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Metilación del ADN y caracterización del módulo mir156-SPLs en el cambio de fase vegetativa de *Eucalyptus globulus* Labill.

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Resumen

En el ciclo de vida de algunas especies arbóreas, se observa el fenómeno denominado heteroblastia, que consiste en una transición abrupta y marcada de rasgos juveniles a adultos. Las variaciones en el tiempo o tasa de esta transición se conocen como heterocronía. El tipo de variación heteroblástica mayormente descrita, es el cambio de fase vegetativa, que se caracteriza por cambios en la morfología, fisiología y bioquímica que son específicos para cada especie. *Eucalyptus globulus* Labill. es un buen modelo genético para estudiar la heteroblastia debido a un cambio de fase delimitada.

Los análisis moleculares han demostrado que la transición de juvenil a adulto, está regulado por miR156 y sus dianas los factores de transcripción de SQUAMOSA PROMOTOR PROTEIN-LIKE (SPL) implicados en los rasgos adultos. Trabajos recientes han revelado que la vía miR156 / SPLs está sujeta a regulación epigenética, a través de las modificaciones de histonas y la remodelación de la cromatina.

En esta tesis, me enfoque como objetivo principal en: Contrastar la expresión del módulo miR156-SPLs regulador del cambio de fase vegetativa con sus perfiles de patrón de metilación en *Eucalyptus globulus* Labill.

Se encontró en el caso de MIR156, diferencias muy sutiles entre hojas juveniles y adultas en su patrón de metilación y un evidente disminución en su abundancia durante esta transición, para SPL3 y SPL9 mostraron diferencias marcadas, en los tejidos juveniles mostraron un patrón de metilación basal mayor que los tejidos adultos, con un evidente aumento en su abundancia durante el cambio de fase.

Adicionalmente evaluamos las zonas proximales y distales de ambos tejidos, se observó una mayor transcripción de miR156/SPLs en las zonas distales en distintos estadios del desarrollo, lo que nos da indicios de cómo los procesos transcripcionales y celulares podrían estar interconectados, por lo que el origen molecular del cambio de fase vegetativa puede deberse a una combinación de mecanismos.

Abstract

In the life cycle of some arboreal species, the phenomenon called heteroblastic is observed, which consists of a sharp and marked transition from juvenile to adult traits. Variations in the time or rate of this transition are known as heterochrony. The type of heteroblastic variation described is the vegetative phase change, which is characterized by changes in the morphology, physiology, and biochemistry that are specific for each species. *Eucalyptus globulus* Labill. it is a good genetic model to study heteroblasty due to a limited phase change.

Molecular analyzes have shown that the transition from juvenile to adult, is regulated by miR156 and its targets the transcription factors of SQUAMOSA PROMOTOR PROTEIN-LIKE (SPL) involved in adult traits. Recent work has revealed that the miR156-SPLs pathway is subject to epigenetic regulation, through histone modifications and chromatin remodeling.

In this thesis, I focus as the main objective, in contrast, the expression of the module miR156-SPLs regulator of the vegetative phase change with its methylation pattern profiles in *Eucalyptus globulus* Labill.

It was found in the case of MIR156, very subtle differences between juvenile and adult leaves in their methylation pattern and an evident decrease in their abundance during this transition, for SPL3 and SPL9 showed marked differences, in juvenile tissues, showed a basal methylation pattern greater than adult tissues, with an evident increase in their abundance during the phase change.

Additionally, we evaluated the proximal and distal zones of both tissues, a greater transcription of miR156-SPLs was observed in the distal zones at different stages of development, which gives us indications of how the transcriptional and cellular processes could be interconnected, so that the molecular origin of the vegetative phase change may be due to a combination of mechanisms.

Capítulo I:

Introducción General

1.1 Heteroblastia y cambio de fase vegetativa.

El ciclo de vida de las plantas se puede considerar como una sucesión de distintas fases de crecimiento. Se pueden distinguir tres etapas claramente definidas: embrionario, postembrionario y crecimiento. Durante la etapa de crecimiento se identifican tres fases: juvenil vegetativa, adulta vegetativa y reproductiva (Poethig 1990; Poethig 2003). Dicha transición va acompañada de cambios en el tamaño de la hoja, la forma y la distribución de los tricomas (Proveniers 2013), fenómeno llamado desarrollo heteroblástico (Zotz *et al.* 2011). El tiempo o momento en que ocurre el cambio de fase, es crítico para la sobrevivencia de una especie en el medio natural (Taiz *et al.* 2006). En aquellas especies en que el tiempo del cambio de fase varía entre individuos, se habla de heterocronía, fenómeno que hace referencia a todos los cambios en el ritmo de los procesos ontogenéticos. Este fenómeno da lugar a la diferenciación en forma y tamaño de los organismos (Antonelli *et al.* 1993), lo cual ha permitido explicar gran parte de la diversidad filogenética (Geuten and Coenen 2013).

La heteroblastia alude a cambios muy drásticos en la morfología y fisiología de unidades consecutivas compuestas por los entrenudos y hojas (Zotz *et al.* 2011), junto a la integración de señales entre el programa ontogenético y el ambiente (disponibilidad de luz, agua o nutrientes), los que conducen al desarrollo heteroblástico (Poethig 2013; Ostria-Gallardo *et al.* 2015; Forster and Bonser 2009). La heteroblastia ha sido descrita principalmente en la especie modelo *Arabidopsis* (Tsukaya *et al.* 2000) y en algunas especies leñosas de los géneros *Pinus* (Climent *et al.* 2006), *Acacia* (Forster and Bonser 2009) y *Eucalyptus* (James and Bell 2001).

El tipo de variación heteroblástica mayormente descrita, es el cambio de fase vegetativa (Poethig 1990), lo cual implica cambios en las características de las hojas, tallos y brotes (Rasmussen 1986). Estos cambios son específicos de cada especie y van desde la transición de hojas juvenil

a adulta, patrones de ramificación, depósito diferencial de cera epicuticular, patrones de producción de tricomas, la forma celular, los patrones vasculares, la capacidad para la producción de raíces adventicias, presencia o ausencia de antocianina u otros fitoquímicos y resistencia a enfermedades o insectos (Whalen 2005; Poethig 2010; Zotz et al. 2011).

Uno de los indicadores de la transición vegetativa de juvenil a adulta, son las variaciones morfológicas y estructurales de las hojas juveniles y adultas (Zotz et al. 2011; Poethig 2010), como en *Arabidopsis thaliana* L., que posee cambios fenotípicos sutiles durante esta transición, presenta hojas juveniles pequeñas y redondas, pecíolos largos y una roseta simple, las hojas adultas son ligeramente alargadas con márgenes dentados, pecíolos cortos y mayor número de tricomas (Telfer et al. 1997; Rodriguez et al. 2014). Por ello es considerada débilmente heteroblástica (Poethig 2013). En contraste las especies arbóreas son modelos muy favorables para estudiar el cambio de fase vegetativa (Poethig 2010), ya que exhiben cambios fenotípicos muy marcados entre la fase juvenil y la fase adulta, dicha transición puede durar desde algunas semanas a varios años, lo cual las hace fáciles de observar y caracterizar (Forster and Bonser 2009). Se puede distinguir en general, en la base del árbol la etapa juvenil, en la punta la etapa adulta y la zona media estadios intermedios (Wang et al. 2011), estas claras diferencias morfológicas en los diferentes estadios, transforma a las especies arbóreas en excelentes modelos de estudio para el cambio de fase vegetativa.

1.2 Regulación del cambio de fase vegetativa.

1.2.1 Regulación molecular

A. thaliana y *Zea mays* L., se han utilizado como modelos para investigar los mecanismos moleculares que controlan el cambio de fase vegetativa (Chuck et al. 2007; Wu et al. 2009; Yang et al. 2012; Xu et al. 2016a) durante más de tres décadas. Estudios han revelado que el cambio de fase vegetativa está controlado por miR156, el cual es denominado el regulador principal para la juventud (Poethig 2009; Feng et al. 2016; Zhang et al. 2015; Wu et al. 2009).

miR156 está presente en todas las plantas, incluido el musgo (Axtell and Bowman 2008; Wang *et al.* 2011).

Se ha descrito que la señalización que coordina el cambio de fase proviene desde los primordios foliares, ya que se ha observado un claro retraso en el cambio fase, en ausencia de estos tejidos, lo cual se ha correlacionado con un aumento de los niveles de miR156 (Yang *et al.* 2011; Orkwiszewski and Poethig 2000). Los niveles de miR156 son muy altos en los brotes y tejido juvenil, posteriormente disminuye drásticamente durante la transición cambio de fase vegetativa (Yang *et al.* 2013). Se ha determinado que la expresión constitutiva de miR156 prolonga la fase juvenil, mientras que la pérdida de la actividad miR156 promueve el cambio de fase y una floración temprana (Wu and Poethig 2006; Wu *et al.* 2009; Zhang *et al.* 2011; Chuck *et al.* 2011), lo cual se ha observado en plantas leñosas como *Pinus radiata* en donde la defoliación y la poda severa, conlleva a un aumentan la producción de hojas juveniles (Libby and Hood 1975).

miR156 regula el cambio de fase vegetativa, reprimiendo directamente la expresión de los factores de transcripción SQUAMOSA Promoter-Binding Protein-Like (SPL)(Schwab *et al.* 2006), los que desempeñan importantes papeles en diferentes procesos fisiológicos, tales como el cambio de fase, la floración, el desarrollo de frutos, la arquitectura de la planta, la señalización por giberelinas, la esporogénesis, la homeostasis del cobre y la respuesta a toxinas fúngicas (Preston and Hileman 2013; Chen *et al.* 2010; Preston and Hileman 2010; Mathieu *et al.* 2009; Kim and Sung 2012; Usami *et al.* 2009; Unte *et al.* 2003).

Los genes SPLs se expresan diferencialmente en hojas, en estadio juvenil y adulto en algunas herbáceas (Xie *et al.* 2006; Zhang *et al.* 2011; Salinas *et al.* 2012) y en algunas leñosas (Wang *et al.* 2011). Uno de los genes SPLs es SPL3, el cual está asociado a la inducción floral y la aparición de características foliares adultas (Wu and Poethig 2006; Kim and Sung 2012; Yamaguchi *et al.* 2009; Teotia and Tang 2015). El SPL9 promueve el desarrollo de la morfología de las hojas adultas, coordina el desarrollo y la tolerancia al estrés (Grotewold *et al.* 2015; Cui *et al.* 2014; Schwarz *et al.* 2008). Los niveles de SPL3 y SPL9, son inicialmente bajos y aumentan a medida que envejece la planta (Wu and Poethig 2006; Wang *et al.* 2009;

Gandikota *et al.*, 2007), los que son regulados a nivel post-transcripcional por el miR156 (Chen *et al.* 2010; Wu and Poethig 2006; Gandikota *et al.* 2007; Rhoades *et al.* 2002; Kim *et al.* 2012). La transición también implica al miR172, participando en la floración (Wu *et al.* 2009). miR172 exhibe un patrón de expresión complementario con miR156, aumentando con la disminución de miR156 (Schwarz *et al.* 2008). Las secuencias y funciones de miR156, SPLs y miR172 se han descrito conservadas evolutivamente en las plantas (Wang *et al.* 2009; Luo *et al.* 2013; Wu *et al.* 2009; Chuck *et al.* 2007; Wang *et al.* 2011; Xie *et al.* 2012; Zhang *et al.* 2011).

Otra forma descrita para la regulación de los SPLs, es a nivel transcripcional y postranscripcional por la proteína DELLA (Sun 2010; Yoshida *et al.* 2014). A nivel transcripcional, DELLA afecta la activación transcripcional de diferentes genes SPLs en el shoot apex (Galvão *et al.* 2012; Porri *et al.* 2012; Wang and Wang 2015). A nivel post-transcripcional, DELLA interactúan físicamente con los SPLs secuestrándolos (Hyun *et al.* 2016; Yu *et al.* 2012). La caracterización más estudiada de DELLA es la regulación negativa con las giberilas (GA), esta unión promueve la ubiquitinación y proteólisis de ésta, provocando la liberación de los factores de transcripción secuestrado por DELLA (Sun 2011).

1.2.2 Azúcares regulan la expresión de miR156.

Los azúcares regulan la fisiología y el desarrollo de las plantas, por varias vías de señalización diferentes (Smeekens *et al.* 2010; Taiz *et al.* 2006; Gibson 2005). Los azúcares modulan importantes procesos vitales de las plantas como el ciclo celular, la germinación, la diferenciación de los cotiledones durante la germinación, el tiempo de floración, la senescencia y la apoptosis (Lorenz *et al.* 2003; Price *et al.* 2003; Borisjuk *et al.* 2004; Ohto *et al.* 2001; Fujiki *et al.* 2001; Kim *et al.* 2006). Se ha demostrado que los niveles de miR156 están fuertemente correlacionados con los niveles de azúcar internos de las plantas, lo cual ha sido demostrado mediante la eliminación de las primeras hojas, lo que causa un aumento en la expresión de miR156 y retrasa la maduración de la planta (Yang *et al.* 2011; Yu *et al.* 2013). De igual modo los niveles de miR156 se reducen, en respuesta al tratamiento con glucosa, lo que va acompañado de una aceleración de la transición de fase de juvenil a adulto (Yang *et al.* 2011;

Yang *et al.* 2013; Yu *et al.* 2013). Se ha demostrado que el azúcar influye en la abundancia de miR156 en tres niveles: reduciendo la transcripción de genes miR156, degradando pri-miR156 y reduciendo miR156 maduro (Kooiman 2014).

Se ha correlacionado una reducción de miR156 inducida por el azúcar, dependería en parte de la actividad del sensor de azúcar Hexokinasa 1 (HXK1), el cual es un componente principal la vía de señalización del azúcar. En particular las plantas usan la hexokinasa como un sensor de glucosa, para relacionar las redes de señalización de nutrientes, luz y hormonas, para controlar el crecimiento y el desarrollo en respuesta al entorno cambiante (Moore *et al.* 2003). El azúcar exógeno se ha determinado, que prolonga la fase juvenil en *Physcomitrella patens* (Lorenz *et al.* 2003). Se ha identificado que el azúcar producida en los primordios foliares causa la disminuido de miR156, lo cual es coordinado por la HXK1 (Yu *et al.* 2013), la cual funciona como un sensor de azúcar, por medio de la cual puede regular la transcripción génica, en respuesta a las variaciones en la concentración de glucosa (Xiao *et al.* 2000).

Se ha determinado que la glucosa promueve el cambio de fase vegetativa mediante la represión de la acumulación de miR156 y disminuyendo la expresión de los genes *MIR156* (Yang *et al.* 2013), lo cual va acompañada con un aumento en los niveles de transcrito de SPL (Wahl *et al.* 2013). Se ha planteado que el mecanismo es de forma indirecta, que se requiere de la síntesis de proteínas que actuarían a nivel transcripcional (Yu *et al.* 2013), también es posible que el azúcar reprima la acumulación de miR156 maduro, es decir un control a nivel post-transcripcional (Yang *et al.* 2013). Por lo tanto, la acumulación gradual de azúcares, sería un componente clave de la señalización foliar que promueve el cambio de fase vegetativa.

1.2.3 Factores ambientales regulan la expresión de miR156.

Los factores ambientales desempeñan un papel en la regulación de la expresión de miR156. La temperatura regula la expresión de los genes de la familia miR156, los niveles de miR156 son mayores a temperaturas menores a 16 °C y por consiguiente regula negativamente la expresión de los genes SPLs (Kim and Sung 2012; Lee *et al.* 2010; Yu *et al.* 2011). Se ha descrito que elevadas concentraciones de CO₂, llevan a la disminución de la expresión de miR156, a un

aumento de miR172 y de los genes SPLs (May *et al.* 2013). La exposición a la luz azul y UV-A en plántulas de *Brassica rapa* L, se ha correlacionado con una disminución de los niveles de transcrito para miR156 y un aumento de SPL9 (Zhou *et al.* 2016).

Los macronutrientes esenciales como el fósforo contribuyen al crecimiento, desarrollo y reproducción de las plantas (Taiz *et al.* 2006), se ha descrito que altos niveles de fósforo se correlacionan con un aumento de los niveles de miR156 y una disminución de los genes SPLs en raíces. Además miR156 es regulado indirectamente por la deficiencia de nitrógeno que es asimilado a nivel radicular, producto que déficit de este inhibe la fotosíntesis, lo cual afecta los niveles de miR156 lo que regularía la transición de fase (Zhao *et al.* 2012) La salinidad y sequía, aumentan los niveles de miR156, lo cual induce a la planta a mantener el estado juvenil, durante un período relativamente largo, mientras que en condiciones favorables el miR156 se suprime para acelerar la transición de desarrollo (Cui *et al.* 2014).

1.3 Regulación epigenética en plantas.

La epigenética es el estudio de los cambios heredables y transitorios en la expresión genética, que no implican un cambio en la secuencia primaria ADN (Bonasio *et al.* 2010). En *Arabidopsis* se han identificado más de 130 reguladores epigenéticos hasta el momento (Pikaard and Scheid 2014). Estas diferentes vías epigenéticas en las plantas, probablemente pueden contribuir a la plasticidad fenotípica del desarrollo, a la capacidad de sobrevivir y reproducirse de las plantas (Baulcombe and Dean 2014).

Los mecanismos epigenéticos que regulan la expresión génica en plantas son las modificaciones de histonas, ARN no codificantes, complejos remodeladores de cromatina y la metilación de ADN (Temel *et al.* 2015)

1.3.1 Metilación del ADN.

La metilación de la 5-metilcitosina (5mC), en plantas ocurre en los motivos CG, CHG, o CHH (H es A, T, o C) (Chan *et al.* 2005), principalmente en las regiones repetitivas del genoma,

promotores, 5 UTR, primer exón y el cuerpo del gen (Pikaard and Scheid 2014). La metilación en plantas es producida por las metiltransferasas, enzimas que catalizan la transferencia de grupos metilo desde S-adenosilmetionina (SAM o S-AdoMet) hasta los residuos de citosina, generando la metilcitosina y S-adenosilhomocisteína (SAH) (Temel *et al.* 2015).

En plantas se han descrito tres tipos de metiltransferasas de ADN (Zhang *et al.* 2010; Pikaard and Scheid 2014). La metiltransferasa 1 (MET1) es la principal metiltransferasa de mantenimiento del contexto CG y en la metilación de novo. La metiltransferasa reorganizadora de dominios 2 (DRM2), cataliza la metilación de citosinas en todos los contextos de secuencia y participa en la vía de la metilación del ADN dirigida por ARN. La cromometiltransferasa 3 (CMT3) es la enzima principal responsable de la metilación en el contexto CHG de mantenimiento y CMT2 desempeña un papel en el mantenimiento de la metilación CHH en genómica, en las regiones centrales de grandes elementos de transposición (Zemach *et al.* 2013).

