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***Environmentally sensitive molecular switches
control diurnal and seasonal growth in poplar***

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control diurnal and seasonal growth in poplar”***

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ABSTRACT

Environmental variations determine seasonal growth, developmental transitions and reproductive behavior in plants and animals. How these organisms integrate seasonal information at the molecular level remain poorly understood. In perennial plants such as poplar, growth-dormancy cycles guarantee survival and sexual reproduction. They also determine several economically important traits, such as tree annual growth rate and yield. Understanding the molecular mechanisms underlying tree annual phenological events supports the design of strategies for tree biotechnology facing plant adaptation and acclimation to climate change. In the last years, important advances have been made in this field of study. Several regulators of Arabidopsis flowering have been shown to participate in the regulation of poplar growth to dormancy transition. These genes act as environmental sensitive switches controlling diverse aspects of tree annual development. One aspect little investigated is the regulation of poplar acclimation during autumnal season. Moreover, the link between poplar autumnal acclimation and stomatal regulation has not yet been identified.

In this thesis, the functional role of RAV1 transcription factor, a poplar ortholog to the Arabidopsis floral repressor TEMPRANILLO/RELATED TO ABI3 AND VP1 (TEM/RAV1), is investigated during the transition from growth to dormancy period. We found that RAV1 neither represses *FT2* nor plays a role in the regulation of growth cessation, therefore photoperiodic regulation of shoot growth in poplar show conserved and distinct features respect to Arabidopsis flowering. Through a reverse genetic approach, we identified genes transcriptionally controlled by RAV1, revealing its implication in the seasonal regulation of plant response to environmental stresses. We found that RAV1 control the expression of *RESPIRATORY BURST OXIDASE HOMOLOG D1* and *ETHYLENE RESPONSE FACTOR 72-1*, showing a tightly coexpression under short days and low ambient temperature and that showed autumnal induction. *RAV1* overexpressing lines showed higher levels of stomatal conductance, photosynthesis, and biomass than wild-type and *RAV1* knockdown plants. We showed that an increased guard cell-ROS burst in hybrid poplar *RAV1* overexpressing plants is associated with faster stomatal closure after ABA treatment. DPI treatment partially reduced ABA-dependent stomatal closure implicating the RBOH enzyme. Time course changes in stomatal conductance under short days and low ambient temperature

conditions indicated that *RAVI* overexpressing plants showed faster diurnal stomatal movement in comparison with wild-type and *RAVI* knock down lines. We propose that seasonal activation of *RAVI* increases the diurnal stomatal activity contributing to poplar acclimation during autumn.

Moreover, we found that *RbohD* transcription shows diurnal rhythms in poplar and *Arabidopsis*. Importantly, *RbohD* gene diurnal rhythms in *Arabidopsis*, generated by integrating endogenous and environmental cyclic signals, contribute to the magnitude of defense response against pathogen attack.

RESUMEN

Las variaciones ambientales determinan el crecimiento estacional, las transiciones en el desarrollo y el comportamiento reproductivo de plantas y animales. Se conoce muy poco de cómo los organismos integran la información estacional a nivel molecular. En plantas perennes como el chopo, los ciclos anuales de crecimiento-dormancia garantizan la supervivencia y la reproducción sexual. Estos ciclos también determinan varias características de gran importancia económica, como la producción de biomasa y el rendimiento de los cultivos. El conocimiento de los mecanismos moleculares que dan origen a los eventos fenológicos anuales permite el diseño de estrategias biotecnológicas para mejorar la adaptación de los árboles frente al cambio climático. En los últimos años, se han realizado importantes avances en este campo de estudio. Se ha demostrado que varios reguladores de la floración de *Arabidopsis* participan en la regulación de la transición del período de crecimiento al período de dormancia en chopo. Estos genes actúan como interruptores sensibles a los cambios medioambientales controlando diversos aspectos de la fisiología y el desarrollo de los árboles. Sin embargo, se sabe poco sobre los mecanismos que participan en la aclimatación del chopo a las condiciones ambientales del otoño. Así mismo, todavía no se ha identificado la conexión entre la aclimatación otoñal del chopo y la regulación estomática.

En esta tesis, se investiga el papel funcional del factor de transcripción RAV1, un ortólogo del represor floral TEMPRANILLO de *Arabidopsis*, durante la transición del período de crecimiento al período de dormancia invernal. Mostramos que RAV1 no reprime a *FT2* ni desempeña un papel en la regulación del cese de crecimiento. Por lo tanto, la regulación fotoperiódica del crecimiento apical en chopo muestra características conservadas y diferentes con respecto a su función en la floración de *Arabidopsis*. Hemos identificado los genes controlados a nivel transcripcional por RAV1, lo que revela su implicación en la regulación estacional de la respuesta de la planta al estrés ambiental. También demostramos que RAV1 controla la expresión de los genes *RESPIRATORY BURST OXIDASE HOMOLOG D1* (*RbohD1*) y *ETHYLENE RESPONSE FACTOR 72-1* (*ERF72-1*), que muestran una estrecha coexpresión con RAV1 en condiciones de días cortos y baja temperatura, además de durante la inducción otoñal. Las líneas de sobreexpresión de RAV1 mostraron niveles más altos de conductancia estomática, fotosíntesis y biomasa respecto a las plantas silvestres (WT). Por el contrario, las plantas con niveles de expresión mas bajos de RAV1 (*RAV1kd*) no mostraron diferencias con respecto al ecotipo

silvestre. Además, demostramos que la sobreexpresión de RAV1 producía mas especies reactivas de oxígeno (ROS) en las células estomáticas y un cierre más rápido de las estomas después del tratamiento con la hormona ABA. Además, el tratamiento con el inhibidor DPI redujo parcialmente el cierre estomático dependiente de ABA, implicando de esta manera a la enzima RBOH en este proceso. Las medidas de conductancia estomática a lo largo del día en condiciones de día corto y baja temperatura demostraron que las plantas que sobreexpresan *RAV1* tienen un movimiento estomático diurno más rápido y eficaz en comparación con las plantas WT y *RAV1kd*. En este trabajo, proponemos que la activación otoñal del RAV1 aumenta la actividad estomática diurna que contribuye a la aclimatación del chopo a las condiciones del otoño.

Por otra parte, observamos que la transcripción de *RbohD* muestra ritmos diurnos en chopo y *Arabidopsis*. Es importante destacar que los ritmos diurnos del gen *RbohD* en *Arabidopsis*, generados por la integración de señales cíclicas endógenas y ambientales, contribuyen a la magnitud de la respuesta de defensa contra el ataque de patógenos.

ABBREVIATIONS

(In alphabetical order)

ABA= abscisic acid
ABI3= ABSCISIC ACID-INSENSITIVE3
AGL= AGAMOUS-LIKE (AGL)
AIL1= AINTEGUMENTA LIKE 1
Arabidopsis= *Arabidopsis thaliana* (L.) Heynh
ATHB16= ARABIDOPSIS HOMEBOX 16
BBX14= B-BOX DOMAIN PROTEIN 14
BHP= BLUE LIGHT-DEPENDENT H⁺-ATPASE PHOSPHORYLATION
BLUS1= BLUE LIGHT SIGNALING1
BRC1= BRANCHED1
CBF= C-REPEAT-BINDING FACTOR
CCA1= CIRCADIAN CLOCK-ASSOCIATED 1
CCR1= COLD, CIRCADIAN RHYTHM, AND RNA BINDING 1
CDF= CYCLING DOF FACTOR
CDL= critical day length
CDPK or CPK= CALCIUM-DEPENDENT PROTEIN KINASE
CENL1/TFL1= CENTRORADIALIS-LIKE1/TERMINAL FLOWER 1
CHE= CCA1 HIKING EXPEDITION
CHX= cycloheximide
CO=CONSTANS
Col-0= *Arabidopsis thaliana* genotype Columbia
COL13= CONSTANT LIKE 13
COP1= CONSTITUTIVE PHOTOMORPHOGENIC 1
COP1= CONSTITUTIVE PHOTOMORPHOGENIC 1
COR= COLD REGULATED GENES
CRY= CRYPTOCHROME
CT= circadian time
CYCD3:2= CYCLIN D-TYPE 3:2
DAM= DORMANCY ASSOCIATED MADS-BOX
DEX= dexamethasone
DHN= DEHYDRIN
DML= DEMETER LIKE
DPI= Diphenyleneiodonium chloride
EBB1= EARLY BUD-BREAK 1
ELF= EARLY FLOWERING
ERF72= ETHYLENE RESPONSE FACTOR 72
FDL1= FLOWERING LOCUS D-LIKE 1
FKF1= FLAVIN-BINDING, KELCH REPEAT, F-BOX 1
FLC= FLOWERING LOCUS C
Flg22= flagellin 22
FT= FLOWERING LOCUS T

GA= gibberellin
 GI=GIGANTEA
 GR= glucocorticoid receptor domain
 HXK= HEXOKINASE
 KAT1= POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1
 LAP1= LIKE-APETALA 1
 LD= long day
 LEA= LATE EMBRYOGENESIS-ABUNDANT
 LHY= LATE ELONGATED HYPOCOTYL
 LL= continuous light
 LUC= LUCIFERASE
 MAMPs= microbe-associated molecular patterns
 NO= nitric oxide
 NPR1= NON-EXRESSER OF PATHOGENESIS-RELATED GENES 1
 OST= OPEN STOMATA
 PAMPs= pathogen-associated molecular patterns
 PHOT= PHOTOTROPIN
 PHY= PHYTOCHROME
 PLD= PHOSPHOLIPASE D DELTA
 PRR= PSEUDO RESPONSE REGULATOR 7
 PTM= photoperiodic time measurement
 PYR/PYL/RCAR= PYRABACTIN RESISTANCE /PYR1 LIKE/REGULATORY COMPONENT OF ABA RECEPTOR
 QTL= quantitative trait loci
 RAV1= RELATED TO ABI3 AND VIVIPAROUS 1
 RBOHD= RESPIRATORY BURST OXIDASE HOMOLOG D
 ROS= reactive oxygen species
 qRT-PCR= quantitative Real Time Reverse Transcription Polymerase Chain Reaction
 RVE= REVEILLE
 SA= salicylic acid
 SAM= shoot apical meristem
 SD= short day
 SHORT VEGETATIVE PHASE
 SLAC1= SLOW ANION CHANNEL ASSOCIATED 1
 SNP= single nucleotide polymorphism
 SOC1= SUPPRESSOR OF OVEREXPRESSION OF CO1
 TEM= TEMPRANILLO
 TFL2= TERMINAL FLOWER 2
 TOC1= TIMING OF CAB EXPRESSION 1
 TSF= TWIN SISTER OF FT
 TSS= transcriptional start site
 VIP= VERNALIZATION INDEPENDENCE
 WT= wild type
 ZT= Zeitgeber time

INTRODUCTION

Plant and animal life show cyclic pattern of behavior and development linked to the seasons. Seasons result from Earth's orbit around the Sun and Earth's tilted axis. In temperate and cold regions, there are four seasons: spring, summer, autumn and winter. From May to August the North Hemisphere is inclined towards the Sun and the summer occurs because the increased perpendicular solar flux. Vice versa, from December to March the Earth's axis is inclined in the opposite direction to the Sun and this hemisphere experiences the winter season. Then, spring and autumn are intermediate seasons after winter and summer, respectively. In the Southern Hemisphere, the alternation of the seasons is reversed respect to the Northern one (Fig. 1).

Figure 1. Generation of the 4 seasons at the mid-latitudes.

(A) Earth's rotation around the Sun together with its titled axis generates the four seasons at the mid-latitudes. (B) Range of latitudes presenting the 4 seasons, autumn, winter, spring and summer along the year.

To survive to these annual climate variations in their habitat, living organisms developed specific seasonal modulation of behavior, physiology and development. This cyclic adaptation strategy is termed phenology. In temperate and boreal regions, the winter season may cause animals to undergo hibernation or to migrate, and plants to produce seeds or to be dormant (Fig. 2).

Figure 2. Different survival strategies of living organisms to seasonal variations in temperate and boreal regions.

Migration or hibernation in animals and seed production or dormancy in plants, allow them to survive over the winter, in temperate and boreal regions.

