methylation levels were quantified by HPLC in poplar apices dissected under a stereomicroscope to remove the leaflets surrounding the shoot apical meristem. Genomic DNA was purified from poplar samples and prepared for HPLC using a published method (Causevic et al. 2005; Gourcilleau et al. 2010) via RNase A digestion, phenol/chloroform extraction, and ethanol precipitation. Purified DNA was hydrolyzed into

nucleosides using DNase I (700 U, Roche Diagnostics, Meylan, France), phosphodiesterase I (0.05 U, SerLabo Technologies, Entraigues sur la Sorgue, France), and alkaline phosphatase type III (0.5 U, Sigma-Aldrich, Saint-Quentin Fallavier, France) successively. Percentage genomic methylation was determined by HPLC using a GeminiTM column (150 9 4.6 mm, 5 μ m, Phenomenex, Le Pecq, France) and isocratic mobile phase composed of 0.5 % methanol (v/v) and 5 mM acetic acid in water according to the methods of (Gourcilleau et al. 2010). Controls for this procedure included co-migration with commercial standards (Sigma-Aldrich), confirmation by enzyme restriction analysis (Causevic et al. 2005), and tests for RNA contamination through HPLC detection of ribonucleosides. The methylcytosine percentage was calculated following the previously described method (Zhu et al. 2013).

Methylome analysis through whole genome bisulfite sequencing (WGBS)

gDNA extracted from apices of WT, KD2 and KD5 specimens just before bud break were sent to Fasteris DNA sequencing service (<https://www.fasteris.com>) for sequencing after bisulfite treatment using the Hiseq 2500 platform. Unmethylated lambda DNA was added to the total DNA before bisulfite treatment to calculate the conversion rate, which was estimated as 94.16, 93.90 and 90.91 for WT, KD2 and KD5, respectively. The Galaxy platform of the University of Perpignan's server was used for quality control of reads and to obtain the read alignment against the *Populus tremula x alba* INRA clone 717-1B4 genome using Bismark mapper (Krueger & Andrews 2011). Before mapping, the last nucleotide of all reads was trimmed because of its lower sequencing quality (<28). In total, 19.02%, 19.04% and 18.36% of reads were mapped against the 717-1B4 genome in WT, KD2 and KD5, respectively. To identify differentially methylated regions (DMR) in KD2 vs. WT and KD5 vs. WT, the methylkit (Akalin et al. 2012) package of R was used with a minimum difference of 15% in methylation level (R studio software, University of Perpignan's server). We calculated the methylation status for cytosines with a minimum sequence depth of 10. A total of 14616377 (10.24%), 16559883 (11.61%) and 15097166 (10.58%) cytosines (% of cytosines in the genome) were evaluable in WT, KD2 and KD5, respectively. Raw data for whole genome bisulfite sequencing were deposited in Gene Expression Omnibus at NCBI, under GEO accession number GSE87445.

To identify which DEG with hypermethylated DMR in their promoter or transcribed regions showed relatively higher expression in young leaves and SAM, we first crossed them with genes identified in previously published transcriptome analysis performed in the roots, young and mature leaves, nodes and internodes of *Populus trichocarpa* reference genotype Nisqually-1. The expression patterns of the genes in the different tissues from this report were examined in the public database www.popgenie.org, and those mainly expressed in young

leaves were separated. We also identified genes with the Arabidopsis homolog primarily expressed in SAM by crossing DEG with the expression browser tool selecting AtGenExpress_Plus-Extended Tissue Series at Arabidopsis eFP Browser (<http://www.bar.utoronto.ca/>). To identify which of those genes are induced or repress during bud break process, we used expression data of that experiment we performed using poplar apices from January 13 to April 14 generated by RNA-seq, and that are available in phytozome11 website (<https://phytozome.jgi.doe.gov/pz/portal.html>).

4.3 RESULTS AND DISCUSSION

Bud break is preceded by a reduction of genomic DNA methylation in poplar apex while PtaDML10 is induced during ecodormancy and bud break

Changes in DNA methylation have been reported during growth-dormancy transitions in trees (Santamaría et al. 2009; Conde et al. 2013; Kumar et al. 2016). In addition, differential expression of genes implicated in DNA methylation has been described during these cycles in poplar (Shim et al. 2014; Vera et al. 2015). To better understand this process during the transition from dormancy to growth, we performed a high-resolution profiling of genomic DNA methylation by HPLC in poplar SAM from January to the time of bud break in April (**Fig. 4.1a**). A stage of DNA hypermethylation in February was followed by a period of marked hypomethylation, with minimum levels detected on March 24. Later on, bud break (stages 1, 2 and 3 of apical bud development, **Fig. 4.1a,b**), coincided with increased 5mC levels. Similar dynamics of 5mC confer plasticity in environmentally controlled responses such as vernalization in sugar beet (Trap-Gentil et al. 2011). To decipher if the decrease in genomic DNA methylation occurs previously or following a reactivation of the cell cycle, we examined the expression of D-type cyclin *PtaCYCD6:1*, a marker for growth reactivation in poplar apical meristem (Karlberg et al. 2011). *PtaCYCD6:1* mRNA levels rose from March 17 until the last time point in April 14 (bud stage 3) (**Fig. 4.1b**), thus, we can envisage an active DNA demethylation mechanism before bud break in poplar.

Through phylogenetic and protein sequence analysis, we have identified three putative *DML* genes in poplar *PtaDML6*, *PtaDML8* and *PtaDML10*, containing all domains and aminoacids conserved in DMLs, essential for active 5mC excision: (**Fig. S4.1**). The annual expression patterns of the three genes in poplar stems detected that only *PtaDML8* and *PtaDML10* had highest levels during vegetative growth, with *PtaDML10* expression peaked during winter dormancy exit (April) (**Fig. 4.1c and Fig. S4.2a**). A weekly winter-spring expression profile for poplar stems and apices, showed *PtaDML10* induction in late winter dormancy (end of February) with maximum expression levels at bud break (April 1 and 9),

while *PtaDML8* was remained constant in stems and in apices. (**Fig. 4.1d,e and Fig. S4.2b,c**). These observations are consistent with previous data from immunofluorescence and transcriptome analyses, indicating a decreased in global methylation and an increased *PtaDML10* expression during dormancy release in poplar (Conde et al. 2013; Shim et al. 2014; Vera et al. 2015).

Additionally, *PtaDML10* mRNA was localized by FISH in poplar apices. The fluorescence signal was detected in the L1-L2 layers and central SAM, and also in the tips of leaf primordia at bud breaks (April 14) (**Fig. 4.1f**). This spatio-temporal localization is similar to that reported for EARLY BUD-BREAK 1 (EBB1), a putative APETALA2/ethylene responsive transcription factor in poplar (Yordanov et al. 2014). EBB1 overexpression causes early bud break activating the *CYCD3* genes in Japanese Pear, whereas its down-regulation delays this process (Yordanov et al. 2014; Anh Tuan et al. 2016). All together, these data suggests that growth after winter dormancy reinitiates in the L1-L2 layers preceded by an active demethylation by *PtaDML10* 4 weeks earlier than *PtaCYCD6*, the marker for growth reactivation.

Figure 4.1. DNA methylation patterns and DNA demethylases mRNA expression from dormancy to bud break in poplar stem and apices (a) DNA methylation levels (mean values $\pm$ SE, $n=4$) in apical meristems quantified by HPLC in poplar growing under natural conditions from Jan 13 weekly to budburst on Apr 14. Each biological sample consists of a pool of $n=28$ dissected apices. Two independent hydrolyses were performed in each biological replicate and two HPLC analyses were carried out for each hydrolysis. Significant differences in each point were analysed by ANOVA and the TUKEY test. Points with different letters showed significant differences ($P < 0.01$). Numbers indicate the different developmental stages of the apical bud during the period of this experiment according to the developmental stages of bud burst defined by UPOV (1981). (b) Normalized expression of *PtaCYCD6*

obtained by RNA sequencing in poplar apices growing under natural conditions from Jan 13 to bud break on Apr 14. Figures and numbers shown inside the *PtaCYCD6* profile indicate the developmental stage of apices as described in (a). **(c,d)** Quantitative RT-PCR analysis of *DML10* in poplar 2-year-old branches: (c) along the year and (d) during winter dormancy from Oct 31 weekly to bud break on Apr 9. Plotted values and error bar are fold-change means $\pm$ SD recorded in three technical replicates. **(e)** Normalized expression of *PtaDML10* was determined by RNA sequencing in poplar apices growing under natural conditions from Jan 13 to bud break on Apr 14. Numbers shown inside the *PtaDML10* profile indicate the developmental stage of apices as described in (a). **(f)** Fluorescence immunodetection of *PtaDML10* in 20 μ m WT apical bud sections collected at bud break on Apr 14 from poplar trees growing under natural conditions. **(g)** Phenological observation of dormant apices of cuttings taken from poplar trees growing under natural conditions before and after fulfilling the chilling requirement and transferred to LD and 22°C conditions. After 12 days in conditions of LD, 22°C, most postchilling cuttings started bud break, while the apices of prechilling cuttings remained at stage 0. Prechilling cuttings are indicated with a white colour and postchilling cuttings with a blue colour. **(h)** DNA methylation levels (mean values $\pm$ SE, n=8) in dormant apical meristems quantified by HPLC of dormant apices of pre- and postchilling cuttings grown under natural conditions and after 6 and 12 days of exposure to LD, 22°C. Two independent hydrolyses were performed for each biological replicate and two HPLC analyses were carried out for each hydrolysis. Significant differences at each point were analysed by ANOVA and the TUKEY test. Points with different letters showed significant differences ($P < 0.05$). **(i,j)** Quantitative RT-PCR analysis of *PtaDML10* and *PtaCYCD6* in dormant apices of pre- and postchilling cuttings growing was done under the same conditions as in (g). Plotted values and error bars are the fold-change means $\pm$ SD recorded in three technical replicates. Two biological samples were analysed at each point consisting of a pool of n=15 dissected apices.

PtaDML10 active DNA demethylation is required for bud break after chilling fulfilled

After the ecodormancy period, the second stage of dormancy is the endodormancy, when trees acquire maximum adaptation to the cold. During this period, a chilling requirement needs to be fulfilled for growth to resume. Once the chilling period is fulfilled, the trees enter ecodormancy again, allowing bud break and the recovery of active growth under the favourable environmental conditions of spring (Ding & Nilsson 2016). To explore the chilling requirement for *PtaDML10* expression, cuttings were taken from trees grown in natural environment, including a set that had not fulfilled the chilling requirement (prechilling cuttings) and a set of postchilling cuttings. Both sets were transferred to long day (LD) at 22°C conditions in a growth chamber and phenological observations were performed at 0, 6 and 12 days after the transfer (**Fig. 4.1g**). 85% of the postchilling apical buds on day 12 reached stages 1 or 2, indicating the initiation of bud break (**Fig. 4.1g**). The genomic DNA methylation level in the postchilling apical buds was higher than in the prechilling ones, but decreased significantly 12 days after transfer to LD conditions at 22°C, to reach levels similar to those in the prechilling apical buds (**Fig. 4.1h**). Accordingly, *PtaDML10* RNA levels increased 13 times relative to time point 0

(Fig. 4.1i). The resumption of growth in the postchilling samples after 12 days is indicated by the expression of *PtaCYCD6* (Fig. 4.1j). As expected, LD and higher temperatures were insufficient to induce bud break in prechilling apical buds. Instead, the chilling requirement had to be fulfilled to promote *PtaDML10* expression and bud break. In other perennial woody species it was also found that the chilling fulfilment promotes the upregulation of some DNA demethylases (Kumar et al. 2016). Moreover, *PtDML10* was noted to be upregulated in the transition from endodormancy to ecodormancy in poplar (Vera et al. 2015).

Functional studies were performed by generating *PtaDML10* knock-down plants (KD) (Fig. S4.3). We examined the phenological variables in two low-level expressing lines, KD2 and KD5. When grown under LD and 22°C conditions, *in vitro* or glasshouse cultured transgenic plants were phenotypically similar to the WT. Moreover, these lines showed no differences compared to WT in cessation of growth elongation and bud formation when transferred from LD to short day (SD) (Fig. 4.2a,b). These observations rule out a role of *PtaDML10* in dormancy entry. WT and KD plants were subjected to 0, 4 and 8 week in SD and 4°C and the bud burst was monitored in LD and 22°C, using the scale shown in Fig. 4.2c (UPOV (1981)). Without chilling, t=0, there is not bud break, so this indicate that *PtaDML10* doesn't affect the dormancy release. More significant differences in bud burst were obtained after 4 weeks (Fig. 4.2d). Thus, lower levels of *PtaDML10* expression led to a significant retardation in growth reactivation in the transgenic trees after dormancy, indicating a role of *PtaDML10* in bud break phenology. In addition, HPLC of genomic DNA from dissected apices showed 4 and 2% higher 5mC levels in KD2 and KD5, respectively, than in the WT (Fig. 4.2e). In summary, all above data reveal that *PtaDML10* participates in the chilling-dependent genomic DNA demethylation, needed for accurate bud break after winter dormancy.

Figure 4.2. *PtaDML10* is required for bud break (a,b) Growth cessation and bud set in response to SDs, 19°C. WT, KD2 and KD5 plants were grown in LD16h, 22°C conditions for 4 weeks and then height and bud scores were monitored in response to SDs, 19°C. Mean heights (cm) $\pm$ SE and mean bud scores are represented for the wild type (n = 24), KD2 (n = 23) and KD5 (n = 25). **(c)** Apex stages during bud break. The scale used to monitor growth reactivation after inducing apical bud formation and fulfilling the chilling requirement was developed by UPOV (1981). **(d)** Timing of bud burst after dormancy. Timing of bud burst was examined in response to LD and warm temperature after dormancy induction. WT, KD2 and KD5 plants were treated as shown in (a,b). Following a shift to SD and 4°C, plants were transfer to LD and 22°C at three different times: t=0, t=4wk and t=8wk. Here we show the apical growth reactivation monitored in the 4w experiment. Mean bud burst scores $\pm$ SE were measured for the wild type (n = 7), KD2 (n = 7) and KD5 (n = 7). Significant differences between KDs and WT were analysed using the Wilcoxon Rank Sum test in R: * P<0.05. **(e)** DNA methylation level in apices of WT, KD2 and KD5. DNA methylation levels (mean values $\pm$ SE, n=4) in dormant apical meristems quantified by HPLC in WT, KD2 and KD5. Shoot apical meristems were collected at the time point close to bud burst. Each biological sample consists of a pool of n=15 dissected apices. Two independent hydrolyses were performed for each biological replicate and two HPLC analyses for each hydrolysis. Significant differences between KDs and the WT were analysed by the Student's t-test: ** P<0.01; *** P<0.001.