La desmetilación en plantas ocurre por medio de la acción de las glicosilasas, a través de la escisión de 5-metilcitosina, ésta en plantas es catalizada por el represor del silenciamiento 1 (ROS1), DEMETER (DME) o DEMETER-like (DML2, DML3) (Agius *et al.* 2006), la reparación de la escisión de la base es rellenada por la ADN polimerasa y ADN ligasa (Martínez-Macías *et al.* 2012). La metilación participa en la regulación de la expresión génica según la región en que se encuentre está, si es en la zona promotora, 5 UTR o primer exón, da lugar a la inhibición de la transcripción (Brenet *et al.* 2011; Zilberman *et al.* 2007). Si la metilación es en el cuerpo del gen está asociada a genes altamente expresados (Vaughn *et al.* 2007; Zilberman *et al.* 2007; Zhang *et al.* 2006).

1.3.2 Desarrollo y epigenética.

La regulación epigenética juega un papel importante en múltiples aspectos del desarrollo de las plantas (Bräutigam *et al.* 2013). La metilación del ADN es el mecanismo epigenético más relevante, ya que sirven como una guía para el establecimiento y el mantenimiento de otros mecanismos epigenéticos (Fuks *et al.* 2003). Además de la tendencia a ser heredada, a través de múltiples generaciones, parece ser una de las principales diferencias, entre la metilación del

ADN y otras modificaciones epigenéticas (Vanyushin and Ashapkin 2011). La metilación del ADN en plantas desempeña papeles fundamentales en distintos procesos:

- La vernalización es un sistema regulador epigenético, que impide la floración en otoño y permite la floración en condiciones mucho más favorables en primavera (Sheldon *et al.* 1999; Sheldon *et al.* 2002; Jean Finnegan *et al.* 2005; Heo and Sung 2011).
- En el control de transposones, en donde la metilación del ADN participa en la supresión de éstos, evitando que se produzcan disrupciones en otras secuencias del genoma que pudiesen alterar los patrones de expresión de éstos (Fukuda *et al.* 2004). Se han identificado variaciones en los niveles de metilación, en transposones durante el cambio de fase en maíz (Li *et al.* 2010).
- El imprinting genómico en donde la metilación está asociada con el silenciamiento génico de ciertos alelos de determinados genes, cuya expresión varía a lo largo del desarrollo del embrión (Henderson and Jacobsen 2007; Makarevich *et al.* 2008).
- Capacidad adaptativa como en *Picea abies L. Karst*, la cual se adapta rápidamente a los cambios de su entorno, debido a su memoria epigenética, la que adquiere durante su embriogénesis (desarrolla la semilla) (Yakovlev *et al.* 2010), para el establecimiento de esta memoria, es fundamental la metilación del ADN (Yakovlev *et al.* 2016).
- Asociada a productividad como por ejemplo en híbridos *Populus deltoides x P. nigra*, se han identificados variaciones en los niveles de metilación del ADN, lo que se ha correlacionado con la productividad de biomasa y el crecimiento altura de éstos (Gourcilleau *et al.* 2010).

1.4 Regulación epigenética del cambio de fase vegetativa.

En los últimos años se ha profundizado como la vía miR156-SPLs, que es el regulador principal de la transición de fase vegetativa y como los componentes de esta vía están sujetos a regulación

epigenética, cómo la metilación del ADN, modificaciones de histonas y remodelación de cromatina. Son esenciales en el control de este evento clave en el ciclo de vida de las plantas.

1.4.1 Metilación del ADN en los genes del módulo miR156-SPL

Se han reportado altos niveles de metilación del ADN nuclear en los tejidos meristemáticos adultos en contraste con los tejidos meristemáticos juveniles en *Prunus persica* (Bitonti *et al.* 2002). En plantas de *Arabidopsis* de cinco semanas de edad, mutantes (*met1* y *drm1 drm2 cmt3*) (Zhang *et al.* 2006) y plantas silvestres de 25 días (Zilberman *et al.* 2007), se han contrastado mediante análisis de metilación del ADN genómico. En dicho estudio se han identificado metilaciones diferenciales del tipo no CG sólo en el gen SPL10 (Xu *et al.* 2018).

1.4.2 Modificaciones de histonas en los genes del módulo miR156-SPL.

En *Arabidopsis* han identificado un aumento de la trimetilación de la histona H3 lisina 27 (H3K27me3), mediada por el remodelador de cromatina CHD3 PICKLE (PKL) (Zhang *et al.* 2008), podría contribuir en la adición de H3K27me3 a los genes MIR156, lo que se correlaciona con una disminución de transcrito de éstos. Esto se ha asociado con una disminución temporal de la acetilación de H3K27, lo cual contribuiría a la disminución de miR156 durante el cambio de fase vegetativa (Xu *et al.* 2015).

La transición de juvenil a adulto en *Arabidopsis*, se asocia a una disminución de la transcripción de *MIR156*, la cual se relaciona con un aumento en la unión del complejo PRC2 a estos, lo que lleva a un aumento de la marca H3K27me3 en su promotor, así como una disminución en la marca H3K27ac en la región posterior al inicio de la transcripción (TSS) (Xu *et al.* 2016a,b). Para el complejo PRC1 que reconoce la marca H3K27me3 para establecer una represión transcripcional (Lund and van Lohuizen 2004).

Se ha demostrado que PRC1 está involucrada en la transición de juveniles a adultos en *Arabidopsis*, participando en la represión de miR156 acelerando la transición de juvenil a adulto,

también se reportado que una disminución de la mono ubiquitinación de la histona 2A (H2Aub) y H3K27me3 en la región TSS de MIR156, lo cual prolonga la fase juvenil (Picó *et al.* 2015). Los complejos formados por RING1-PRC1 y EMF1-PRC1 reprimen los SPLs, la disminución de la marca H2Aub en SPL3 y SPL9, acelera la aparición de rasgos adultos (Li *et al.* 2017). En *Arabidopsis* se codifican tres H3K4 metiltransferasa TRITHORAX1 (ATX1), ATX2 y ATXR7, de las cuales ATXR7 se une a una región adyacente al TSS de MIR156 y depositando la H3K4me3, lo cual activa la transcripción MIR156 (Xu *et al.* 2018).

También se ha reportado que la regulación epigenética asociada a factores ambiente (luz y fotoperiodo), juegan un papel importante en la regulación de los genes SPLs y en la inducción de miR156, mediante la acetilación de acetiltransferasas de histonas (HAT) del tipo Spt-Ada-Gcn5-acetiltransferasa (SAGA), podría ser un aspecto importante en la transición de fase vegetativa, dicho mecanismo de control, es previo a la regulación post-transcripcional mediada por miR156 en el cambio de fase (Kim *et al.* 2015).

1.4.3 Remodelación de cromatina

Se ha descrito que la transcripción de MIR156 es promovida por el remodelador nucleosomal, BRAHMA (Xu *et al.* 2016c) y por el complejo SWR1, que intercambia la variante de histona H2A.Z por H2A (Choi *et al.* 2016). Se ha encontrado en mutantes para el complejo SWR1 una reducción en la expresión de MIR156, con una aceleración en el cambio de fase vegetativa, lo que indica que H2A.Z promueve la identidad vegetativa juvenil, también H2A.Z promovería la expresión de MIR156 al facilitar la deposición de H3K4me3 (Xu *et al.* 2018).

Como se mencionó anteriormente la proteína remodeladora de nucleosomas PICKLE (PKL), está asociada a los complejos de remodelación y desacetilación de nucleosomas (Ho *et al.* 2013), este se une a la región adyacente TSS de MIR156 reprimiendo su transcripción (Xu *et al.* 2016b).

1.5 *Eucalyptus globulus* Labill como modelo estudio del cambio de fase vegetativa.

Los mecanismos epigenéticos son claramente importantes, durante el desarrollo, por ello ahondar en cómo está regulando el cambio de fase vegetativa, permitirá generar información valiosa al respecto, para lo cual utilizaremos como modelo estudió *Eucalyptus globulus* Labill, que es un excelente modelo del cambio fase vegetativa (James and Bell 2001), ya que posee claras diferencias morfológicas entre sus hojas (Johnson, 1926).

Permite cuantificar el momento en el que ocurre en cambio de fase vegetativa, que se determina a la altura del árbol, en la cual se observa la primera hoja peciolada o adulta (altura del cambio de fase ACF). A nivel poblacional en el centro de origen de *E. globulus* (Australia), se observan diferencias de entre 2 a 5 m en la ACF, según la zona geográfica de crecimiento (Jordan *et al.* 2000). Se ha verificado que la ACF tiene una correlación con el incremento en diámetro de los individuos (Hamilton *et al.* 2011). Por otra parte, diferentes procedencias de *E. globulus* cultivadas en un jardín común, conservan los patrones de ACF que presentaban, según el lugar de su procedencia, por lo que es considerado un carácter estable y heredable (Hamilton *et al.* 2011; Dutkowski and Potts 1999). Además el cambio de fase está asociado con efectos fisiológicos, como mayor o menor resistencia a enfermedades (Farrow *et al.* 1994; Dungey *et al.* 1997), cambios en la capacidad fotosintética (James and Bell 2000; James and Bell 2001) y diferencias en la tasa crecimiento, se ha descrito que las hojas juveniles favorecen el crecimiento en diámetro (Jordan *et al.* 2000). También posee un componente genético, asociado al cambio de fase vegetativa en *E. globulus*, por medio de la detección de un QTL (locus de carácter cuantitativo) altamente significativo, el cual explicaría el 62,8% de variaciones fenotípicas, asociadas al cambio de fase (Hudson *et al.* 2014). Dicho locus está asociado al gen del EglMIR156.5 (Hudson, 2012), lo que ha sido descrito para la especie modelo *A. thaliana* (Wu *et al.* 2009; Wu and Poethig 2006; Wang *et al.* 2009).

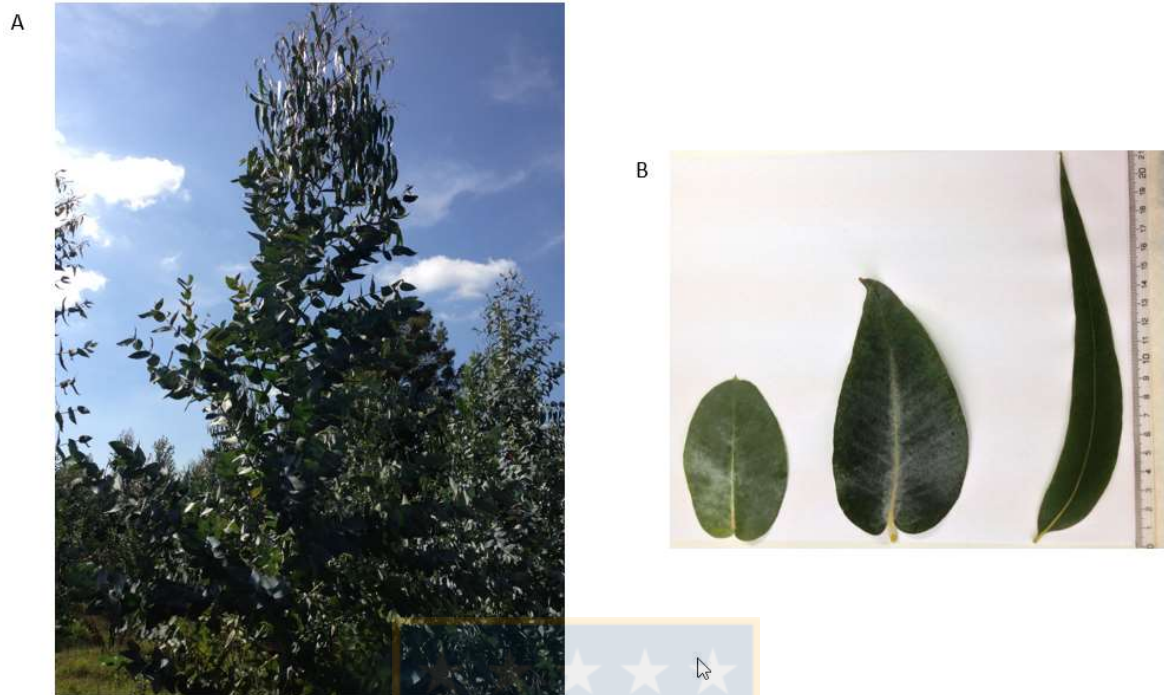


Figura 1 Variación heteroblástica en *Eucalyptus globulus* (A). Cambio drástico en la morfología del follaje (B) Hojas juveniles, intermedias y adultas (de izquierda a derecha).

En este contexto anteriormente expuesto, la presente investigación buscó responder las siguientes preguntas:

¿Está correlacionado el patrón de metilación de los genes del módulo miR156-SPLs con su expresión génica al contrastar hoja juvenil y adulta de *Eucalyptus globulus* Labill?

¿Existen diferencias espaciales dentro de las hojas que indiquen que el módulo miR156-SPLs responde a gradientes al comparar hoja juvenil y adulta de *Eucalyptus globulus* Labill?

La presente investigación persiguió el siguiente objetivo general:

Contrastar la expresión del módulo miR156-SPLs regulador del cambio de fase vegetativa con sus perfiles de patrón de metilación en *Eucalyptus globulus* Labill.

Objetivos específicos:

1. Contrastar la expresión del módulo miR156-SPLs con sus perfiles de patrón de metilación en *Eucalyptus globulus*.
2. Identificar los elementos reguladores en las regiones diferencialmente metiladas de las zonas promotoras putativas del módulo miR156-SPLs en *Eucalyptus globulus*.
3. Analizar la expresión del módulo miR156-SPLs en el eje distal proximal en hojas juveniles y adultas de *Eucalyptus globulus*.

Hipótesis:

Hipótesis 1: El cambio de fase vegetativa de hoja juvenil a adulta en *Eucalyptus globulus* correlaciona los niveles de expresión del módulo miR156-SPLs con la presencia de zonas hiper e hipo metiladas alrededor de las regiones promotoras putativas y cuerpos genéticos del módulo miR156-SPLs.

Hipótesis 2: Hojas juveniles y adultas no muestran diferencias en los niveles de expresión del módulo miR156-SPLs en el eje distal proximal de la lámina folial en *Eucalyptus globulus*.



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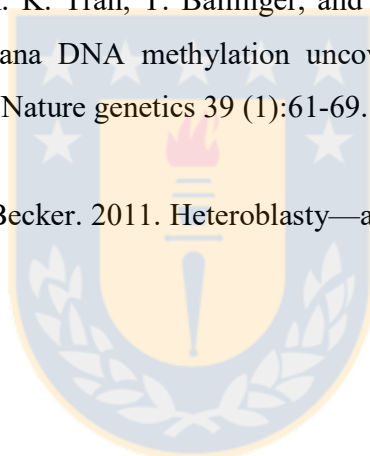
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Capítulo II:

Differential Methylation of Genomic Regions Associated with Heteroblasty Detected by M&M Algorithm in the Nonmodel Species *Eucalyptus globulus* Labill.

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2.1. Abstract

Epigenetic regulation plays important biological roles in plants, including timing of flowering and endosperm development. Little is known about the mechanisms controlling heterochrony (the change in the timing or rate of developmental events during ontogeny) in *Eucalyptus globulus*. DNA methylation has been proposed as a potential heterochrony regulatory mechanism in model species, but its role during the vegetative phase in *E. globulus* has not been explored. In order to investigate the molecular mechanisms governing heterochrony in *E. globulus*, we have developed a workflow aimed at generating high-resolution hypermethylome and hypomethylome maps that have been tested in two stages of vegetative growth phase: juvenile (6-month leaves) and adult (30-month leaves). We used the M&M algorithm, a computational approach that integrates MeDIP-seq and MRE-seq data, to identify differentially methylated regions (DMRs). Thousands of DMRs between juvenile and adult leaves of *E.*

globulus were found. Although further investigations are required to define the loci associated with heterochrony/heteroblasty that are regulated by DNA methylation, these results suggest that locus-specific methylation could be major regulators of vegetative phase change. This information can support future conservation programs, for example, selecting the best methylomes for a determinate environment in a restoration project.

2.2. Introduction

Tree species are usually able to tolerate a wide range of environmental conditions. In fact, they are able to tolerate a wide range of environmental conditions and, in many cases, extreme seasonal changes [1]. Some of these organisms are able to manifest different phenotypes depending on the environment in which they grow. This phenomenon, called phenotypic plasticity, has been defined as a change in the phenotype expressed by a single genotype in different environments [2, 3]. Phenotypic plasticity evolves to maximize fitness when the environment is variable and increases with latitude [4–6].

Leaf heteroblasty is a significant and abrupt change in form and function that occurs over the maturation process (phase change from seedlings to reproductive individuals) of certain plants [7]. Characteristics commonly affected include leaf form, size, and arrangement. The earlier and later stages of leaf development are named juvenile and adult, respectively. In contrast to phenotypic plasticity, heteroblastic development does not depend on environmental cues. However, the timing or rate of heteroblastic changes, which can be referred to as a type of heterochrony—change of relative timing of events throughout development—can be modified by the environment [8]. Heterochrony has been implicated in plant evolution, because it can impact the physiology, phytochemistry, or resistance to pests and disease of certain plants [9, 10]; however it is a plastic response largely underexplored.

Phenotypic plasticity and heterochrony may interact to produce a pattern of variation in the leaf phenotype, even in organisms with little or no genetic diversity. Most endangered species have

lower genetic diversity than related nonendangered species [11], and phenotypic plasticity and heterochrony together can increase the possibilities of adaption/persistence. Therefore, knowledge of the molecular mechanisms regulating both processes could open new alternatives to assist conservation programs.

Epigenetic regulation, in particular DNA methylation, plays an important biological role in plants, including timing of flowering and endosperm development [12]. Transgenerational inheritance of DNA methylation can mediate phenotypic plasticity via novel epialleles and phenotypes within populations/species [13]. In a review by Pascual et al. [3] it was shown that the coordination of genetic and epigenetic mechanisms mediated phenotypic variation in different plants. For example, in populations of *Arabidopsis thaliana* with experimental alteration of DNA methylation, the overall patterns of variability among the genotypes indicated that epigenetic changes could affect not only the short-term environmental responses of plants, but also the evolutionary potential of important traits and their plasticity [14]. Similarly, a recent study in invertebrates proposed that the absence of germline DNA methylation in genes involved in the response to fluctuating conditions facilitates phenotypic variation, which could contribute to increased adaptive potential [15]. In conifers it has been reported that environment influences a differential DNA methylation during embryogenesis, inducing differential priming of the embryos that causes differential capabilities to adapt to environment [16].