1. Overview of growth-dormancy cycles in poplar

Plants have evolved several phenological strategies to enhance their adaptation to seasonal changes. Most of the annual plants optimize its flowering time to the adequate environmental conditions and generate seeds for overwintering. Woody perennials cope with winter freezing temperatures developing a process called winter dormancy, which involves the inactivation of all plant meristematic activity. Annual growth-dormancy cycle of these perennials is tightly controlled by photoperiod and/or temperature signals (Allona et al., 2008; Cooke et al., 2012; Ding and Nilsson, 2016; Singh et al., 2016; Maurya & Bhalerao, 2017). In autumn, when the photoperiod falls below the critical day length (CDL) poplars undergo growth cessation, being the CDL the length of daylight required for shoot growth. CDL can vary considerably among the ecotypes depending of their geographical origin (Böhlenius et al., 2006). At the shoot apical meristem (SAM), growth cessation involves a progressive reduction of leaf organogenesis that takes approximately 4 weeks. Afterward, SAM initiates a winter bud formation stage that takes 4-6 more weeks under short days (SDs). Winter bud is a protective structure for the SAM and leaf primordia that remains the whole winter and represents a precondition for the establishment of dormancy (Fig. 3; Rohde et al., 2002).

SD also causes cessation of stem growth. After SD perception, the stem continues normal rate of elongation until the 4th week and progressive decreased until the formation of the autumn bud (Böhlenius et al., 2006; Ruttink et al., 2007), while leaves continues doing photosynthesis along growth cessation and bud formation stages (Rinne, Welling,

& van der Schoot, 2010). Growth cessation leads to the decreased demand from sink tissues, generating the redistribution of the excess of photosynthates to storage tissues such as roots, stems and developing buds (Fege and Brown, 1984; Rinne et al., 1994). These photosynthates are converted into starch, fatty acids and proteins necessary for cold acclimation (Sauter and van Cleve, 1991; Rinne et al., 1994; Sauter et al., 1996; Stitt and Hurry, 2002). The importance of photosynthesis as the main source of storage compounds during growth cessation has been demonstrated because leaf abscission impairs cold acclimation and survival (Fuchigami et al., 1971). This storage phase continues until dormancy is established (Keskitalo et al., 2005). Storage has a double function during dormancy, conferring cold hardiness through the accumulation of cryoprotective metabolites and providing energetic sources to sustain cell division and growth in absence of photosynthesis in the early steps of bud break (Rinne, Welling, & van der Schoot, 2010).

From induction of growth cessation, the tree enters in the ecodormancy state, where shoot apical growth could be resumed by placing the plant under favorable conditions. Further exposure to SD and low temperature over the autumn, initiates the endodormancy state, and returning the plant to favorable conditions cannot resume growth. To break the endodormancy, a plant must accumulate a determined number of hours around 4°C, which it has been defined as the chilling requirement. After passing this period in cold conditions, the plant enters again in ecodormancy, where reactivation of growth and bud break is produced under long days (LDs) and warm temperature (Lang, 1987).

Figure 3. Environmental signals are essential for growth-dormancy cycle in poplar.

Schematic representation of an annual growth-dormancy cycle in poplar. From the spring poplars grow until the shortening of photoperiod in the early autumn induces growth cessation. Then, prolonged exposure to SDs and low temperature promote bud set and the establishment of dormancy. In winter, a determined number of hours are required to break dormancy (chilling requirement), which allows bud break when LDs and warm temperature occur during the spring. A representative image of SAM, bud set and bud break are shown.

2. Molecular orchestration of growth-dormancy transition in poplar

2.1. A photoperiodic time measurement (PTM) mechanism determine the timing of growth cessation

Photoperiod is the most regular environmental signal that drives seasonal development in many insects, birds, other animals and plants (Nelson et al., 2010). The external coincidence hypothesis, initially proposed by Bünning (1936) and later modified by Pittendrigh and Minis (1964), has been the prevailing photoperiodic time measurement (PTM) model tested in many organisms (Goldman, 2001; Pegoraro et al., 2014; Song et al., 2015). This model predicts that seasonal physiological responses are created when, by coincidence, diurnal endogenous rhythms match the external photoperiod. Accordingly, day/night duration is measured through photoperiod-dependent modulation of the activity of an endogenous oscillatory component, which controls seasonal physiological and developmental responses (Pittendrigh and Minis, 1964). Three basic components are required to create a day length measurement mechanism: a photosensory system which includes photoreceptors, an endogenous oscillator, which has been identified as the circadian clock system, and an endocrine effector or mobile photoperiodic signal that translates photoperiod information from the photosensory system to the target organs. In plants, the molecular nature of these components was firstly identified for *Arabidopsis* flowering time (Yanovsky and Kay, 2002).

2.1.1 Phytochromes as players in the poplar PTM mechanism

It is assumed that the photoperiodic signal is perceived in the leaf through discrimination of light quality information by photoreceptors, which have been linked to growth regulation in poplar (Howe et al., 1996; Olsen et al., 1997; Frewen et al., 2000; Ingvarsson et al., 2008; Ruonala et al., 2008; Kozarewa et al., 2010). In detailed-designed night-break

experiments, subjecting plants to red light illumination caused the suppression of short day-induced growth cessation in poplar. This effect was reversed by red light followed by far red light treatment in night-break experiment, indicating that phytochromes participate in the photoperiodic regulation of poplar shoot apical growth (Howe et al., 1996). One *phytochrome A* (*PHYA*) and two *phytochrome B* (*PHYB*) genes, *PHYB1* and *PHYB2*, were identified in poplar (Howe et al., 1998). The overexpression of *oat PHYA* in poplar (*Populus tremula x tremuloides*) showed relative insensitivity to SD-induced growth cessation (Olsen et al., 1997). Conversely, poplar showing downregulation of *PHYA* has been noted to cease growth earlier than control plants subjected to photoperiod-inducing conditions below CDL (Kozarewa et al., 2010). Poplar *PHYB1* but not *PHYB2* has been observed to complement an Arabidopsis *phyB* mutant indicating that *PHYB1* maintains the molecular function of Arabidopsis *PHYB*, while *PHYB2* shows divergent molecular features (Karve et al., 2012). However, quantitative trait loci (QTL) and single nucleotide polymorphism (SNP) analyses have genetically linked the timing of photoperiod-induced bud set to *PHYB2* function, but not to *PHYB1* (Frewen et al., 2000; Ingvarsson et al., 2008). Thus, *PHYA* and *PHYB2* could play a photosensory function during poplar shoot apical growth and may participate in the PTM mechanism. Some authors have explored the link between *PHYA* and the circadian clock. Poplar *PHYA* antisense gave rise to a longer period of leaf movement rhythms than wild type (WT) plants indicating that the circadian clock period depends on the *PHYA* level (Kozarewa et al., 2010).

2.1.2 The circadian clock in plants and its role in the PTM mechanism in poplar

Every living organism has an endogenous molecular mechanism, known as the circadian clock, that creates rhythmic oscillations with a period of 24 hours (Millar, 2003). The function of the circadian clock is to anticipate daily and seasonally predictable changes such as day/night cycle or other environmental challenges in order to inform the rest of the cell/body about the hour of the day, making possible to fit every physiological function to an optimum time (Schultz and Kay, 2003). In plants, the uncoupling of the clock and environment affected the physiology of the organism (Dodd et al., 2005). The optimal amount of plant response to a given external or endogenous stimuli mainly depends on the circadian clock function. In other words, the magnitude or the occurrence of the response of circadian-controlled pathways depends on the time of day. This

property of the clock to modify the responsiveness to environmental or endogenous stimuli is known as ‘gating’ (Greenham and McClung, 2015). Most knowledge about the plant circadian clock has been obtained in *Arabidopsis*. Components of the clock core are described based on the timing of their gene expression patterns resulting in morning-, day- and evening-phased components. To simplify the complexity of the circadian clock, below we provide a schematic view of the main genes belonging to the 3 feedback loops of the core of the clock based on the *Arabidopsis* information (Fig. 4). The morning-phased genes are two MYB-like transcription factors *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) which transcript and protein levels are highly abundant in the morning. Then, *PSEUDO RESPONSE REGULATOR 9* and *7* (*PRR9* and *PRR7*) together with *REVEILLE 8* (*RVE 8*) are the daytime-phased genes, while the evening loop is composed by *PRR5*, *TIMING OF CAB EXPRESSION 1* (*TOC1*), *CCA1 HIKING EXPEDITION* (*CHE*), *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 3* and *4* (*ELF3* and *ELF4*) (Hsu & Harmer, 2014). These genes interact each other to generate complex transcriptional feedback loops which keep in timing the expression of each gene and their downstream targets throughout the day. Moreover, post-transcriptional and post-translational modifications together with protein turnover contribute to the complexity and the maintenance of these feedback loops (Hsu & Harmer, 2014).

Figure 4. Transcriptional regulation of the Arabidopsis clock.

Schematic representation of the main components of morning, day and evening loops which compose the plant circadian clock.

Among the function of the circadian clock in plants, such as seasonal control of photoperiodic flowering, the diurnal gating of abiotic and biotic stresses-related responses and metabolism (Greenham and McClung, 2015), the implication of the biological clock in the control of poplar growth cessation and bud set has recently emerged (Ibáñez et al., 2010). The first clock genes identified in a woody perennial species were chestnut *LHY* and *TOC1*, orthologs of essential components of the Arabidopsis circadian clock (Ramos et al., 2005). Later, the homologous of Arabidopsis *PRR* genes, *PRR9*, *PRR7*, and *PRR5* were identified in chestnut and showed daily peak expression after *LHY* in the order *PRR9* → *PRR7* → *PRR5* → *TOC1*, in a similar serial manner that have seen in Arabidopsis (Ibáñez et al., 2008). Poplar has two copies of the *LHY* transcription factor, denoted *LHY1* and *LHY2* (Takata et al., 2009). The daily gene expression pattern of poplar *LHY1* and *LHY2* shows a morning expression peak similar to Arabidopsis and chestnut (Ramos et al., 2005; Takata et al., 2009; Ibáñez et al., 2010; Ramos-Sánchez et al., 2017). Moreover, the poplar clock has several *PRR* orthologs of Arabidopsis *PRR5*, *PRR7*, *PRR9* and *PRR1/TOC1*, which display a similar daily gene expression pattern to that previously reported in Arabidopsis and chestnut (Ramos et al., 2005; Ibáñez et al., 2008; Ibáñez et al., 2010; Filichkin et al., 2011). Pioneering functional analyses of poplar *LHY* and *TOC1* clock genes pointed that downregulation of *LHY* or *TOC1* caused a drastic delay in growth cessation respect to the WT under SDs, indicating that circadian clock function is necessary to measure photoperiodic time (Ibáñez et al., 2010).

2.1.3 FT2 acts as a mediator of photoperiodic signaling to control poplar shoot apical growth

The mobile protein FLOWERING LOCUS T (FT) transmits photoperiod information from the leaf to the apex controlling Arabidopsis flowering (Corbesier et al., 2007). *FT* gene expression is extremely sensitive to photoperiodic changes in Arabidopsis. LDs enhance *FT* transcription and SDs drastically reduce it. *FT* shows a robust diurnal gene expression pattern under LD conditions showing a peak of mRNA accumulation at the end of the day. This temporal pattern is critical to distinguish photoperiod information for flowering time (Krzymuski et al., 2015). The combined actions of circadian clock-controlled activators and repressors sustain this tight *FT* diurnal expression in Arabidopsis (Song et al., 2015). Among them, CONSTANS (CO) and GIGANTEA (GI) are well-known activators of *FT* transcription, while TEMPRANILLO (TEM) and

CYCLING DOF FACTOR (CDF) transcription factors repress *FT* gene expression (Suárez-López et al., 2001; Castillejo and Pelaz, 2008; Fornara et al., 2009; Sawa & Kay, 2011; Song et al., 2012). The balance between the activators and repressors activity is critical to maintain adequate *FT* mRNA levels in Arabidopsis. *CO* expression is controlled by the circadian clock, and diurnal transcriptional activation of *CO* precedes the activation of *FT* under LD conditions in Arabidopsis (Suárez-López et al., 2001). Moreover, mutations in *lhy-7* and *lhy-20* clock mutants, which shorten the clock period, have been detected to induce the early timing of *CO* expression and high levels of *FT* (Park et al., 2016). Hence, the diurnal matching of *CO* and *FT* expression patterns is important to control Arabidopsis flowering. *CO* protein levels are also diurnally regulated. Accordingly, during daylight hours, *CO* protein levels are maintained, while in the dark, *CO* levels are reduced contributing to *FT* downregulation (Valverde et al., 2004). Moreover, the repression of *FT* is, in part, transcriptionally determined by *TEM* genes, transcription factors belonging to the RELATED to ABI3 and VIVIPAROUS1 (RAV) family, which directly bind the *FT* promoter (Castillejo and Pelaz, 2008). *TEM* and *FT* are coexpressed, showing peak mRNA accumulation at the day-night transition under LDs in Arabidopsis (Castillejo and Pelaz, 2008; Osnato et al., 2012).