Gene targets of PtaDML10 during bud break

The delayed bud break phenology observed in our *PtaDML10* KD lines led us to hypothesize that *PtaDML10* downregulation causes inefficient reactivation of growth promoting genes or, alternatively, that *PtaDML10* KD lines failed to turn off the dormancy genes. To identify genes demethylated by *PtaDML10* in poplar apices, we undertook whole genome bisulfite sequencing in WT, KD2 and KD5 lines, in parallel with a genome wide RNA sequencing transcriptome analysis. The data and experimental flow through are described in **Fig. 4.3 and Tables S4.1-4.3**.

As we expected that growth-promoting genes, targets of *PtaDML10*, should be highly expressed in young proliferating aerial tissues and induced during bud break we decided to select, from the DEG that were hypermethylated in the three context either in their promoter or transcribed region, and downregulated in KD plants compared to WT, the ones that were relatively more expressed in young leaves and SAM. Between these, we looked for the ones that were also induced at the time of bud break based in our RNA sequencing in poplar apices growing under natural conditions from Jan 13 to bud break on Apr 14, available in the geneAtlas (<https://phytozome.jgi.doe.gov/pz/portal.html>) (**Table S4.4 and S4.5**). As shown in **Fig. 4.4a-c**, of the 19 and 60 genes respectively, almost 75% grouped into four Gene Ontology biological processes related to cell metabolism: protein translation, chloroplast metabolism and

light reaction, secretory pathway and mitochondrial metabolism. An open question for further studies will be to explore the role in bud break of the 7 genes belonging to the chromatin remodelling and transcription regulation biological process. Remarkably, between them we found poplar homolog to Arabidopsis genes with pivotal role in meristem development and maintenance, like *SHOOTMERISTEMLESS* (Byrne et al. 2003), *ULTRAPETALA 1* (Fletcher 2001) or *KNOTTED1-like homeobox gene 6* (Belles-Boix et al. 2006) indicating *PtaDML10* promotes reactivation of meristematic activity.

To identify dormancy genes repressed under *PtaDML10* control, we selected from the DEG hypermethylated in the promoter or transcribed region, and upregulated in KD plants compared to WT, the ones that were suppressed during bud break in our time series RNAseq profiling deposited in the geneAtlas (**Table S4.4 and S4.5**). As shown in **Fig. 4.4d-f**, of the 3 and 41 genes respectively, more than 38 % are unknown genes, indicating the broad lack of knowledge about the regulation and maintenance of the dormant state that could only be studied in perennial plants.

Figure 4.3. Analysis pipeline and the identification of DEG with hypermethylated DMR in promoter or transcribed region, of KD lines respect WT. (a) Flowchart of RNA-seq analysis pipeline.

Transcriptome comparisons revealed 2151 differentially expressed genes (DEG) in KD lines with respect to WT, among which 764 were upregulated and 1387 downregulated. **(b)** Flowchart of WGBS analysis pipeline. **(c)** Flowchart of the identification of DEG with hypermethylated DMR in KDs vs. WT. The identification of DEG with hypermethylated DMR either in their promoter or transcribed region, comparing KD2 vs. WT and KD5 vs. WT was performed. Next, common DEG in both datasets was identified. **(d, e)** The distribution of methylation context in DEG with hypermethylated DMR in the promoter are shown in a Venn diagram. A set of 83 common DEG, 55 down (d) and 28 up regulated (e), with hypermethylated DMR in the promoter region were detected mostly in CG and CHG contexts. **(f, g)** The distribution of methylation context in DEG with hypermethylated DMR in the transcribed region are shown in a Venn diagram. We found 524 DEG, 254 down (f) and 270 up regulated (g), with hypermethylated DMR common to both transgenic lines located mainly in a CG context.

Figure 4.4. Genes targets of PtaDML10 during bud break. **(a)** Model showing the inefficient reactivation of growth promoting genes in KD plants during bud break. **(b,c)** Biological processes for DEG genes hypermethylated in their promoter region (b) or in their transcribed region (c) and downregulated in KD lines compared to WT, primarily expressed in young leaves or SAM and induced during bud break. **(d)** Model showing the inefficient repression of dormancy genes in KD plants during bud break. **(e,f)** Biological processes of DEG genes hypermethylated in their promoter region (e) or in their transcribed region (f), upregulated in KD lines compared to WT and repressed during bud break.

4.4 CONCLUSION

All these data, suggest that the active DNA demethylation carried out by PtaDML10 is required for bud break and occurs as two simultaneous processes: enabling the induction of cell metabolism genes and key regulators of meristem development and maintenance needed for the reactivation of growth and triggering the downregulation of genes more highly expressed during bud dormancy. Most hypermethylated and downregulated genes in *PtaDML10* KD plants were those involved in cellular metabolic processes, indicating that PtaDML10 is an important

regulator of spring bud break. In apple, Kumar et al. (2016) found that reduced genomic DNA methylation during bud break affects the expression of genes involved in cellular metabolic processes, suggesting a conserved regulatory mechanism controlling growth-dormancy cycles in deciduous trees. Although a role for *DEMETER-LIKE* genes in Arabidopsis embryo development has been long established (Hsieh et al. 2009), mutants in Arabidopsis DMLs showed little or no ulterior developmental alterations (Gong et al. 2002; Penterman et al. 2007; Yamamuro et al. 2014) suggesting that active DNA demethylation is not critical for normal development in this species. The participation of these genes in postembryonic development has recently emerged in crops. It has been shown that active 5mC demethylation by DEMETER proteins is required for transcriptional reprogramming during tomato fruit maturation (Liu et al. 2015), symbiotic organogenesis uses DME-dependent epigenetic regulation to reprogram gene expression in specific host cells (Satgé et al. 2016), and as observed here, to restore sensitivity to growth-promoting stimuli for bud break in poplar. To date, the molecular regulation of bud break is poorly understood. So far, only a molecular cascade triggered by EBB1 has been implicated in the regulation of this process (Yordanov et al. 2014; Busov et al. 2016). No overlapped was found when we crossed the putative direct target genes of PtaDML10 with the putative direct targets of EBB1. Hence, it seems that PtaDML10 and EBB1 mechanisms act via separate pathways to control bud break. Our functional study is the first to associate a tree DNA demethylase, PtaDML10, with the chilling-dependent genomic DNA demethylation needed to control bud break and the reactivation of cell proliferation in the SAM after winter dormancy.

4.5 REFERENCES

- Abascal F, Zardoya R, Posada D. 2005. ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics* 21: 2104–2105.
- Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, Mason CE. 2012. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome biology* 13: R87.
- Anh Tuan P, Bai S, Saito T, Imai T, Ito A, Moriguchi T. 2016. Involvement of EARLY BUD-BREAK, an AP2/ERF Transcription Factor Gene, in Bud Break in Japanese Pear (*Pyrus pyrifolia* Nakai) Lateral Flower Buds: Expression, Histone Modifications and Possible Target Genes. *Plant and Cell Physiology* 57: 1038–1047.
- Belles-Boix E, Hamant O, Witiak SM, Morin H, Traas J, Pautot V. 2006. KNAT6: an Arabidopsis homeobox gene involved in meristem activity and organ separation. *The Plant cell* 18: 1900–7.

Blankenberg D, Kuster G Von, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. 2010. Galaxy: A web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology*.

Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O. 2006. CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science (New York, N.Y.)* 312: 1040–3.

Busov V, Carneros E, Yakovlev I. 2016. EARLY BUD-BREAK1 (EBB1) defines a conserved mechanism for control of bud-break in woody perennials. *Plant signaling & behavior* 11: e1073873.

Byrne ME, Groover AT, Fontana JR, Martienssen R a. 2003. Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. *Development (Cambridge, England)* 130: 3941–3950.

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

Conde D, González-Melendi P, Allona I. 2013. Poplar stems show opposite epigenetic patterns during winter dormancy and vegetative growth. *Trees - Structure and Function* 27: 311–320.

Ding J, Nilsson O. 2016. Molecular regulation of phenology in trees-because the seasons they are a-changin'. *Current Opinion in Plant Biology* 29: 73–79.

Fletcher JC. 2001. The ULTRAPETALA gene controls shoot and floral meristem size in Arabidopsis. *Development* 128: 1323–1333.

Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, *et al.* 2005. Galaxy: A platform for interactive large-scale genome analysis. *Genome Research* 15: 1451–1455.

Goecks J, Nekrutenko A, Taylor J. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome biology* 11: R86.

Gong Z, Morales-Ruiz T, Ariza RR, Roldán-Arjona T, David L, Zhu JK. 2002. ROS1, a

repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. *Cell* 111: 803–814.

Gourcilleau D, Bogeat-Triboulot M-B, Le Thiec D, Lafon-Placette C, Delaunay A, El-Soud WA, Brignolas F, Maury S. 2010. DNA methylation and histone acetylation: genotypic variations in hybrid poplars, impact of water deficit and relationships with productivity. *Annals of Forest Science* 67: 208.

Howe GT, Horvath DP, Dharmawardhana P, Priest HD, Mockler TC, Strauss SH. 2015. Extensive Transcriptome Changes During Natural Onset and Release of Vegetative Bud Dormancy in Populus. *Frontiers in plant science* 6: 1–28.

Hsieh T-F, Ibarra CA, Silva P, Zemach A, Eshed-Williams L, Fischer RL, Zilberman D. 2009. Genome-wide demethylation of Arabidopsis endosperm. *Science (New York, N.Y.)* 324: 1451–4.

Ibañez C, Ramos A, Acebo P, Contreras A, Casado R, Allona I, Aragoncillo C. 2008. Overall alteration of circadian clock gene expression in the chestnut cold response. *PLoS ONE* 3.

Karlberg A, Bako L, Bhalerao RP. 2011. Short day-mediated cessation of growth requires the downregulation of AINTEGUMENTALIKE1 transcription factor in hybrid aspen. *PLoS Genetics* 7.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* 30: 772–780.

Krueger F, Andrews SR. 2011. Bismark: A flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* 27: 1571–1572.

Kumar G, Rattan UK, Singh AK. 2016. Chilling-Mediated DNA Methylation Changes during Dormancy and Its Release Reveal the Importance of Epigenetic Regulation during Winter Dormancy in Apple (*Malus x domestica* Borkh.). *Plos One* 11: e0149934.

Leida C, Conejero A, Arbona V, Gómez-Cadenas A, Llácer G, Badenes ML, Ríos G. 2012. Chilling-dependent release of seed and bud dormancy in peach associates to common changes in gene expression. *PLoS ONE* 7.

Letunic I, Bork P. 2007. Interactive Tree Of Life (iTOL): An online tool for phylogenetic tree display and annotation. *Bioinformatics* 23: 127–128.

Liu R, How-Kit A, Stammadti L, Teyssier E, Rolin D, Mortain-Bertrand A, Halle S, Liu M, Kong J, Wu C, *et al.* 2015. A DEMETER-like DNA demethylase governs tomato fruit ripening. *Proceedings of the National Academy of Sciences of the United States of America* 112: 10804–9.

Mohamed R, Wang CT, Ma C, Shevchenko O, Dye SJ, Puzey JR, Etherington E, Sheng X, Meilan R, Strauss SH, *et al.* 2010. Populus CEN/TFL1 regulates first onset of flowering, axillary meristem identity and dormancy release in Populus. *Plant Journal* 62: 674–688.

Moreno-Cortés A, Hernández-Verdeja T, Sánchez-Jiménez P, González-Melendi P, Aragoncillo C, Allona I. 2012. CsRAV1 induces sylleptic branching in hybrid poplar. *New Phytologist* 194: 83–90.

Penterman J, Zilberman D, Huh JH, Ballinger T, Henikoff S, Fischer RL. 2007. DNA demethylation in the Arabidopsis genome. *Proceedings of the National Academy of Sciences of the United States of America* 104: 6752–6757.

Penuelas J, Filella I. 2001. Responses to a Warming World. *Science* 294: 793–794.

Rios G, Leida C, Conejero A, Badenes ML. 2014. Epigenetic regulation of bud dormancy events in perennial plants. *Frontiers in Plant Science* 5: 247.

Rohde A, Storme V, Jorge V, Gaudet M, Vitacolonna N, Fabbrini F, Ruttink T, Zaina G, Marron N, Dillen S, *et al.* 2011. Bud set in poplar - genetic dissection of a complex trait in natural and hybrid populations. *New Phytologist* 189: 106–121.

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

Satgé C, Moreau S, Sallet E, Lefort G, Auriac M-C, Remblière C, Cottret L, Gallardo K, Noirot C, Jardinaud M-F, *et al.* 2016. Reprogramming of DNA methylation is critical for nodule development in Medicago truncatula. *Nature Plants* 2: 16166.

Shim D, Ko J-H, Kim W-C, Wang Q, Keathley DE, Han K-H. 2014. A molecular framework for seasonal growth-dormancy regulation in perennial plants. *Horticulture Research* 1: 14059.

Singh RK, Svystun T, Aldahmash B, Maria A, Bhalerao RP. 2016. Tansley review control of phenology in trees – a molecular perspective.

Stamatakis A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690.

Trap-Gentil MV, Hèbrard C, Lafon-Placette C, Delaunay A, Hagge D, Joseph C, Brignolas F, Lefebvre M, Barnes S, Maury S. 2011. Time course and amplitude of DNA methylation in the shoot apical meristem are critical points for bolting induction in sugar beet and bolting tolerance between genotypes. *Journal of Experimental Botany* 62: 2585–2597.

Tylewicz S, Tsuji H, Miskolczi P, Petterle A, Azeez A, Jonsson K, Shimamoto K, Bhalerao RP. 2015. Dual role of tree florigen activation complex component FD in photoperiodic growth control and adaptive response pathways. *Proceedings of the National Academy of Sciences of the United States of America* 112: 3140–5.