There is little evidence linking epigenetic regulation and heterochrony. Only a few studies focusing on epigenetic changes during leaf differentiation and development have been developed in *Arabidopsis* [17–19], rice [20], or pine [21] but none of these species are strongly heteroblastic. Environmental cues are perceived as input signals for the microRNA156/SQUAMOSA promoter-binding protein-like (SPL or SBP box) module and act as a quantitative developmental clock of phase transitions in plants [22, 23]. The evidence shows that sugars promote vegetative phase change through their effect on miR156 [24], but other endogenous factors could play additional important roles. The same authors proposed that heritable epigenetic modification of the miR156 precursor and/or additional chromatin structure alterations could regulate heteroblasty [24].

Eucalyptus globulus, a tree of Australian origin but cultivated worldwide, is strongly heteroblastic with clear differences between its juvenile and adult leaves [25]. Jordan et al. [26] found that genetic association of the timing of vegetative phase change with growth rate ranged from positive to negative at different sites. Early phase change may be favored in warm, wet environments to reduce damage produced by leaf fungi, but it may also be favored on exposed dry sites to increase form or plant structure by which it is protected from desiccation [27, 28]. Genome-wide DNA methylation maps of many model organisms have been reported, but in nonmodel organisms like *Eucalyptus* spp. the methylation patterns remain poorly studied. These types of maps can be applied to a wide range of biological problems, using the analysis of methylation differences between ecotypes or individuals within species [29–31].

The aim of this study is to set up the required methodology and assess the epigenetic changes (hypermethylation and hypomethylation) related to heteroblasty in *E. globulus*. Specifically, we want to detect differentially methylated regions (DMRs) between juvenile and adult leaves of *E. globulus*. DMRs are stretches of genomic DNA that have different DNA methylation patterns. Their natural variation could guide the conservation management of the species or the selection of individuals with potentially adequate methylomes set of modifications of nucleic acid methylation in the genome of an organism for a discrete environmental condition. We expect that the results and experience from this work could be used for the discovery of key regulators of heterochrony in future studies, which could be used to assist conservation programs of threatened species.

2.3. Materials and Methods

To investigate the molecular mechanisms governing heterochrony in *Eucalyptus globulus*, we applied the M&M algorithm [32] to identify DMRs related to heteroblasty of vegetative growth. We used the previously sequenced X46 clone (Mininco SA JGI Project ID: 401875). Ten ramets of the X46 clone were produced by cuttings, and a genetic trial was established in the commune

of Renaico, province of Malleco, region of La Araucanía, Chile (latitude -37.67 , longitude -72.59). Juvenile leaf material from five plants was harvested after 6 months at nodes 8 to 10, mixed, and stored at -70°C . Plants were grown until the vegetative phase change was evident (after more than 2 years of growth, average 45 nodes), and adult leaf material was collected. Genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN Inc.).

The methylation profiles were determined by DNA sequencing of enriched genomic libraries: (i) hypermethylome (the methylated part of the genome) using immunoprecipitation of methylated DNA (MeDIP-seq) and (ii) hypomethylome (the nonmethylated part of the genome) based on restriction enzymes sensitive to methylation (MRE-seq).

2.3.1. MeDIP-seq and MRE-seq Library Generation and Sequencing.

MeDIP-seq is a large-scale purification technique used to enrich libraries for methylated DNA sequences. It consists of isolating methylated DNA fragments via an antibody raised against 5-methylcytosine (5mC). MRE-seq utilizes a combination of methyl sensitive enzymes to enrich libraries for unmethylated DNA sequences.

Libraries were generated as previously described in Li et al. [32], with minor modifications. For MeDIP-seq, 3500 ng of isolated DNA was sonicated using 26 pulses of 10 s ON/20 s OFF (Sonic Dismembrator model 100, Fisher Scientific) to a fragment size of 100–500 bp, end processed, and ligated to paired-end adapters using NEXTflex PCR free DNA Sequencing Kit (Bio Scientific). After size selection of 166–566 bp using Agencourt AMPure XP (Beckman Coulter), DNA was heat denatured and then immunoprecipitated using Methylated DNA IP Kit (Zymo Research), using a mouse monoclonal anti-5-methylcytosine antibody according to manufacturer's instructions. DNA was then purified with Agencourt AMPure XP (Beckman Coulter) and eluted in 25 mL resuspension buffer (10 mM Tris-HCl, pH 8.5). DNA was amplified by 12 cycles of PCR with the standard Illumina index primers. For MRE-seq, three digestion reactions (HpaII, Acil, and Hin6I; Fermentas) were performed in parallel, each with 600 ng of DNA. Ten units of enzyme (except Acil, which uses five units) were initially

incubated with DNA for 3 h, and then additional five units of enzyme were added to the digestion for a total of 6 h of digestion time. Digested DNA from the different reactions was combined and purified using ChiP DNA Clean & Concentrator™ (Zymo Research). The purified DNA was end processed and ligated to single-end adapters using NEXTflex PCR free DNA Sequencing Kit (Bioo Scientific). After size selection (166–566 bp) with Agencourt AMPure XP (Beckman Coulter), the DNA was amplified by PCR for six cycles.

MeDIP and MRE libraries were sent to the DNA Sequencing Facility of the Biotechnology Center at the University of Wisconsin. Samples were sequenced on an Illumina HiSeq machine, yielding a total of 204 million MeDIP-seq reads and 236 million MRE-seq reads. The reads were mapped to the latest *Eucalyptus grandis* genome assembly (v2.0) [33], using BWA-MEM Li [34] with default settings.

2.3.2. Use of M&M Algorithm to Detect DMRs.

We used an algorithm named M&M [32] that integrates data from both MeDIP-seq and MRE-seq to detect DMRs. M&M is available as an R package called “methylMnM.” Briefly, M&M integrates MeDIP-seq and MRE-seq by dynamically scaling, normalizing, and combining the datasets and provides exact p value and q-value for different sample comparison. The coverage of MeDIP and MRE sequencing data and genomic CpG information were calculated for each 2000 bp genomic bin. Before applying the M&M method, we generated two input files: (1) CpG sites of each window and (2) MRE-CpG sites of each window. To generate file (1) we used the script `fasta2bed.py` (Computational Genomics Analysis Tools CGAT 0.2.3), and to calculate (2) we used a script created specifically for this project (discussed at <https://www.biostars.org/p/86480/>). DMRs between developmental stages were identified using the M&M algorithm with default parameters in the R environment (version v.2.12.1). Briefly, the coverage of MeDIP and MRE sequencing data and genomic CpG information were calculated for each 2000 bp genomic bin. Scaffolds were excluded from the analysis. DMRs with a q-value of $1E - 7$ were selected for analysis. Several statistical functions are implemented in the methylMnM package: “MnM.test()” to calculate the probability that the methylation

levels of the two samples within each bin were different, “MnM.qvalue()” to estimate q-values based on all the p values, and “MnM.select-DMR()” to select significant DMRs based on a cutoff of q-value $< 1E - 4$. The output files contained genomic locations of statistically significant DMRs and their MeDIP-seq and MRE-seq values (in RPKM), as well as p values and q-values. The absolute values of genomic regions are negative log₁₀-transformed q-values. If the value is negative, it represents hypermethylation in the vegetative juvenile sample and hypomethylation in the vegetative adult; if the value is positive, it represents hypomethylation in the vegetative juvenile sample and hypermethylation in the vegetative adult. Mapping results and detected DMRs were visually inspected with the GenomeView software [35] using default parameters.

2.4. Results and Discussion

2.4.1. Sequencing Results

For each developmental stage, we constructed one sequencing library using two complementary technologies: MeDIP-seq and MRE-seq. These libraries were sequenced to generate 446 million reads in total (Table 1), of which 387 million were mapped to the *E. grandis* genome covering at least 50%. We note that MeDIP-seq data has less mapping efficiency. Li et al. [32] explained this because relatively more MeDIP-seq reads are derived from repetitive regions of the genome, which are often heavily methylated. Some reads from repetitive regions cannot be mapped uniquely. In line with expectations MRE-seq scores were inversely correlated with MeDIP-seq scores.

Table 1 Summary of mapping statistic from MeDIP-seq and MRE-seq libraries of juvenile and adult leaves.

	Total fragments (Tf)	Mapped fragments	% of Tf	Uniquely mapped fragments	% of Tf	Non-redundant uniquely mapped fragments	Genome coverage (%)
MeDIP-seq-juvenile	1.37E+8	1.17 E+8	85.4	6.74E+7	49.1	6.79E+6	52.3
MeDIP-seq-adult	0.70E+8	0.57E+8	82.0	2.48E+7	35.4	6.72E+6	38.3
MRE-seq-juvenile	1.14E+8	1.02E+8	89.5	7.91E+7	69.4	9.6E+6	57.0
MRE-seq-adult	1.25E+8	1.11E+8	88.8	8.83E+7	70.6	6.34E+6	49.2
Total	4.46E+8	3.87E+8	86.7	2.60E+8	58.3	2.95E+7	

The two sequencing signals covered nonoverlapping regions as expected, but some adjacent genomic regions showed overlapping signals (Figure 1).

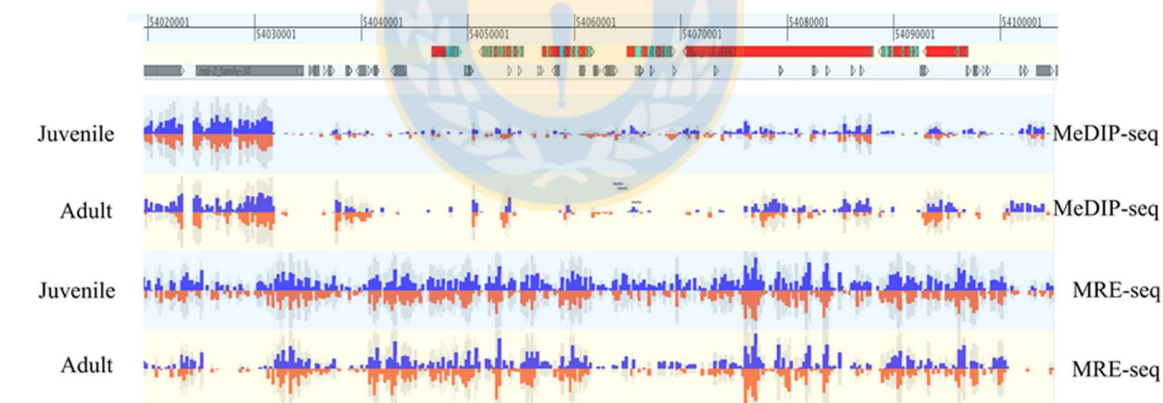


Figure 1 Epigenome view (GenomeView software) of a 138.7 kb region of chromosome 07 (54,020,001–54,100,001) of *Eucalyptus grandis* reference genome. MeDIP-seq libraries and MRE-seq libraries covered largely nonoverlapping and short overlapping regions.

2.4.2. Identification of DMRs

The main aim of this work was the determination of the local DNA methylation changes between two developmental phases (i.e. vegetative juvenile and vegetative adult). We only considered CpG methylation and applied the M&M algorithm to our data to identify DMRs. This allowed us to find a total of 1090 putative DMRs ($q\text{-value} < 1E - 4$) between the two developmental stages. More than 70% of DMRs showed increasing DNA methylation levels from vegetative growth phases juvenile to adult. These preliminary results suggest that locus-specific methylation patterns could be an important feature of vegetative heteroblasty control. This increment in the number of methylated regions agrees with the reduction in the number of genes and proteins that is observed in mature leaves (when they reach their full physiological competence) compared to leaves in proliferative stage [36, 37] which have been also related to an increase of global DNA methylation in conifers [21, 38] and Arabidopsis [39].

In future work, candidate DMRs related to genes involved in plastic responses will be validated using more genotypes of *E. globulus* with heterochronic responses in the expression of heteroblasty. Using quantitative techniques for quantifying methylation (e.g. bisulfite/sequencing of specific genomic locus) we hope to find DMRs that could explain or predict heterochronic responses. Knowledge of genomic loci that regulate heterochrony in *E. globulus* will improve our understanding of molecular mechanism of this process and can support future conservation programs, for example, selecting the best methylomes for a specific environment in a restoration project. This complete workflow could be easily applied to other nonmodel species with the only requirement of a reference genome. Given that the cost of DNA sequencing is falling [40] and bioinformatics facilities for assembly of massive sequences are increasing quickly [41], generating a reference genome for almost any species is becoming more affordable. The latter also is valid for threatened species.

The introduction of a workflow allowing the study of the epigenetic regulation at genome level will provide new insight that goes further than the mere description of the genes that are regulated during plant development or environmental adaption. For instance, having the possibility of tracking epigenetic changes in the form of metastable epialleles alleles that are variably expressed in genetically identical individuals due to epigenetic modifications

established during early development and are thought to be particularly vulnerable to environmental influences will allow us to add a new source of variation, which is particularly important for threatened species, which usually exhibit a very low genetic diversity [42, 43]. Although these studies can be done routinely in model species [44, 45], it is particularly difficult to adapt wet and in silico methodologies for nonmodel like trees, since tissues are rich in polyphenols and other contaminant molecules requiring optimizations (e.g. [46–48]) and at the same time bioinformatics pipelines (gene prediction, characterization, and annotation algorithms) still require improvements [49]. In this study we describe a workflow aiming at achieving results comparable to those obtained in *Arabidopsis* in terms of quality of libraries and quantity of reads. This procedure will allow future studies employing a higher number of samples and experimental situations that permit addressing important issues related to conservation and management of threatened species such as restoration and translocation. Furthermore, the integration of this regulatory layer together with other omic levels has been proved to be useful for explaining adaptive divergences (see Meijón et al. [50] for an example of the power of high-throughput approaches to explore natural variation in tree species).

2.5. Conclusion

These preliminary results suggest that locus-specific methylation process could be an important feature of vegetative heteroblasty control in *E. globulus*. The workflow set up in this project opens a promising future for discovering DNA methylation patterns among different tissue types, cell types, and individuals that will help us to explain phenotypic plasticity and adaption capabilities through the basis of a divergent epigenetic regulation. High-throughput epigenomic technology and analytical tools used in this study could be applied to population-based studies of nonmodel plants but principal challenges are experimental design, data analysis, and interpretation of results. The implementation of epigenetic fingerprinting as a support tool in restoration and conservation projects of threatened species requires the discovery of loci involved in adaptive variation.

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Capítulo III

Relación de los genes implicados en el cambio de fase vegetativa y sus perfiles de metilación en *Eucalyptus globulus* Labill.

En preparación.

Carolina Iturra

3.1. Resumen

En el ciclo de vida de algunas especies arbóreas se observa el fenómeno de heteroblastía, el cual se caracteriza por una marcada transición de los rasgos juveniles a adultos. Como es el caso de *Eucalyptus globulus* Labill, que se caracteriza por cambios en la morfología y su fisiología durante esta transición. Además es un buen modelo genético para el estudio de la heteroblastía, debido a su cambio de fase vegetativa claramente delimitado. El control molecular de la heteroblastía sigue siendo poco claro, aunque se ha demostrado en *Arabidopsis thaliana* L. que hay mecanismos epigenéticos relacionados con la acetilación y metilación de histonas, regulando éste. Entre los mecanismos descritos como reguladores de este proceso está el miR156 y sus genes asociados directamente a él, los factores de transcripción SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) e indirectamente la hexokinasa y DELLA. Para determinar la relación entre la expresión de estos genes reguladores del cambio de fase vegetativa y su posible control epigenético en *E. globulus*, contrastamos los perfiles de expresión de los genes seleccionados con sus perfiles de metilación, comparando hojas adultas y juveniles. Los resultados muestran que la expresión de miR156 disminuye durante el cambio de fase vegetativa, que coincide con el aumento de la expresión de los genes SPL3 y SPL9 en tejidos adultos. Comparando los patrones de expresión relativa de SPL3 y SPL9, con los perfiles de metilación en tejidos adultos y juveniles, se encontró una aparente relación con la metilación de las regiones promotoras, en la que hay más metilación en los tejidos juveniles, coincidiendo

con una menor expresión de estos. En el caso de miR156 no se observó diferencias en los perfiles de metilación. Para la hexokinasa se determinó una mayor expresión en tejido adulto, lo cual se correlacionó con una menor metilación en la zona promotora. Además para el gen DELLA su patrón expresión fue mayor en tejido juvenil, lo cual se asocia con una disminución de su perfil de metilación en la zona promotora. Estos resultados nos proporcionan un marco para futuros estudios sobre la regulación epigenética en el cambio de fase vegetativa, con la finalidad de una mejor comprensión sobre el tema.

3.2. Introducción

Las plantas están sometidas a una serie de transiciones en su ciclo de desarrollo. Podemos hablar de tres etapas claramente definidas: embrional, postembrional y crecimiento. Durante la etapa de crecimiento se identifican tres fases: juvenil vegetativa, adulta vegetativa y reproductiva (Poethig 1990; Poethig 2003). Durante esta transición de fases, se observan cambios en el tamaño de la hoja, la forma y la distribución de los tricomas (Proveniers 2013), fenómeno llamado desarrollo heteroblástico (Zotz *et al.* 2011). El tiempo o momento en que ocurre el cambio de fase (tiempo de cambio de fase) es crítico para la sobrevivencia de una especie en el medio natural (Taiz *et al.* 2006). En aquellas especies en que el tiempo del cambio de fase varía entre individuos, se habla de heterocronía, fenómeno que hace referencia a todos los cambios en el ritmo de los procesos ontogenéticos. Este fenómeno da lugar a la diferenciación en forma y tamaño de los organismos (Antonelli *et al.* 1993), lo cual ha permitido explicar gran parte de la diversidad filogenética (Geuten *et al.* 2013).

La heteroblastía ha sido descrita para especies leñosas de los géneros *Pinus* (Climent *et al.* 2006), *Acacia* (Forster *et al.* 2009) y *Eucalyptus* (James *et al.* 2009). El tipo de variación heteroblástica mayormente descrita es el cambio de fase vegetativa (Poethig 1990), la cual implica cambios en las características de hojas, tallos y brotes (Rasmussen 1986). Estos cambios son específicos de cada especie y van desde la transición de hojas juvenil a adulta, patrones de ramificación, depósito diferencial de cera epicuticular, patrones de producción de tricomas,

forma celular, patrones vasculares, capacidad para la producción de raíces adventicias, presencia o ausencia de antocianina u otros fitoquímicos y resistencia a enfermedades o insectos (Zotz *et al.* 2011; Whalen 2005; Poethig 2010). En el género eucaliptus, el momento de cambio de fase vegetativa está bajo un fuerte control genético y puede variar notablemente tanto dentro como entre especies (Jordan *et al.* 1999; Hamilton *et al.* 2011). Se ha descrito la presencia de un componente genético asociado al cambio de fase vegetativa para *E. globulus*, por medio de la detección de un QTL asociado al cambio de la fase vegetativa precoz, en las poblaciones en las que el cambio de la fase vegetativa se retrasó varios años, se identificó la expresión del microRNA EglMIR156.5 como responsable de la variación heterocrónica en el cambio de la fase vegetativa en *E. globulus* (Hudson 2012; Hudson 2014), lo que ha sido descrito para especies modelos como *A. thaliana* (Wu *et al.* 2006; Wu *et al.* 2009; Wang *et al.* 2009). Sin embargo, las bases moleculares de estas variaciones en *E. globulus*, aún están sin explorar.