Two poplar orthologs of Arabidopsis *FT* have been identified (Hsu et al., 2011). They show different spatio-temporal pattern of gene expression whereby *FT1* is mainly expressed in stem and flower bud tissues at the end of winter, and *FT2* expression occurs during the growing season mainly in leaf tissues (Pin and Nilsson, 2012). Remarkably, poplar *FT2* mRNA levels indicate conserved diurnal expression and photoperiod responses as in Arabidopsis (Böhlenius et al., 2006; Ibáñez et al., 2010; Hsu et al., 2011). Moreover, natural variation across a latitudinal gradient suggests diurnal *FT2* expression is locally adapted to the day length regime (Böhlenius et al., 2006). Further, the ectopic expression of *FT2* in poplar has been noted to induce a dramatic delay in growth cessation. Conversely, *FT2* downregulation significantly speeds up growth cessation (Böhlenius et al., 2006; Hsu et al., 2011). These studies have shown the conserved role of poplar *FT2* as a mediator of the photoperiod signalling required for shoot apical growth (Fig. 5).

Figure 5. Photoperiodic time measurement mechanism controls shoot apical growth in poplar.

Schematic representation of the leaf-localized photoperiod measurement mechanism in poplar.

(1) Photosensory pathway, (2) circadian clock system, and (3) mobile photoperiodic mediator signal.

Among the regulators of *FT2*, poplar orthologs of *CO* have been identified and functionally implicated in the photoperiodic regulation of poplar shoot growth and flowering (Böhlenius et al., 2006). Simultaneous downregulation of poplar *CO1* and *CO2* correlates with reduced levels of poplar *FT2* leading to accelerated growth cessation under SD conditions (Böhlenius et al., 2006). In Arabidopsis, the ectopic expression of *CO* gives rise to constitutively high levels of *FT* leading to early flowering under conditions of both LDs and SDs. Consequently, in these plants, high-levels of *CO* expression are sufficient to promote flowering (Putterill et al., 1995). In contrast, the overexpression of poplar *CO1* or *CO2* orthologs was found neither to upregulate *FT2* nor to delay growth cessation under conditions of SDs in poplar (Hsu et al., 2012), suggesting that *FT2* repression is dominant under SD conditions. Recently, Ding et al. (2018) showed that GIGANTEA (*GI*), contributes to poplar photoperiodic control of shoot apical growth via strong direct *FT2* activation, whereas in Arabidopsis, *GI* strongly activates both *CO* and *FT* (Sawa and Kay, 2011; Ding et al., 2018). Poplars overexpressing *GI* showed delayed SD-induced growth cessation indicating its predominant role as an activator of *FT2* over *CO* (Ding et al., 2018).

Downregulation of *FT2* is necessary but not sufficient for poplar growth cessation. Resman et al. (2010) showed that the timing of growth cessation involved additional downstream events to *FT2*. Photoperiod signaling downstream from *FT2*, controls the rate of shoot apical cell proliferation (Karlberg et al., 2010). *FT2* interacts with FLOWERING LOCUS D-LIKE 1 (*FDL1*) and this protein complex targets a poplar ortholog of Arabidopsis *APETALA1*, *LIKE-API* (*LAPI*) gene (Tylewicz et al., 2015).

LAP1 activity maintains shoot vegetative growth via direct regulation of *AINTEGUMENTA LIKE 1 (AIL1)* transcription factor (Azeez et al., 2014). AIL1 directly activates the expression of *CYCLIN D-TYPE 3:2 (CYCD3:2)* gene, promoting the cell cycle and, thus, the growth (Karlberg et al., 2010). When the photoperiod is below the CDL, *FT2* downregulation produces the suppression of this growth-promoting pathway inducing growth cessation.

2.2 Short days promote acclimation to winter conditions during bud formation

To summarize, SD perception through PTM mechanism triggers *FT2* downregulation and growth cessation in poplar. Moreover, prolonged exposure to SDs promotes bud formation and cold acclimation following a sequential molecular remodeling (Ruttink et al., 2007). The transcriptional and metabolomic profiling of poplar shoot apices and stem tissues has been performed to investigate the molecular signatures underlying SD-driven adaptive responses. The results revealed that SD conditions significantly alter the transcription of many genes respect to LD (Rohde et al., 2007; Ruttink et al., 2007; Karlberg et al., 2010; Hoffman et al., 2010; Resman et al., 2010). Firstly, the transcription of several light signaling- and circadian clock-regulated genes is affected during 1-2 weeks under SDs indicating that these genes mediate in the first step of SD-induced growth cessation (Ruttink et al., 2007). Later, SDs induces genes related to dehydration and cold acclimation, such as *LATE EMBRYOGENESIS-ABUNDANT (LEA)*, *DEHYDRIN (DHN)*, *COLD REGULATED GENES (COR)*, and a set of transcription factors that mediate responses to abiotic stresses, among others (Ruttink et al., 2007; Karlberg et al., 2010). After that, continued SD exposure stimulates ethylene and abscisic acid (ABA) signaling (Rohde et al., 2002; Ruttink et al., 2007; Karlberg et al., 2010). Ethylene and ABA are abiotic stress-related phytohormones required for plant adaptive responses and are also functionally associated to the regulation of many aspects of bud development in poplar and birch as we will see along this introduction (Rohde et al., 2002; Ruonala et al., 2006; Shi and Yang, 2014; Müller and Munné-Bosch, 2015; Tylewicz et al., 2015; Trivedi et al., 2016; Tylewicz et al., 2018). These transcriptional profiles suggest that SDs are important to induce the acclimation of the plant to the oncoming decrease of temperature. Irrefutable experimental evidence for the short day (SD) requirement to cold acclimation comes from studies of photoperiod-insensitive *oat PHYA* overexpressing poplars (Junttila and Kaurin, 1990; Olsen et al., 1997; Welling et

al., 2002). When transgenic and WT plants grown during SDs were subjected to freezing conditions, poplars overexpressing *oat PHYA* did not develop cold hardiness (Olsen et al., 1997; Welling et al., 2002). In contrast, neither transgenic nor WT poplars grown during LDs were able to survive freezing temperatures (Junttila and Kaurin, 1990). This indicates that exposure to SDs acclimates poplars to cold weather conditions, and possibly other environmental stresses, which suggests that SDs may activate acclimation pathways.

Thus, acclimation to winter conditions is mediated by the temporal orchestration of SD-induced transcriptional responses that partly originate from light signaling dependent pathways.

2.3 Temperature modulates growth cessation and bud set

The effect of temperature on growth cessation and bud set has been poorly investigated at the molecular level. Earlier physiological studies have shown that growth cessation and bud set are thermosensitive processes in trees (reviewed in Tanino et al., 2010). Furthermore, recent phenotyping studies, using clonally replicated poplars grown under natural conditions, show that the timing of growth cessation and bud set depends on the local climate (Rohde et al., 2011; Evans et al., 2014). Rohde et al. (2011) proposed that temperature modifies CDL for growth cessation and influences bud formation. Thus, photoperiod and temperature interact each other to determine poplar local adaptation. Moreover, decrease of temperature during autumn contributes to cold acclimation. It has been shown that chestnut clock-related genes display arrhythmic expression under winter conditions (Ramos et al., 2005; Ibañez et al., 2008) and it has been suggested that this circadian clock disruption may facilitate bud set under cold temperatures (Johansson et al., 2015). Similarly, cold-induced disruption of circadian rhythms caused wide transcriptional rearrangement of cold response genes in *Arabidopsis* (Bieniawska et al., 2008). Additionally, when exposed to freezing temperatures, *LHY* knock down transgenic poplars showed far more severe stem injuries than control plants. This result indicates that the circadian clock is pivotal in the development of cold hardiness during bud set (Ibañez et al., 2010). For instance, the transcription of well-known cold tolerance genes in *Arabidopsis*, C-REPEAT BINDING FACTOR (CBF) genes, follow circadian rhythms (Fowler et al., 2005; Dong et al., 2011). *CBF1-3* is a small family of three transcription factors which are induced by low temperature and enhance freezing tolerance in

Arabidopsis (Jaglo-Ottosen et al., 1998; Medina et al., 1999). *CBF* induction is followed by the activation of CBF-targeted cold-regulated (COR) genes, known as the CBF regulon (Barrero-Gil & Salinas, 2018). The function of CBFs in cold hardiness is conserved in poplar and birch (Benedict et al., 2006; Welling et al., 2008). Nevertheless, the functional implications of cold-induced and clock-regulated pathways during bud set needs further investigation.

2.4 Role of hormones in growth cessation, bud formation and dormancy establishment

Prolonged SD exposure after growth cessation and bud set results in dormancy establishment in various plants (Heide, 1974; Espinosa-Ruiz et al., 2004; Ruttink et al., 2007; Maurya and Bhalerao, 2017). Plant hormones such as gibberellins (GA), ethylene and ABA are required for growth cessation, bud formation and dormancy establishment. For instance, poplars overexpressing enzymes involved in GA biosynthesis, GA20 oxidases, showed a delayed growth cessation and bud set without affecting *FT2* levels, indicating that GA could work in a *FT2*-independent growth-promoting pathway (Eriksson et al., 2014). In birch, ethylene-insensitive plants overexpressing the dominant negative *etr1-1* allele failed to develop apical buds under SD conditions (Ruonala et al., 2006). Moreover, the molecular timetable of poplar bud formation revealed several *ETHYLENE RESPONSE FACTORS* (*ERFs*) to be differentially regulated under SDs respect to LDs, suggesting a potential role of ethylene in SD-induced bud set (Ruttink et al., 2007). In poplar, *ABSCISIC ACID-INSENSITIVE3* (*ABI3*) is specifically expressed after the perception of CDL in buds and the overexpression or downregulation of *ABI3* affected bud maturation (Rohde et al., 2002; Ruttink et al., 2007). Moreover, *ABI3* interacts with *FDL1* to regulates the expression of adaptive response genes under SDs (Tylewicz et al., 2015; Fig. 6). Very recently, abscisic acid-insensitive poplars overexpressing the dominant negative *abi1-1* allele failed to establish dormancy under SDs (Tylewicz et al., 2018). The authors conclusively demonstrated that ABA signal is required for plasmodesmata closure in SAM and it is critical for dormancy establishment because inhibits the movement of growth-promoting signals to the SAM, such as *FT* (Tylewicz et al., 2018).

Bud maturation is also characterized by the accumulation of lignin, starch, anthocyanins, phenols and fats (Perry, 1971), which explains the red-brown coloration of

bud scales and the upregulation of the genes-encoded enzymes participating in flavonoid biosynthesis (Rohde, 2002; Ruttink et al., 2007). Recently, the function of a DEMETER-LIKE DNA demethylase has been reported in poplar bud maturation. Poplars overexpressing *CsDML* showed early bud maturation and increased flavonoid synthesis whether flavonoid production is related to bud maturation is unknown (Conde et al., 2017a).

2.5 Chilling requirement, dormancy release and bud break

As mentioned earlier, dormancy establishment makes the SAM impermeable to growth-promoting signals, even under LD conditions. Release from dormancy requires that buds are exposed to low temperature (LT) below 4°C for a period that depends on the ecotypes, also defined as the chilling requirement (Saure, 1985; Hannerz et al., 2003; Brunner et al., 2014; Fu et al., 2015).

How temperature regulates dormancy release remains a poorly studied topic. The exposure of chilled buds to warm temperature is necessary to induce dormancy release and subsequent bud break. It has been suggested that LT treatment leads to the opening of plasmodesmata because some of the genes needed for the removal of plasmodesmatal dormancy sphincters by degrading the deposited callose, such as 1,3- β -glucanases (GH17), are upregulated after chilling treatment (Rinne et al., 2011). GAs may promote *GH17s* expression to reopen the plasmodesmata and thus restart the symplastic, growth-promoting cell-to-cell signaling within the SAM (Fig. 6). Moreover, after chilling treatment, the expression of *CENTRORADIALIS-LIKE1/TERMINAL FLOWER 1* (CENL1/TFL1), *FT1* and *EARLY BUD-BREAK 1 (EBB1)* genes increases in poplar apical buds. CENL1 is negative regulator of dormancy release, while FT1 and EBB1 promote bud break (Mohamed et al., 2010; Yordanov et al., 2014; Fig. 6). Together, these genes interact each other to determine the proper timing of bud break (Rinne et al., 2011; Yordanov et al., 2014). Similar expression pattern has been shown for poplar *DEMETER LIKE 10* (DML10) gene, which is upregulated in poplar apical buds after chilling treatment. Transgenic poplars showing downregulation of *DML8/10* caused delayed bud break. Genome-wide transcriptome and methylome analyses revealed that DML8/10 remove repressive epigenetic markers to allow the transcription of genes that are genetically associated with bud break (Conde et al., 2017b).