UPOV (1981) Guidelines for the Conduct of Tests for Distinctness, Homogeneity and Stability Populus L. International Union for the Protection of New Varieties of Plants, Geneva

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview Version 2-A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25: 1189–1191.

Yamamuro C, Miki D, Zheng Z, Ma J, Wang J, Yang Z, Dong J, Zhu J-K. 2014. Overproduction of stomatal lineage cells in Arabidopsis mutants defective in active DNA demethylation. *Nature communications* 5: 4062.

Yordanov YS, Ma C, Strauss SH, Busov VB. 2014. EARLY BUD-BREAK 1 (EBB1) is a regulator of release from seasonal dormancy in poplar trees. *Proceedings of the National Academy of Sciences of the United States of America* 111: 10001–6.

Zhu J-K. 2009. Active DNA demethylation mediated by DNA glycosylases. *Annual review of genetics* 43: 143–66.

Zhu R, Shevchenko O, Ma C, Maury S, Freitag M, Strauss SH. 2013. Poplars with a PtDDM1-RNAi transgene have reduced DNA methylation and show aberrant post-dormancy morphology. *Planta* 237: 1483–1493.

4.6 SUPPORTING INFORMATION FOR CHAPTER 4

Figure S4.1. Phylogenetic tree analyses of DML proteins from Arabidopsis and poplar. **(a)** Maximum likelihood tree obtained using the RAXML tool with 100 bootstrap replicates. (Only bootstraps higher than 70 are shown). Arabidopsis protein sequences of DMLs were retrieved using the TAIR10 website (www.arabidopsis.org). These sequences were used as queries to BLAST the Populus trichocarpa proteome (Phytozome v10.3, www.phytozome.net) and identify homolog proteins in this organism. These protein were aligned using MAFFT (E-INS-i algorithm) (Kato & Standley 2013) and edited in Jalview 2.8 (Waterhouse et al. 2009). The best evolutionary model of the alignment was highlighted using ProtTest 2.4 (Abascal et al. 2005). The maximum likelihood tree was inferred with the RAXML tool (Stamatakis, 2006) and visualized in the iTOL website <http://itol.embl.de> (Letunic & Bork 2007). Bootstrap values were generated with 100 replicates. **(b)** Scheme showing 5mC DNA glycosylase conserved domains in Arabidopsis and poplar. Domains were assembled in the PROSITE website (<http://prosite.expasy.org/mydomains/>). PtDML6, PtDML8 and PtDML10 conserve all the domains required for 5mC DNA glycosylase activity. Orange oval represents the lysine-rich domain. Green rectangles represent the motifs of the DNA glycosylase domain. Blue pentagon represents the c-terminal domain. **(c)** MAFF alignment of the 5mC DNA glycosylase domain of Arabidopsis and poplar. Black

boxes indicate identical aminoacids. Black asterisk indicates the non-conserved linker sequence between the two motifs of the DNA glycosylase domain. Arrows indicate the position of the aminoacids necessary for the 5mC demethylation activity of AtROS1, AtDME and AtDML2-3 (green: required for base 5mC excision, blue: required for preference for 5mC over T mismatches, red: involved in control of context preference for 5mC). Red boxes indicate HhH-GPD and [4Fe-4S] motifs. PtaDML6, PtaDML8 and PtaDML10 show all the aminoacids that have been described to be essential for DMLs activity.

Figure S4.2. *PtaDML8* mRNA expression from dormancy to bud break in poplar. (a,b) Quantitative RT-PCR analysis of *DML8* in poplar 2-year-old branches: (a) along the year and (b) during winter dormancy from Oct 31 weekly to bud break on Apr 9. Plotted values and error bar are fold-change means $\pm$ SD recorded in three technical replicates. (c) Normalized expression of *PtaDML8* was determined by RNA sequencing in poplar apices growing under natural conditions from Jan 13 to bud break on Apr 14.

Figure S4.3. Characterization of *PtaDML10* RNAi transgenic lines. Quantitative RT-PCR analysis of *PtaDML8* and *PtaDML10* in WT plantlet leaves. Samples were collected from plantlets growth in a greenhouse for 5 weeks in conditions of LD, 22°C. Plotted values and error bars are the fold-change means $\pm$ SD recorded in three technical replicates.

a

PtaDML8 forward	5'-TTTCAACAGAAGGAGTTCAGCA-3'
PtaDML8 reverse	5'-AGCTTGCCATGTCTATTAACAAA-3'
PtaDML10 forward	5'-ACCAACAATGCCAGTCCAGAGA-3'
PtaDML10 reverse	5'-CAAACCTCTCCAAAAGCAGAAC-3'
PtaCYCD6:1 forward	5'-AAGGGTTTCTTGCAACTTCG-3'
PtaCYCD6:1 reverse	5'-CATGGCTTTGGTTGAGGAAT-3'
18S forward	5'-TCAACTTTCGATGGTAGGATAGTG-3'
18S reverse	5'-CCGTGCAGGATTGGTAATTT-3'

b

<i>PtDML10</i> -RNAi forward	5'GGGGACAAGTTTGTACAAAAAGCAGGCTGAGATTGGAGAGTAGGCTTTCTG3'
<i>PtDML10</i> -RNAi reverse	5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCGGTAGATTCAGTAGTTTAGTTGC3'

Figure S4.4. (a) The gene-specific primers used for the analyses of their expression by real-time PCR. **(b)** Primers with attB sites used to generate the RNAi fragment for downregulated *PtaDML10* gene expression.

5. A DNA DEMETHYLASE OVEREXPRESSION PROMOTES EARLY APICAL BUD MATURATION IN POPLAR THROUGH THE BIOSYNTHESIS AND ACCUMULATION OF FLAVONOIDS

5.1 ABSTRACT

The transition from active growth to dormancy is critical for the survival of perennial plants. We identified a *DEMETER-like* (*CsDML*) cDNA from a winter-enriched cDNA subtractive library in chestnut (*Castanea sativa* Mill.), an economically and ecologically important species. Next, we explored the role of this DNA demethylase and its putative orthologue in the more experimentally tractable hybrid poplar (*Populus tremula x alba*), under the signals that trigger bud dormancy in trees. We performed phylogenetic and protein sequence analysis, gene expression profiling and 5mC immunodetection studies to evaluate the role of *CsDML* and its homologue in poplar, *PtaDML6*. Transgenic hybrid poplars overexpressing *CsDML* were produced and analyzed. SD and cold temperatures induced *CsDML* and *PtaDML6*. Overexpression of *CsDML* accelerated SD-induced bud formation while acquiring the red-brown coloration earlier than wild type (WT) plants, alongside with the upregulation of flavonoid biosynthesis enzymes and accumulation of flavonoids in the SAM and bud scales. Our data shows that the *CsDML* gene induces bud formation needed for the survival of the apical meristem under the harsh conditions of winter. This study provides *in planta* evidence implicating chromatin remodeling by DNA demethylation during SD induction of bud maturation through the induction of flavonoids biosynthesis.

Keywords: bud formation, chestnut, DEMETER, DNA demethylase, poplar, winter dormancy

5.2 INTRODUCTION

Coordinating growth and reproduction with the environment is essential for the survival of perennial plants in temperate and boreal latitudes (Peñuelas & Filella 2001). In these regions, deciduous and periodic growth habits evolved into a single trait known as winter dormancy. This process begins with the growth cessation through the arrest of meristems activity and subsequent enclose of the apical meristem into a dormant winter bud, a process called bud set (Rhode & Bhalerao 2007, Paul et al. 2014). This is a precondition for the establishment of dormancy in the meristem. Considerable time may elapse before full winter dormancy is achieved, considered it as the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favorable conditions (Rhode et al. 2002, Rhode & Bhalerao 2007). For some perennial plants such as poplar, the perception of SD is sufficient to promote cessation of growth and bud formation (Ding & Nilsson 2016). These processes may be positively influenced by synergic crosstalk between SD and temperature drops in autumn (Rohde et al. 2011a).

Early insights into the molecular mechanisms of growth cessation and bud set induced by SD were gained through the analysis of the poplar homologues of *Arabidopsis* (*Arabidopsis thaliana* L.) *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) (Böhlenius et al. 2006). Downregulation of the *FT2* gene by SD was shown to be an early event promoting growth cessation (Böhlenius et al. 2006; Hsu et al. 2011). Downstream of *FT2*, and during the subsequent downregulation of *FLOWERING LOCUS D-LIKE1* (*FDL1*), MADS box transcription factors Like-APETALA1 (*LAP1*) and AINTEGUMENTA LIKE1 (*AIL1*) were found to play a role in growth cessation under SD conditions in poplar apices (Karlberg et al. 2010; Petterle et al. 2013; Azeez et al. 2014; Tylewicz et al. 2015). Phytochrome photoreceptors and circadian clock core components upstream of these genes have also been implicated in the early regulation of growth cessation by SD (Kozarewa et al. 2010; Ibañez et al. 2010; Ding & Nilsson 2016). While shoot elongation ceases, apical buds develop. During this process, leaf primordia become bud scales enclosing the apical meristems and preformed embryonic shoots. These bud scales accumulate phenolic compounds, leading to their characteristic red-brown color (Rohde et al. 2002). *ABI3* is involved in this processes and ethylene resistant birch shows an altered bud development (Rohde et al. 2002; Ruonala et al. 2006). SD signals also influence growth by modulating gibberellic acid and indole-acetic acid pathways (Eriksson et al. 2000; Petterle et al. 2013).

Several transcriptome studies in *Populus spp.* and other perennial plants have correlated the expression patterns of genes linked to DNA methylation with the transition from active growth to dormancy of the apical and stem meristems (Howe et al. 2015; Karlberg et al. 2010;

Rios et al. 2014; Shim et al. 2014;). Genomic DNA methylation is known to be involved in numerous aspects of chromatin function, particularly gene expression (Law & Jacobsen 2010). In plants, three classes of DNA methyltransferases catalyze 5-methyl-cytosine methylation (5mC) in symmetrical (CG or CHG) and nonsymmetrical (CHH) contexts (H represents A, T or C) (Zhang et al. 2010). However the opposite process, regulation of 5mC DNA demethylation, is less well understood. It may take place passively through the synthesis of unmethylated strands during DNA replication (Law & Jacobsen 2010), or actively through the actions of enzymes that remove methylated bases. Plants have evolved specific enzymes to remove methylated cytosines, and these include a subgroup of DNA glycosylase-lyases known as DEMETER LIKE DNA demethylases (DMLs). Methylome studies performed in *Arabidopsis* and *Populus trichocarpa* (Torr & Gray) have demonstrated that DNA methylation in promoter and upstream sites near the transcriptional start site (TSS) suppress gene expression (Zhang et al. 2006; Vining et al. 2012; Lafon-Placette et al. 2013; Liang et al. 2014). In *Arabidopsis*, active DNA demethylation by DMLs reduces extensive DNA methylation near TSS and the 3' end of the transcribed region in hundreds of discrete *loci* of the genome (Penterman et al. 2007; Zhu et al. 2007; Ortega-Galisteo et al. 2008). Prior work in *Arabidopsis*, tomato (*Solanum lycopersicum* L.) and tobacco (*Nicotiana tabacum* L.) has assigned DMLs a main role as transcriptional activators of their target genes. Indeed, gene activation by DMLs is necessary for the response of plants to biotic and abiotic stresses (Yu et al. 2013; Le et al. 2014; Bharti et al. 2015) and for plant development (Gehring et al. 2006; Liu et al. 2015). Epigenetic regulation of adaptive responses of forest tree species to the environment, such as changes in DNA methylation, have been implicated in the switch from active growth to dormancy (Santamaría et al. 2009, Conde et al. 2013; Zhu et al. 2013; Kumar et al. 2016), possibly one of the most critical developmental transitions for the survival of perennial plants. However, functional studies have not confirmed this hypothesis. Here we report the identification, phylogenetic and expression analyses and phenological studies of a chestnut 5mC DNA demethylase that triggers apical bud maturation under SD conditions in poplar.

5.3 MATERIAL AND METHODS

Plant materials, growth conditions and assays

To monitor gene expression throughout the year, 2-year-old branches were harvested monthly from adult European chestnut trees (*Castanea sativa* Mill.) growing under natural conditions in Zarzalejo (Madrid, Spain 4°11'W, 40°35'N). At each time point, samples were collected from three different chestnut tree individuals. To evaluate gene expression induction by short day (SD) conditions, plants were first grown under long day (LD) (16h light/8h darkness) at 22°C during 8 months, and then changed to SD (8h light/16h darkness) at 22°C for

6 weeks. In each time point, stems of 4 plants were sampled and pooled. To evaluate gene expression induction by low temperatures (4°C), plants were grown under LD conditions at 22°C during 8 months and then changed to LD and 4°C during 7 weeks. In each time point, stems of 4 plants were sampled and pooled. For the dormancy release experiment, 11-month-old dormant chestnut plantlets grown under natural conditions were transferred to a growth chamber under LD and 22°C before the chilling requirement was fulfilled. Stems from 3 plantlets were sampled and pooled at the indicated time points. Two biological replicates were analyzed. The details of these experiments have been described elsewhere (Ramos et al. 2005).

To evaluate gene expression induction by SD in poplar (*Populus tremula x alba* INRA clone 717 1B4), plants were first grown under LD and 22°C during 6 weeks, and then changed to SD and 22°C during 7 weeks. In each time point, stems of 2 plants were sampled and pooled. To evaluate the induction by cold temperatures in poplar, plants were first grown under LD, 22°C conditions during 8 weeks and then changed to 4°C during 7 weeks. In each time point, stems of 3 plants were sampled and pooled.