Los mecanismos del control molecular de la heteroblastia descritos hasta ahora, están asociados a dos microARNs (miRs) muy conservados entre especies, miR156 y miR172 (Poethig 2003; Wu *et al.* 2009; Huijser *et al.* 2011; Feng *et al.* 2016; Yang *et al.* 2011; Levy *et al.* 2014). Se ha descrito que el miR156 está presente en altos niveles después de la germinación y disminuye durante el desarrollo, lo cual promueve la expresión de rasgos adultos (Huijser *et al.* 2011). Los genes blancos directos del miR156 son la familia de factores de transcripción SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPLs) (Wu and Poethig 2006; Wang and Wang 2015; Xie *et al.* 2006; Schwab *et al.* 2006). Éstos poseen secuencias complementarias al miR156 en la zona 3' UTR de los SPLs (Wu and Poethig 2006), lo que reprime su expresión por medio de la represión directa de su transcripción (Schwab *et al.* 2005), o por medio de la represión de traducción (Xu *et al.* 2016). Esto último se traduce en una disminución de los niveles de proteínas SPLs (Xie *et al.* 2006). Los genes SPLs son conservados y se expresan diferencialmente en tejido juvenil y adulto en herbáceas (Zhang *et al.* 2011; Salinas *et al.* 2012), así como en plantas leñosas (Wang *et al.* 2011). El miR156 reprime los niveles SPL3 (Schwab *et al.* 2005), el cual está asociado a la inducción floral y regulación del cambio de fase vegetativa en *Arabidopsis thaliana* (Wu and Poethig 2006) y también reprime al miR172 a través de su efecto sobre la expresión de SPL9. Este último gen ha sido relacionado a la morfología de las

hojas (Chitwood *et al.* 2014). Se ha descrito como regulador del miR156, a los azúcares glucosa y sacarosa (Fig. 1) producidos durante la fotosíntesis (Wu *et al.* 2006; Wang *et al.* 2009; Xu *et al.* 2016), los cuales se van acumulando y reprimen la expresión de éste (Yu *et al.* 2013; Yang *et al.* 2013). Esta represión es dependiente de la actividad de señalización de Hexokinasa 1, que también contribuye a la disminución de los niveles de miR156, lo cual ocurriría mediante la regulación de la transcripción (Yang *et al.* 2013). De igual forma se ha asociado a este miRNA, al control de rasgos de importancia económica, como la producción de madera, el diámetro del tallo, la producción de raíces adventicias, crecimiento de ramas, tiempo de floración, la arquitectura de inflorescencia, además de la forma, tamaño y expansión de las hojas (Poethig 2010; Fu *et al.* 2012).

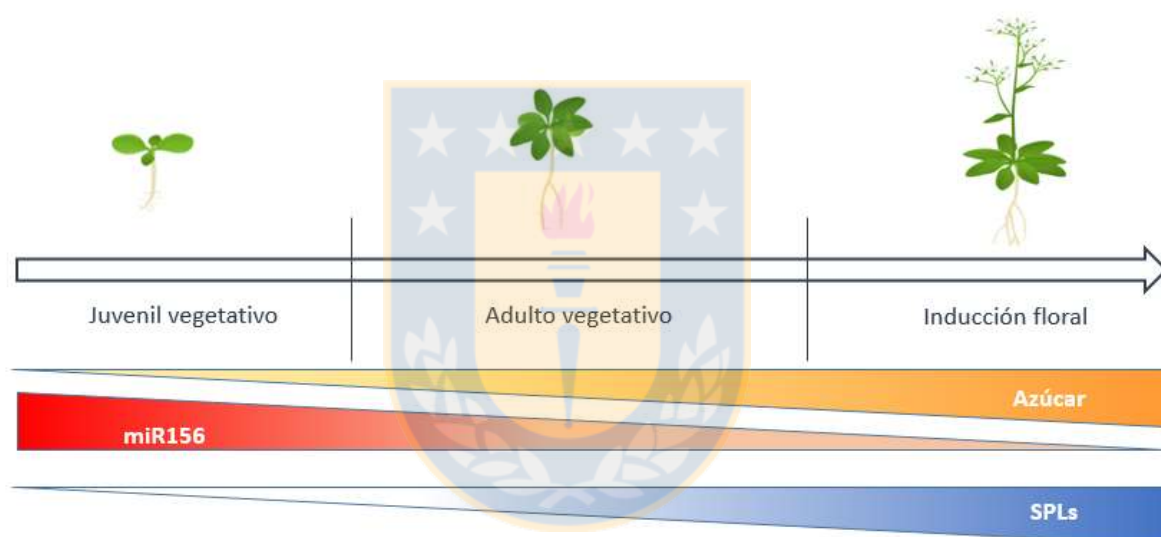


Figura 1: Esquema resumen de los principales mecanismos moleculares descrito para el control de cambio de fase vegetativa (Adaptado de Xu *et al.* 2016; Wu and Poethig 2006; Proveniers 2013; Yang *et al.* 2013).

Se ha identificado recientemente que el cambio de fase, posee un fuerte componente epigenético en su regulación, el cual sería determinante, ya que ocurre en forma previa a la regulación dada por miR156. De esta forma, la regulación epigenética asociada al componente ambiental juega un papel clave en la regulación de los genes SPLs y su mecanismo de control, a través de la acetilación de acetiltransferasas de histonas (HAT) del tipo Spt-Ada-Gcn5-acetiltransferasa

(SAGA), lo cual es previo a la posterior regulación post-transcripcional por miR156 (Kim *et al.* 2015). De igual forma se asocia el cambio de fase con un aumento en el nivel de histona H3 trimetilada en la lisina 27 (H3K27me3), la cual determina al empaquetamiento de la cromatina (Xu *et al.* 2015), pero aún son desconocidas las posibles regulaciones epigenéticas asociada a los genes que controlan el cambio de fase.

Los mecanismos de control genéticos y epigenéticos previamente descritos han utilizado como modelo de estudio *Arabidopsis thaliana* (L.) Heynh, la cual posee una sutil diferencia morfológica, de sus hojas entre el estado juvenil y adulto (Telfer *et al.* 1997). En cambio en especies leñosas la transición de fase juvenil a adulta, posee ventajas como modelo de estudio, ya que presentan una clara diferencia entre las fases (Wang *et al.* 2011) y la transición es variable en el tiempo, es decir heterocrónica (Lawson and Poethig 1995). A la fecha existen muy pocos estudios en especies leñosas, destacando los resultados observados en *Acacia confusa* Merr, *Acacia colei* Maslin & L.A.J. Thomson, *Eucalyptus globulus* Labill, *Hedera hélix* L. *Quercus acutissima* Carruth y *Populus canadensis* Moench, que muestran niveles significativamente altos de miR156 en hojas juveniles en comparación con hojas adulta (Wang *et al.* 2011).

De las especies leñosas previamente mencionadas, *Eucalyptus globulus*, es un excelente modelo del cambio fase vegetativa (James and Bell 2001). Porque permiten cuantificar el momento en el que ocurre en cambio de fase vegetativo, en especies leñosas es la altura del árbol en la cual se observa la primera hoja peciolada o adulta (altura del cambio de fase ACF). A nivel poblacional en el centro de origen de *E. globulus* (Australia), se observan diferencias de entre 2 a 5 m en la ACF, según la zona geográfica de crecimiento (Jordan *et al.* 2000) Se ha verificado que éste tiene un efecto posterior en el incremento en diámetro de los individuos, observándose una relación positiva con la ACF (Hamilton *et al.* 2011). Por otra parte, diferentes procedencias de *E. globulus* cultivadas en un jardín común, conservan los patrones de ACF que presentaban según el lugar de su procedencia, por lo que es considerado un carácter estable y heredable (Hamilton *et al.* 2011; Dutkowski and Potts 1999).

Estudios previos de nuestro laboratorio han generado mapas de alta resolución de la metilación, de las dos etapas de la fase de crecimiento vegetativo: juvenil y adulto (Hasbún et al. 2016). A partir de lo anteriormente expuesto, surge la pregunta si existe correlación entre los perfiles de metilación de los principales genes implicados en el cambio de fase vegetativa y los niveles de transcrito de estos.

3.3. Materiales y métodos.

3.3.1. Material vegetal.

Para los ensayos de qRT-PCR, se colectaron hojas en sus estadios juvenil, intermedia y adulta, a partir de tres individuos que presentaban los tres estadios, los muestreos se realizaron en la temporada de verano. Las muestras fueron almacenadas a -80°C.

3.3.2. Extracción ARN total y síntesis cADN.

Para la extracción de ARN, se realizó utilizando el método de CTAB (Chang et al. 1993), para ello se utilizaron hojas juveniles, intermedias y adultas de *E. globulus*. La integridad se evaluó mediante gel de agarosa 1% y la cuantificación se realizó mediante Qubit (Invitrogen).

El ARN resultante se trató con DNasa 1 (Fermentas Kit) y fue retrotranscrito utilizando Revertir™ H Minus Kit (Fermentas). La reacción se realizó usando las condiciones indicadas por el proveedor, utilizando para el proceso primers hexaméricos de secuencias aleatorias. La reacción se llevó a cabo en un termociclador T100 BIO-BAD® con un ciclo de 25°C por 10 minutos, 37°C por 120 minutos y 85°C durante 5 segundos.

3.3.3. Diseño de partidores.

Los partidores para qRT-PCR fueron diseñados en base a secuencias almacenadas en la base de datos Phytozome para *Eucalyptus grandis* W.Hill, utilizando las regiones conservadas de cada

gen para el diseño de los partidores, éstos fueron elaborados mediante la aplicación Primer 3 (disponible en el sitio <http://frodo.wi.mit.edu/primer3/>), para los genes Actina, EF1a, Hexokinasa y DELLA. Los partidores para miR156, SPL3 y SPL9, fueron utilizados los descritos en literatura (Hudson et al. 2016).

3.3.4. Cuantificación de transcritos por qRT-PCR.

El mix de reacción comprendió de 5µL de ADNc (20ng), 2,5µL de cada partidor (5mM) y 10µL de “SYBR Green PCR Master Mix” (Applied Biosystem). Se utilizó un equipo de detección para qRT-PCR Rotor-Gene Q (QIAGEN). Todas las reacciones de qRT-PCR se llevaron a cabo con el siguiente programa: 10min a 95°C, 40 ciclos de 15s a 95°C y 1min a 60°C. Se utilizaron partidores correspondientes a los genes housekeeping factor de elongación 1α (EF1α), actina (ACT) y los de los genes en estudio miR156, Hexokinasa 1, DELLA, SPL3 y SPL9, para la evaluación del nivel de expresión.

3.3.5 Identificación de genes candidatos y variaciones en su perfil de metilación.

Para la investigación de los mecanismos moleculares que regulan el cambio de fase en *E. globulus*, se utilizaron datos previos de una biblioteca de alta resolución de hipermetilación de *E. globulus* (Hasbún et al. 2016). A partir de ella se identificaron regiones diferencialmente metiladas (DMRs), asociadas a los perfiles de metilación de hojas juvenil y adulta de *E. globulus*, estas DMRs se asociaron con los genes de las vías descritas para la regulación cambio fase vegetativa, lo cual nos permitió generar una ruta, que correlacionó los potenciales genes candidatos regulados por mecanismos epigenéticos. Se evaluó para cada candidato los cambios en la metilación en el cuerpo del gen y su promotor putativo (2000 pb corriente arriba del inicio transcripción).

3.4. Resultados

3.4.1. Identificación de genes candidatos y variaciones en su perfil de metilación.

Se generó una ruta con los potenciales genes candidatos, regulados por mecanismos epigenéticos hexokinasa, DELLA, SPL3 y SPL9 (Fig. 2). Se evaluó para cada candidato los cambios en la metilación en el cuerpo del gen y su promotor putativo, se identificaron cambios en los perfiles de metilación en la zonas promotoras para los genes Hexokinasa, DELLA (Fig. 3), SPL3 y SPL9, en la zona 5 UTR y primer exón para SPL9, de igual forma se observaron variaciones en los niveles de metilación en el cuerpo del gen, lo cual queda de manifiesto para los genes hexokinasa, DELLA, SPL3 y SPL9. No se identificaron variaciones en el perfil de metilación, para el gen del miR156 evaluado.

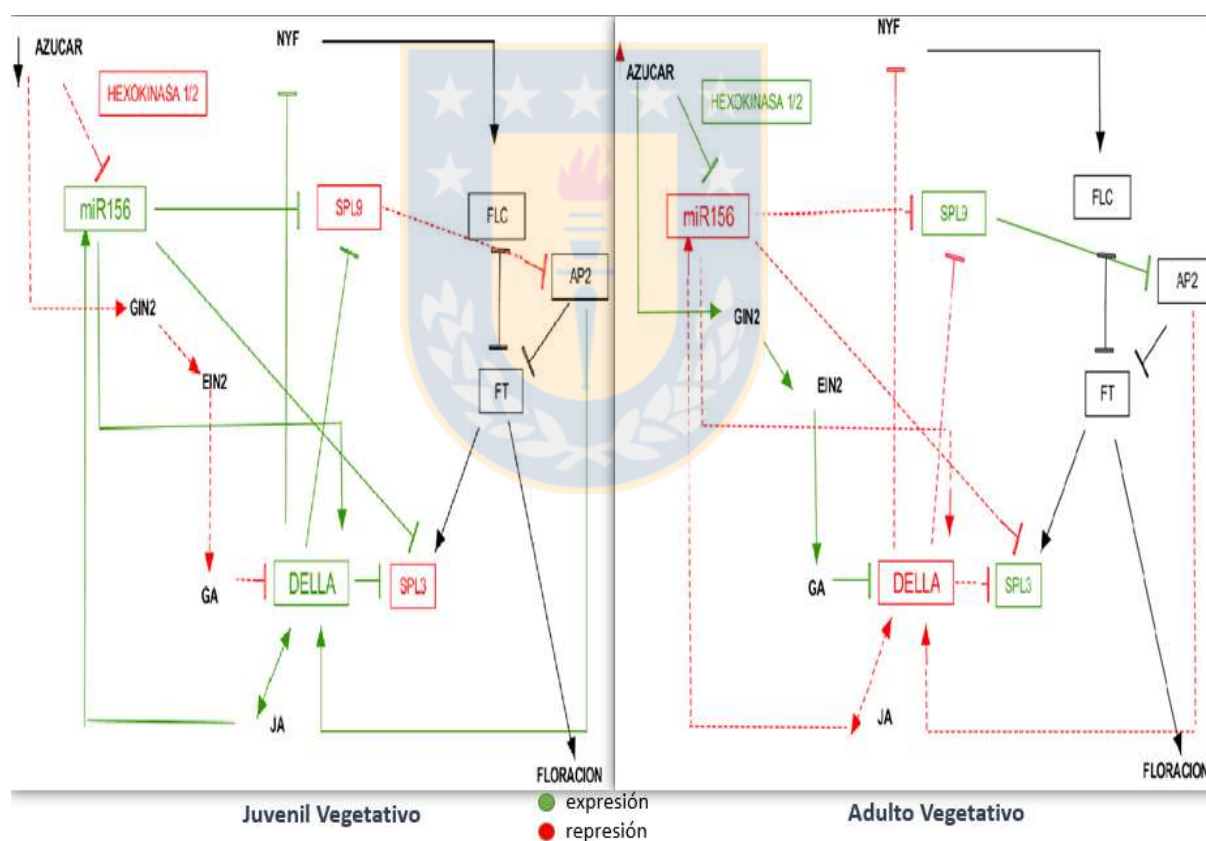


Figura 2. Ilustración de genes seleccionados, asociados al cambio del fase estadio juvenil vegetativo, genes seleccionados miR156, Hexokinasa, DELLA, SPL3 y SPL9 (adaptación de Huijser and Schmid 2011, Poethig, 2014, Wang and Wang 2015, Teotia and Tang 2015).

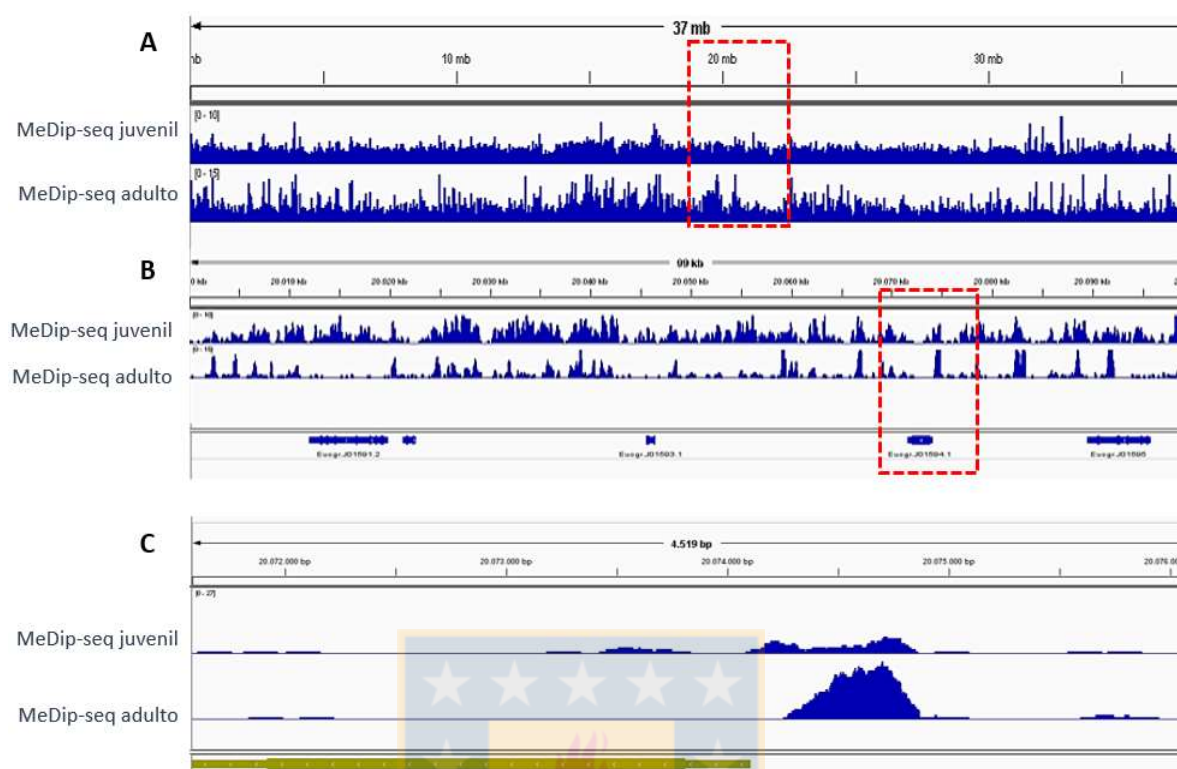


Figura 3. A. Vista del nivel metilación entre los tejidos adulto y juvenil (librería MeDIP-seq) del cromosoma 10, B identificación del gen DELLA, C perfil de metilación de la zona promotora y cuerpo del gen de DELLA.