The process of dormancy release in trees is comparable to vernalization in *Arabidopsis* (Chouard, 1960). Many of the MADS-box transcription factors – termed DORMANCY ASSOCIATED MADS-BOX (DAM) – are known to be induced in the dormant buds of many plants and are similar to SHORT VEGETATIVE PHASE (SVP) and AGAMOUS-LIKE (AGL) transcription factors which control flowering in *Arabidopsis* (Ríos et al., 2014). Similar to FLOWERING LOCUS C (FLC) in *Arabidopsis*, these DAM genes are epigenetically silenced after cold treatment suggesting that these genes are repressors of dormancy release (Shim et al., 2014; Howe et al., 2015). Very recently, it has been shown that SHORT VEGETATIVE PHASE-LIKE (SVL), a poplar ortholog of *Arabidopsis* SVP, acts as a central regulator of dormancy release and bud burst in poplar. SVL and ABA constitute a positive feedback loop that ensure dormancy maintenance. SVL negatively regulates dormancy release and bud break by promoting and repressing the expressions of negative and positive regulators of growth, respectively, after cold treatment (Singh et al., 2018).

Figure 6. Photoperiodic and temperature control of bud set, dormancy establishment and bud break. Short days promote bud set via activation of FDL1 and ABI3 as well as the ethylene pathways. ABA plays an essential role in dormancy establishment by promoting plasmodesmata closure. Increased exposure to chilling temperatures is necessary to the downregulation of ABA. Dormancy release increases gibberellin levels, mediates plasmodesmata opening and activates bud break-promoting factors such as FT1, EBB1, and DML10. LDs and warm temperatures restore shoot apical growth.

3. Regulation of stomatal movement

3.1 Role of stomata in plant

Photosynthesis in plants depends on the assimilation of atmospheric CO₂ at the leaf. At the same time, plant has to prevent the excessive loss of water due to the leaf transpiration which represents a driving force to ensure the water flux and nutrients from roots to the aerial parts (Jones *et al.*, 1998; Schroeder *et al.*, 2001). Leaf transpiration also permits to regulate the internal temperature of the plant (Jones *et al.*, 1998). Leaves are surrounded by an impermeable layer to both CO₂ and water, the cuticle. Thus, to ensure gas exchanges between plant and environment and guarantee photosynthesis and transpiration, plants display pores located on the leaf surface, the stomata.

Stomata is constituted by two guard cells, specialized cells that control the aperture of the stomatal pore. Adjacent to guard cells there are subsidiary cells, which do not participate in the opening or closing of stomata but may assist guard cell functioning (Bergmann & Sack, 2007). Stomatal pore aperture is controlled by an osmotic response produced in the guard cells. Increasing osmotic pressure causes increased guard cell volume and stomata to open (guard cell turgid), while a decrease in pressure leads to closure (guard cell flaccid). The property of guard cells to control their own osmotic state is conferred by the presence of ion channels, such as K⁺ and Cl⁻, in the plasma and vacuolar membranes (Pandey *et al.*, 2007).

Guard cells are continuously sensing and integrating endogenous and environmental signals in order to control their turgor pressure in a proper manner. A wide range of environmental stimuli contribute to stomatal aperture, such as light, darkness, temperature, humidity, CO₂ concentration, abiotic and biotic stresses (Schroeder *et al.*, 2001; Melotto *et al.*, 2006; Agurla *et al.*, 2018). Among endogenous signals, ABA is the main inductor of stomatal closure in plant, but other phytohormones such as auxin, cytokinin, ethylene, brassinosteroids, jasmonates, and salicylic acid have a role in stomatal activity (Kim *et al.*, 2010). Through stomatal pore aperture regulation plants acclimate abiotic stresses, prevent pathogen infection or maximize photosynthesis for optimal growth conditions. Thus, regulation of stomatal aperture strongly depends on the adaptation of plants to their environment (Kim *et al.*, 2010).

3.2 ABA-induced stomatal closure under abiotic and biotic stresses

Temperate and cold region plants close their stomata at dusk to avoid water loss during the night. Moreover, stomatal closure represents also a protective mechanism in response to abiotic and biotic stress in plant. Indeed, drought, elevated CO₂ and ozone

levels, pathogen attack or low temperature induce stomatal closure. ABA is the main inductor of stomatal closure which is produced under drought as well as cold (Agurla et al., 2018). High [CO₂] also induces stomatal closure and requires ABA and ABA signaling (Chater et al., 2015). Moreover, ABA signaling is also required for stomatal closure under pathogen attack (Melotto et al., 2006).

The presence of ABA triggers the efflux of anions and K⁺ through plasma membrane ion channels, resulting in water efflux and stomatal closure. ABA-induced stomatal closure is mediated by many signalling components like cytoplasmic pH, reactive oxygen species (ROS), nitric oxide (NO), cytosolic Ca²⁺, G-proteins, protein kinases, protein phosphatases, phospholipases, and sphingolipids (Agurla et al., 2018). ABA-signaling starts with the ABA binding to its receptor PYRABACTIN RESISTANCE /PYR1 LIKE/REGULATORY COMPONENT OF ABA RECEPTOR (PYR/PYL/ RCAR) inducing a conformational change in the core receptor complex. In this conformation, the ABA-receptor complex inhibits the type 2C protein phosphatases (PP2Cs) activity to trigger ABA downstream responses into the guard cells (Fig. 7). PP2Cs inhibition by PYR/PYL/RCAR-ABA complex removes the suppression on OPEN STOMATA 1 kinase (OST1/SnRK2), which in turn phosphorylates and activates the SLOW ANION CHANNEL ASSOCIATED 1 (SLAC1) anion channel and the RESPIRATORY BURST OXIDASE HOMOLOGUE D/F NADPH oxidases (AtRBOHD/F) to trigger anion efflux and ROS burst (Sirichandra et al., 2009; Geiger et al., 2009; Lee et al., 2009; Fig. 7). ABA induces ROS burst in the apoplast, via AtRBOHD and AtRBOHF, which in turn activate Ca²⁺ channels in the plasma membrane of guard cells generating Ca²⁺ influx (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). Ca²⁺ binds to EF hand motifs of RBOHD protein which in turn induces conformational change in the EF-hand region and ROS production (Ogasawara et al., 2008) resulting in a positive feedback loop (Fig. 7). The intracellular transient increase of Ca²⁺ elicits CALCIUM-DEPENDENT PROTEIN KINASES (CDPKs or CPKs), which contribute to activate SLAC channels leading to Cl⁻ efflux, the decrease of turgor and stomatal closure (Fig. 7; Kim et al., 2010).

In addition to ROS and Ca²⁺, NO is an important second messenger in ABA-induced stomatal closure (Gayatri et al., 2013). The generation of NO in guard cells is attributed to the activity of nitrate reductase (NR) which reduces nitrate to nitrite and then to NO, using NADPH as reductive power (Baudouin, 2011). The increased NO levels in guard cells modulates the activity of phospholipases, such as PLDα1 and PLDδ, which in turn generate phosphatidic acid (PA). PA contributes to stomatal closure by inhibiting

K^+ channels and by promoting the generation of ROS by RBOHD/F proteins (Zhang et al., 2009; Distéfano et al., 2012; Uraji et al., 2012; Fig. 7). Moreover, the group of ETHYLENE RESPONSE FACTORS type VII are transcription factors acting as sensors of oxygen. In presence of NO and O₂, ERFs VII are targeted for destruction through the N-end rule pathway in Arabidopsis (Licausi et al., 2011; Gibbs et al., 2011). The degradation or stability of ERFs VII has been implicated in several developmental and physiological responses in Arabidopsis (Gibbs et al., 2014). Among them, ERFs VII degradation by NO accumulation induced stomatal closure (Gibbs et al., 2014).

A functional role in stomatal closure has been attributed to sucrose. Addition of exogenous sucrose induces stomatal closure in several plant species compared with the osmotic control, suggesting its role as a signalling molecule in the regulation of stomatal closure (Kottapalli et al., 2018). How sucrose induce stomatal closure is not clear yet. A possible mechanism involves sucrose breakdown, which involves invertases (INVs), and subsequent phosphorylation of hexose by the HEXOKINASE (HXK). Then, sucrose-derived hexoses are converted in glucose-6-phosphate to be transported to the chloroplast for starch synthesis resulting in an osmotic response. (Daloso et al., 2016a; Fig. 7). Another experimental evidence suggests that addition of exogenous sucrose induce NO production and ABA signalling which is repressed in the presence of HXK inhibitor (Kelly et al., 2013).

Figure 7. ABA-mediated stomatal closure under abiotic and biotic stresses.

Schematic representation showing main cellular and molecular events occurring during ABA-induced stomatal closure. Arrows and T-bar represent positive and negative regulation, respectively. The P in the yellow circles indicates phosphorylation. Stars represents hexoses generated by sucrose breakdown.

The role to transcriptional regulation in stomatal closure has been reported mainly in the control of ABA signalling (Cominelli et al., 2010). Several MYB transcription factors, such as MYB61, MYB44 and MYB15, have been identified to promote stomatal closure (Liang et al., 2005; Jung et al., 2008; Ding et al., 2009).

As it was mentioned before, stomata represent also a barrier against pathogen attack. Thus, stomatal closure is induced after perception of pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) by plasma membrane receptors (Melotto et al., 2006). Part of the components involved in the induced-stomatal closure in response to abiotic stress also participate in the pathogen-induced stomatal closure, such as ABA, OST1, the production of ROS via RBOHD/F, NO and Ca^{2+} as secondary messengers, the heterotrimeric G protein and the regulation of K^{+}_{in} channels (Melotto et al., 2017). Besides shared signalling components, bacterium- and MAMP-triggered stomatal closure also requires plant hormone salicylic acid (SA), its receptor NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1 (NPR1) and mitogen-activated protein kinases (MPKs) which contribute to the phosphorylation cascade required for the activation of anion channels (Melotto et al., 2017). Very recently, NPR1 has been demonstrated to play an essential role in cold acclimation by regulating cold-induced gene expression independently of SA in Arabidopsis (Olate et al., 2018). These findings reinforce the idea that abiotic and biotic stresses converge in common molecular sets required for plant responses to diverse environmental challenges.

3.3 Light-induced stomatal opening

At dawn, stomatal opening enhances photosynthetic CO_2 fixation by removing stomatal limitation for CO_2 uptake. After perception of both red and blue light, the accumulation of K^{+} into the guard cell generates the decrease of water potential, water uptake and consequently increased guard cell volume resulting in the opening of stomata (Talbot & Zeiger, 1998). Perception of blue light induces hyperpolarization of the guard cell plasma membrane by the H^{+} -ATPase-dependent extrusion of H^{+} from guard cells to apoplast. The genetic evidence supporting the role of H^{+} -ATPases in stomatal opening

was provided by the *ost2* mutants, which codifies for the OPEN STOMATA 2 (OST2), an H⁺-ATPase. These mutations produce constitutively activated H⁺-ATPases, constant stomatal opening, and ABA insensitivity. Membrane hyperpolarization activates inward-rectifying K⁺_{in} channels and induces K⁺ influx together with the counteranions Cl⁻, nitrate, and malate²⁻. Among guard cell K⁺ channels, POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1) is the major responsible of K⁺ influx (Kwak et al., 2001).

Phototropin 1 and 2 (PHOT1 and PHOT2), together with cryptochrome 1 and 2 (CRY1 and CRY2) have been identified as blue-light receptors in Arabidopsis guard cells controlling stomatal opening (Fig. 8). *phot1 phot2* double mutant did not open stomata in response to blue light (Kinoshita et al., 2001). Moreover, the *cry1 cry2 phot1 phot2* quadruple mutant showed higher stomata closure respect to *phot1 phot2* double mutant under blue light treatment, suggesting that CRYs function synergistically with PHOTs to promote stomatal opening in response to blue light (Mao et al., 2005). Stomatal opening was resembled in *phot1 phot2 cop1* and *cry1 cry2 cop1* triple mutants, indicating that CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) is a repressor of stomatal opening acting downstream PHOTs and CRYs signalling (Mao et al., 2005).

Blue light induces autophosphorylation of two serine residues in the kinase activation-loop of phototropin molecules (Inoue et al., 2008). The phosphorylated phototropins directly phosphorylate another protein kinase BLUE LIGHT SIGNALING1 (BLUS1) which together with BLUE LIGHT-DEPENDENT H⁺-ATPASE PHOSPHORYLATION (BHP) transmits the signal to type 1 protein phosphatase (PP1) and its regulatory subunit PRSL1 (Takemiya et al., 2006, 2013a, 2013b; Takemiya and Shimazaki, 2016; Hayashi et al., 2017). The signal generated by BLUS1 finally activates the H⁺-ATPase OST2 in guard cells through phosphorylation of a penultimate threonine in the C terminus with subsequent binding of a 14-3-3 protein (Shimazaki et al., 2007; Hayashi et al., 2011; Yamauchi et al., 2016; Fig. 8).