For the phenological assays, *in vitro*-cultivated poplar plantlets (*P. tremula x alba* INRA clone 717-1B4) of wild type (WT) and two selected independent *CsDML*-overexpressing lines, *CsDML* OX1 and OX3, were transferred to 3.5 L pots containing blond peat, pH 4.5 (Moreno-Cortés et al. 2012). Before any photoperiodic treatments, plants were grown in a chamber under controlled conditions: LD, 22°C, 65% relative humidity and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux). After 4 weeks these plants were exposed to a SD, 19°C regime for 10 weeks to induce bud set. Bud set progression was graded by scoring from stage 3 (fully growing apex) to stage 0 (fully formed apical bud) according to Rohde et al. (2011b). Apical buds for immunofluorescence were sampled at 0 and 24 days after SD induction; buds used for transcriptome analysis were sampled 28 days after SD induction; apical buds for quercetin fluorescence detection were sampled at 28 and 48 days after SD induction and apical buds for flavonoids absorbance measurements by spectrophotometry were sampled after 48 days in SD, in three independent assays. To break dormancy and allow further bud burst under good conditions (LD, 22°C), poplars plants were subjected to a SD, 4°C regime for 4 weeks. The regrowth was scored according to the six developmental stages of bud burst (stage 0 to stage 5) according to UPOV (International Union for the Protection of New Varieties of Plants, 1981) and Ibáñez et al. (2010). Throughout the entire experiment, photosynthetic photon flux and relative humidity were kept at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 65%, respectively. Statistical significance on differences in the phenological apex stages between *CsDML* OX and WT plants was tested through pairwise comparisons using the Wilcoxon Rank Sum test after Shapiro-Wilk

confirmation of the non-normal distribution of data. All statistical tests were run in R (www.r-project.org).

To evaluate the expression levels of *PtaCHS*, *PtaDFR* and *Pta3GT* in transgenic and WT poplar plants, *CsDML* OX1, *CsDML* OX3 and WT in 3 weeks old plants were grown under SD conditions during 3 weeks. A pull of 3 apical buds was taken at this time point to analyze gene expression in each sample. Two biological replicates, three technical replicas for each one, were performed.

Isolation of full-length CsDML cDNA

A 464-bp-long fragment of chestnut *DEMETER-like* (*CsDML*) cDNA was obtained from a winter-enriched cDNA subtractive library (PCR-Select cDNA Subtraction Kit, Clontech Laboratories, Mountain View, CA, USA) prepared from 2-year-old branches of adult trees (Ramos et al. 2005). Full-length cDNA was obtained using the SMARTer RACE cDNA amplification kit (Clontech Laboratories). *CsDML* nucleotide sequence was deposited in the GenBank sequence databases with the accession number KX575710.

Phylogenetic analysis of HhH glycosylase family members and in-silico identification of the active site for DNA demethylation in the CsDML protein

Sequences of proteins containing the hairpin-helix-hairpin (HhH) glycosylase domain, a characteristic feature of the *CsDML* protein, were retrieved from *Arabidopsis thaliana* genome using the TAIR10 website (www.arabidopsis.org). These sequences were used as queries to BLAST the *Populus trichocarpa* proteome (Phytozome v10.3, www.phytozome.net) and identify homologue proteins in this organism. All the protein sequences retrieved from *Arabidopsis* and poplar, together with *CsDML*, were aligned using MAFFT (E-INS-i algorithm) (Katoh & Standley 2013) and edited in Jalview 2.8 (Waterhouse et al. 2009). The best evolutionary model of the alignment was highlighted using ProtTest 2.4 (Abascal et al. 2005). The maximum likelihood tree was inferred with the RAxML tool (Stamatakis 2006) and visualized in the iTOL website <http://itol.embl.de> (Letunic & Bork 2007). Bootstrap values were generated with 100 replicates. Putative members of the 5mC DNA glycosylase family were named PtDML, followed by a number corresponding to the chromosome where they are located.

An additional alignment using MAFFT (E-INS-i algorithm) (Katoh & Standley 2013) was performed with 5mC DNA glycosylases of *Arabidopsis thaliana* (AtDMLs), *Populus trichocarpa* (PtDMLs) and *Castanea sativa* (*CsDML*). The result was visualized in Jalview 2.8 (Waterhouse et al. 2009). The conserved regions of these proteins were assembled in the

PROSITE website (<http://prosite.expasy.org/mydomains/>), allowing identification of the catalytic center residues in the CsDML protein.

Quantitative RT-PCR expression analysis

The procedures used for total RNA extraction, synthesis of single-stranded cDNA, design of gene-specific primers, quantitative real-time PCR (qRT-PCR) and data analysis have been previously described in Ramos et al. (2005) & Ibáñez et al. (2008). 18S was previously used as reference gene (Ramos et al. 2005; Ibáñez et al. 2008; Berrocal-Lobo et al. 2011, Hsu et al. 2011) showing to be stably expressed in all the different conditions used for this study both in chestnut and poplar. The primers used are described in **Fig. S5.1a**.

Generating constructs in a binary vector and poplar transformation

The coding sequence (CDS) of the *CsDML* gene was amplified from chestnut cDNA using PfuUltra Hotstart High-Fidelity DNA polymerase (Agilent Technologies, La Jolla, CA, USA) and the primers used are described in **Fig. S5.1b**. The *attB*-flanked PCR product was purified, inserted in the pDONR222 vector (Life Technologies, Carlsbad, CA, USA) and verified by sequencing. The insert was transferred into the destination binary vector pGWB15, which includes a cauliflower mosaic virus (CaMV) 35S promoter and adds 3 copies of hemagglutinin (3xHA) at the N-terminal protein end (Nakagawa et al. 2007). The generation and selection of hybrid poplars overexpressing *CsDML*, hereafter referred to as *CsDML* OX lines, was carried out as described in Moreno-Cortés et al. (2012). Based on qRT-PCR analysis of *CsDML* transgene expression in these plants, we selected 2 lines, *CsDML* OX1 and *CsDML* OX3, for further study.

Immunofluorescence of 5mC in SD-induced shoot apices

Overall DNA methylation in 5mC residues was examined by immunofluorescence in SD-induced (for 24 days) and non-induced shoot apices of *CsDML* OX and WT hybrid poplars (*CsDML* OX1, *CsDML* OX3 and WT). These apices were formaldehyde-fixed and preserved as previously described in Conde et al. (2013) before embedding in paraffin wax. For this, the specimens were washed twice in PBS (30 min each) and dehydrated in a series of 30%, 50%, 70% (1 h each) and 95% ethanol overnight. Specimens were then washed in 100% ethanol for 12 h. Ethanol was then replaced with a mixture 50% histoclear (HC)-50% ethanol overnight and 100% HC for 12 h (twice). Vials containing the apices were filled up to a quarter of their volume with paraplast and incubated at 42°C for 12 h. Next, another quarter of volume was added, followed by incubation at 60°C overnight. Paraplast in HC was replaced with melted paraffin wax (60°C), and this was replenished every 12 h for 5 days. Finally, the apices were

placed in molds and, after solidification at room temperature, they were kept at 4°C. Sections of 15-20 µm were obtained in a microtome Leica 2055 (Leica Microsystems, Wetzlar, Germany) and the paraffin was removed in HC for 15 min and then in 100% ethanol for 15 min. The sections were rehydrated in a series of 96% and 70% ethanol for 10 min each and, finally, in PBS for 15 min. Images of immunolocalization were acquired using a Leica TCS-SP8 confocal microscope under the laser excitation line of 561nm. Gain and offset conditions were maintained in all captures for comparison purposes. Signal quantification in apex sections were performed as reported in (Conde et al. 2013). Three independent samples were analyzed for each genotype and time point.

Probe synthesis and Fluorescence in situ hybridization (FISH)

A specific probe to the mRNA of *PtaDML6* was synthesized from a fragment of 1100-pb-long of the 5' of the Untranslated Terminal Region (UTR). The *PtaDML6* DNA was cloned into pGem-T (Promega, Madison, WI, USA). The probe was *in vitro* transcribed to obtain digoxigenin-UTP (DIG) labeled RNA probes using T7 and Sp6 RNA polymerases for the sense and the anti-sense mRNA probes, respectively (Roche Applied Science, Mannheim, Germany). The probe of 1100-pb-long was subjected to a mild carbonate hydrolysis step to obtain approx. 100-pb-long RNA fragments before hybridization (Cox et al. 1984).

The specimens for *FISH* were collected from WT poplar trees growth under SD at time points 0 and 24 days. Apexes were fixed, paraffin-wax embedded and cut as described before for the immunolocalization of global 5mC. After deparaffination, dehydration and rehydration, as previously described, bud sections were treated with 2% cellulase and proteinase K (1 µg ml⁻¹) at 37°C, for 1 hour each. Afterwards, 30 ng µl⁻¹ of either the sense or the antisense probe diluted in the hybridization mixture (50% formamide, 10% dextrane sulfate, 10 mM Pipes, 1 mM EDTA, 300 mM NaCl, 1000 ng µl⁻¹ tRNA) was applied to the sections and incubated at 50°C overnight. After hybridization the slides were washed in 1X Saline Sodium Citrate buffer (SSC) for 30 minutes at 50°C. DIG detection and fluorescence visualization were carried out with a primary anti-DIG (Sigma) antibody, diluted 1/3000 in 3% BSA in PBS for 1 hour at room temperature, and a secondary anti mouse 546 (ALEXA, Molecular Probes) antibody, applied 1/25 in 3% BSA in PBS for 1 hour at room temperature in the dark. Images were acquired using a Leica TCS-SP8 confocal microscope under the laser excitation line of 561 nm. Gain and offset conditions were maintained in all captures for comparison. Three independent samples were analyzed for each genotype and time point.

Quercetin fluorescence detection

Quercetin accumulation was examined by fluorescence in apical buds of *CsDML* OX1, *CsDML* OX3 and WT plants after 28 and 48 SD, using diphenylboric acid-2-aminoethyl ester (DPBA, Sigma-Aldrich) dye (0.25% in water). Quercetin-DPBA fluorescence was collected on a Leica MZ10F stereomicroscope with a GREEN filter (excitation BP 546/10, emission LP 590). Images were recorded with CCD Leica DFC 420C camera. The specificity of the signal was already tested for this flavonoid (Silva-Navas et al. 2016). Three independent samples were analyzed for each genotype and time point.

Flavonoids measurements

To determine the total flavonoid content of *CsDML* OX1, *CsDML* OX3 and WT plants after 48 days in SD, 100 mg of fresh weight of a pull of 4 apical buds for each sample, were homogenized in HCL:methanol (1:99, v/v). The tissue was shaken at 4°C overnight. The supernatant absorbance was determined in a Ultraspec 3300 Pro spectrophotometer (Amersham Biosciences) after diluting to ¼ with HCL:methanol (1:99, v/v) at 300 nm (Nogués & Baker, 2000). Three biological replicates were performed for this experiment for each genotype and time point.

RNA sequencing in SD-induced shoot apices and identification of differentially expressed transcripts

Expression profiles were examined by RNA sequencing (RNA-Seq) in SD-induced shoot apices of *CsDML* OX and WT hybrid poplars. At 28 day in SD, 25 apices were collected from 25 individuals of WT, *CsDML* OX1 and *CsDML* OX3 trees. Previous to RNA extraction, apices were dissected under a stereomicroscope to remove the leaflets surrounding the shoot apical meristem. RNA extraction was performed following earlier published protocol (Ibáñez et al. 2008). Libraries were prepared using 1 µg of RNA in the NEBNext® Ultra™ Directional RNA Library Prep Kit following the supplier's instructions (New England Biolabs, Ipswich, MA, USA). Sequencing was done with an Illumina NextSeq500 in high-throughput mode (2×150 cycles) at the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL, USA). Raw data for the RNAseq was deposited in the Gene Expression Omnibus database at NCBI, with GEO accession number GSE87155 (Edgar et al. 2002; Barret et al. 2013).

Pre-processing and assembly of sequence data were conducted using the web-based platform Galaxy at <https://usegalaxy.org/> (Giardine et al. 2005; Blankenberg et al. 2010; Goecks et al. 2010), which includes tools to remove reads containing N blurs, adapter sequences, and sequences shorter than 15 nucleotides. Quality control of reads was carried out with the FastQC Galaxy tool (Andrews 2010). Removal of rRNA, if needed, was performed locally with the

SortMeRNA tool (Kopylova et al. 2012). The TopHat Galaxy tool was used to map clean reads to the *Populus tremula* x *P. alba* INRA clone 717-1B4 genome using the default settings (Kim et al. 2013). We used the *summarizeOverlaps* function (packages GenomicAlignments of R) to generate the matrix of read count for each gene from BAM files. Differentially expressed genes (DEG) lists for *CsDML* OX vs. WT were obtained using the function *exactTest* (edgeR package of R) (Robinson et al. 2010). For DEG analyses, *CsDML* OX1 and *CsDML* OX3 transcriptomes were considered as biological replicates when comparing to WT, using a *FDR* < 0.1. Gene annotations for DEG were obtained from the *Populus trichocarpa* annotation table v.3.0. <http://www.phytozome.net>. Gene ontology (GO) categories were analyzed using the web-based platform agriGO at <http://bioinfo.cau.edu.cn/agriGO> (Du et al. 2010). Enriched GO categories were identified using an FDR-adjusted value of ≤ 0.05 as the cutoff for significance.

5.4 RESULTS

Identification of a DEMETER-LIKE gene during autumn in Castanea sativa

To identify candidate genes regulating the winter dormancy process, we selected a cDNA showing similarity to a DNA glycosylase that was preferentially expressed at the beginning of autumn (**Fig. S5.2a**). InterPro analysis of the full-length sequence revealed the presence of a conserved HhH-GPD domain (helix-hairpin-helix and Gly/Pro rich loop followed by a conserved aspartate). The HhH-GPD domain belongs to the superfamily of the DNA glycosylases, which excise oxidized and methylated bases thus correcting base pairing mismatches (Denver et al. 2003). Phylogenetic analyses of the *Arabidopsis* and poplar homologues of this chestnut DNA glycosylase showed that the protein clusters in the clade of 5mC DNA glycosylases (5mC), closer to the *Arabidopsis* DEMETER and poplar PtDML6 (**Fig. 5.1**). Based on this finding, the chestnut protein was named *CsDML* (*Castanea sativa* DEMETER-like). Biochemical and structural analyses have shown that *Arabidopsis* DEMETER (AtDME), DEMETER LIKE 2/3 (AtDML2, AtDML3) and REPRESSOR OF SILENCING 1 (AtROS1), all grouping in this cluster, are DNA glycosylases that specifically remove 5mC from DNA (Zhu 2009). The identification of *CsDML* in this cluster led us to look for the presence of a conserved 5mC DNA glycosylase active site in this protein.