3.4.2. Expresión relativa de miR156, DELLA, Hexokinasa, SPL3 y SPL9 en relación con estadio desarrollo foliar en *E. globulus*.

Se identificó al miR156 altamente expresado en tejido juvenil, mientras que en tejido de desarrollo intermedio y adulto fue prácticamente indetectable. Para determinar si la variación en la expresión miR156 es funcionalmente importante, evaluamos SPL3 y SPL9, en consonancia con la abundancia relativa de miR156 presente en los tejidos, encontrando una correlación entre el cambio en fase vegetativa y el nivel de transcrito de estos genes, siendo mayor en los estadios adultos, donde miR156 disminuye. De igual manera evaluamos los perfiles de expresión de hexokinasa, la cual está asociada al control de miR156, mediante su inhibición, los datos muestran altos niveles del transcrito en estadio adulto y menores niveles en

los estadios intermedio y juvenil. Además se evaluó el perfil del gen DELLA, asociada al control de genes SPL3 y SPL9, los resultados de expresión confirman mayores niveles de expresión de DELLA en tejidos juveniles, en comparación con los otros estadios de desarrollo (Fig. 4).

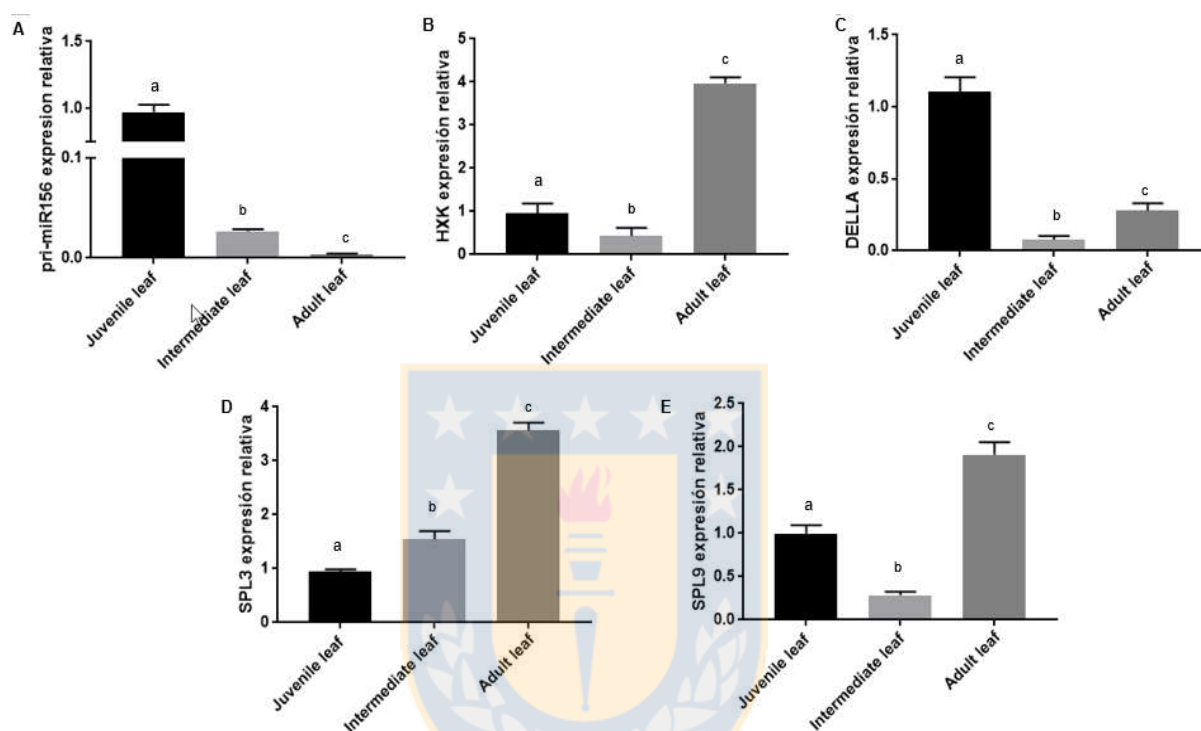


Figura 4. Expresión de miR156, hexokinasa, DELLA, SPL3 y SPL9, en hojas juveniles (J), intermedias (I) y adultas (A). La expresión se normalizó con EF1a. Se muestran los promedios de tres repeticiones técnicas y tres árboles para cada tejido. $p < 0,05$.

3.5. Discusión

Nuestros resultados confirman para *E. globulus*, una disminución de miR156 durante el desarrollo foliar, el cual ha sido identificado, como regulador del desarrollo vegetativo en especies como *A. thaliana* (Wu *et al.* 2009), *Zea mays* L (Chuck *et al.* 2007), *Physcomitrella paten* (Cho *et al.* 2012)] y *Nicotiana tabacum* L.(Feng *et al.* 2016; Zhang *et al.* 2015). Ésto está

relacionado con un aumento en los niveles de transcrito durante el desarrollo, de sus genes asociados a esta modulación SPL3 y SPL9, los cuales están directamente relacionados con el cambio de fase (Xu *et al.* 2016; Preston and Hileman 2013). Consideramos que la presencia de transcrito de los genes SPLs en el estadio juvenil, como evidencia indirecta de que miR156 regula la expresión de estos, principalmente a través de su efecto sobre la traducción, por sobre su efecto sobre la transcripción. Esto ya ha sido sugerido en otros estudios en *A. thaliana*, en donde se ha propuesto que el miR156 no solo actuaría a nivel transcripcional sino también a nivel de represión de traducción (Xu *et al.* 2016; Gandikota *et al.* 2007; Lei *et al.* 2016).

La presencia detectada de transcrito en estadio juveniles de SPL3, sería necesaria para un desarrollo adecuado de la hoja (Usami *et al.* 2009; Wu and Poethig 2006) y posteriormente el aumento del transcrito en estadio adultos, estaría asociado a un desarrollo vegetativo adecuado (Gandikota *et al.* 2007). Para SPL9 el transcrito detectado en estadio juvenil, se asociaría a su función en la forma de la hoja (Usami *et al.* 2009) y su aumento en estadios adulto, se vincula a su actividad en la fase reproductiva promoviendo la inducción floral (Xu *et al.* 2016).

Los altos niveles de transcrito para Hexokinasa (HK) detectados en tejido adulto, se conecta con un aumento de los azúcares, producto de una mayor eficiencia en la actividad fotosintética en hojas adultas en *E. globulus* (Velikova *et al.* 2007). Los azúcares poseen funciones de señalización en las plantas importantes para el desarrollo (Yang *et al.* 2013). Esta relación entre los azúcares y desarrollo, sería a través de la vía de señalización HK1, la cual regularía la transición de la fase juvenil a adulta (Poethig 2013), mediante señales a larga distancia que reprimen la expresión de miR156 (Zotz *et al.* 2011), a nivel transcripcional y post transcripcional, a través de la degradación de éste (Yu *et al.* 2013).

Al evaluar la expresión de la proteína DELLA, se detectaron altos niveles en estadio juvenil, lo cual se asocia a lo descrito en otras investigaciones, donde se ha propuesto que DELLA puede interactuar directamente con los factores de transcripción SPLs secuestrándolos, lo que ayudaría en la regulación del crecimiento y el inicio del cambio de fase vegetativa (Evans and Poethig

1995; Yu *et al.* 2012). Dicho mecanismo estaría actuando en forma paralela a la regulación miR156-SPLs (Beydler 2014).

Al correlacionar los niveles de transcrito de los diferentes genes evaluados en este estudio, con los diferentes patrones de metilación de los tejidos juvenil y adulto, se encontró una clara relación entre el estado de metilación de la zona promotora de tres de los genes evaluados y su nivel expresión, el rol de la metilación en la zona promotora ya ha sido descrito en numerosos estudios, como un mecanismo epigenético clave en la represión de la transcripción de diversos genes (Newell *et al.* 2000; Suzuki and Bird 2008; Chicoine *et al.* 2002). Esto se vió reflejado en un mayor grado de metilación en la zona promotora en el tejido juvenil para los genes hexokinasa, SPL3 y SPL9 y su asociación con un menor nivel de transcrito para éstos. Para el gen DELLA, no se observó una correlación evidente entre el nivel de metilación visualizado y su nivel de transcrito, por lo tanto deberá ser investigado con mayor profundidad en futuros trabajos. De igual modo se observaron diferencias en los perfiles de metilación de las zonas 5 UTR y primer exón para SPL9, lo cual se conecta con un menor nivel de transcrito de éste, el papel de dicha marca epigenética, se ha asociado a represión de la transcripción génica (Brenet *et al.* 2011).

Por consiguiente estos resultados nos están dando claros indicios de un posible control epigenético sobre la expresión de estos genes (Zilberman *et al.* 2007). Además es de destacar, la existencia de diferencias en los perfiles de metilación en el cuerpo del gen, entre los perfiles juveniles y adultos para los genes hexokinasa, SPL3 y SPL9, para los cuales se observó una mayor metilación en estadio juvenil, lo que se relaciona con un menor nivel de transcrito de dichos genes, lo cual se reafirmaría lo planteado en algunos estudios, donde la pérdida de metilación en el cuerpo de un gen, conduciría a una mejor transcripción (Zilberman *et al.* 2007; Takuno *et al.* 2012).

La evaluación en conjunto de estos genes, no había sido abordada en otros estudios, por ello dilucidar cómo estas vías están interconectadas y cómo los mecanismos epigenéticos posiblemente están regulando cambio de fase vegetativa en *E. globulus*, nos permite una mejor comprensión de éstas, así como nuevas interrogantes, las cuales serán abordadas en futuros estudios.

3.6. Conclusiones

Se logró establecer una clara relación entre los perfiles de metilación detectados en los principales genes asociados al cambio de fase vegetativa y sus niveles de transcrito. Estos resultados preliminares sugieren que el proceso de metilación específica de estos genes, podría ser una característica importante en el control de la heteroblastía en *E. globulus*.

Estos resultados nos proporcionan un marco para futuros estudios sobre la regulación epigenética del cambio de fase vegetativa, con la finalidad de una mejor comprensión sobre el tema.

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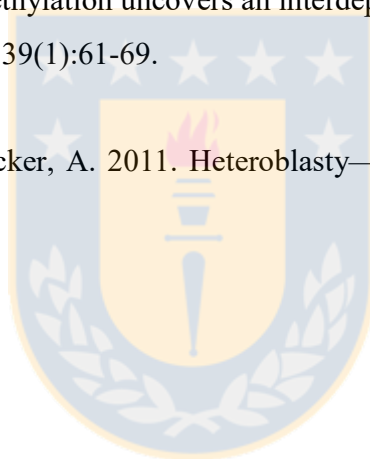
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Capítulo IV

Changes in the expression of miR156 -SPL and HXK1 genes during the ontogenetic leaf transition from juvenile-to-adult in *Eucalyptus globulus* Labill are in part explained by modulations of DNA methylation

Enviada a Frontiers Plant Science

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4.1 Abstract

In plants life cycle, the juvenile-to-adult transition can occur with an abrupt and marked transition called heteroblasty. *E. globulus* is a non-model species with a clear transition in its heteroblastic leaves. To study the importance of conserved and key molecular mechanisms, we selected some of the central gears of the developmental pathway of microRNA156 (miR156), SQUAMOSA promoter binding-protein like (SPL) transcription factors SPL3 and SPL9 and the glucose-sensing enzyme Hexokinase 1 (HXK1). We correlated expression profiles from selected genes with the genome-wide methylation profiles from adult and juvenile leaves of *E. globulus*. Expression of miR156 decreased during transition in contrast to the increase observed for SPL3, SPL9 and HXK1, in accordance with subtle changes on the methylated putative promoter zones of miR156 and the hypermethylation on putative promoter zones from SPL3, SPL9 and HXK1 during transition. We identified a gradual increase in miR156 and SPLs expression on juvenile and adult whole leaves. When leaves were divided into proximal and distal zones, more miR156 and SPL3/SPL9 transcription was observed in distal zones, which might be essential for regulating the temporal expression of genes involved in leaf development. We also demonstrated that the exogenous application of glucose (but not manitol) decreased the abundance of miR156 in *E. globulus* seedlings, without affecting SPLs levels. Our results show key indicators of development, a link between DNA methylation and molecular mechanisms in juvenile-to-adult transition, contributing to the possible improvement of plants micropropagation processes avoiding accelerated ageing.

Keywords: Plant development, epigenetics, hexokinase, glucose, heteroblasty, miR156, SPL.

4.2 Introduction

Plants experience manifold developmental transitions during their lives, from embryonic to post-embryonic, vegetative phase change (growth stage) and reproductive transitions (acquisition of reproductive traits, reproductive stage). Vegetative phase change transition consists in anatomical, morphological, and physiological changes in the different parts of the shoots (Poethig, 1990; Poethig, 2003). During this process, leaves undergo anatomical, morphological, and physiological changes (Poethig, 2013; Proveniers, 2013). When these changes are easily detectable, the transition is called heteroblasty (Zotz et al., 2011), and usually implies drastic changes in the morphology and physiology of the consecutive units composed of internodes and leaves (Zotz et al., 2011). This is consequence of the integration of signals between the ontogenetic program of the plant and the environment (availability of light, water or nutrients), which lead to heteroblastic development (Poethig, 2013; Ostria-Gallardo et al., 2015; Forster and Bonser, 2009). Vegetative phase change is the most described type of heteroblastic variation so far (Poethig, 1990; He et al., 2018).

Recent studies have revealed the existence of major regulators of vegetative phase change: microRNA miR156 and its direct targets the SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SBP/SPL) family of transcription factors (Poethig, 2013; Wang et al., 2011; Wu and Poethig, 2006). The interaction between miR156 and SPL factors was identified as a key event of the aging pathway clock that underlies the plants vegetative transition, where miR156 regulates vegetative phase change transition by directly repressing SPLs expression (Schwab et al., 2006). SPL genes are differentially expressed in juvenile and adult leaves in herbaceous (Xie et al., 2006; Zhang et al., 2011; Salinas et al., 2012) and woody plants (Wang et al., 2011). It has been shown the pivotal role of miR156 in the maintenance of the juvenile vegetative phase and the role of its decrease, promoting early flowering (Wu and Poethig, 2006; Wu et al., 2009; Zhang et al., 2011; Chuck et al., 2011). Among SPL gene family only two of them are related to leaf morphology. SPL3, implied in the appearance of adult foliar characteristics and also related to floral induction (Wu and Poethig, 2006; Kim and Sung, 2012; Yamaguchi et al., 2009; Teotia and Tang, 2015), and SPL9 which promotes the development of adult leaf morphology, coordinates development, and mediates in stress tolerance (Grotewold

et al., 2015; Cui et al., 2014; Schwarz et al., 2008). This system is regulated by sugar availability and is mediated by hexokinase 1 (HXK1). HXK1 catalyzes glucose phosphorylation to yield glucose-6-phosphate, during the first step of glycolysis. Indeed, HXK1 has dual roles in glucose homeostasis because also functions as a sugar sensor, and can regulate gene transcription in response to changes in glucose concentration (Moore et al., 2003; Rolland et al., 2006). Working with mutated forms of HXK1 revealed that the signaling function of HXK1 is independent of its enzymatic activity because the lacking of the glucose phosphorylation capacity are still able to mediate glucose signaling (Moore et al., 2003). It has been shown that HXK1 promotes vegetative phase change by repressing the accumulation of miR156 and its effect can be attributed to a decrease in the expression of miR156 genes in *Arabidopsis* (Yang et al., 2013), consequently increasing the expression of SPLs transcription factors (Schwab et al., 2005; Wu et al., 2009).

Silencing of miR156, essential for phase shift control, is mediated by epigenetic mechanisms through trimethylation of histone H3 lysine 27 (H3K27me3), associated with gene repression catalyzed by the chromatin remodeler CHD3 PICKLE (PKL) (Köhler et al., 2012; Kim and Sung, 2012; Xu et al., 2015). The increase in H3K27me3 is correlated with a temporary decrease in the acetylation of H3K27, fact which could also contribute to the reduction of miR156 expression levels (Xu et al., 2015). On the other hand, the transcription of miR156 is promoted by the nucleosomal remodeler BRAHMA (Xu et al., 2016a) and by the SWR1 complex, exchanging the histone variant H2A.Z by H2A (Choi et al., 2016), which would indicate that H2A.Z promotes juvenile vegetative identity, also H2A.Z would promote the expression of miR156A/ miR156C to facilitate the deposition of H3K4me3 (Xu et al., 2018).

Despite other herbaceous and woody species also reported significant differences of miR156 expression levels between juvenile and adult leaves, the epigenetic mechanisms regulating this expression levels (described above) have only been described in *Arabidopsis* (Wang et al., 2011). This characterization can be considered a first step towards the full understanding of leaf transition in complex plant systems such as trees. The analysis of the epigenetics of forest tree species such as *E. globulus* will significantly improve our understanding of the mechanisms that influence development and adaptation to the environment.

Among woody species, *Eucalyptus globulus* is an excellent model of the module juvenile-to-adult transition since it allows to predict when the heteroblastic transition will occur based on the height of the tree (phase change height). Phase change height also exhibit natural variation (between 2 to 5 m) depending on the different native populations (Jordan et al., 2000), which is also correlated to an increase of the diameter of individuals (Hamilton et al., 2011). The fact that the different populations showed different phase change height, pointed to a genetic base of this mechanisms. Interestingly, a QTL marker which explains 62.8% of phenotypic variation associated with this transition (Hudson et al., 2012) is close to the miR156 loci (Hudson, 2014). However, despite the description of this strong association and the recent description of the differential methylomes of juvenile and adult leaves of *E. globulus* (Hasbun et al, 2016), the epigenetic mechanisms involved in the heteroblasty regulation in this non-model species are still unknown.

To fill this gap, our investigation prompt to establish a relationship between the miR156 and related genes (SPL3, SPL9, HXK1) expression levels with their methylation profiles during the heteroblastic transition from juvenile to adult leaves in *E. globulus*. Moreover, we studied the expression of these genes at different concentrations of glucose to investigate the potential regulatory role of sugars signaling, and its relations with DNA methylation during the ontogenetic processes.

4.3 Results

Changes in DNA methylation are correlated with the miR156-SPLs module regulation of vegetative phase change in *E. globulus*.