Regarding red light-dependent stomatal opening, it seems to work synergistically with blue light to promote stomatal opening by stimulating photosynthesis and reducing internal CO₂ concentration. Consequently, ATP and NADH are generated and promote H⁺ pumping and malate²⁻ formation needed as a necessary counterion for K⁺, respectively (Shimazaki et al., 2007; Fig. 8).

In the phosphorylation cascade induced by blue light perception that leads to the H⁺-ATPase activation, it has been reported the participation of Ca²⁺ (Shimazaki et al.,

1999). These results appear to be in conflict with those that demonstrate Ca^{2+} as a second messenger in ABA-induced stomatal closure (Schroeder et al., 2001). It has been demonstrated that ABA and high concentration of external Ca^{2+} causes the oscillation of cytosolic concentration of Ca^{2+} with a defined range of frequency, duration and amplitude (Allen et al., 2001). If cytosolic Ca^{2+} increased continuously without oscillation, or the intervals between oscillations were too short or too long, the Ca^{2+} response did not cause sustained stomatal closure, indicating that such oscillations were required for the maintenance of stomatal closure (Allen et al., 2001). This non-oscillatory increase of Ca^{2+} might encode information for stomatal opening (Shimazaki et al., 2007).

A similar contradictory story has been reported for ROS function in stomatal movement. As we see above, ROS are generated by RBOHD/F downstream ABA signaling and they contribute to Ca^{2+} release for stomatal closure. In the study carried out by Shang and colleagues, it was demonstrated that RBOHD/F-dependent ROS burst, Ca^{2+} influx and H^+ efflux was required in extracellular ATP-induced stomatal opening. Moreover, ROS showed a double effect on stomatal movement, promoting stomatal opening at a low concentration and stomatal closure at a high concentration (Hao et al., 2012). Thus, the same signal, such as Ca^{2+} and ROS, could lead to opposite physiological responses which probably depend on the nature of upstream stimuli, their concentration or oscillation in the cytosol and the cross-talk between various combination of downstream signals.

In addition to Ca^{2+} and ROS, sucrose has also a double effect on the regulation of stomatal pore aperture. Transgenic plants overexpressing enzymes involved in sucrose synthesis displayed higher stomatal conductance without the accumulation of the products of its degradation, glucose and fructose, indicating that the role of sucrose in stomatal opening is primarily energetic and not osmotic (Daloso et al., 2016b). Starch and sucrose breakdown may help the light-induced stomatal opening providing an energetic source during dark-to-light transition. Recently, it has been showed that blue light-dependent starch degradation in guard cells improves stomatal opening (Horrer et al., 2016). However, as we described above, addition of exogenous sucrose induces stomatal closure. A possible explanation about these contradictory results may be related with the energetic state of the plant cell. Mesophyll-derived sucrose enters into the guard cells and is degraded into hexoses which are then phosphorylated by HXK inducing stomatal opening or closure, depending probably on the photosynthetic rate of the mesophyll and sucrose concentration in guard cells (Daloso et al., 2016a; Fig. 8).

Figure 8. Light-dependent stomatal opening.

Schematic representation showing the main cellular and molecular events occurring during light-dependent stomatal opening. Arrows positive regulation. The P in the yellow circles indicates phosphorylation. Stars represents hexoses generated by sucrose breakdown.

As reported for stomatal closure regulation, transcriptional regulation contributes also to the regulation of stomatal opening. In *Arabidopsis*, *MYB60* and *ERF7* transcription factors have been described to promote stomatal opening (Cominelli et al., 2005; Song et al., 2005). Moreover, the signaling components of the photoperiodic flowering and circadian clock genes including *GI*, *CO*, *ELF3*, *FT*, *CCA1*, *TWIN SISTER OF FT (TSF)*, and *SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)* control light-induced stomatal opening, but the molecular mechanisms undergo this regulation are still poorly understood (Kinoshita et al., 2011; Ando et al., 2013; Kimura et al., 2015; Hassidim et al., 2017). Taken together, these transcriptional regulations contribute to enhance stomatal opening.

As we have shown in this introduction, the coordination of growth and development with seasonal environmental changes is critical for the survival of trees adapted to temperate and boreal regions. How trees integrate seasonal information at molecular level depends of unknown factors sensitive to photoperiod and temperature. In perennials, habitat adaptation determines several economically important traits, such as tree annual growth rate and fruit development. In poplar, a tree model system, environmental control of growth-dormancy cycles determines annual growth rate and guarantee survival and sexual reproduction. Understanding the molecular mechanisms underlying growth-dormancy cycles envisages the design of strategies for tree adaptation and acclimation to climate change. In the last years, several important works have shed light on the regulation of the timing of growth-dormancy cycles (Ramos et al., 2005; Tylewicz et al., 2015; Tylewicz et al., 2018; Singh et al., 2018). However, molecular mechanisms controlling poplar acclimation during autumnal season remain poorly investigated. In this sense, the link between poplar autumnal acclimation and stomatal regulation has not yet been identified.

Members of RELATED OF ABI3 AND VP1 (RAV) family are transcription factors have been shown to have a versatile function associated to regulation of plant biotic and abiotic stress as well as seasonal development (Gutiérrez et al., 2002; Fowler et al., 2005; Lee et al., 2005; Sohn et al., 2006; Kagaya & Hattori, 2009; Li et al., 2011; Woo et al., 2010; Zhao et al., 2008, Castillejo & Pelaz, 2008, Moreno-Cortés et al., 2012; Moreno-Cortés et al., 2017). Our working hypothesis is that environmentally sensitive molecular switches, such as RAV proteins, contribute to poplar phenology. In this thesis, we investigated the role of poplar ortholog of TEMPRANILLO floral repressor, RAV1 transcription factor, and its transcriptional gene network during transition from growth to dormancy in poplar.

Figure 9. Environmentally sensitive molecular switches drive poplar phenology.

Explanatory scheme that illustrates the working hypothesis underlying this thesis. Environmental seasonal and daily variations must be perceived and integrated by the expression/activity of molecular switches (in red) which in turn modulate physiological and developmental processes.

OBJECTIVES

1. Analyse the role of RAV1 as repressor of *FT2* and photoperiodic shoot apical growth. Predict new environmental sensitive potential regulators of shoot apical growth and bud set in poplar.
2. Investigate the implication of RAV1 during the transition from growth to dormancy in poplar.
3. Shed light on the transcriptional regulation of *RbohD* in poplar and Arabidopsis.

MATERIALS AND METHODS

Plant material and growth conditions

Hybrid poplar *Populus tremula x alba* 717-1B4 was used as the WT control and to generate transgenic plants. Plants were grown at 21°C under long days, LD, (16 hours of light, 8 hours of darkness; 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity) or short days, SD, (8 hours of light, 16 hours of darkness; 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity) at 21°C and 15°C. Transplanted plants in 15 cm² pots were fertilized (Peters Professional allrounder 20-20-20+TE plus WELGRO MICROMIX, 1g*L⁻¹ of H₂O) only under LD conditions. 4-week-old *in vitro* grown poplars were used for gene expression analysis, RNA-seq experiments, stomatal closure and opening assays and H₂O₂ measurements. For phenological and physiological analysis, 4-week-old *in vitro* grown poplars were transplanted in 15cm² pots and grown under LD for 3 weeks. When plant height was *c.a.* 20 cm, they were moved to SD conditions at 21°C or 15°C.

Arabidopsis thaliana accession Col-0 and the reporter line *pRbohD::LUC* derived from it were grown on a soil-vermiculite 3:1 mixture under a 10:14 hours day/night photoperiod, with a daytime temperature of 24°C and a night temperature of 22°C, under relative humidity of 65 % and light intensity of 120 $\mu\text{E m}^{-2} \text{sec}^{-1}$.

Wild-type Col-0 plants were transformed with the *p35S::CsRAVI::tNOS* (Moreno-Cortés et al., 2012) construct by the standard floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MS agar plates with 50 $\mu\text{g ml}^{-1}$ kanamycin and homozygous plants were obtained from lines with single insertion events. The presence of the transgene was analysed by qRT-PCR.

Generation of vector constructs and transformation

The RAV1 coding sequence (CDS; Potri.010G129200) was amplified from *Populus tremula x alba* cDNA using specific primers (Appendix – Supplementary Table 1). For PCR, we used Pfu Ultra Hotstart High-Fidelity DNA Polymerase (Agilent) and the PCR fragments were purified and cloned into pCR-Zero-Blunt vector (Invitrogen).

To generate *p35::GR:EGFP:RAVI::tNOS* construct, firstly, GR sequence including StuI site at 5' end was amplified by PCR, gel purified and ligated into pCR_Blunt cloning vector to generate the construct pCRII_blunt-GR. Similarly, pCRII_blunt-EGFP construct carrying 5' StuI and 3' NotI restriction sites was cloned, as well as pCRII_blunt-RAVI construct carrying 5' NotI and 3' XbaI restriction sites, respectively. Next, the

pCRII_blunt-GR and pCRII_blunt-EGFP construct were digested with StuI and NotI restriction enzymes, gel purified and ligated to make a pCRII_blunt-GR:EGFP fusion construct into the cloning vector. Finally, the pCRII_blunt-GR:EGFP and pCRII_blunt-RAV1 construct were digested using NotI and XbaI restriction enzymes, respectively. The products of restriction reaction were gel purified and ligated to generate pCRII_blunt-GR:EGFP:RAV1 cloning vector, which was used as a template for amplifying the construct with Gateway tails. The fragment amplified was then cloned into a pDONOR207 Gateway vector. This vector was used to generate plant expression pGWB2 vector with the Gateway cloning system (Nakagawa et al., 2007). To generate the *p35S::3xHA:RAV1::tNOS* construct, the *RAV1* CDS was cloned into the pENTR222. This vector was used to generate plant expression pGWB15 vector with the Gateway cloning system (Nakagawa et al., 2007). The poplar leaf explants were transformed via an Agrobacterium-mediated protocol previously described (Gallardo et al., 1999). pGWB2-*p35S::GR:EGFP:RAV1::tNOS* and pGWB15-*p35S::3xHA:RAV1::tNOS* vectors were used to generate stable poplar transgenic lines via callus regeneration in medium with kanamycin. Finally, the shoots resulting after shoot induction treatment were transferred to propagation medium with kanamycin and cefotaxime until the seedlings regenerated. Rooted seedlings were selected and characterized as independent transgenic line.

For transactivation assays, we used GoldenBraid (GB) technology (Sarrion-Perdigones et al., 2011) to generate an overexpression construct. RAV1 CDS was amplified using primers pUPD from *Populus tremula x alba* cDNA samples. Fragments were purified and cloned in a pUPD vector. Then, we generated a complete transcriptional unit using 35S promoter, the coding sequence of RAV1, the tNOS terminator and YFP fused to de C-terminus, in alpha expression vector (pGreen derived vector).

Construct	Plasmid	Selection Marker	Cloning Technique	Observation
p35S::GR:EGFP:RAV1	pGWB2	Kan	Gateway	
p35S::RAV1	pGWB15	Kan	Gateway	
p35S::hpRAV1	pHELLSGATE12	Kan	Gateway	Moreno–Cortes et al., 2012
p35S::CsRAV1	pGWB15	Kan	Gateway	Moreno–Cortes et al., 2012
p35S::RAV1:YFP	pGreen	Kan	GoldenBraid	Trionzi et al., 2018
p35S::YFP	pGreen	Kan	GoldenBraid	Trionzi et al., 2018
pRbohD::LUC	pLUCTRAP–3	Kan	Gateway	Morales et al. 2016

Table 1. Construct used in this thesis.

Electrophoretic mobility shift assay (EMSA)

RAV1 CDS was cloned into the gateway-adapted MBP vector (Jurado et al., 2010; New England Biolabs) by LR recombination to obtain the maltose binding protein fused to N-terminus of RAV1 (MBP:RAV1). *E.coli* B21 cells were transformed with the pMAL-c2-MBP:RAV1 vector, grown until OD 0.6 and induced with 1 mM Isopropyl β -D-thiogalactoside (IPTG). Protein extraction was carried out using a protein extraction buffer (20 mM Tris-HCl pH 7.5, 0.2 M NaCl, 1 mM EDTA) and protein was purified on amylose resin (E8021S, New England Biolabs). DNA fragments containing the predicted RAV1 binding sites in the *ERF72* and *RbohD* promoter regions were designed (30 bp) in the forward and reverse directions, respectively, and synthesized. Double-stranded probes were prepared by annealing forward and reverse DNA fragments. The EMSA assay was performed in Binding buffer (10 mM Tris-HCl pH 8, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 16% glycerol) at room temperature for 30 min. Increasing volumes of MBP:RAV1 purified protein were used to test the binding to *RbohD1* and *ERF72-1* promoters. Once the optimal concentration of binding was established, this concentration was used to test the loss of binding affinity to the *RbohD1* mutated probe. The DNA/protein complex samples were loaded onto 6% polyacrylamide native gels (Bio-Rad) and run at 100 V for 1 h. The DNA in the gel was stained using SYBR® Safe Stain (Invitrogen) and visualized using an Imager Gel Doc XR system (Bio-Rad). The DNA probes used for EMSA are listed in Table S1.