Figure 5.1. Phylogenetic tree analyses of glycosylase proteins from *Arabidopsis*, poplar and chestnut. Maximum likelihood tree obtained using the RAXML tool with 100 bootstrap replicates. Gray clades grouped protein sequences closer to proteins of known function: AlkA: alkyladenine glycosylases that excise 3-methyladenine (Begley et al. 1999); Nth: endonuclease III and OggI: 8-oxoguanine glycosylase I, which remove oxidative damaged bases from the DNA backbone (Rosenquist et al. 1997); U: specific for the excision of uracil (Córdoba-Cañero et al. 2010); mA: excise 3-methyl adenine (Santerre & Britt 1994); MutM: formamidopyrimidine-DNA glycosylase (Duclos et al. 2012); 5mC: the group of 5mC DNA glycosylase (Zhu 2009). CsDML and 5 poplar DML proteins clustered with AtDME, AtROS1, AtDML2 and AtDML3. Only bootstraps higher than 70 are shown.

Comparative analyses of the 5-methyl cytosine DNA glycosylase domain suggests a functional catalytic site for CsDML and PtDML6

A MAFFT protein alignment was carried out using the 5mC DNA glycosylases sequences of our phylogenetic tree. The alignment revealed that CsDML, PtDML6, PtDML8 and PtDML10 protein sequences show the three characteristic domains of AtDME/AtDMLs/AtROS1: a lysine-rich domain, a DNA glycosylase domain and a C-terminal

domain (**Fig. 5.2a**). In AtDME/AtDMLs/AtROS1, the catalytic DNA glycosylase domain consists of two conserved non-contiguous segments connected through a linker region which is highly variable in sequence and length (Ponferrada-Marín et al. 2010). These segments are highly conserved in CsDML, PtDML6, PtDML8 and PtDML10, but partially conserved in PtDML1 and PtDML3 (**Fig. 5.2a,b**).

Structural studies and site-directed mutagenesis of AtROS1 and AtDME have identified the amino acids required for excision of 5mC in the second segment of the DNA glycosylase domain (Gong et al. 2002; Mok et al. 2010; Ponferrada-Marín et al. 2011; Parrilla-Doblas et al. 2013; Brooks et al. 2014). This domain includes the HhH-GPD motif, the invariant aspartate and the iron sulfur [4Fe-4S] cluster loop motif (**Fig. 5.2b**). CsDML, PtDML6, PtDML8 and PtDML10 contain all the residues required for the excision of 5mC, suggesting they have a functional catalytic site.

Figure 5.2. Comparative analysis of the 5mC DNA glycosylase domains of *Arabidopsis*, poplar and chestnut DMLs. (a) Scheme showing the 5mC DNA glycosylase conserved domains in *Arabidopsis*, poplar and chestnut. CsDML, PtDML6, PtDML8 and PtDML10 conserve all the domains required for 5mC DNA glycosylase activity. Orange oval represents the lysine-rich domain. Green rectangles represent the motifs of the DNA glycosylase domain. Blue pentagon represents the C-terminal domain. **(b)** MAFF alignment of the 5mC DNA glycosylase domain of *Arabidopsis*, poplar and chestnut. Black boxes indicate identical aminoacids. Black asterisk indicates the non-conserved linker sequence between the two motifs of the DNA glycosylase domain. Arrows indicate the position of the aminoacids necessary for the activity of AtROS1/AtDME/AtDML2-3 (green: required for base 5mC excision, blue: required for preference for 5mC over T mismatches, red: involved in control of context preference for 5mC). Purple boxes indicate HhH-GPD and [4Fe-4S] motifs. Purple asterisk show the position of the lysine residue necessary for bifunctional glycosylase/lyase activity and the conserved aspartic acid of the DNA glycosylase family.

SD and cold temperatures promotes CsDML and PtaDML6 expression

Day length shortening and temperature dropping in autumn are the environmental cues driving seasonal growth cessation and bud development in trees (Cooke et al. 2012). Given the seasonal expression of *CsDML*, preferentially at the beginning of autumn, we analyzed the expression of this gene under each inductive condition, separately (SD and cold temperature). By qRT-PCR, we determined that *CsDML* increased its expression in response to both signals (**Fig. S5.2b,c**). qRT-PCR analysis also indicated that *CsDML* mRNA expression is not required for dormancy maintenance, since its expression decreased in dormant plants that have not fulfilled the chilling requirement, when these plants were transferred to good conditions (**Fig. S5.2d**). Likewise, qRT-PCR experiments showed that expression of *PtaDML6* increased in response to SD and cold temperature in poplar stems (**Fig. S5.2f,g**). The SD-induced expression of *PtaDML6* was assessed by fluorescence *in situ* hybridization (FISH) on sections of poplar apical buds. After 24 days under SD, *PtaDML6* mRNA was detected in the shoot apical meristem (SAM) and leaf primordial but not in LD conditions (**Fig. 5.3a,b**). This result showed the accumulation of *PtaDML6* mRNA is SD dependent in the apex. Taking together these data suggest that these genes play a role in the regulation of bud set.

Figure 5.3. *PtaDML6* fluorescence *in situ* hybridization. *PtaDML6* mRNA detection in 20 μ m sections of paraffin-wax embedded WT apical buds at SD time points 0 and 24 days (n=3). The highest intensity of fluorescence was observed in cytoplasmic areas of cells in the meristem and leaf primordia at 24 days under SD conditions. Arrows point at the SAM.

Apical bud maturation is accelerated in CsDML OX poplars

The role of *CsDML* during dormancy was investigated by overexpressing it in the model species poplar. *CsDML* OX plants showed no phenotypic variation compared to the WT when grown under LD conditions. Given their higher expression levels (**Fig. S5.3**), *CsDML* OX1 and OX3 lines were chosen to examine growth and apical bud phenology in growth chambers, under photoperiodic and temperature conditions that mimic seasonal transitions autumn-winter-spring (Rohde et al. 2011a). Once leaf emergence from the apex ceased and

green bud scales were formed (stage 1) in response to SD, apical buds from both *CsDML* OX1 and OX3 lines underwent maturation more rapidly, reaching stage 0 earlier than WT buds (**Fig. 5.4a**). A deviation in the bud maturation rate was observed from the 30th day of SD exposition onwards. Thus, while WT took 30 days more to reach stage 0, *CsDML* OX1 and OX3 needed 19 and 23 days, respectively ($P < 0.01$) (**Fig. 5.4a**). Accordingly, bud scales in *CsDML* OX1 and OX3 plants turned red earlier than in WT plants (**Fig. S5.3**). These results indicate that constitutive expression of *CsDML* in poplar accelerates the transition from stage 1 to 0 during bud set. These results were confirmed by a second experiment, during which we sampled WT, *CsDML* OX1 and OX3 plants at time points $t=0$, $t=24$ and $t=50$ days under SD conditions (data not shown). Plant height measurements revealed that WT, *CsDML* OX1 and OX3 plants arrested their growth on 28th day of SD exposition (**Fig. 5.4b**). These results indicate that cessation of growth elongation is not affected by *CsDML* overexpression.

Once the chilling requirement had been fulfilled, WT, *CsDML* OX1 and OX3 plants were placed under LD, 21°C conditions and bud burst was monitored. After 18 days, all three genotypes showed greenish buds (stage 1 to 2), and after 30 days all three genotypes had elongated their shoots (stages 3 to 5) (**Fig. 5.4c**). These results suggest that constitutive expression of *CsDML* in poplar may not play a role in bud burst.

Figure 5.4. Phenological studies of *CsDML* OX plants. (a) Temporal dynamics of bud stage during the induction of apical bud formation by SD. WT, *CsDML* OX1 and OX3 plants were grown under LD, 22°C conditions for 4 weeks and then the buds were scored over the following 13 weeks after transferring the plants to SD, 19°C conditions. Mean bud scores $\pm$ SE are indicated for WT ($n=24$), *CsDML* OX1 ($n=21$) and *CsDML* OX3 ($n=25$). Significant differences between *CsDML* OX1 and WT at $t=49$ d of SD and between *CsDML* OX3 and WT at $t=53$ d of SD were analyzed by the Wilcoxon Rank Sum test in R, ***: $P<0.01$. (b) Growth cessation in response to SD. Heights of WT, *CsDML* OX1 and OX3 plants were measured under SD conditions for 44 days. Height means (cm) $\pm$ SE are indicated for WT ($n=24$), *CsDML* OX1 ($n=21$) and *CsDML* OX3 ($n=25$). (c) Timing of bud burst after dormancy. WT, *CsDML* OX1 and *CsDML* OX3 lines were treated as shown in (a), following the shift from SD, 4°C to LD, 22°C, apical growth reactivation was monitored. Mean bud burst scores $\pm$ SE are indicated for WT ($n=7$), *CsDML* OX1 ($n=7$) and *CsDML* OX3 ($n=7$).

5mC immunodetection studies suggest a locus-specific role for DNA demethylase during bud maturation

Overall changes in 5mC levels were compared in poplar apex sections of WT, *CsDML* OX1 and OX3 plants during SD induction of bud formation in growth chamber. By immunolocalization we examined overall DNA methylation levels under LD conditions ($t=0$) and after 24 days of growth under SD conditions ($t=24$), that is, six days before the apices of *CsDML* OX lines began to show faster apical bud maturation than WT plants (**Fig. 5.5a-f**). This experiment revealed that at $t=0$, DNA methylation levels are restricted to the central zone of the SAM (Paul et al. 2014) both in WT and *CsDML* OX apices (**Fig. 5.5a-c**). At $t=24$, DNA methylation spread to the rest of the SAM and other bud regions (Paul et al. 2014), with the same pattern in WT and *CsDML* OX apices (**Fig. 5.5d-f**). Quantification of the fluorescence derived from DNA methylation indicated that the ratio of levels in the central zone of the meristem and the remaining SAM was stable in the different genotypes at both time points (**Fig. 5.5g-i**). All these findings indicate *CsDML* overexpression does not affect the overall DNA methylation changes induced by SD, thus suggesting a locus-specific activity for this gene during bud development.

Figure 5.5. Immunodetection of 5mC in *CsDML* OX and WT. Immunofluorescence detection of 5-methylcytosine in 20 μ m sections of paraffin-embedded poplar apical buds obtained from the WT ($n=3$) (a,d) and lines *CsDML* OX1 ($n=3$) (b,e), *CsDML* OX3 ($n=3$) (c,f) (4-week-old trees). Apical bud showing the 5mC signal both in LD conditions ($t=0$) (a, b, c) and after 24 days of growth under SD conditions ($t=24$ d) (d, e, f). Histogram represents the ratio between fluorescence signals emitted by the central zone (CZ) and the whole SAM of WT (g), *CsDML* OX1 (h), *CsDML* OX3 (i) at $t=0$ vs. at $t=24$ d (means $\pm$ SE). Differences between each time point and genotype were assessed by one-way ANOVA. *** indicates significant differences between the ratio $t=24$ d to $t=0$ in each genotype ($P<0.01$).

CsDML OX plants differentially express genes involved in bud formation

To investigate the molecular basis causing the acceleration of bud maturation in *CsDML* OX plants, a genome wide transcriptome analysis was performed by RNA sequencing. WT and *CsDML* OX1 and OX3 plants were first grown in LD conditions during 4 weeks and then in SD conditions for additional 4 weeks. Apices were collected on day 28, just before the early bud maturation took place in *CsDML* OX plants (**Fig. 5.4a**). Total RNA was extracted from 25 pooled apices per genotype and RNA-seq libraries were generated and sequenced.

Transcriptome comparisons identified 318 DEG in *CsDML* OX lines compared to the WT, among which 79 were found to be upregulated and 239 downregulated (**Table S5.1**). To examine their molecular functions, GO analysis of these DEGs was conducted (**Table S5.2**). Upregulated genes in *CsDML* OX lines did not show significant enrichment in any biological categories. Downregulated genes showed enrichment in the following categories: cellular processes, cell cycle and cell division, DNA and chromatin modifications, anatomical structure development, and developmental processes (**Table S5.2**).

Upregulation of flavonoid biosynthesis have been shown as molecular markers of bud maturation (Ruttkink et al. 2007; Tylewicz et al. 2015). Our transcriptome analyses revealed three upregulated genes in *CsDML* OX plants encoding putative enzymes belonging to the flavonoids biosynthetic pathway. They were a chalcone synthase (CHS), a quercetin 3-O-glucosyltransferase (3GT) and a dihydroflavonol 4-reductase (DFR) (**Table S5.1**). CHS and DFR enzymes are known to be involved in the early steps of flavonoid biosynthesis (Ono et al. 2010), so upregulation of these genes in *CsDML* OX plants could correlates with their early red coloring during bud maturation (Perry 1971; Tylewicz et al. 2015).

To deep inside the flavonoids role in bud maturation, we determined by qRT-PCR that *PtaCHS*, *PtaDFR* and *Pta3GT* mRNAs, codifying for enzymes belonging to the flavonoids biosynthetic pathway, accumulated differentially after 21 days under SD conditions in apices of *CsDML* OX plants compared to WT plants. Expression of *PtaCHS* was 2.7 and 2.3 times higher in *CsDML* OX1 and *CsDML* OX3 than in WT. *PtaDFR* was 9 and 4.6 higher in *CsDML* OX1 and *CsDML* OX3 than in WT and *Pta3GT* was 3.7 and 2.1 higher in *CsDML* OX1 and *CsDML* OX3 than in WT (**Fig. 5.6a**).

CsDML OX accumulates flavonoids earlier than WT in SAM and bud scales

Towards determine whether an increment of flavonoid biosynthetic genes correlates with flavonoid accumulation, we quantified the absorbance of flavonoids in apical buds of WT, *CsDML* OX1 and *CsDML* OX3 after 48 days in SD. Both transgenic lines, showed an increment of the absorbance of flavonoids compared to WT, indicating the involvement of *CsDML* in the accumulation of flavonoids (**Fig. 5.6b**).