The presence of hyper- and hypomethylated zones around the putative promoter regions and gene bodies of miRNA156, SPL3, SPL9, and HXK1 was discovered analyzing the *E. globulus* methylome performed by our group (Hasbún et al, 2016). In the case of miR156 gene, subtle differences between juvenile and adult leaves were detected. On the other hand, SPL3 and SPL9, shows differential patterns. Juvenile leaves had higher basal methylation than adult leaves, and

in particular, SPL9 shows a differentially-methylated region about 1Kb downstream. Similar, but less prominent pattern changes were observed at the HXK-1 locus (Figure 1).

Relative expression of miR156 in juvenile / adults leafs and shoot apex of *E. globulus*.

The miR156 transcript levels in distal and proximal area of juvenile and adult leaves of *E. globulus* was quantified by RT-qPCR. In juvenile leaves, the miR156 transcript levels were significantly higher than in adult leaves (Figure 2A), and the transcripts were concentrated at the distal zone of leaves. In regard of these results, the abundance of miR156 was also analyzed in the shoot apex of juvenile and transitional trees, showing the first significantly higher miR156 transcripts levels (Figure 2D).

Relative expression of SPL3 and SPL9 in juvenile and adults leaves of *E. globulus*.

To determine if the variation in the miR156 expression is functionally relevant, the abundance of SPL3 and SPL9 genes transcript was studied in the distal and proximal area of leaves from juvenile and adult trees. Unlike the expression pattern of miR156, the abundance of SPL3 and SPL9, was significantly higher in adult leaves (Figure 2B/C). In all cases, the level of transcripts at the distal tissue of the leaves was significantly higher than in the proximal area and the differential was wider in adult leaves (Figure 2B, C).

Sugar signal involvement in the regulation of miR156/SPLs module via HXK during vegetative phase change in *E. globulus*.

Regarding the HXK transcripts levels, neither juvenile nor adult leaves showed differences between distal and proximal area, but the levels of transcripts were significantly higher in adult leaves, in accordance with miR156 pattern described in the Figure 3A.

To get insights about the participation of sugar signal and sensors in the regulation of the miR156/SPLs-mediated vegetative phase change, we assessed miR156, SPL3, SPL9 and HXK-1 transcript levels under different Glucose concentrations (Fig 4). Transcript levels of SPL3, SPL9, and HXK-1 remain unchanged (Figure 4 B/C/D). Nevertheless, the abundance of miR156 decreases in presence of glucose, showing a dose-dependent response (Figure 4A). Interestingly, this response is suppressed when glucose was replaced by manitol (S2 complementary).

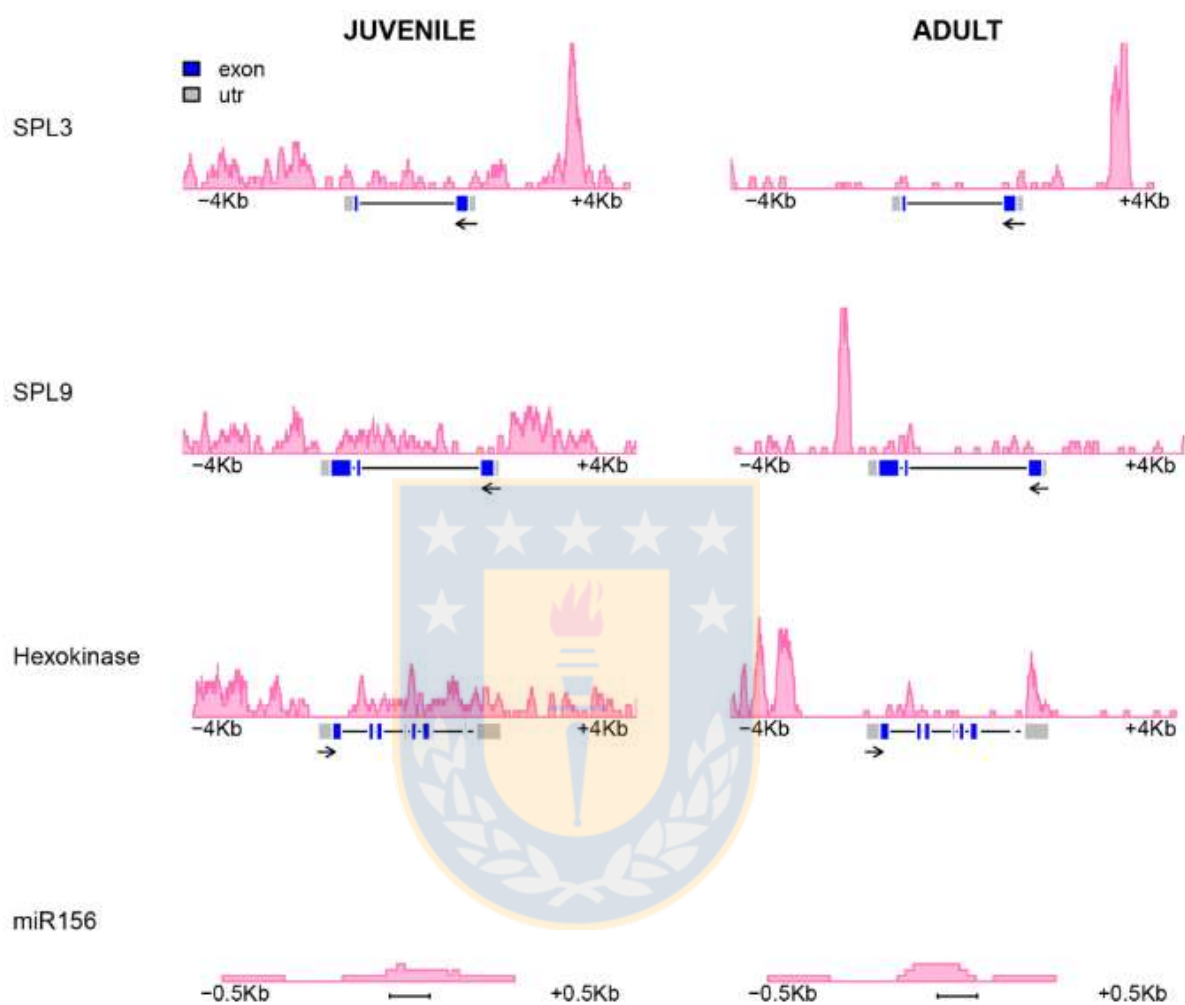


Figure 1. Methylation pattern at vegetative phase control genes. Normalized signal plots (pink, MEDIP) for adult (left) and juvenile (right) leaves. Arrows indicate direction of transcription.

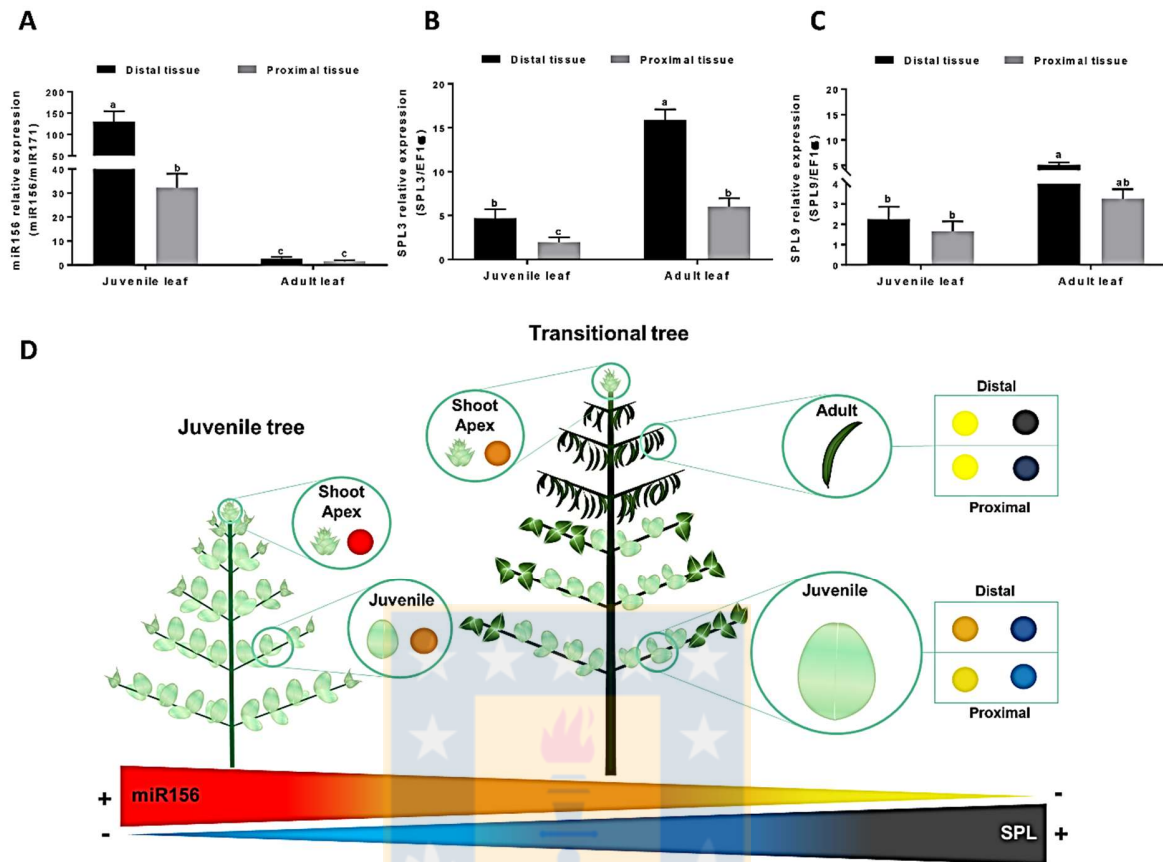


Figure 2. A. Expression of miR156 in juvenile and adult leaves of *E. globulus*, distal zone and proximal zone. The expression was normalized with miR171a. B. expression of SPL3 in juvenile and adult leaves of *E. globulus*, distal zone and proximal zone. The expression was normalized with EF1a and actin. C. expression of SPL3 in juvenile and adult leaves of *E. globulus*, distal zone and proximal zone. The expression was normalized with EF1a and actin. Averages of three replicates and three trees are shown for each tissue, two-way ANOVA. $p < 0.05$. D. Expression of miR156 in tissue shoot apex. Individuals showing vegetative phase change and individuals control without phase change of *E. globulus*. One-way ANOVA, $p < 0.05$ ($n=3$).

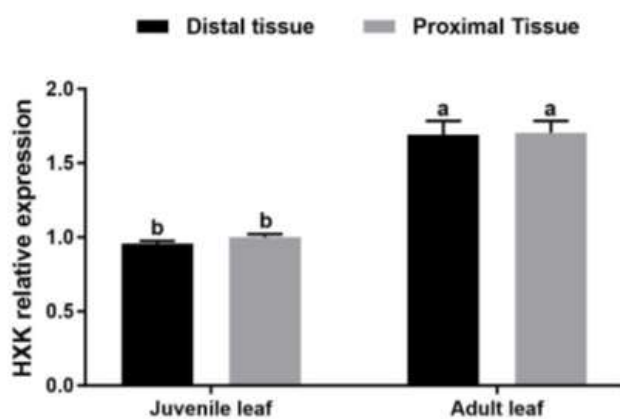
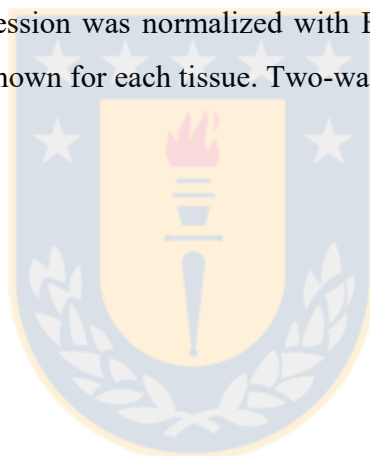


Figure 3. Expression of Hexokinase1 in juvenile and adult leaves of *E. globulus*, distal zone and proximal zone. The expression was normalized with EF1a and actin. Averages of three replicates and three trees are shown for each tissue. Two-way ANOVA, $p < 0.05$ ($n=3$).



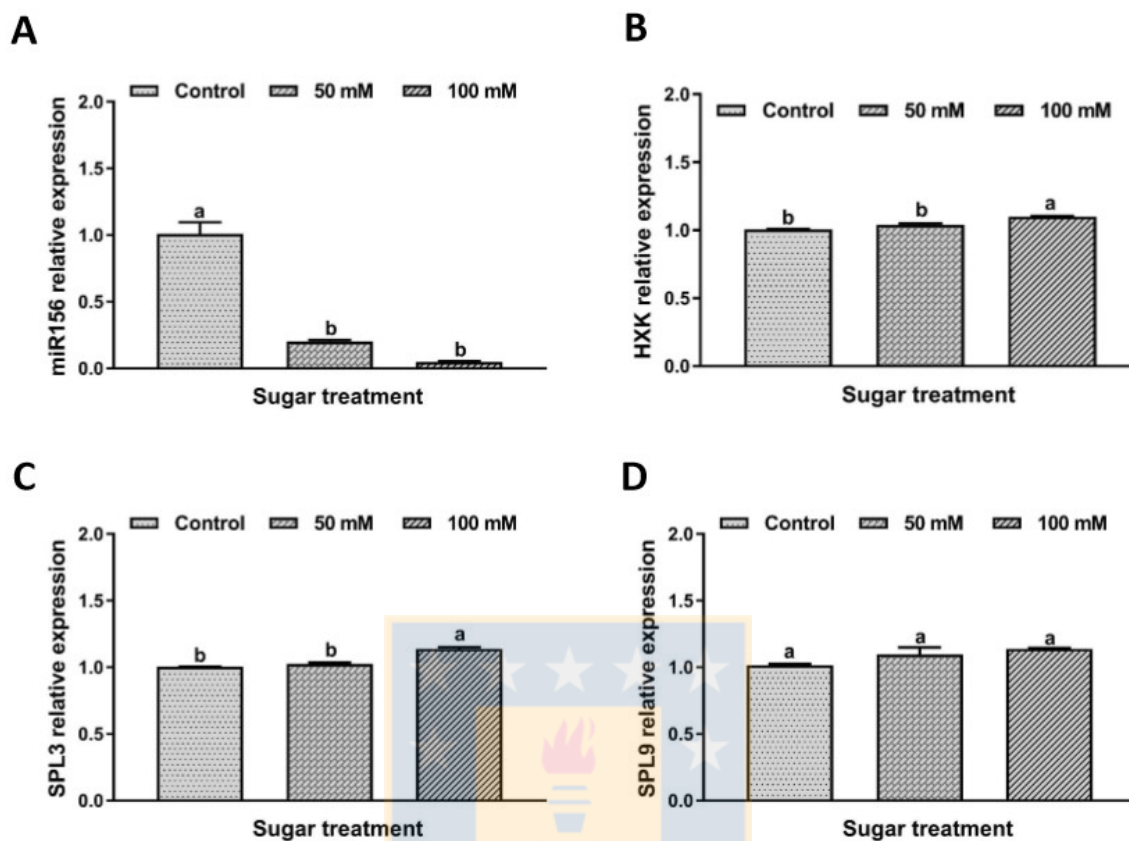


Figure 4. A. Expression of miR156 in seedlings of 31 days of *E. globulus*, added 50mM of glucose, 100mM of glucose and C control. The expression was normalized with miR171a. **B** /**C**/**D**. Expression of hexokinase 1, SPL9 and SPL3 in *E. globulus*, 50mM of glucose, 100mM of glucose and C control. The expression was normalized with EF1a and actin. Averages of three technical repetitions and three independent trials for each tissue are shown. One-way ANOVA, $p < 0.05$ ($n=3$).

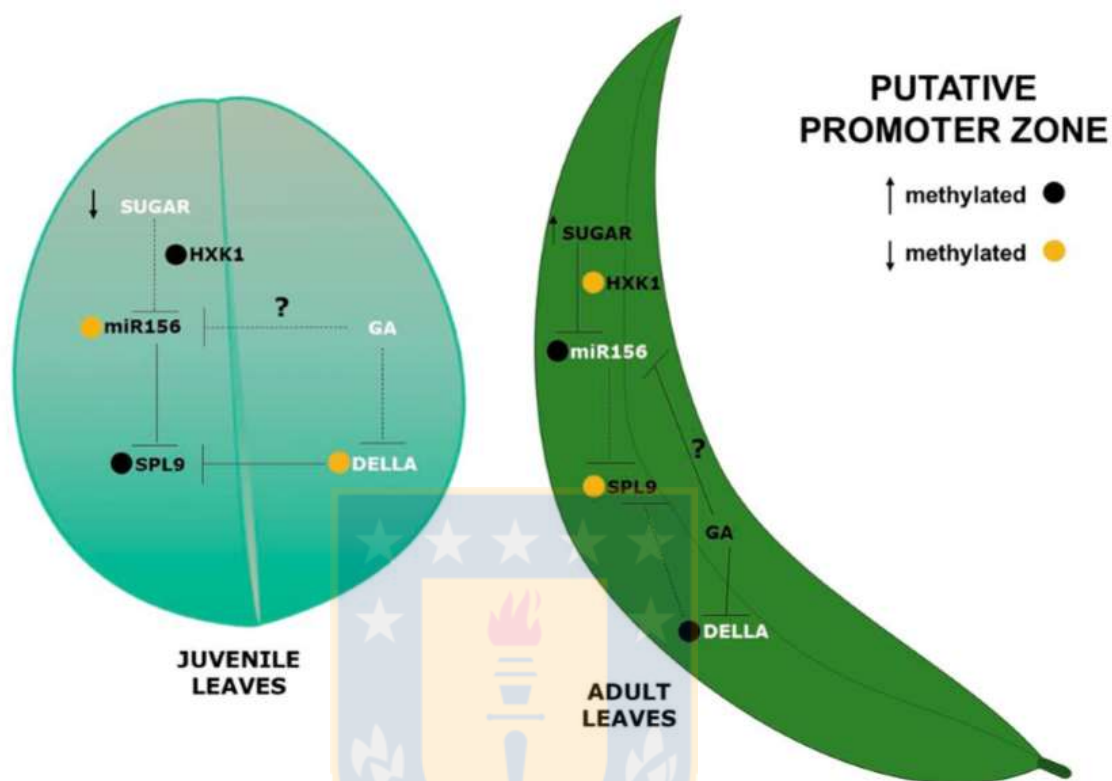


Figure 5. Schematic representation of the developmental pathway in juvenile and adult leaves in *E. globulus*. Active genes or molecules are represented in white and inactive genes or molecules are represented in black. The circles represent methylated zones, the orange circles show hypomethylated zones and black circles show hypermethylated zones. The white genes represent active genes or molecules, and black represent inactive genes or molecules.