Protein extraction and Western blot

To analyze the RAV1 protein annual pattern, 2-year-old poplar branches were sampled during the year in the field. Protein purification and detection were performed following the protocol described in Moreno-Cortés et al., 2017.

Confocal Microscopy

To test the reliability of the GR system in poplar, we performed a DEX treatment (Tween20 0,05%, 30 μ M dexamethasone) compared to MOCK treatment (Tween20 0,05%, ethanol) in poplar root tissue. Roots from *in vitro* plantlets were submerged in DEX or control solution for 4 hours. After that, root samples were excised and prepared on an object slide. These root samples were analyzed and pictures taken using a Leica

TCS-SP8 confocal microscope with an excitation of 488 nm. Gain and offset conditions were maintained in all captures for comparison.

Gene expression analysis by quantitative RT-PCR

4-week-old *in vitro* transgenic and WT plantlets were grown under LD and then transferred to the LD or SD conditions for 1 week. For daily gene expression analysis, leaves from three different individuals were collected every 3 hours for the 24h-time course experiment. For transgene expression analysis, leaves were collected at one time point from three different individuals. Plant material was ground to powder in liquid nitrogen. RNA extraction was performed using the Nucleospin RNA Plant Kit (Machery-Nagel) according to the manufacturer's instructions. cDNA synthesis was carried out using Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermoscientific) according to the manufacturer's instructions. qRT-PCR analyses were carried out with a Roche LightCycler 480 instrument.

Microarray analysis

We analyzed the global gene expression in two-year-old branches tissue during 12 months in field conditions (Madrid 2007, 28040, 40°27'02.2"N 3°43'21.8"W 40.450611, -3.722717). Two sets of independent target mRNAs were prepared from branches of WT *Populus alba* as previously described (Moreno-Cortés et al., 2012). GeneChip™ Poplar Genome Array was used for the analysis (*Populus trichocarpa*, v1.1). Details of the microarray hybridization experiment and the complete dataset are available at Gene Expression Omnibus at NCBI under GEO accession number GSE126760.

RNA sequencing analysis

WT, *p35S::RAV1::tNOS* and *p35S::GR:EGFP:RAV1::tNOS* plants were used to carry out RNA sequencing (RNA-seq) experiments. 4-week-old *in-vitro* plantlets were transferred to SD for 10 days and the shoot apical part from 10 independent plantlets were collected after 4 hours of darkness (ZT 12). In the case of the GR system, plants were sprayed with a MOCK solution (Tween20 0,05%, ethanol, 50 µM cycloheximide) or a

DEX solution (Tween20 0,05%, 30 μ M dexamethasone, 50 μ M cycloheximide) at ZT8 and after 4 hours the shoot apical part from 10 independent plantlets were collected. Collected material was ground in liquid nitrogen and the powder was used for RNA extraction as described above. The quality of RNA samples was assaying using a bioanalyzer (Agilent). For library preparation, we used 1 μ g of RNA in the NEBNext® UltraTM Directional RNA Library Prep Kit following the supplier's instructions (New England Biolabs, Ipswich, MA, USA). Sequencing was done with an Illumina NextSeq500 in high-throughput mode at the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL, USA). After Illumina next generation sequencing, adapters were removed from raw data using Trimmomatic (Bolger et al., 2014). Sequences were mapped to the *Populus tremula x alba* 717-1B4 genome using STAR (Dobin et al., 2013). Reads counting was executed using HTseq software (Anders et al., 2015). For identifying differentially expressed genes (DEGs) analysis we used the DESeq2 package (Love et al., 2014) with R software. Three biological replicates were used for these analyses for each genotype or treatment using a P adjusted value <0.1. Details of the RNA-seq experiments and the complete dataset are available at Gene Expression Omnibus at NCBI under GEO accession number GSE126764.

Phylogenetic analysis

ERF type VII transcription factors and RBOH protein sequences were obtained from the *Arabidopsis thaliana* genome using the TAIR10 website (www.arabidopsis.org). These sequences were used as queries to BLASTP against *Populus trichocarpa* genome using Phytozome12 database. We identified the most similar hits to perform the phylogenetic analysis. Once we selected the Poplar ID (Potri) of the ERFs and RBOHs, we identified the protein sequences in the *Populus tremula x alba* genome (<http://aspendb.uga.edu>). We used protein sequences to do phylogenetic trees. We used MUSCLE algorithm to do the alignments (100 iterations) and Maximum Likelihood to infer phylogenetic trees. We firstly calculated the best evolutionary model of the alignments (ProtTest software). To carry out this work we used MEGA7 application (Kumar et al., 2016).

Induction of bud set and growth cessation

WT and *RAV1kd* and *RAV1oe* plants were grown 3 weeks under LD conditions and then shifted to SD at 21°C or SD at 15°C for 6 weeks. The stage of bud formation was scored as previously described (Rohde et al., 2002). Growth cessation was monitored by recording height in different individuals per genotype every week.

Physiological and biomass measurements

In the youngest fully expanded leaves, we measured chlorophyll content (SPAD-50s Meter), stomatal conductance (SC-1 Leaf Porometer; Decagon Devices) and net photosynthesis and transpiration rate (LICOR 6400). Photosynthetic determinations were performed with a 6 cm² leaf area chamber making the measurements at 60% of humidity and 400 ppm CO₂. Then, the leaf was excised, fresh weight (FW) immediately recorded and leaf water potential (Ψ_w) was determined using a Scholander pressure chamber following the procedure described by (Scholander et al., 1965). Afterwards, the leaves were dried at 80°C for 48 hours and dry weight (DW) determined. Water content was calculated as $[(FW - DW)/FW] \times 100$. Total biomass of the aerial part of the plant was calculated by measuring DW of stem and leaves after 6 weeks of SD at 15°C. To determine progressive water loss from detached leaves, leaves incubated at 25°C and 60% RH were weighed every 30 min for 4 hours.

Stomatal movement assays and guard cell H₂O₂ measurement

For stomatal closure assays, we adapted the protocol from (Kwak et al., 2003). Leaf discs were cut from fully expanded leaves and placed with the abaxial side down in a petri plate with “opening buffer” (50 mM KCl, 10 mM CaCl₂, Tween 0,01%, 10 mM MES-KOH, pH 6,15). Then, leaf discs were placed back into the growth chamber for three hours. After this time, 5 μ M of ABA was added to induce stomatal closure. After 2 hours, leaf discs were placed on microscope slides and were visualized with a microscope with bright-field optics (40X objective). For stomatal opening assay, we adapted a protocol from (Allen et al., 1999). Leaf discs were cut from fully expanded leaves in darkness and stomatal aperture was recorded. Then, leaf discs were placed with the abaxial side down in a petri plate with opening buffer (50 mM KCl, 10 mM KOH, 10 mM MES-KOH, pH

6.15) in the light. After 2 hours, stomatal aperture was measured again. Stomatal aperture was calculated as width/length of guard cell pores using ImageJ software.

H₂O₂ production in guard cells was analyzed with 2,7-dichlorofluorescein diacetate (H₂DCF-DA) (Sigma-Aldrich). Leaf discs from 4-week old poplars were incubated in buffer containing 10 mM MES-KOH (pH 6.15), 50 mM KCl, and 10 mM CaCl₂. After 3 hours, 30 μ M H₂DCF-DA was added to the buffer. After another 20 min, the leaf discs were washed three times with MES-KOH buffer to remove the excess dye. ABA (50 μ M) was added to the buffer for 20 min, and then the fluorescence of guard cells was detected using a Leica TCS-SP8 confocal microscope with an excitation of 488 nm and emission of 500/550 nm. Fluorescence intensity in guard cells (n>100) was measured using ImageJ software and calculated as Corrected Total Cell Fluorescence (CTCF; Hansen et al., 2017). Fluorescence intensities for each treatment and genotype were normalized to mock-treated WT and represented as percentage of CTCF.

Transactivation assays

Transient poplar transformation was performed following the protocol reported previously (Takata and Eriksson, 2012; Ramos-Sánchez et al., 2017). Poplar leaf agroinfiltrated with the constructs *p35S::YFP::tNOS* and *p35S::RAVI::YFP::tNOS* were identified by GFP fluorescence after 3 days post-infiltration. Leaf area showing GFP fluorescence were harvested and frozen in liquid nitrogen and total RNA was extracted to perform qRT-PCR analysis as described above.

Primers

The primers used in this study are listed in Table S2 (Appendix).

Luciferase reporter assays

To perform continue monitoring luciferase activity, we used leaves tissue from 4 weeks old plants and 2-week-old seedlings carrying the *pRbohD::LUC* transcriptional reporter (Morales et al., 2016). The leaf discs were done using a leaf disc cutter leaving a total area of 30 mm² approximately. After this, they were left in a 96-well plate with Murashige and Skoog 1B with vitamins (MS1B, Duchefa Biochemie) medium (pH 5.7) with or

without sucrose. Seedlings were grown into the 96-well plate with MS1B supplemented with 1% of sucrose (~30mM). Before assays, MS1B + 1% sucrose medium was removed, seedlings washed and replaced with the proper medium. Fifty microlitres of D-Luciferin 1mM were added into every well. A qPCR film was used to cover the plate, making three holes in every well to avoid condensation with a syringe needle. The TriStar2 LB 942 luminometer (Berthold Technologies, Bad Wildbad, Germany) was used to register the luciferase activity measuring 5 second per well every 2h.

H₂O₂ production measurement

5 mm diameter leaf discs were collected from fully expanded leaves from 4-week-old plants and washed over night with 150 ml of water. For H₂O₂ measurement, water was exchanged for a solution containing 10 µg/ml horseradish peroxidase (Sigma-Aldrich) and 100 µM luminol (SIGMA). H₂O₂ production was triggered with 500 nM flg22. Luminescence was measured as relative light units with a TECAN Genius Pro luminescence reader over a period of 40 minutes. All H₂O₂ measurements were repeated three times with similar results.

Statistical analysis

For testing homogeneity of variance and normal distribution, we used Levene's test and Shapiro-Wilk test, respectively. In case of pairwise comparisons, two tails Student's *t*-test was performed. For multiple comparisons, Kruskal-Wallis test followed by a pairwise Wilcox test (Benjamini-Hochberg method) were calculated. P-values<0.05 were considered as significant. We did plots and statistical analysis using (RStudio Team, 2016). To infer the period and the relative amplitude error we use Spectrum Resampling application (Costa et al., 2013).

RESULTS

Chapter 1

A brief introduction

In perennial plants from temperate and boreal regions, such as poplar, growth-dormancy cycles determine annual growth rate and guarantee survival over winter. The control of growth to dormancy transition in poplar shows similarities to Arabidopsis flowering. Several poplar orthologs to Arabidopsis flowering regulators have been implicated in the regulation of poplar growth-dormancy transition pointing to a conserved molecular framework. In this chapter, we tested the function of poplar ortholog of TEMPRANILLO floral repressor, RAV1 transcription factor, as a photoperiodic regulator of shoot apical growth. Moreover, based on Genome Wide Association Studies (GWAS) and diurnal patterns of gene expression obtained from public databases, we identified candidate genes for photoperiodic control of shoot apical growth in poplar. In addition to this, we identified genes sensitive to temperature variations which could be involved in the thermoregulation of growth to dormancy transition.

Poplar orthologs of CONSTANS and TEMPRANILLO show diverged features in the regulation of *FT* between poplar and Arabidopsis

The gene *FT* shows a conserved role as photoperiodic integrator of Arabidopsis flowering and poplar shoot apical growth. The expression analysis of poplar *FT2* gene for 48 hours showed a robust diurnal gene expression pattern under LDs with a maximal mRNA accumulation at the end of the day (Fig. 10A; Böhlenius et al., 2006). The *FT2* diurnal expression pattern is conserved between poplar and Arabidopsis as well as its role as the main photoperiodic integrator (Fig. 10B; Böhlenius et al., 2006; Krzymuski et al., 2015).

Figure 10. Temporal expression patterns of *FT*, *CO* and *TEM* in poplar and Arabidopsis under LDs.

(A) Time course experiment showing the relative *FT2* mRNA expression detected by qRT-PCR during 48 hours in poplar under LD (B) mRNA expression pattern of Arabidopsis *FT*, *CO* and *TEM* under LD. Values of gene expression have been taken from data already published (Suárez-López et al., 2001; Castillejo & Pelaz, 2008; Krzymuski et al., 2015). (C, D) Time course experiment showing the relative *CO1* (C) and *RAV1* (D) mRNA expression detected by qRT-PCR during 48 hours in wild-type leaves poplar under LD. Time is expressed as Zeitgeber time (ZT), being ZT0 when light turned on. Dark panels represent night duration. Values represent the mean ± SD of three technical replicates.