The spatio-temporal accumulation of flavonoids was explored by detecting quercetin, a flavonoid of the flavonol subgroup that can be identified by fluorescence after staining with DPBA, in apical buds at two time points. We localized and quantified quercetin in apical buds of *CsDML* OX1, *CsDML* OX3 and WT plants, after 28 and 48 days of SD induction. Apical buds were already formed after 28 days under SD conditions, although still immature, in WT, *CsDML* OX1 and *CsDML* OX3 trees. At this time point, the red fluorescence emitted by

quercetin-DPBA is primarily expressed in SAM, being its intensity higher in *CsDML* OX1 and *CsDML* OX3 SAM than in WT (**Fig. 5.6c,d**). After 48 days, bud maturation occurred earlier in the OX than in WT plants, as indicated by their higher red-brown coloration (**Fig. 5.4 and S5.3**). In the SAM of the three genotypes, fluorescence was higher after 48 days than after 28 days, suggesting an accumulation of quercetin in SAM under SD (**Fig. 5.6c-f**). Accordingly, fluorescence intensity was higher in the SAM of the transgenic lines SAM than in WT. After 48 days in SD, quercetin-DPBA fluorescence is also highly detected in external bud scales of both transgenic lines (**Fig. 5.6e,f**). The localization of quercetin after SD induction, matched with the *PtaDML6* spatial expression pattern suggesting the conserved functions between *CsDML* and *PtaDML6* promoting flavonoid biosynthesis.

Altogether, these results showed that *CsDML* could be involved in the induction of flavonoid biosynthesis enzymes, needed for apical bud maturation by the accumulation of flavonoids in the SAM and bud scales during SD induced bud formation.

Figure 5.6. Flavonoids accumulation assays in *CsDML* OX and WT plants apices. (a) Quantitative RT-PCR analysis of *PtaCHS*, *PtaDFR* and *Pta3GT* in *CsDML* OX1, *CsDML* OX3 and WT apices after 21 days in SD. Plotted values and error bars are fold-change means $\pm$ SE recorded in two biological replicates. (b) Absorbance measurements of flavonoid (at 300nm) in *CsDML* OX1, *CsDML* OX3 and WT apex extracts after 48 days in SD. Values are the means of three biological replicates, and bars indicate SE. Significant differences between genotypes were analyzed by ANOVA and the TUKEY test. Different letters showed significant differences ($P < 0.05$). (c) Localization of Quercetin-DPBA fluorescence emission in *CsDML* OX1, *CsDML* OX3 and WT apices after 28 days in SD. White arrows indicate the

5. A DNA demethylase overexpression promotes early apical bud maturation

SAM areas. **(d)** Quantification of Quercetin-DPBA fluorescence in the SAM in *CsDML* OX1, *CsDML* OX3 and WT apices after 28 days in SD. Values are the means of three independent samples, and bars indicate SE. Significant differences in genotype were analyzed by ANOVA and the TUKEY test. Different letters showed significant differences ($P < 0.05$). **(e)** Localization of Quercetin-DPBA fluorescence emission in *CsDML* OX1, *CsDML* OX3 and WT apices after 48 days in SD. White arrows indicates the SAM areas and blue arrows indicate the external bud scales. **(f)** Quantification of Quercetin-DPBA fluorescence in the SAM in *CsDML* OX1, *CsDML* OX3 and WT apices after 48 days in SD. Values are the means of three independent samples, and bars indicate SE. Significant differences between genotypes were analyzed by ANOVA and the TUKEY test. Different letters showed significant differences ($P < 0.05$).

DEGs analyses revealed the temporal effects of CsDML under SD-inductive conditions

Temporal transcriptional profiling under SD-inductive conditions revealed that 6,001 out of 16,495 genes analyzed were differentially expressed throughout apical bud development in poplar (Ruttink et al. 2007). When we crossed our list of DEGs in *CsDML*-OX with the time-lapse expression tables reported by Ruttink et al. (2007), only 18 of the up regulated and 67 of the down regulated genes in the *CsDML* OX versus WT plants, were previously analyzed in this study. From these genes, 12 upregulated and 44 downregulated (two thirds of the genes) were differentially expressed during apical bud development (**Fig. S5.5a**). According to those expression tables, average expression values for upregulated and downregulated genes were represented on a 6-weeks long SD treatment duration (**Fig. S5.5b**). We observed that upregulated genes in *CsDML* OX plants were also upregulated during the first 4 weeks of the SD treatment, while downregulated genes were also downregulated during that time (**Fig. S5.5b and Table S5.3**). So, molecular changes caused by overexpression of *CsDML* enhance the molecular changes previously published by Ruttink et al. (2007) under SD-inductive conditions.

5.5 DISCUSSION

A locus-specific 5mC DNA demethylase is involved in bud maturation

Here we show that *CsDML* is preferentially expressed at the beginning of autumn in response to SD and cold temperatures. Our qRT-PCR and RNA FISH studies confirm the induction by SD of the poplar closest orthologue to chestnut *CsDML*, *PtDML6*, in the apical meristem. Previous RNA-seq studies had associated *PtDML6*, with dormancy inductive period, in poplar stem and apex (Shim et al. 2014; **Fig. S5.4**). These results suggest that activation of a 5mC demethylase during bud maturation is conserved both in chestnut and poplar. SD treatment promotes an overall increase in 5mC DNA methylation in SAM and leaf primordial tissues. Such increased levels of 5mC DNA methylation have been previously observed during winter in

chestnut and poplar (Santamaría et al. 2009; Conde et al. 2013). Also, the spatial pattern of the *PtDML6* transcript in apical buds detected by FISH overlaps with that of 5mC immunofluorescence methylation observed under SD-induction conditions. Overall 5mC levels in apical buds were unaffected by *CsDML* overexpression in poplar, both under LD conditions and following 24 days of exposure to SD. Other authors have reported a locus-specific 5mC DNA demethylation in response to stress for *Arabidopsis* ROS1, DML2 and DML3 (Zhu et al. 2007; Yu et al. 2013; Le et al. 2014). On the basis of these results, we propose that *PtDML6* and *CsDML* might act as locus-specific 5mC DNA demethylases during bud maturation.

CsDML accelerates apical bud maturation

During SD-induced bud development, two concatenated events can be identified: cessation of shoot elongation and apical leaf formation, and the progressive arrest of cell proliferation while the buds are formed (Rohde et al. 2002). The later includes differentiation of leaf primordia into bud scales alongside with the formation of the embryonic shoot. Bud scales accumulate flavonoid components leading to their characteristic red-brown color (Rohde et al. 2002; Tylewicz et al. 2015). In addition to the above-mentioned transformations, the SD signal promotes the acquisition of cold and desiccation tolerance in apical and cambial meristems before they become dormant (Rohde et al. 2002; Rohde & Bhalerao 2007; Ruttink et al. 2007; Cooke et al. 2012).

So far, published functional data for the regulation of bud formation were obtained by modifying ethylene and abscisic acid (ABA) signaling pathways (Rohde et al. 2002; Ruonala et al. 2006). Phenological studies on ethylene-insensitive birch trees (*Betula pendula*) showed that these trees do not form apical buds under inductive conditions, indicating that ethylene signaling is essential for bud formation but not for the cessation of shoot elongation (Ruonala et al. 2006). In poplar, downregulation of *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) or its interactor *FDL1*, promotes early bud formation without affecting the cessation of shoot elongation (Rohde et al. 2002; Tylewicz et al. 2015). Anatomical analysis of *PtABI3* overexpressing and knockdown plants have revealed deficiencies in size and ratio of embryonic leaves and bud scales under SD conditions, pointing to a role of ABA in these processes (Rohde et al. 2002). Our observations suggest that overexpression of *CsDML* only impacts the formation of the bud, while growth arrest continues unaltered. This suggests that the function of the *CsDML* protein requires other SD dependent factors (or that must first be removed) for DML to function. In our transcriptional profiling of *CsDML* OX compared to WT, we did not detect enrichment either for ABA or for ethylene pathway enzymes, even though several ABA-related genes such as *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) were found among the DEG (Table S1). Therefore, we propose that our 5mC DNA demethylase would control bud

formation separately from ethylene and may act downstream or independently of ABI3. Because our *CsDML* OX plants did not differ phenotypically from WT plants until the 4th week of SD treatment, *CsDML* may act jointly with the ABA signal, which is accumulated in apical buds at the same time after SD induction (Ruttink et al. 2007).

Overexpression of CsDML promotes flavonoid biosynthesis

The transition from active growth to dormancy has been associated to a decreased cellulose biosynthesis, concomitant to the generation of phenylpropanoids (lignin and flavonoids) (Perry 1971, Rohde et al. 2002; Tylewicz et al. 2015). Accordingly, our comparative RNA-seq analyses of SD-induced apical buds revealed three upregulated flavonoid biosynthesis genes and two downregulated cellulose synthase-like genes in *CsDML* OX plants compared to WT. By qRT-PCR we confirmed that *CHS*, *DFR* and *3GT* expression was upregulated in transgenic plants, and the measurement of flavonoids absorbance revealed higher levels of these compounds in transgenic plants apices, concurrent with the earlier accumulation of red color in apical buds that was observed in *CsDML* OX earlier than in WT plants. Flavonoids accumulation as part of the adaptive response and bud maturation changes occurred in response to seasonal dormancy (Ruttink et al. 2007; Tylewicz et al. 2015). These compounds could act as a sun screen protecting agents against photooxidative damage of the organs present in buds (bud scales, embryonic leaves, leaf primordia and SAM) (Falcone Ferreyra et al. 2012). CHS and DFR are involved in the first steps of the flavonoid biosynthesis pathway implying high levels of synthesis of anthocyanins, flavonols and flavanols (Ono et al. 2010), suggesting that DML accelerated the accumulation of flavonoids during apical bud formation. The regulation of the flavonoid pathway through DNA methylation has been recently reported in tobacco (Bharti et al. 2015). Tobacco plants overexpressing *AtROS1* showed a higher expression of flavonoid biosynthesis enzymes, including CHS, CHI and DFR, and lower levels of 5mC in their promoters than in WT plants (Bharti et al. 2015). This scenario was enhanced under salt stress, suggesting that a stimulation of *AtROS1* activity under these conditions activates flavonoid pathway genes (Bharti et al. 2015). The increased levels of flavonoid biosynthetic enzymes detected in tobacco *AtROS1* and poplar *CsDML* overexpressors indicated that this pathway is activated by 5mC demethylation.

Downregulation of cell proliferation genes precedes meristem inactivation at the final stage of bud development (Ruttink et al. 2007). Several cell cycle regulators were downregulated in *CsDML* OX plants compared to the WT when they were exposed to 28 days of SD. A pivotal role of flavonoids in the cell cycle arrest has been reported in both plants and animals (Taylor & Grotewold 2005; Woo et al. 2005; Butelli et al. 2008). The build-up of flavonoids, such as quercetin and other molecules synthesized in the first steps of the flavonoid

biosynthesis pathway, is known to block polar auxin transport, required for cell division and differentiation in SAM tissues (Kuhn et al. 2011). According to this, the flavonol quercetin was found earlier accumulated in the SAM and bud scales of *CsDML* OX than in WT plants. The accumulation of quercetin in SAM tissues undergoing cell arrest could be a consequence of auxin transport blockage triggered by flavonoid. In future studies, the downregulation of cell proliferation genes during bud formation should be explored as a consequence of the build-up of flavonoids.

5.6 CONCLUSION

Based on our observations, we propose the following model for the implication of a DML in the SD-mediated control of bud maturation (**Fig. 5.7**). As the day-length shortens, *DML* mRNA is accumulated in poplar SAM and leaf primordial. DML might act as a locus-specific 5mC DNA demethylase, activating genes encoding enzymes of the flavonoids biosynthesis pathway. These flavonoids will be implicated in bud maturation through: the acquisition of the characteristic red-brown color, acting as a sunscreen protecting the organs enclosed within the buds against photo oxidative damage and the repression of cell proliferation, which ultimately could be triggering the acceleration of bud formation. This study provides *in planta* evidence for a role of 5mC DNA demethylase activity in the regulation of bud maturation through the biosynthesis of flavonoids.

Figure 5.7. A Model for the implication of a DML in SD induced bud maturation in trees.

5.7 REFERENCES

- Abascal F, Zardoya R, Posada D. 2005. ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics* 21: 2104–2105.
- Andrews S. 2010. FastQC: A quality control tool for high throughput sequence data. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>:
<http://www.bioinformatics.babraham.ac.uk/projects/>.
- Azeez A, Miskolczi P, Tylewicz S, Bhalerao RP. 2014. A tree ortholog of APETALA1 mediates photoperiodic control of seasonal growth. *Current Biology* 24: 717–724.
- Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M. 2013. NCBI GEO: archive for functional genomics data sets--update. *Nucleic Acids Res* 41(Database issue): D991-5.
- Begley TJ, Haas BJ, Noel J, Shekhtman A, Williams WA, Cunningham RP. 1999. A new member of the endonuclease III family of DNA repair enzymes that removes methylated purines from DNA. *Current Biology* 9: 653–656.
- Berrocal-Lobo M, Ibañez C, Acebo P, Ramos A, Perez-Solis E, Collada C, Casado R, Aragoncillo C, Allona I. 2011. Identification of a homolog of Arabidopsis DSP4 (SEX4) in chestnut: Its induction and accumulation in stem amyloplasts during winter or in response to the cold. *Plant, Cell and Environment* 34: 1693–1704.
- Bharti P, Mahajan M, Vishwakarma AK, Bhardwaj J, Yadav SK. 2015. AtROS1 overexpression provides evidence for epigenetic regulation of genes encoding enzymes of flavonoid biosynthesis and antioxidant pathways during salt stress in transgenic tobacco. *Journal of Experimental Botany* 66: 5959–5969.
- Blankenberg D, Kuster G Von, Coraor N, Ananda G, Lazarus R, Mangan M, Nekrutenko A, Taylor J. 2010. Galaxy: A web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology*.
- Böhlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O. 2006. CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science (New York, N.Y.)* 312: 1040–1043.
- Brooks SC, Fischer RL, Huh JH, Eichman BF. 2014. 5-methylcytosine recognition by Arabidopsis thaliana DNA glycosylases DEMETER and DML3. *Biochemistry* 53: 2525–2532.

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J, *et al.* 2008. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature Biotechnology* 26: 1301–1308.

Conde D, González-Melendi P, Allona I. 2013. Poplar stems show opposite epigenetic patterns during winter dormancy and vegetative growth. *Trees - Structure and Function* 27: 311–320.

Cooke JEK, Eriksson ME, Junttila O. 2012. The dynamic nature of bud dormancy in trees: Environmental control and molecular mechanisms. *Plant, Cell and Environment* 35: 1707–1728.