4.4 Discussion

Changes in DNA methylation are correlated with the miR156-SPLs module juvenile-to-adult transition in *Eucalyptus globulus* Labill.

Recent studies have revealed that epigenetic mechanisms play important roles in regulating the expression of some components in the developmental pathway during juvenile-to-adult transition in plants (Xu et al., 2018). To date, the only known epigenetic regulators associated with the miR156/SPLs module are involved in mechanisms that have been associated with the control of phase change. In *Arabidopsis*, silencing mediated by the trimethylation of lysine 27 of histone H3 (H3K27me3) has been identified as regulator of several miR156 genes (Lafos et al., 2011; Picó et al., 2015). This has also been correlated with a temporary decrease of the acetylation on the lysine 27 in histone H3 (H3K27), which could contribute to the decrease in expression from some of the miR156 family members such as miR156a and miR156c during vegetative phase change (Xu et al., 2015). However, there are no reports about DNA methylation of the genes involved in the miR156/SPLs module during juvenile-to-adult transition. Nevertheless, we explored for difference in the methylation profile in a previously constructed methylome (Hasbún et al., 2016). Subtle differences in the DNA methylation profiles in the putative promoter zone between juvenile and adult leaves for the miR156 gene were identified. On the other hand SPL3 and SPL9 shows marked differences between the DNA methylation profiles between adult and juvenile leaves. The same trend was observed in HXK-1 gene showing a clear DNA methylation pattern. These genes showed specific DNA methylation patterns, with a low abundance of this mark in the promoter and increased in the ORF. This methylation pattern was previously associated to gene expression in *Arabidopsis*, soybean and rice (Zemach et al., 2010; An et al., 2017; Bartels et al., 2018).

The *in silico* analysis of the putative promoter regions of the studied genes identified regulatory elements responding to light, hormones and temperature (Supplementary Table 1), as described previously about HXK1 in *Arabidopsis* (Moore et al., 2003). In the promoter zone of miR156, elements of response to light and gibberellin were recently identified. It has been reported in *Arabidopsis* that darkness induces a rapid accumulation of phytochrome-interacting factors

(ISF) proteins, which bind to the promoter region of miR156, inhibiting its expression, thus promoting the elevation in the expression of SPL3 (Xie et al., 2017). At the level of regulation in the expression of the genes of the miR156-SPL module, the detection of variations in methylation levels between juvenile and adult tissues, in areas where regulatory elements were identified. It would be indicating that said epigenetic mechanism would allow the integration of signaling networks of nutrients, light and hormones, which would control the vegetative phase change in response to the changing environment.

Relative expression of miR156 in juvenile and adults leaves of *E. globulus*.

There is a growing amount of detailed information exhibiting that miR156 plays a repressive role in phase transition conserved throughout plant species. In model and non-model plants it has been shown that the miR156 level is higher in juvenile phase and significantly decreased in adult phase, this suggesting that miR156 regulation could act as a master switch of juvenile-to-adult transition (Wang and Wang et al., 2015). Our results demonstrate a decrease of miR156a during juvenile-to-adult transition in *E. globulus*, consistent with previous reports, in which the levels of miR156 have been assessed by analysis of RNA blot in juvenile and adult tissue (Wang et al., 2011) and by RT-qPCR analysis in which the abundance of the precursor EglMIR156.5 was assessed in juvenile tissue (Hudson et al., 2014).

The established accumulation gradient of miR156 transcript between distal and proximal areas of juvenile leaves that was found could be establishing a regulation of the transcription factors SPL3, agrees with the recent description of a slight proximal-distal gradient of SPL3 in adult tissues of *Arabidopsis* (Nguyen et al., 2017). The regulation of the vegetative phase change could be coordinated by the transition from cell proliferation to cell expansion and differentiation, which as a whole has been proposed to drive changes in development time (Buendía-Monreal and Gillmor 2018), could explain these differences observed in the proximal distal axis.

Moreover, high levels of miR156 transcript were detected at juvenile stage (trees without juvenile-to-adult transition), in contrast to the shoot apex tissues from individuals in transition (with transitional traits from vegetative phase change). In *Arabidopsis*, miR156 transcript are

enriched at the apex of the outbreak, and decreases when the plant matures, which promotes the development transition to the adult stage (Wu and Poethig, 2006, Chuck et al., 2007, Wu et al., 2009).

Furthermore, the progressive reduction of miR156 in *Arabidopsis* leaves developing successively on the shoot confers the gradual transition from juvenile-to-adult phase (Hyun et al., 2017). Leaf ablation increases the levels of miR156 delaying the juvenile-to-adult transition, suggesting that an unknown mobile signal derived from the leaves could be responsible for the repression of the transcription of miR156 in the meristematic tissues (Yang et al., 2011). This could be supported with our findings in the tip from juvenile leaves with marked, abrupt and higher levels of miR156 than in the base, who could also be suggesting an apparent connection between shoot apical meristem and the tip from leaves in juvenile phase. As the meristematic tissue is crucial in foliar identity signal, it is involved in the maintenance of a stem cell population and in the formation of a predictable regular pattern of leaves, from the periphery of the meristems (Golz et al., 2006). Results shown in this work are coherent with previous investigations about the abundance of miR156 and its declining in the timing process during juvenile-to-adult transition.

Relative expression levels of SPL3 and SPL9 are associated to juvenile-to-adult transition in *E. globulus* leaves.

During leaf juvenile-to-adult transition, increased in SPLs transcript abundance levels in adult tissues were detected. Similar phenomenon was already reported by Hudson et al. (2014), being directly related to the development of foliar traits experienced in the juvenile-to-adult transition (Preston et al., 2013; Xu et al., 2016b; Usami et al., 2009). For both genes adult tissues showed a 4-fold increase in its expression levels in comparison to the juvenile leaves. This can be associated to a reduction of the expression of SPL3 and SPL9 consequence of increased abundance of miR156, as previously described in *Populus* hybrids (Wang et al., 2011). SPL3 overexpression would accelerate the characteristic traits of adult leaves in collaboration with SPL9 promoting most of the traits associated with the adult phase (Wu and Poethig, 2006).

The abundance of transcribed genes SPL3 and SPL9 at the tip and base area of juvenile and adult leaves demonstrated the functional relevance of miR156. A higher expression of this miRNA was detected in the distal area, which could be establishing a regulation on the target transcription factors SPL3 and SPL9, being this regulation not present at distal area, where the abundance of SPL3 and SPL9 peaked in adult leaves. This gradient of miR156 could be establishing a spatial expression contributing to the space-temporal expression of SPLs during foliar development.

Sugar signaled regulation of miR156 expression in seedlings of *E. globulus*.

Glucose might have a key role modulating developmental switches and the glucose-sensing enzyme HXK1 seems to be on the eye of the storm, especially during early stages of development. HXK1 experienced an up-regulation during the transition between juvenile and adult phase, reflecting differences in abundance of glucose, acting as signaling mechanisms during leaf maturation. To test the importance of glucose-based signaling during juvenile-to-adult transition, seedlings of *E. globulus* were grown under different glucose concentrations and expression levels of HXK1, miR156, SPL3, SPL9 were determined. Results showed that HXK1, SPL3 and SPL9 expression remained unaffected, while miR156 was silenced displaying a dose-dependent response. This effect seems to be glucose specific, since the replacement of this sugar by manitol did not have effect over the abundance of miR156 (Supplementary Figure 1) as it was previously proposed (Wingler, 2018). Moreover, this irregular behavior of miR156 levels of expression has been also shown in studies of *Prunus* sp. plants rejuvenated and propagated *in vitro*, which suggested that tissue culture alters the aging pathway regulation (Bastías et al., 2016), indicating that the glucose present in the propagation media could accelerate the stages of development by affecting the levels of miR156.

HXK1 directly promotes the transcription of miR156 genes (Yang et al., 2013), but the reason why glucose levels did not affected the transcript levels of HXK1 remains unclear. A possible explanation is that HXK1 expression is only affected by strong changes in sugar concentration (Moore et al., 2003), having a consequences in multiple biological functions such as photosynthesis, absorption of minerals (Granot et al., 2013), closure of stomata (Kelly et al.,

2013) or senescence (Wingler et al., 2012). Another factor to be considered is the plant capacity to orchestrate quickly and effectively the transcriptional state of genes needed to respond to low concentrations of sugar (Kunz et al., 2014). This fact would explain the absence of variation on the levels of SPLs and HXK1 at different concentrations of glucose. These findings and the experimental observations shown above support the idea of an specific signaling pathway related to nutrition performance is acting (Moore et al., 2003).

Interestingly transcript levels of SPL3 and SPL9 were not affected by glucose concentration and miR156 abundance, pointing to a second regulatory layer. DELLA (Figure complementary S1) transcription factors were proposed to be directly involved in SPLs kidnapping, which attenuates the transcription activities of the SPLs (Evans and Poethig, 1995; Yu et al., 2012; Yamaguchi et al., 2014), helping in the regulation of the vegetative phase change in parallel with miR156-SPLs system (Beydler, 2014).

4.5 Conclusion

Juvenile to adult transition in *E. globulus* courses along with a miR156 decrease coincident with an increase of the expression of SPL3, SPL9 and HXK1. The expression levels of SPL3 and SPL9 seemed to be regulated by DNA methylation, with the highest methylation found in the promoter zones of adult leaves, demonstrating its repressive effect. Similar pattern was shown by HXK-1. When evaluating miR156, juvenile levels had higher transcript levels than adults. In both tissues, distal parts of the leaf showed higher levels than proximal, in an opposite way than SPL3 and SPL9 genes. The distal zone apparently could be transporting key signals through the phloem to the whole plant, to control the transition from juvenile to adult in *E. globulus* and other plants. Acquisition of photosynthetic competence may be regulating juvenile to adult transition, since glucose signaling regulation in early stages of the development of *E. globulus* was demonstrated by the drastic decrease of miR156 after the treatment of seedlings with high concentrations of glucose. Interestingly, this treatment did not affect other studied genes, suggesting the existence of new regulatory pathways to be discovered. Summing up our results

revealed useful epigenetic and molecular indicators in the miR156-SPL developmental pathway showing the role of the main participants in the juvenile-to-adult transition orchestra.

4.6 Methods

Experimental Design

Growing conditions and Plant material

For the understand the role of key genes (miR156, HXK1, SPL3 and SPL9) involved in the transition of juvenile to adult stage, both juvenile (without adults leaves) and transitional (with juvenile and adult leaves) trees were used in this work. Trees of three years old were grown under controlled/natural conditions in the city of Concepción in the Bío-Bío region, Chile. and shoot apex were collected from both juvenile and transitional trees, while juvenile and adult leaves were obtained from transitional trees. All samples were collected from 3 different individuals of *E. globulus* (n=3) at the beginning of autumn, on April 21, 2017 at 2:00 p.m. Samples were frozen in liquid nitrogen and stored at -80 ° C for posterior analysis.

Glucose experiment

For the evaluation of the effect of glucose on transcript levels of miR156, HXK1, SPL3 and SPL9, 28 days old seedlings of *E. globulus* were subjected to different concentrations of glucose: Controls (without glucose) 50mM and 100mM. Seedlings were grown and kept under long day photoperiod (18:6 day: night) at 22 °C during all the experiment.

After 8 or 12 days of glucose treatment, seedlings were maintained in darkness for 3 days before the RNA extraction (Yu et al., 2013). Three individuals per treatment were sampled.

Total RNA extraction

Leaves were first separated into distal and proximal zones, then ground to powder in liquid nitrogen, and total RNA was extracted using the RNeasy mini kit (QIAGEN) according to

manufacturer's instructions. RNA integrity was evaluated in 1% agarose gels, and concentration was determined by fluorometric assays employing Qubit (Invitrogen).

cDNA synthesis and RT-qPCR transcript quantification.

miRNAs were retrotranscribed employing stem-loop pulsed reverse transcription as described by Varkonyi-Gasic *et al.* 2007 starting from total RNA solution 40 ng/ μ L.

In the case of the hexokinase 1, SPL3, SPL9, elongation factor 1 α (EF1 α) and actin (ACT), hexameric primers were used and DNase I (Fermentas kit) was used and retrotranscribed with Revert H Minus kit (Fermentas). Elongation factor 1 α (EF1 α) and actine (ACT) were used as housekeeping genes. miR156 expression was normalized employing miR171a.

The qPCR was prepared with 5X HOT FIREPol EvaGreen qPCR Mix Plus (ROX) Solis Biodine according to the supplier's instructions, with a RT-qPCR Rotor-Gene Q (QIAGEN) detection device. A 10-min program was performed at 95 ° C, 40 cycles of 15 s at 95 ° C and 1 min at 60 ° C.

RT-qPCR primer design.

qRT-PCR primers were designed with sequences extracted from Phytozome database for *Eucalyptus grandis* W.Hill, using each gene conserved regions. Primers were elaborated with Primer 3 tool (available on: <http://frodo.wi.mit.edu/primer3/>), for Actine, EF1a and Hexokinase 1, pre-miR156, SPL3 and SPL9 primers were extracted from literature (Pappas *et al.*, 2011; Hudson *et al.*, 2014).

For the miRNAs primer design, the mature sequences were indexed in the stem-loop primers designer online tool (Castro *et al.*, 2016) (available on: <http://www.plantbiotech.cl/amir319edesigner/stemloop/>). The *Eucalyptus* conserved miRNAs identification by similarity against miRBase sequences described in literature (Pappas *et al.*, 2011), was used as a reference to use mature sequences from miRBase. To identify an endogenous housekeeping miRNA gene on plants leaf tissues with constant expression through development, we used mirEX² online tool (Zielezinski *et al.*, 2015) (available on:

<http://www.combio.pl/mirex2>). The mature sequences of miRNAs were extracted from miRBase. Mature miRNAs sequences from the Sanger Institute miRBase Sequence Database, designed primers for miR156 and the housekeeping gene miR171a.

Table 1 Sequences of the primers used in this study.

miR156a	Mature sequence	UGACAGAAGAGAGUGAGCACA	designed
	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTGTGCT	
	Forward primer	GTATACTGACAGAAGAGAGTG	
	Reverse primer	GTGCAGGGTCCGAGGT	
miR171a	Mature sequence	UGAUUGAGCCGUGCCAAUAUC	designed
	RT-primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGATATT	
	Forward primer	GTATACTGATTGAGCCGTGCC	
	Reverse primer	GTGCAGGGTCCGAGGT	
EglSPL3	Forward primer	GGGCCGCCACTAGGTCCACCA	Hudson <i>et al.</i> 2014
	Reverse primer	TGACAGCTCGTGGAACCTGCTA	
EglSPL9	Forward primer	GATCTGAGCGATGCCAAGGC	Hudson <i>et al.</i> 2014
	Reverse primer	GTGGTGTCTCAAATATAGAGG	
Hexokinase	Forward primer	CGGGAGTAAGCTCAAGATGC	designed
	Reverse primer	ATCAACGCAACAACCACAC	
Actine	Forward primer	AGATGACCCAGATTATGTTTGAGACCTTC	designed
	Reverse primer	ACCATCACCAGAATCCAACACAATACC	
EF1a	Forward primer	GATGATTCCCACCAAGCCCATG	designed
	Reverse primer	CCGGTTGGGTCCCTTCTCTC	

Correlation between candidate genes and their methylation profiles.

To get deep into the molecular regulation of VPC on *E. globulus*, we used previously published hypermethylation high resolution libraries *E. globulus* (Hasbún *et al.*, 2016). This data provided information for the identification of differentiated methylated regions (DMRs) in juvenile and adult leaves from *E. globulus*, these DMRs were correlated with candidate genes involved in VPC, the methylation profiles were evaluated in the gene body and the putative promoter region (2000bp upstream transcription initiation) in order to search for a methylation pattern.

Stability in reference genes

The stability of the candidate reference genes was analyzed using three tools, geNorm from qbase + (version 3.1) of Biogazelle (Vandesompele *et al.*, 2002), NormFinder (version 0.953) (Andersen *et al.*, 2004) and BestKeeper (version 1.0) (Pfaffl *et al.*, 2004). Raw expression data of the qPCR were analyzed according to instruction manuals in distal-proximal gradient of adult and juvenile leaves separately and in adults with juveniles of three individuals to validate the genes not only in proximal distal gradient but also during development. All the tools were used to generate rankings of the most stable genes by assessing stability values or in the case of BestKeeper the standard deviation. In all the tools used, higher values indicate less stable genes and lower values indicate greater stability. Finally, NormFinder was used to search for the best pair of reference genes with their respective stability values. Finally, the values of the means of the C_q were used to support the data generated by the tools used.

Statistical analysis.

The gene expression of the genes evaluated were submitted to the analysis of before ANOVA, the data of normality and homogeneity of variances were verified. The differences between the values ($p < 0.05$) were determined by a two-way ANOVA and ANOVA one-way as corresponded the analysis. All statistical tests were performed with InfoStat version 2017e.

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Capítulo V

Discusión General

Aquí, proporcionamos un marco conceptual integrado de los mecanismos moleculares descritos en la transición de juveniles a adultos y la metilación del ADN, como un componente clave en el desarrollo, de la especie heteroblástica *Eucalyptus globulus* Labill.

Identificamos una disminución de pri-miR156 durante el desarrollo foliar de *E. globulus*, concordante con otros trabajos, en los cuales se ha evaluado los niveles de miR156 mediante análisis de RNA blot en tejido juvenil-adulto (Wang *et al.* 2011) y mediante análisis de RT-qPCR donde se evaluó la abundancia del precursor EglMIR156.5 en hoja juvenil (Hudson *et al.* 2014). El rol de miR156 como controlador del desarrollo vegetativo, ya ha sido reportado en la especie modelo *A. thaliana*, los cuales indican que miR156 es necesario para mantener la fase juvenil, actúa reprimiendo la expresión de genes SPLs (Wu *et al.* 2009), lo que ha sido reportado en otras especies como *Zea mays* L (Chuck *et al.* 2007) y *Nicotiana tabacum* L. (Feng *et al.* 2016; Zhang *et al.* 2015).

Se logró establecer un aumento de los genes blanco de miR156 los SPL3 y SPL9 en tejido adulto en *E.globulus*, lo cual concuerda con lo informado para esta especie, un aumento gradual desde los tejidos juveniles a adultos (Hudson *et al.* 2014). Lo que está directamente relacionados al desarrollo de rasgos foliares que se experimentan en la transición de fase vegetativa (Preston *et al.* 2013; Xu *et al.* 2016; Usami *et al.* 2009). En híbridos de *Populus* se ha demostrado que la sobreexpresión de miR156 reduce la expresión de SPL3 y SPL9 (Wang *et al.* 2011). La sobreexpresión SPL3 acelera los rasgos característicos de las hojas adultas y SPL9 promueve la mayoría de los rasgos asociados con la fase adulta (Wu and Poethig 2006). La presencia detectada de SPL3/9 en estadio juvenil en el presente trabajo, podría deberse al establecimiento del punto de partida para la fase adulta.