As we described in the introduction, CO protein is the major activator of *FT* transcription and flowering in Arabidopsis. *CO* mRNA accumulates before *FT* gene expression at the end of the day in Arabidopsis (Fig. 10B; Suárez-López et al., 2001). Moreover, diurnal expression pattern of poplar *CO1*, which overlaps with poplar *CO2*, showed an anti-phase temporal pattern relative to *FT2* (Fig. 10C; Triozzi et al., 2018). Although CO play a conserved function as photoperiodic activator of *FT* (Böhlenius et al., 2006), this expression analysis shows that Arabidopsis and poplar CO have different diurnal expression patterns suggesting a different regulation of *FT* in these plant species.

On the other hand, *TEM* genes have been shown to represses *FT* and flowering in Arabidopsis (Castillejo & Pelaz, 2008). Previous work of our laboratory identified poplar and chestnut RAV1 transcription factors, orthologs of TEM (Moreno-Cortés et al., 2012). To analyse *RAVI* diurnal regulation, we performed 24h time course gene expression studies of poplar *RAVI* under LD conditions. qRT-PCR expression revealed a different diurnal pattern of *RAVI* as it was reported for Arabidopsis *TEM1* (Fig. 10B and D). Poplar *RAVI* shows two mRNA peaks, the smaller one in the morning and the bigger one during the night, on the other hand, *TEM1* shows only a night peak under this condition (Fig. 10D). This expression pattern does not support a role of RAV1 as a repressor of *FT2* as it was shown for Arabidopsis *TEM1*. To test if RAV1 represses *FT2*, we performed a transactivation assay in poplar leaf tissues. The assay revealed that the overexpression of poplar *RAVI* led to *FT2* activation (Fig. 11A). Moreover, the overexpression of the chestnut *RAVI* (*CsRAVIoe*) in Arabidopsis did not delay flowering compared to WT plants, as it was reported for *TEM* overexpression in Arabidopsis transgenic lines (Fig. 11B and C; Castillejo and Pelaz, 2008; Triozzi et al., 2018). Therefore, this result suggests that *CsRAVI* does not repress *FT*. All together, these studies indicate that poplar and chestnut RAV1 do not conserve the function as repressor of *FT*.

Figure 11. Divergent function of poplar RAV1 respect to TEM1.

(A) *FT2* mRNA expression at ZT15 determined by qRT-PCR performed on poplar leaf samples transiently expressing *p35S::YFP::tNOS* (control) and *p35S::RAV1:YFP::tNOS* (*RAV1:YFPoe*) constructs. Data are represented as the mean ± SEM of three independent experiments. Asterisk indicates significant differences between the *RAV1:YFPoe* construct and control (Student's *t*-test, **P*<0.05). (B) Picture showing the flowering of WT and *p35S::CsRAV1* (*CsRAV1oe*) Arabidopsis plants grown for 5 weeks under LDs. (C) Flowering time of WT and *CsRAV1oe* Arabidopsis plants obtained by counting the total leaf number at bolting transition of plants grown under LDs (*n*=12). Data are represented as the mean ± standard deviation. n.s., not significant (Student's *t*-test, *P*>0.05).

As we described in the introduction, *FT2* is the main photoperiodic integrator that controls poplar shoot apical growth. After perception of SD condition, *FT2* transcription is strongly downregulated representing one of the first molecular signature of poplar growth cessation (Fig. 12A). Poplar *FT2* overexpressing lines, showing higher levels of *FT2* expression under SD conditions, retained full shoot apical growth under SDs (Böhlenius et al., 2006; Hsu et al., 2011). Thus, we investigated *FT2* expression levels in *RAV1* overexpressing lines (*RAV1oe*; Appendix, Fig. S1) compared with WT after 5 days under SDs. We found that WT as well as *RAV1oe* showed a strong downregulation of *FT2* expression compared to the control under LDs, indicating that *RAV1* overexpression do not sustain *FT2* activation under SD (Fig. 12B).

Figure 12. SD-induced downregulation of *FT2* gene expression is dominant on *RAV1oe*-induced *FT2* activation.

(A) Time course experiment showing the relative *FT2* mRNA expression detected by qRT-PCR under LD and after 1 week of SD conditions in wild type poplar. Time is expressed as Zeitgeber time (ZT), being ZT0 when light turned on. Dark panels represent night duration. Values represent the mean $\pm$ SD of three technical replicates. **(B)** Relative *FT2* gene expression obtained by qRT-PCR of WT poplar under LDs and WT, *RAV1oe3* and *RAV1oe4* lines under SDs. Values represent the mean $\pm$ SEM (n=3 biological replicates). Statistical analysis was performed using a Student's *t*-test and asterisks represent statistically differences between the means (***P*<0.01, ****P*<0.001).

This result also suggests that the SD-induced repression of *FT2* transcription has a dominant effect on *RAV1oe*-induced *FT2* activation under LDs, similar effect have been also reported for *CO1* and *CO2* poplar overexpressing lines (Hsu et al., 2012). Even more, poplar overexpressing *CsRAV1* as well as poplar *RAV1* and knock down lines (*RAV1kd*; Moreno-Cortés et al., 2012) did not show differences in the timing of growth cessation and bud set compared with WT plants under SDs at 21°C (Fig. 13A and B). All together these results indicate that poplar and chestnut *RAV1* do not play a role in the photoperiodic regulation of poplar growth cessation and bud set.

Figure 13. *RAV1* does not affect the timing of growth cessation and bud formation in poplar.

(A) Line plot showing a comparative analysis of shoot apical growth under SDs determined by scoring tree height of poplar WT plants poplar lines overexpressing chestnut and poplar *RAVI*. Tree height scores were collected during 5 weeks under SDs at 21°C. Values represent the means $\pm$ SEM (n=6). (B) Box plot showing the distribution of bud stage of poplar WT plants poplar lines overexpressing chestnut and poplar *RAVI* after 5 weeks under SDs at 21°C. Box plots show two biological replicates for each genotype (n=6). Picture of representative bud for each genotype is shown. Statistical analysis was performed using a Kruskal-Wallis test followed by pairwise Wilcox tests (BH method).

Previous results of our laboratory reported that overexpression of *CsRAVI* in poplar increased axillary bud outgrowth under LDs (Moreno-Cortés et al., 2012; Moreno-Cortés et al., 2017). Similarly, we found that poplar *RAVIoe* increased axillary bud outgrowth under LD conditions (Fig. 14A and B). Because FT together with TWIN SISTER OF FT (TSF) promote axillary bud outgrowth in Arabidopsis (Hiraoka et al., 2013) and poplar *RAVI* transient overexpression resulted in *FT2* activation, we checked if the increased shoot branching in *RAVIoe* lines correlates with *FT2* increased expression levels. We found that *RAVIoe* lines showed higher levels of *FT2* gene expression compared to WT plants under LD condition (Fig. 14C). Therefore, high levels of *FT2* positively correlates with increased shoot branching under LDs in poplar.

Figure 14. *RAVI* overexpression increases axillary bud outgrowth and *FT2* gene expression under LD conditions in poplar.

(A) Representative picture of axillary bud outgrowth of poplar WT, *RAVIkd1*, *RAVIoe3* and *oe4* lines under LDs. White arrows indicate primordia of branches. (B) Branching score calculated as number of branches/height. Values represent the mean $\pm$ SEM (n=15). (C) Relative *FT2* gene expression obtained by qRT-PCR from poplar WT, *RAVIkd1*, *RAVIoe3* and *oe4* lines under LDs. Statistical analysis was performed using a Kruskal-Wallis test followed by pairwise Wilcox tests (BH method). Groups of significance are indicated with different letters (P<0.05).

Taken together, these results indicate that regulation and function of the poplar and chestnut RAV1 show diverged and particular features respect to its Arabidopsis ortholog TEM. We find that poplar RAV1 does not play a role as a photoperiodic regulator of shoot apical growth through *FT2* transcriptional regulation under SD conditions. However, *RAV1* overexpression causes high levels of *FT2* expression under LD condition which is associated to the increased poplar shoot branching.

Diurnal gene expression of height-associated SNPs uncovers potential photoperiodic regulators of poplar shoot apical growth

From the studies of the poplar ortholog of CO and TEM flowering regulators, we have learned that protein sequence homology between Arabidopsis flowering regulators and poplar candidates do not account for a conserved gene regulation or function. Thus, we explored poplar orthologs of Arabidopsis flowering genes showing photoperiodic regulation and potential functional implication in shoot apical growth using data obtained from public databases.

Daily cycles of *FT2* gene expression are important to sustain poplar shoot apical growth and repression of *FT2* initiates growth cessation (Böhlenius et al., 2006). Therefore, *FT* expression defines the annual extension of shoot apical growth and tree height. In Arabidopsis, many flowering regulators, activators and repressors, of *FT* transcription show a circadian and/or diurnal rhythmicity in their gene expression which is important to regulate *FT* transcription. Thus, we hypothesized that putative *FT2* regulators should exhibit daily oscillations of gene expression in poplar as well. A recent work in poplar has provided a list of genes associated to important adaptive traits (Evans et al., 2014). These genes show single nucleotide polymorphisms (SNPs) which correlate with phenotypic variations such as height, bud development and bud break.

Thus, to identify putative regulators of *FT2* and poplar shoot apical growth, we examined the diurnal patterns of poplar genes showing height-associated SNPs using the Diurnal database (www.diurnal.mocklerlab.org; Mockler et al., 2007; Filichkin et al., 2011; Evans et al., 2014). The 17% of genes presenting SNPs associated with height trait showed robust diurnal rhythms of transcription (cutoff = 0.8) over a 48h period under photothermocycles (LDHC, 12:12 h light/dark and 25:12 °C day/night; Appendix, Dataset S1) indicating the requirement of diurnal regulation of gene expression for poplar

shoot apical growth. We detected clusters of genes showing all possible diurnal expression patterns, indicating no specific phase (time of the day showing the maximum of gene expression) enrichment in diurnally-controlled genes involved in shoot apical growth (Fig. 15A). Individual clusters were examined for poplar orthologs of genes that have been attributed a regulating role in the transition from vegetative growth to flowering in *Arabidopsis*. This led to the identification of 14 genes, whose altered expression in *Arabidopsis* affects *FT* gene expression and gives rise to a variation in flowering time (Fig. 15B). Among them, we found poplar orthologs to the *Arabidopsis* clock-controlled *GI*, *PRR7*, *FLAVIN-BINDING*, *KELCH REPEAT*, *F-BOX 1 (FKF1)*, and *RVE6* (Park et al., 1999; Song et al., 2012; Gray et al., 2017; Hayama et al., 2017) confirming the role of the circadian clock in the regulation of poplar shoot apical growth (Ibañez et al., 2010; Fig. 15A and B). Phase differences in daily rhythms reflect local environmental constraints, which prompt diversification of molecular function contributing to phenotypic variation in natural populations (de Montaigu and Coupland, 2017). The poplar *GI* phase is delayed only 3 h compared with *Arabidopsis GI* and is synchronous with the daily timing of poplar *FT2* activation (Fig. 15B).

Figure 15. Diurnal oscillations of poplar genes showing height-associated SNPs unravel potential regulators of the photoperiodic control of shoot apical growth.

(A) Clusters of poplar genes showing height-associated SNPs based on the phase of peak expression from the Diurnal database (diurnal.mocklerlab.org). A total of 12 clusters were obtained covering the 24 h of the day and grouping phases every 2 h starting at cluster 1 (including phases 0 and 1). For each cluster, red line shows the total mean and vertical dotted lines indicate the phase of gene expression. The central circadian circle shows the temporal succession of the clusters including identified poplar orthologs of known Arabidopsis flowering time regulators. (B) List of poplar genes orthologous to Arabidopsis flowering time regulators. Arabidopsis mutations in these genes gave rise to altered expression levels of *FT*. Poplar and Arabidopsis expression phases are shown (LDHC, cutoff = 0.8).