Córdoba-Cañero D, Dubois E, Ariza RR, Doutriaux MP, Roldán-Arjona T. 2010. Arabidopsis uracil DNA glycosylase (UNG) is required for base excision repair of uracil and increases plant sensitivity to 5-fluorouracil. *Journal of Biological Chemistry* 285: 7475–7483.

Cox KH, DeLeon D V., Angerer LM, Angerer RC. 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Developmental Biology* 101: 485–502.

Denver DR, Swenson SL, Lynch M. 2003. An evolutionary analysis of the helix-hairpin-helix superfamily of DNA repair glycosylases. *Molecular Biology and Evolution* 20: 1603–1611.

Ding J, Nilsson O. 2016. Molecular regulation of phenology in trees-because the seasons they are a-changin'. *Current Opinion in Plant Biology* 29: 73–79.

Du Z, Zhou X, Ling Y, Zhang Z, Su Z. 2010. agriGO: A GO analysis toolkit for the agricultural community. *Nucleic Acids Research* 38.

Duclos S, Aller P, Jaruga P, Dizdaroglu M, Wallace SS, Doublié S. 2012. Structural and biochemical studies of a plant formamidopyrimidine-DNA glycosylase reveal why eukaryotic Fpg glycosylases do not excise 8-oxoguanine. *DNA Repair* 11: 714–725.

Edgar R, Domrachev M, Lash AE. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30(1):207-10

Eriksson ME, Israelsson M, Olsson O, Moritz T. 2000. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology* 18: 784–788.

Falcone Ferreyra ML, Rius SP, Casati P. 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in plant science* 3: 222.

Gehring M, Huh JH, Hsieh TF, Penterman J, Choi Y, Harada JJ, Goldberg RB, Fischer RL. 2006. DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. *Cell* 124: 495–506.

Giardine B, Riemer C, Hardison RC, Burhans R, Elnitski L, Shah P, Zhang Y, Blankenberg D, Albert I, Taylor J, *et al.* 2005. Galaxy: A platform for interactive large-scale genome analysis. *Genome Research* 15: 1451–1455.

Goecks J, Nekrutenko A, Taylor J. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome biology* 11: R86.

Gong Z, Morales-Ruiz T, Ariza RR, Roldán-Arjona T, David L, Zhu JK. 2002. ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. *Cell* 111: 803–814.

Howe GT, Horvath DP, Dharmawardhana P, Priest HD, Mockler TC, Strauss SH. 2015. Extensive Transcriptome Changes During Natural Onset and Release of Vegetative Bud Dormancy in Populus. *Frontiers in plant science* 6: 1–28.

Hsu C-Y, Adams JP, Kim H, No K, Ma C, Strauss SH, Drnevich J, Vandervelde L, Ellis JD, Rice BM, *et al.* 2011. FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. *Proceedings of the National Academy of Sciences of the United States of America* 108: 10756–10761.

Ibañez C, Ramos A, Acebo P, Contreras A, Casado R, Allona I, Aragoncillo C. 2008. Overall alteration of circadian clock gene expression in the chestnut cold response. *PLoS ONE* 3.

Ibañez C, Kozarewa I, Johansson M, Ogren E, Rohde A, Eriksson ME. 2010. Circadian clock components regulate entry and affect exit of seasonal dormancy as well as winter hardiness in Populus trees. *Plant physiology* 153: 1823–33.

Karlberg A, Englund M, Petterle A, Molnar G, Sjödin A, Bako L, Bhalerao RP. 2010. Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology* 27: 1–16.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* 30: 772–780.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology* 14: R36.

Kopylova E, Noé L, Touzet H. 2012. SortMeRNA: Fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28: 3211–3217.

Kozarewa I, Ibañez C, Johansson M, Ögren E, Mozley D, Nylander E, Chono M, Moritz T, Eriksson ME. 2010. Alteration of PHYA expression change circadian rhythms and timing of bud set in Populus. *Plant Molecular Biology* 73: 143–156.

Kuhn BM, Geisler M, Bigler L, Ringli C. 2011. Flavonols accumulate asymmetrically and affect auxin transport in Arabidopsis. *Plant physiology* 156: 585–595.

Kumar G, Rattan UK, Singh AK. 2016. Chilling-Mediated DNA Methylation Changes during Dormancy and Its Release Reveal the Importance of Epigenetic Regulation during Winter Dormancy in Apple (*Malus x domestica* Borkh.). *Plos One* 11: e0149934.

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 Phytologist* 197: 416–430.

Law JA, Jacobsen SE. 2010. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nature reviews. Genetics* 11: 204–20.

Le T-N, Schumann U, Smith NA, Tiwari S, Au P, Zhu Q-H, Taylor JM, Kazan K, Llewellyn DJ, Zhang R, *et al.* 2014. DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in Arabidopsis. *Genome Biology* 15: 458.

Letunic I, Bork P. 2007. Interactive Tree Of Life (iTOL): An online tool for phylogenetic tree display and annotation. *Bioinformatics* 23: 127–128.

Liang D, Zhang Z, Wu H, Huang C, Shuai P, Ye C-Y, Tang S, Wang Y, Yang L, Wang J, *et al.* 2014. Single-base-resolution methylomes of Populus trichocarpa reveal the association between DNA methylation and drought stress. *BMC genetics* 15 Suppl 1: S9.

Liu R, How-Kit A, Stammiti L, Teyssier E, Rolin D, Mortain-Bertrand A, Halle S, Liu

M, Kong J, Wu C, *et al.* 2015. A DEMETER-like DNA demethylase governs tomato fruit ripening. *Proceedings of the National Academy of Sciences of the United States of America* 112: 10804–9.

Mok YG, Uzawa R, Lee J, Weiner GM, Eichman BF, Fischer RL, Huh JH. 2010. Domain structure of the DEMETER 5-methylcytosine DNA glycosylase. *Proceedings of the National Academy of Sciences of the United States of America* 107: 19225–30.

Moreno-Cortés A, Hernández-Verdeja T, Sánchez-Jiménez P, González-Melendi P, Aragoncillo C, Allona I. 2012. CsRAV1 induces sylleptic branching in hybrid poplar. *New Phytologist* 194: 83–90.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of bioscience and bioengineering* 104: 34–41.

Nogués S, Baker NR. 2000. Effects of drought on photosynthesis in Mediterranean plants grown under enhanced UV-B radiation. *Journal of experimental botany* 51: 1309–1317.

Ono E, Homma Y, Horikawa M, Kunikane-Doi S, Imai H, Takahashi S, Kawai Y, Ishiguro M, Fukui Y, Nakayama T. 2010. Functional differentiation of the glycosyltransferases that contribute to the chemical diversity of bioactive flavonol glycosides in grapevines (*Vitis vinifera*). *The Plant cell* 22: 2856–2871.

Ortega-Galisteo AP, Morales-Ruiz T, Ariza RR, Roldán-Arjona T. 2008. Arabidopsis DEMETER-LIKE proteins DML2 and DML3 are required for appropriate distribution of DNA methylation marks. *Plant Molecular Biology* 67: 671–681.

Parrilla-Doblas JT, Ponferrada-Marín MI, Roldán-Arjona T, Ariza RR. 2013. Early steps of active DNA demethylation initiated by ROS1 glycosylase require three putative helix-invading residues. *Nucleic Acids Research* 41: 8654–8664.

Paul LK, Rinne PLH, Van der Schoot C. 2014. Shoot meristems of deciduous woody perennials: Self-organization and morphogenetic transitions. *Current Opinion in Plant Biology* 17: 86–95.

Penterman J, Uzawa R, Fischer RL. 2007. Genetic interactions between DNA demethylation and methylation in Arabidopsis. *Plant physiology* 145: 1549–1557.

- Peñuelas J, Filella I. 2001. Responses to a Warming World. *Science* 294: 793–794.
- Perry TO. 1971. Dormancy of trees in winter. *Science (New York, N.Y.)* 171: 29–36.
- Petterle A, Karlberg A, Bhalerao RP. 2013. Daylength mediated control of seasonal growth patterns in perennial trees. *Current Opinion in Plant Biology* 16: 301–306.
- Ponferrada-Marín MI, Martínez-Macías MI, Morales-Ruiz T, Roldán-Arjona T, Ariza RR. 2010. Methylation-independent DNA binding modulates specificity of repressor of silencing 1 (ROS1) and facilitates demethylation in long substrates. *Journal of Biological Chemistry* 285: 23032–23039.
- Ponferrada-Marín MI, Parrilla-Doblas JT, Roldán-Arjona T, Ariza RR. 2011. A discontinuous DNA glycosylase domain in a family of enzymes that excise 5-methylcytosine. *Nucleic Acids Research* 39: 1473–1484.
- Ramos A, Pérez-Solís E, Ibáñez C, Casado R, Collada C, Gómez L, Aragoncillo C, Allona I. 2005. Winter disruption of the circadian clock in chestnut. *Proceedings of the National Academy of Sciences of the United States of America* 102: 7037–42.
- Rios G, Leida C, Conejero A, Badenes ML. 2014. Epigenetic regulation of bud dormancy events in perennial plants. *Frontiers in Plant Science* 5: 247.
- Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139–140.
- Rohde A, Bastien C, Boerjan W. 2011a. Temperature signals contribute to the timing of photoperiodic growth cessation and bud set in poplar. *Tree Physiology* 31: 472–482.
- Rohde A, Bhalerao RP. 2007. Plant dormancy in the perennial context. *Trends in Plant Science* 12: 217–223.
- Rohde A, Prinsen E, De Rycke R, Engler G, Van Montagu M, Boerjan W. 2002. PtABI3 impinges on the growth and differentiation of embryonic leaves during bud set in poplar. *The Plant cell* 14: 1885–901.
- Rohde A, Storme V, Jorge V, Gaudet M, Vitacolonna N, Fabbrini F, Ruttink T, Zaina G, Marron N, Dillen S, *et al.* 2011b. Bud set in poplar - genetic dissection of a complex trait in natural and hybrid populations. *New Phytologist* 189: 106–121.
- Rosenquist TA, Zharkov DO, Grollman AP. 1997. Cloning and characterization of a

mammalian 8-oxoguanine DNA glycosylase. *Proceedings of the National Academy of Sciences of the United States of America* 94: 7429–34.

Ruonala R, Rinne PLH, Baghour M, Moritz T, Tuominen H, Kangasrvi J. 2006. Transitions in the functioning of the shoot apical meristem in birch (*Betula pendula*) involve ethylene. *Plant Journal* 46: 628–640.

Ruttink T, Arend M, Morreel K, Storme V, Rombauts S, Fromm J, Bhalerao RP, Boerjan W, Rohde A. 2007. A molecular timetable for apical bud formation and dormancy induction in poplar. *The Plant cell* 19: 2370–90.

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

Santerre A, Britt AB. 1994. Cloning of a 3-methyladenine-DNA glycosylase from *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 91: 2240–2244.

Shim D, Ko J-H, Kim W-C, Wang Q, Keathley DE, Han K-H. 2014. A molecular framework for seasonal growth-dormancy regulation in perennial plants. *Horticulture Research* 1: 14059.

Silva-Navas J, Moreno-Risueno MA, Manzano C, Téllez-Robledo B, Navarro-Neila S, Carrasco V, Pollmann S, Gallego FJ, Del Pozo JC. 2016. Flavonols mediate root phototropism and growth through regulation of proliferation-to-differentiation transition. *Plant Cell* 28: 1372–1387.

Stamatakis A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690.

Taylor LP, Grotewold E. 2005. Flavonoids as developmental regulators. *Current Opinion in Plant Biology* 8: 317–323.

Tylewicz S, Tsuji H, Miskolczi P, Petterle A, Azeez A, Jonsson K, Shimamoto K, Bhalerao RP. 2015. Dual role of tree florigen activation complex component FD in photoperiodic growth control and adaptive response pathways. *Proceedings of the National Academy of Sciences of the United States of America* 112: 3140–5.

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.

UPOV (1981). Guidelines for the Conduct of Tests for Distinctness, Homogeneity and Stability Populus L. International Union for the Protection of New Varieties of Plants, Geneva

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview Version 2-A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25: 1189–1191.

Woo HH, Jeong BR, Hawes MC. 2005. Flavonoids: From cell cycle regulation to biotechnology. *Biotechnology Letters* 27: 365–374.

Yu A, Lepère G, Jay F, Wang J, Bapaume L, Wang Y, Abraham A-L, Penterman J, Fischer RL, Voinnet O, *et al.* 2013. Dynamics and biological relevance of DNA demethylation in Arabidopsis antibacterial defense. *Proceedings of the National Academy of Sciences of the United States of America* 110: 2389–94.

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

Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SWL, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, *et al.* 2006. Genome-wide High-Resolution Mapping and Functional Analysis of DNA Methylation in Arabidopsis. *Cell* 126: 1189–1201.

Zhu J-K. 2009. Active DNA demethylation mediated by DNA glycosylases. *Annual review of genetics* 43: 143–66.

Zhu J, Kapoor A, Sridhar V V., Agius F, Zhu JK. 2007. The DNA Glycosylase/Lyase ROS1 Functions in Pruning DNA Methylation Patterns in Arabidopsis. *Current Biology* 17: 54–59.

Zhu R, Shevchenko O, Ma C, Maury S, Freitag M, Strauss SH. 2013. Poplars with a PtDDM1-RNAi transgene have reduced DNA methylation and show aberrant post-dormancy morphology. *Planta* 237: 1483–1493.

5.8 SUPPORTING INFORMATION FOR CHAPTER 5

a

<i>PtaDML6</i> forward	5'-AGTTGCAGCTTACATGAATGTG-3'
<i>PtaDML6</i> reverse	5'-CAATTTGAGAATTTTCTTCCCGT-3'
<i>CsDML</i> forward	5'-GCACCTCGTCCTCTAATGG-3'
<i>CsDML</i> reverse	5'-TCATCCGTGTTACCCTTTGTC-3'
<i>PtaCHS</i> forward	5'-AGAAATAACAGCAGTGACATTTTCGT-3'
<i>PtaCHS</i> reverse	5'-CAGCTCAAACAAAGGCTTCTCG-3'
<i>PtaDFR</i> forward	5'-AGGAGGATCAGAGAGACTACAAG-3'
<i>PtaDFR</i> reverse	5'-TCCTTGGATAACTACTTCCTCG-3'
<i>Pta3GT</i> forward	5'-TCTCTTCTTGTTCCTTCTTCCAG-3'
<i>Pta3GT</i> reverse	5'-GTGGAGAAGGTGACAAGGAAG-3'
18S forward	5'-TCAACTTTCGATGGTAGGATAGTG-3'
18S reverse	5'-CCGTGTCAGGATTGGGTAATTT-3'

b

<i>CsDML</i> forward	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGTTAAAGAAGGCCAAGATG-3'
<i>CsDML</i> reverse	5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAATCATCCGTGTTACCCTTTGTC3'

Figure S5.1. List of primers used in the present study. (a) List of primers used for the analyses of gene expression by RT-PCR. **(b)** Primers used for amplification the CDS of *CsDML* gene with attB sites to generate the constructions needed for the overexpression of *CsDML* in poplar.