Al evaluar HXK1 se identificaron mayores niveles de está en tejidos adultos de *E. globulus*, lo que estaría conectado a un aumento de los azúcares, debido a una mayor eficiencia en la actividad fotosintética en hojas adultas de *E. globulus* (Velikova *et al.* 2007). Existen varias teorías de cómo HXK1 participa en este proceso, la primera es que actuaría como intermediario en la vía reprimiendo la transcripción de MIR156A y MIR156C (Yang *et al.* 2013). También se ha propuesto que los azúcares derivados desde las hojas fotosintéticamente activas, generarían señales de larga distancia que reprimen la expresión de miR156 a nivel de los primordios foliares en *Arabidopsis* (Proveniers 2013).

Los niveles identificados en tejidos completos de DELLA, fueron altos niveles de transcritos en tejido juvenil, lo cual nos permite inferir de acuerdo a lo descrito en literatura donde DELLA podría estar interactuando físicamente con las SPLs y evitando su transcripción (Hyun *et al.* 2016, Yu *et al.* 2012), lo cual estaría ayudando la actividad de miR156 en este tejido.

Los resultados anteriormente expuestos para SPLs, HXK1, DELLA y pri-miR156 correspondientes al capítulo 1, se evaluaron en hojas completas juveniles y adultas, sin encontrar una tendencia clara para los tejidos intermedios. Al considerar los niveles de diferenciación celular que están ocurriendo en los tejidos evaluados, se decidió dividir las hojas en la zona proximal y distal, identificándose un gradiente espacial en el eje proximal-distal de miR156 en las hojas juveniles de *E. globulus*, detectando un mayor número de transcrito en la zona distal. Lo que no ha sido reportado en otros trabajos, dando como indicio una regulación de los factores de transcripción blancos SPL3 y SPL9 que estarían produciéndose en dicha zona del tejido. En tejido adulto se identificó mayor nivel de transcrito en la zona distal para SPL3 y SPL9, lo cual concuerda con lo recientemente reportado en *Arabidopsis* en tejido adulto (Nguyen *et al.* 2017).

Se ha propuesto que el cambio de fase vegetativa, no solo está controlado por las vías genéticas, sino también está asociada al control metabólico, hormonal, azúcares y señales redox (Jia *et al.* 2017), lo cual asociado al control temporal de la proliferación celular, la expansión celular y la diferenciación celular, regularán este proceso (Buendía-Monreal and Gillmor 2018). Por ello se hacen necesarias investigaciones adicionales sobre estas vías.

En la especie modelo *Arabidopsis*, la detención del ciclo celular comienza en la parte distal y se mueve hacia la zona proximal en el tejido juvenil (Donnelly *et al.* 1999; Czesnick and Lenhard 2015), en el caso de *E. globulus* no existe reporte de cómo ocurre esto, en la familia de las Myrtaceae a la cual pertenece *E. globulus*, se ha reportado diferentes patrones en la diferenciación celular, esto ocurre distal-proximal, proximal- distal o desde la zona intermedia hacia los extremos (Gupta and Nath 2015). Se ha informado que para la adquisición de capacidad fotosintética, es necesaria para iniciar el cambio de la división celular a la expansión celular (Andriankaja *et al.* 2012).

En el caso de *E. globulus* hay diferencias en la capacidad fotosintética entre la zona distal y proximal en hoja juvenil, con mayores niveles en la zona distal (Pereira *et al.* 1992). En base a lo anterior podemos proponer que las diferencias de los niveles de transcritos detectados en el eje distal proximal, para el módulo miR156-SPLs podría estar asociado a los procesos de diferenciación celular, lo que llevaría a un aumento de los niveles de azúcar en la zona distal, que conlleva a un aumento de los niveles de SPLs, lo cual estaría tratando de ser controlado por miR156 en esta zona en una primera instancia, hasta que los niveles de miR156 comienzan a decaer producto del aumento de los azúcares, mediados por HXK1 y trehalosa-6-fosfato (T6P) (Wahl *et al.* 2013; Yang *et al.* 2013), lo cual permitiría el aumento SPLs en la zona distal del tejido adulto.

Al evaluar la otra fuente de miR156 reportada el tejido shoot apex de individuos en etapa juvenil (sin cambio de fase vegetativa), en contraste individuos en transición (con cambio de fase), Los niveles de miR156 disminuyen en comparación al estadio juvenil. Esto concuerda con lo descrito en *Brassicaceae* en donde los niveles de miR156 en meristema apical del brote (SAM) de plantas jóvenes son muy altos (Hyun *et al.* 2017). El aumento de miR156 por ablación de sus hojas, retrasa la transición de fase vegetativa, lo que sugiere que una señal móvil desconocida derivada de las hojas podría ser responsable de la represión de la transcripción de miR156 en los tejidos meristemático (Yang *et al.* 2011).

Respecto a la cuantificación de miARNs por RT-qPCR en plantas, existen varios estudios en los que se discute la importancia de normalizar los datos de expresión relativa de miARNs, con genes de referencia que sean miARNs. Se han descrito miARNs estables en distintos estadios de desarrollo (Machado *et al.* 2015), en condiciones estrés biótico y abiótico (Feng *et al.* 2012). Para el caso de miR171, ha sido utilizado en experimentos bajo frío, sequía, salinidad, metales pesados, radiación UV y aplicación de ácido abscísico (ABA), cuyos resultados indican que miR171 es estable, lo cual sugiere que es un buen gen de referencia (Borowski *et al.* 2014; Yang *et al.* 2016). Nuestros resultados confirman la información emergente en los últimos años muestra sobre los miARNs como genes de referencia. Hemos identificado a uno de los miembros de la familia de miR171, miR171a, mantiene una expresión estable en tejidos juveniles, intermediarios y adultos, además también en el gradiente de eje distal-proximal de tejido foliar en *E. globulus*, lo cual es un aporte en este tipo de investigaciones.

Al evaluar los niveles de transcrito en el eje distal-proximal para HXK1, no se identificamos variaciones significativas. En mutantes de *Arabidopsis*, que carecen de HXK1, en diferentes etapas de desarrollo, han revelado que el nivel de transcripción de miR156 en la etapa juvenil es parcialmente dependiente de la señalización de HXK1, mientras que la expresión de miR156 en la etapa posterior es independiente de HXK1 (Yu *et al.* 2013; Yang *et al.* 2013).

Con el fin de complementar los resultados anteriores, evaluamos el efecto de concentraciones crecientes de glucosa en plántulas de *E. globulus*, al medir los niveles de miR156 se identificó un efecto dosis dependiente (a mayor concentración de glucosa menor nivel de transcrito de miR156), en plantas de *Arabidopsis* tratadas con glucosa, se ha demostrado que el azúcar puede desencadenar la degradación del pri-miR156A/C (Yu *et al.* 2013; Yang *et al.* 2013; Proveniers 2003). El papel del azúcar en la transición heteroblástica, ha demostrado que la adición de azúcar al medio de cultivo da como resultado la formación temprana de hojas adultas, dicho proceso podría ser revertido al transferir a un medio libre azúcar (Allsopp 1954). De igual modo evaluamos el efecto de la adición de manitol en concentraciones crecientes en plántulas de *E. globulus* no observándose variaciones en los niveles del miR156. Existe un reporte en *Pino marítimo* propagados mediante embriogénesis somática, en los cuales se reportó la pérdida progresiva de la capacidad embriogénica temprana (proceso de envejecimiento) (Breton *et al.*

2005), lo cual es un claro indicio del efecto glucosa presente en los medios propagación, podría estar acelerando los estadios del desarrollo en estas condiciones, al afectar los niveles de miR156 asociado a la juvenilidad. De igual modo existen reportes en plantas de *Prunus* sp. que han sido rejuvenecidas y propagadas por métodos in vitro, muestran un comportamiento más errático en los niveles miR156, lo cual ha sugiriendo que el cultivo de tejidos altera el control normal de la vía del envejecimiento (Bastías *et al.* 20016), lo cual podría ser un claro efecto de las concentraciones de glucosa que se adicionan a los medios de cultivos.

De manera complementaria evaluamos los niveles de transcrito de HXK1, SPL9 y SPL3, identificándose diferencias leves en estos dos últimos. Las bajas diferencias identificadas pueden deberse a otros actores como DELLA, que estaría interactuando directamente con los factores de transcripción SPLs secuestrándolos (Yamaguchi *et al.* 2014; Yu *et al.* 2012), lo que ayudaría en la regulación del cambio de fase vegetativa. Dicho mecanismo estaría actuando en forma paralela a la regulación miR156-SPLs (Beydler 2014). Otra explicación estas diferencias tenues identificadas, puede deberse a las condiciones de oscuridad experimentales, ya que en el análisis in silico de las zonas promotoras de SPLs presentan cajas de luz. En el caso de HXK1, esto se puede deber a que la hexokinasa posee múltiples funciones biológicas como sobre la fotosíntesis, la absorción de minerales (Granot *et al.* 2013), el cierre de estomas (Kelly *et al.* 2013) y senescencia (Moore *et al.* 2003).

El cambio de fase vegetativa, es un evento clave del desarrollo en el ciclo de vida de la planta, aquí abordamos el módulo miR156/SPL el principal regulador conservado entre plantas, de igual modo indagamos en algunos reguladores descritos para este módulo, como DELLA asociada al control SPLs en forma complementaria a miR156, también indagamos en la regulación de miR156 asociada a los azúcares, para ello evaluamos los niveles de HXK1 y los efectos a concentraciones crecientes de glucosa, en tejidos completos, todos estos puntos en conjunto no habían sido abordados en otros estudios, lo cual nos permitió generar una visión integrada de la transición en una especie arbórea como *E. globulus*, los diferentes niveles de transcritos de los genes anteriormente mencionados, se asociaron a los diferentes niveles de metilación del ADN en sus zonas promotoras putativas, encontrando una clara correlación para

SPL9, SPL3 y DELLA, para el caso de MIR156 observamos diferencias sutiles y para HXK1 un patrón similar al observado para SPLs, pero menos prominente en el locus HXK-1. Estos resultados sugieren que los genes en sentido ascendente o descendente de la vía, así mismo como miR156/SPL, podrían estar regulados por la metilación del ADN.

Al dividir los tejidos en las zonas proximales y distales, identificamos un mayor nivel de transcrito de miR156 y SPL3 en las zonas distales, para el primero en el estadio juvenil y para los SPL3 en el estadio adulto, lo que podría ser esencial para regular la expresión temporal de los genes implicados en el desarrollo foliar. De tal forma creemos que los mecanismos moleculares y celulares están interrelacionados, los cuales en conjunto conducen el cambio de fase vegetativa. Lo que debería ser ahondado en futuras investigaciones en conjunto con las implicancias fisiológicas de estas.

Con la aplicación de glucosa exógena, generó una disminución de la abundancia de miR156 en plántulas de *E. globulus*, mientras que la manitol no lo hizo. Estos resultados nos hacen cuestionarnos de posibles aplicaciones en mejoras en los procesos de micropropagación, esta técnica es ampliamente utilizada en la propagación de plantas, en la cual se utilizan grandes cantidades de glucosa, ignorando los procesos que están siendo afectados como es el caso de alteración de los niveles miR156, por ello conocer más profundamente estas implicaciones nos permitiría controlar algunos problemas de esta técnica como es el envejecimiento precoz.

La generación de conocimiento de todos los elementos reguladores del cambio de fase vegetativa en forma integrada, nos permitirá diseñar herramientas biotecnológicas aplicadas, en diferentes áreas de la producción como la micropropagación, mejor rendimiento de especies forestales así como control de la fase juvenil de los árboles frutales.

Conclusiones Generales

Este estudio proporciona una base integrada de las bases moleculares del cambio de fase vegetativa en conjunto con la metilación del ADN en *Eucalyptus globulus* Labill. Se concluye que:

1. La expresión del módulo miR156-SPLs se correlacionó con sus perfiles de patrón de metilación, identificándose para el gen miR156, diferencias sutiles entre los perfiles las hojas juveniles y adultas, para SPL3 y SPL9 muestra patrones claramente diferenciados entre los perfiles las hojas juveniles y adultas de *Eucalyptus globulus*.
2. Se identificaron los elementos reguladores en las regiones diferencialmente metiladas de las zonas promotoras putativas del módulo miR156-SPLs, para miR156, se identificaron elementos de respuesta a la luz y la giberelina. Para SPLs se identificaron elementos de respuesta a la luz, hormonas y estrés en *Eucalyptus globulus*.
3. El análisis de expresión del módulo miR156-SPLs en el eje distal proximal, permitió establecer un gradiente proximal-distal para miR156 en hojas juveniles y un gradiente proximal-distal de SPL3 en tejidos adultos de *Eucalyptus globulus*.

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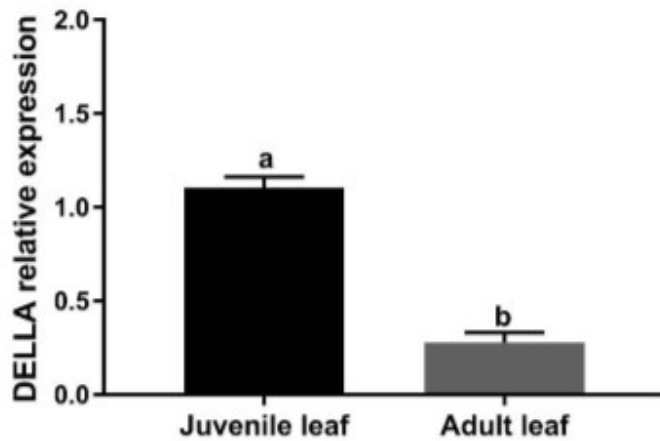
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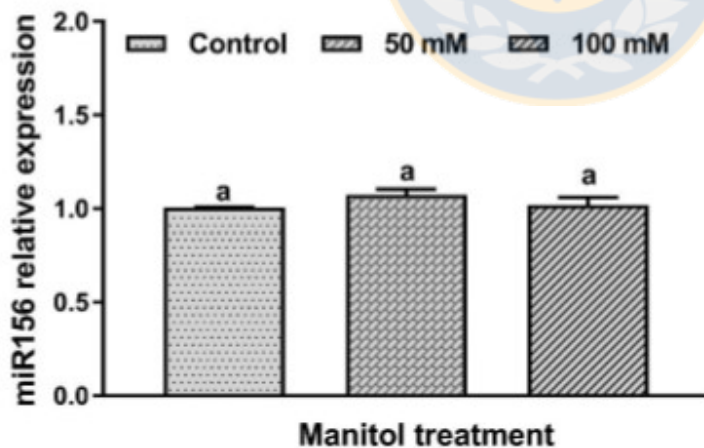
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Anexo 1. Supplementary Materials



S1 Figure 1 Expression of DELLA in juvenile and adult leaves of *E. globulus*. The expression was normalized with EF1a and actin. Averages of three replicates and three trees are shown for each tissue. one-way ANOVA, $p < 0.05$



S2 Figure 2. Expression of miR156 in seedlings of 31 days of *E. globulus*, added 50mM of manitol, 100mM of manitol and C control. The expression was normalized with

miR171a. Averages of three technical repetitions and three independent trials for each tissue are shown.

S3 Table 1. List of principal TF identified in the zone putative promotor of evaluated genes.

Gene	Regulatory element	Answer	Reference
MIR156	G-box /GAG-motif	Light	Gangappa <i>et al.</i> 2013, Li <i>et al.</i> 2013
	Gare-motif	Gibberellin	Bastian <i>et al</i> 2010
SPL3	Sp1/G-box/GAG-MOTIF GT1-motif/ AE box CAAT-BOX Gap-box	Light	Dolferus <i>et al.</i> 1994; Gangappa <i>et al.</i> 2013 Li <i>et al.</i> 2013 Park <i>et al.</i> 1996
	TGACG-motif	MeJA-responsiveness	Terzaghi and Cashmore 1995
	HSE	Element of stress in cis	Rouster <i>et al.</i> 1997
	Gare-motif	Gibberellin	LEE <i>et al</i> 1997.
	AAA-C MOTIF	Light	Bastian <i>et al</i> 2010 Schindler <i>et al.</i> 1992
SPL9	SARE	Salicylic acid	Ulmasov <i>et al</i> 1994
	Sp1/chs-CMA2a/AE- box/BOX1/BOXII/GT1- motif/lbox/MRE/TGG- motif/G-box/GA-box/GAG- motif/GT1-motif	Light	Yihong <i>et al</i> 2016 Wingender <i>et al.</i> 1990 Donald and Cashmore 1990 Hartmann <i>et al</i> 1990 Mongkolsirawatana <i>et al.</i> 2009 Kumar <i>et al</i> 2009.
	TGACG-motif	MeJA-responsiveness	Terzaghi and Cashmore 1995
	ERE	Ethylene	Solano <i>et al.</i> 1998
HXK1	G-box/box4/CATT-motif	Light	Gangappa <i>et al.</i> 2013 Yamada <i>et al.</i> 1994 Li <i>et al.</i> 2013
	ABRE	Stress	Narusaka <i>et al</i> 2003
	TGACG-motif	MeJA-responsiveness	Terzaghi and Cashmore 1995
	Chs-CMA1a	Circadian control	Thain <i>et al</i> 2002.
	LTR	Low temperature	Hughes and Dunn 1996
	P-box	Gibberellin	Mena <i>et al.</i> 2002
	TCA-element	Salicylic acid	Hong <i>et al.</i> 2013
	TGA-element	Auxin	Guilfoyle <i>et al</i> 1993

Anexo 2: Congresos

Hasbún Rodrigo, **Iturra Carolina**, Beltrán Francisca, Arellano Macarena, Rivas Paulina, Stuardo Marcela, Bravo Soraya, Salas Alexis, Rebolledo Boris, Valledor Luis. Differentially methylated genomic regions between juvenile and adult leaves discovered applying the algorithm methylMnM; application to a non-model species *Eucalyptus globulus* Labill. International Plant & Animal Genome XXIII / January 10-14, 2015 San Diego, CA, USA. Poster.

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Anexo 3: Publicaciones

Iturra C, Olivares S, Bravo S, Rebolledo-Jaramillo B, Amoroso A, Duguet J, Bascuñán-Godoy L, Bertin A, Valledor L, Hasbún R. Changes in the expression of miR156 -SPL and HXK1 genes during the ontogenetic leaf transition from juvenile-to-adult in *Eucalyptus globulus* Labill are in part explained by modulations of DNA methylation. *Frontiers in Plant Science* 2018.

Enviada

Hasbún R, **Iturra C**, Bravo S, Rebolledo-Jaramillo B, Valledor L Differential Methylation of Genomic Regions Associated with Heteroblasty Detected by M&M Algorithm in the Nonmodel Species *Eucalyptus globulus* Labill. *Int J Genomics* 2016.

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