Figure S5.2. Characterization of *CsDML* gene expression in chestnut trees. Quantitative RT-PCR analysis of: **(a)** *CsDML* annual expression pattern in 2-year-old chestnut branches from adult trees growing under natural conditions; **(b)** *CsDML* cold induction expression in chestnut stems from 8-months-old plants grown in a controlled-environment growth chamber (LD, 22°C) and subsequently transferred to LD, 4°C for 7 weeks; **(c)** *CsDML* SD induction expression in chestnut stems from 8-month-old plants grown in a controlled-environment growth chamber (LD, 22°C) and subsequently transferred to SD, 22°C for 6 weeks; **(d)** *CsDML* expression levels in chestnut stems from 11-month-old endodormant plants grown under natural conditions, and transferred before chilling fulfillment to a controlled-environment growth chamber in standard conditions (LD, 22°C); **(e)** *PtaDML6* cold induction expression in poplar stems from 8-weeks-old plants grown in a controlled-environment growth chamber (LD, 22°C) and subsequently transferred to LD, 4°C for 7 weeks; **(f)** *PtaDML6* SD induction expression in poplar

stems from 6-weeks-old plants grown in a controlled-environment growth chamber (LD, 22°C) and subsequently transferred to SD, 22°C for 7 weeks. In a,b,c,e,f, plotted values and error bars are the fold-change means $\pm$ SD recorded in three technical replicates. In d, plotted values and errors bars are the fold-change means $\pm$ SD recorded in two biological replicates.

Figure S5.3. Characterization of the *CsDML* OX overexpressing poplar trees. (a) Quantitative RT-PCR analysis of *CsDML* in the *CsDML* OX (line OX1) transgenic plantlet leaves. Samples were obtained from plantlets grown in a greenhouse for 5 weeks in conditions of LD, 22°C. Plotted values and error bars are the fold-change means $\pm$ SD recorded in three technical replicates. (b) b1 and b2 indicate WT and *CsDML* OX (line OX1) plants after growth for 4 weeks in conditions of LD, 22°C. At this point no phenotypic differences, compared to WT, were observed. b3 and b4 indicate WT and *CsDML* OX (line OX1) plants after growth for 49 days in conditions of SDs, 19°C. At this point, bud maturation was advanced in *CsDML* OX compared to the WT. The same occurs for the other OX line.

Figure S5.4. Expression of poplar DMLs in apex. FPKM values of *PtDML6*, *PtDML8* and *PtDML10* in public RNA seq analyses performed in *Populus trichocarpa* apex during different developmental stages along the year; FOB: fully open bud; PDI-PDII: predormant bud I and II; ED: early dormant bud; LD: late dormant bud. These data are available in phytozome11 website (<https://phytozome.jgi.doe.gov/pz/portal.html>).

Figure S5.5. Molecular timetable of DEG during bud development. (a) Venn diagram showing the common gene set between differentially expressed genes in both *CsDML* OX transgenic lines versus WT, and the genes differentially expressed in WT apices during SD-induced dormancy (Ruttink et al. 2007). Only 18 of the up regulated and 67 of the down regulated genes in the *CsDML* OX versus WT plants were previously analyzed in Ruttink et al. (2007). From these genes, 12 upregulated and 44 downregulated were differentially expressed during apical bud development in this study (b) Trends in expression of the common gene set during the 6 weeks of SD induction of dormancy period (Ruttink et al.

5. *A DNA demethylase overexpression promotes early apical bud maturation*

2007). Data points and error bars represent means $\pm$ SE over the averaged expression of the common genes. R² represents the coefficient of goodness of fit for the calculated polynomial regression of the trend line. In (a) and (b), genes upregulated and downregulated in *CsDML* OX trees are indicated as “up” and “down,” respectively.

6. GENERAL DISCUSSION

The results presented in this thesis are structured in three chapters (chapters 3, 4 and 5). The chapter 3 describes the overall changes in the chromatin functional states between winter dormancy and active growth periods. Immunolocalization assays detected an increase of 5mC in dormant stems, which suggest a relatively transcriptional inactivation as compared with the growth period. The chapter 4 confirmed the importance of 5mC dynamics in apical buds during the winter, and demonstrated that active 5mC demethylation by PtaDML10 is required for bud break after winter dormancy. In a third work (chapter 5) we showed that chestnut CsDML, the putative homolog of PtaDML6, accelerates bud maturation by the activation of the flavonoids biosynthetic pathway, likely playing a role in the arrest of cell growth.

To assess an epigenetic control of growth-dormancy transitions in hybrid poplar, we localized two functionally different epigenetic marks by immunofluorescence in poplar stems. Quantification of fluorescence revealed a twofold higher intensity of 5mC (an epigenetic mark of condensed chromatin) in winter than in summer specimens, while measurements of the acetylated lysine 8 of histone H4 (an epigenetic mark of open chromatin) revealed the opposite seasonal pattern. Similar results were obtained in chestnut and apple apices (Santamaría et al. 2009; Kumar et al. 2016). Therefore, the relative high levels of 5mC during winter dormancy in apple, chestnut and poplar suggest that chromatin rearrangement is a common situation in these tree species. It also correlates with the general transcriptional and metabolic shutdown described during dormancy (Karlberg et al. 2010). The epigenetic control of gene expression can be linked to the cell's energy status since there is a relation between the levels of SAME, the donor of a methyl group to a cytosine, and the NAD^+/NADH ratio. Thus, under low energy situations, such as those of winter dormancy, the higher levels of NAD^+ will promote SAME synthesis and DNA methylation alongside with the histone deacetylation reactions produced by Sirtuins.

Once determined a possible epigenetic control in the transition between dormancy and the growth period in the stem, we looked for the dynamics of 5mC in the SAM. gDNA was extracted from apical buds weekly, at 14 time points from January to the time of bud break in April, and 5mC was quantified by HPLC, the most sensitive technique to this aim (Gentil & Maury 2007). A hyper-hypo methylation wave was found during this period, with the maximum level of methylation during February and the minimum just before bud break in April. Kumar et al. (2016) showed a decrease of 5mC in the transition from the dormant bud to the initial stages of fruit set, but only when the trees were grown under conditions that fulfill the chilling requirement. To our knowledge, only a similar study was performed to evaluate the link between 5mC and bolting in sugar beet. This work showed that in sugar beet SAM vernalization promotes a hypermethylation-hypomethylation wave, being the amplitude and the period of the

5mC wave correlated with the variations in the vernalization requirements for bolting of the different genotypes analyzed (Trap-Gentil et al. 2011). The sugar beet work and the results of Kumar et al. (2016) established a putative link between DNA methylation dynamics and vernalization or chilling requirement, respectively. Hence, the hyper-hypo methylation wave that we found in our time course in hybrid poplar SAM could be produced by the endogenous factors involved in quantifying the chilling period. Then, we put forward that the maximum of 5mC coincides with the transition from endo to ecodormancy that occurs once the chilling requirement is fulfilled, before bud break, although more experimental data are needed to confirm this hypothesis.

During dormancy, the cell cycle in the SAM is arrested, thus, DNA methylation dynamics cannot be explained by a passive mechanism since there is no replication. Therefore, the decrease of 5mC levels before bud break might be due to active DNA demethylation. By phylogenetic and protein sequence analyses we identified three DMLs in hybrid poplar. In the stem, the expression of two of them, *PtaDML8* and *PtaDML10*, was higher during the growing season than in the winter dormancy period. In the apex, *PtaDML10* expression increased from February, when the 5mC begins to decrease in the SAM, until April, at the time of bud break, whereas *PtaDML8* was constant during this period. Functional studies were carried out in KD lines with downregulated expression of *PtaDML10*. Bud break was delayed in the transgenic plants in comparison to the WT. DEG were identified by RNA-seq and DMR by WGBS in KD vs. WT plants. Crossing RNA-seq and WGBS data, we found that *PtaDML10* actively demethylates growth-promoting genes, which are required for bud break. FISH experiments localized *PtaDML10* mRNA in the L1 and central zone of the SAM, which is consistent with the reactivation of cell proliferation. Conversely, growth suppression genes were downregulated.

In the fifth chapter we characterized *CsDML*, the chestnut homolog to *PtaDML6*, which had been previously found in our laboratory in a winter subtractive hybridization, and whose expression is induced by SD and cold conditions. So far, there are no publications of any DML responding to external environmental signals. Transgenic lines of hybrid poplar overexpressing *CsDML* were generated. Our immunofluorescence results showed that 5mC levels increased in apices from transgenic and WT plants under SD, as occurred during chestnut bud maturation (Santamaría et al. 2009). Thus *CsDML*, and *PtaDML6*, would be needed to demethylate certain loci required for bud maturation during dormancy in a context of global DNA methylation. *PtaDML6* mRNA was localized by FISH in the SAM and leaf primordia under SD conditions. Earlier apical bud maturation was observed under dormancy inductive conditions in the *CsDML* OX lines as compared with the WT. Our transcriptomic analysis showed that the expression of

genes encoding enzymes involved in flavonoids biosynthesis was upregulated in these transgenic plants. Based on Ruttink et al. (2007) the enzymes for the biosynthesis of phenylpropanoids, which accumulate in bud scales during apical bud formation in autumn, reach the maximum expression after 3-4 weeks under SD. Flavonoids could act as a sun screen protecting the meristems from photooxidative damage during dormancy (Falcone Ferreyra et al. 2012). Accordingly, quercetin and other flavonoids were induced in the SAM under SD conditions, and their accumulation occurred earlier in *CsDML* OX vs. WT trees. Cell proliferation genes were found downregulated in *CsDML* OX lines, so flavonoids accumulation could be involved in the early cease of proliferation, upon apical bud formation. Flavonoids have been already related with cell cycle arrest in both plants and animals (Taylor & Grotewold 2005; Woo et al. 2005; Butelli et al. 2008), although their molecular mechanism is not well understood. Quercetin and other flavonoids are known to block polar auxin transport, required for cell division and differentiation (Kuhn et al. 2011).

Altogether, the results presented in this thesis provide new evidences of the importance of DNA methylation dynamics in response to the environmental signals that control active growth-winter dormancy transitions during the annual cycle of trees. Active DNA demethylation by PtaDML10 is required for the expression of genes involved in cellular metabolism, needed for the reactivation of growth during bud break after winter dormancy. *CsDML*, and likely its poplar homolog PtaDML6, is required for the activation of the flavonoid biosynthetic pathway, which is related to cell proliferation cease and apical bud maturation during winter dormancy entrance.

References

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J, *et al.* 2008. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature Biotechnology* 26: 1301–1308.

Falcone Ferreyra ML, Rius SP, Casati P. 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in plant science* 3: 222.

Gentil M-V, Maury S. 2007. Characterization of epigenetic biomarkers using new molecular approaches. In: Varshney R, Tuberosa R, eds. Genomic assisted crop improvement, Vol. 1. Berlin: Springer, 351–370.

Karlberg A, Englund M, Petterle A, Molnar G, Sjödin A, Bako L, Bhalerao RP. 2010. Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology* 27: 1–16.

Kuhn BM, Geisler M, Bigler L, Ringli C. 2011. Flavonols accumulate asymmetrically and affect auxin transport in Arabidopsis. *Plant physiology* 156: 585–595.

Kumar G, Rattan UK, Singh AK. 2016. Chilling-Mediated DNA Methylation Changes during Dormancy and Its Release Reveal the Importance of Epigenetic Regulation during Winter Dormancy in Apple (*Malus x domestica* Borkh.). *Plos One* 11: e0149934.

Ruttink T, Arend M, Morreel K, Storme V, Rombauts S, Fromm J, Bhalerao RP, Boerjan W, Rohde A. 2007. A molecular timetable for apical bud formation and dormancy induction in poplar. *The Plant cell* 19: 2370–90.

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

Taylor LP, Grotewold E. 2005. Flavonoids as developmental regulators. *Current Opinion in Plant Biology* 8: 317–323.

Trap-Gentil MV, Hèbrard C, Lafon-Placette C, Delaunay A, Hagge D, Joseph C, Brignolas F, Lefebvre M, Barnes S, Maury S. 2011. Time course and amplitude of DNA methylation in the shoot apical meristem are critical points for bolting induction in sugar beet and bolting tolerance between genotypes. *Journal of Experimental Botany* 62: 2585–2597.

Woo HH, Jeong BR, Hawes MC. 2005. Flavonoids: From cell cycle regulation to biotechnology. *Biotechnology Letters* 27: 365–374.

7. CONCLUSIONS

1. The immunofluorescence results suggest an epigenetic control of winter dormancy in hybrid poplar stem. Epigenetic marks of low transcriptional activity (DNA methylation and histone H4 hypoacetylation) were detected during the winter, and those compatible with gene expression (DNA hypomethylation and histone H4 acetylation) were found in the growing season.
2. The high-resolution profile of genomic DNA methylation by HPLC, obtained in poplar SAM from January to the time of bud break in April alongside with the expression of the cell proliferation marker during this period, revealed that the reactivation of the cell cycle after winter dormancy requires DNA demethylation.
3. Active DNA demethylation by PtaDML10 is required for bud break in two ways: inducing the expression of cell metabolism genes needed for the reactivation of growth and downregulating bud dormancy-specific genes.
4. CsDML, the chestnut homolog to PtaDML6, acts as a locus-specific 5mC DNA demethylase activating genes encoding enzymes of the flavonoids biosynthesis pathway, which would have a role in bud maturation as a sunscreen protecting the organs enclosed within the buds against photo oxidative damage and downregulating cell proliferation genes during bud formation.