Additionally, we have identified 3 poplar CCT (CO, CO-like, and TOC1) domain-containing transcription factors (CO, CO-like, and TOC1) designated *CONSTANT LIKE 13* (*COL13*), *B-BOX DOMAIN PROTEIN 14* (*BBX14*), and *CCT*, showing robust diurnal expression (Fig. 15B). Proteins carrying the CCT domain have been implicated in the photoperiodic regulation of *FT* in Arabidopsis (Robson et al., 2001; Wang et al., 2014;

Sheng et al., 2016). Moreover, the CCT motif has been shown to bind DNA and participate in protein-protein interactions (Wenkel et al., 2006; Gendron et al., 2012). Poplar *COL13* and *CCT* showed a phase similar to *GI* (Fig. 15B). However, it remains to be determined whether they are also involved in the transcriptional activation of *FT2* in poplar. A poplar ortholog of Arabidopsis *CYCLING DOF FACTOR 2* (*CDF2*) was also found within cluster 11 (Fig. 15A). This factor has been shown to transcriptionally repress *CO* and *FT* in Arabidopsis (Fornara et al., 2009; Song et al., 2012; Fig. 15B). A poplar ortholog of *ARABIDOPSIS HOMEBOX 16* (*ATHB16*), which is a member of the HD-ZIP family of plant transcription factors, showed a similar expression phase in both plant species (Fig. 15B). Functional studies have shown that *ATHB16* acts as a negative regulator of flowering time in Arabidopsis (Fig. 15B). Genetics studies have located *ATHB16* downstream of the blue light signalling pathway (Wang et al., 2003). However, it is still not known if this pathway contributes to the control of poplar shoot apical growth. We also identified a poplar ortholog of the Arabidopsis transcription factor *MYB56*, which has been shown to be a negative regulator of photoperiodic flowering time and *FT* expression in Arabidopsis (Chen et al., 2015). Interestingly, this poplar ortholog shares identical diurnal gene expression with the poplar ortholog of *CDF2* and could thus be a negative regulator of poplar *FT2* and growth cessation (Fig. 15B). Contrary to findings in poplar, the diurnal expression of *MYB56* showed no significant rhythmicity in Arabidopsis suggesting its different regulation in trees. Remarkably, poplar orthologs of Arabidopsis *VERNALIZATION INDEPENDENCE 4* and *5* (*VIP4* and *VIP5*), *AGAMOUS-like* (*AG-like*) and *TERMINAL FLOWER 2* (*TFL2*), have also shown robust diurnal rhythms in poplar. However, diurnal variations in Arabidopsis *VIP4*, *VIP5*, *AG-like*, and *TFL2* were found not to be significant, indicating their orthologous poplar genes have acquired robust diurnal expression (Fig. 15B). Arabidopsis *VIP4*, *VIP5*, *AG-like*, and *TFL2* have been attributed roles in flowering time and vernalization through the epigenetic regulation of *FLOWERING LOCUS C* (*FLC*) and *FT*, particularly for *TFL2* (Kotake et al., 2003; He et al., 2004; Oh et al., 2004; Yoo et al., 2011).

These results together with the reported regulators of poplar *FT2* allowed us to propose a hypothetical molecular model underlying poplar shoot apical growth (Fig. 16).

Figure 16. Schematic representation of poplar known and predicted *FT2* regulators.

Continuous lines represent experimentally supported links in poplar. Dashed lines represent experimentally supported links in Arabidopsis.

Temperature modulates the timing of growth to dormancy transition in poplar

The shortening of photoperiod is accompanied by drop of temperature during autumn-winter transition. The effect of temperature on the transition from growth to dormancy has been poorly investigated at the molecular level. Earlier physiological studies have shown that growth cessation and bud set are thermosensitive processes in trees (Tanino et al., 2010). Furthermore, phenotyping studies using clonally replicated poplars grown under natural conditions showed that the timing of bud set depends on the local climatic conditions (Rohde et al., 2011; Evans et al., 2014). To assess the effect of low ambient temperature on the timing of growth to dormancy transition in poplar, we grown poplars (*Populus tremula* x *P. alba*) under LDs and at 21°C for 1 month and then they were transferred to SDs at 21°C, 18°C or 15°C for 9 weeks. We found that poplar is highly sensitive to small changes in ambient temperature (Fig. 17A and B). Bud set was delayed by approximately 7 days in plants grown at 18°C relative to plants grown at 21°C, and this difference was amplified when the temperature was further reduced to 15°C (Fig. 17A and B). This indicates that ambient temperature variations can impact the SD responses modulating the timing of bud set. Thus, it can be hypothesized that the

activation or repression of thermosensitive genes underlies the regulation of pathways that control SD-induced bud set.

As we observed for poplar height, SNPs associated to poplar bud set trait provide candidate genes which could participate in bud formation. Between them, genes showing sensitive diurnal gene expression to temperature variations could also respond to seasonal variations of temperature. Thus, to identify poplar candidate genes that could affect the timing of bud set acting through the temperature signalling, we investigated how daily photo- and thermoscillations affect diurnal expression of genes showing bud set-associated SNPs (Mockler et al., 2007; Filichkin et al., 2011; Evans et al., 2014). Particularly, we analyzed the diurnal oscillations (cutoff= 0.8) of bud set-associated SNP genes in two different conditions, photothermocycles (LDHC) and photocycles (LDHH; 12:12 h light/dark and 25:25 °C day/night; Appendix, Dataset S2), in *Populus thricocarpa* using the Diurnal database (diurnal.mocklerlab.org; Mockler et al., 2007; Filichkin et al., 2011). A total of 134 genes (~9%) associated with bud set showed robust diurnal transcriptional patterns over a 48h period under LDHC conditions but not under constant temperature (LDHH), which suggests that the lower night temperature is necessary for their rhythmic expression (Fig. 17C). Within the clusters of genes affected by photothermal cycles, we identified diurnal expression in the poplar ortholog of Arabidopsis *PHOSPHOLIPASE D DELTA* (*PLDDELTA*; Potri.007G060300), which is involved in phospholipid metabolism, freezing tolerance and stomatal closure (Fig. 17D; Chen et al., 2008a,b; Distéfano et al., 2012; Uraji et al., 2012). Moreover, the poplar ortholog of *CBF4* (Potri.012G134100), which mediates the response to low temperature in Arabidopsis (Wang and Hua, 2009), is also induced by cold in poplar and birch (Fig. 17E; Benedict et al., 2006; Welling and Palva, 2008). These examples indicate that the rhythmic expression of genes associated with bud set is stimulated by diurnal oscillations in temperature. They also suggest that low temperature might control seasonally these genes during autumn-winter transition which may be required for cold acclimation.

In contrast, we identified 172 genes (~12%) with bud set-associated SNPs that showed diurnal transcriptional patterns under constant temperatures (LDHH) but not under photothermocycles (LDHC). This suggests that reduced night-time temperatures can undermine the rhythmic expression of certain genes (Fig. 17C). Within the clusters of genes with diurnal expression disrupted by photothermocycles, we highlight the poplar ortholog of Arabidopsis *COLD, CIRCADIAN RHYTHM, AND RNA BINDING 1* (*CCR1*; Potri.009G116400) gene (Fig. 17F). CCR1 contains an RNA recognition motif (RRM),

which promotes alternative splicing that is coupled to degradation by nonsense-mediated decay (NMD) (Schöning et al., 2008). *CCR1* transcription is also induced by cold and controlled by the circadian clock in Arabidopsis (Carpenter et al., 1994). Furthermore, we found that the diurnal expression of the poplar ortholog of Arabidopsis *PURIN 7* (*PUR7*; Potri.017G051500) gene (Fig. 17G), which is required to generate purine dependent cofactors in tissues under high rates of cell division, was disrupted by low night-time temperatures (Senecoff et al., 1996).

Taken all together, the results of our phenological studies show that the timing of growth to dormancy transition is highly sensitive to a decrease in low ambient temperature. Our data mining analysis identified genes showing SNPs associated with this transition showing also diurnal cycles of gene expression highly sensitive to temperature. We highlight that key regulators of metabolic processes show diurnal expression and temperature sensitivity that could provide an explanation to thermoregulation to poplar shoot growth.

Figure 17. Bud set and bud set-associated genes in poplar are sensitive to low ambient temperature. (A) Line plot showing the bud set scores for poplar (*Populus tremula* x *P. alba*) plants grown under SDs and three different temperatures (21, 18, and 15°C) for 9 weeks. (B) Comparative analysis of poplar bud

stage progression in plants grown under SDs and three different temperatures (21, 18, and 15°C) over 4 weeks. Asterisks denote significant differences between poplar groups ($n = 8$) grown at different temperatures (Kruskal–Wallis test followed by pairwise Wilcox test; * $P < 0.05$, ** $P < 0.01$). **(C)** Venn diagram representing the intersection between genes with SNPs associated with bud set and that show robust diurnal oscillations (cutoff = 0.8) under light/dark cycles (LD) and constant temperature (25°C, HH) or thermocycles (25:12°C, HC). 172 genes (lilac) show robust diurnal rhythms only under LDHH, 134 genes (pink) show diurnal rhythms only under LDHC and 135 genes (fuchsia) oscillate under both conditions, respectively. **(D)** Clusters of genes that show diurnal expression within 0–11 h under LDHC (D, mean = pink) and **(E)** within 12–23 h under LDHC (E, mean = pink). The line chart shows how photothermocycles (LDHC) promote diurnal oscillation of poplar PDLDELTA (D) and CBF4 genes (E). **(F)** Clusters of genes that show diurnal expression within 0–11 h under LDHH (F, mean = lilac) and **(G)** within 12–23 h under LDHH (G, mean = lilac). The line chart shows how photothermocycles (LDHC) disrupt the diurnal oscillation of poplar CCR1 (F) and PUR7 genes (G).

Chapter 2

A brief introduction

In the chapter 1, we showed that RAV1 do not play a role in the photoperiodic regulation of shoot apical growth. In this chapter, we identified that RAV1 transcription factor is induced by autumnal cues which in turn activates *Respiratory Burst Oxidase Homolog D1* gene (*RbohD1*), responsible of reactive oxygen species (ROS) signalling. *RAV1-RbohD1* could constitute a transcriptional module, induced by low ambient temperature under SD conditions, which regulates diurnal stomatal movements contributing to poplar acclimation during autumn-winter transition.

RAV1 molecular function is related with plant response to environmental stress

RAV1 gene expression was strongly activated by transition from summer to winter in chestnut (Moreno-Cortés et al., 2012). To test if poplar *RAV1* showed a similar environmental regulation, we analyzed poplar *RAV1* annual mRNA and protein accumulation in stem tissue collected throughout the year, under natural conditions. Annual transcriptional profiling (Appendix, Fig. S2; Dataset S3) revealed the upregulation of *RAV1* mRNA in early autumn, with highest expression in winter (Fig. 18A). Immunoblot analyses showed a similar pattern for *RAV1* protein abundance, with increased protein accumulation in early autumn (Fig. 18B). Similarly, transcriptional profiling reported the induction of *RAV2*, *RAV1* paralog, after 2-3 weeks of continuous SDs in poplar (Ruttink et al., 2007; Tarancón et al., 2017). Moreover, we decided to investigate whether *RAV1* diurnal gene expression was sensitive to photoperiodic changes, from LD to SD conditions, as it has been observed for the gene expression of *Arabidopsis TEMs* (Osnato et al., 2012). A 24h time course of mRNA expression indicated that *RAV1* has a different diurnal pattern after one week in SD compared with constant LD conditions (Fig. 18C). *RAV1* gene expression showed a maximum peak at ZT12 under SDs, with an increase of 34% compared with the peak expression at ZT18 observed under LDs (Fig. 18C).

To uncover genes that are transcriptionally regulated by *RAV1* under SDs, poplar *RAV1oe#3* line was grown for 10 days under SDs. Shoot apical tissue was collected at ZT12, the time of highest *RAV1* gene expression under SDs (Fig. 18C). RNA sequencing (RNA-seq) identified 133 differentially expressed genes (DEGs) in *RAV1oe* compared to WT plants (Appendix; Dataset S4). Gene ontology (GO) analysis using the agriGO database revealed GO terms over-represented including response to stimulus, which was the most significant GO term represented and showing an overall tendency to be activated by *RAV1* (Appendix, Fig. S3; Dataset S4; Tian et al., 2017). In addition to *RAV1* overexpression, we developed an inducible system to identify *RAV1* primary response genes. This is an activatable version of *RAV1* obtained by fusing the glucocorticoid ligand receptor domain (GR) to the eGFP:*RAV1* protein (Fig. 18D; Appendix, Fig. S4A and B). Nuclear translocation of eGFP:*RAV1* was evaluated upon addition of dexamethasone (DEX) in *p35S::GR:eGFP:RAV1* poplar lines (*GERoe*; Fig. 18D;

Appendix, Fig. S4C). For transcriptional profiling, *GERoe#9* line was grown for 10 days under SDs and shoot apical tissue was collected after 4hrs of dexamethasone /cycloheximide (DEX/CHX) treatment at ZT12. The protein translation inhibitor CHX was added to identify RAV1 primary response genes. RNA-seq identified 3962 DEGs compared with the control (MOCK/CHX) treatment (Appendix, Dataset S4). Transient GR:eGFP:RAV1 activation revealed massive transcriptional remodeling, which could represent the RAV1-induced transcriptional response under a rapid increment of RAV1 activity. Similar to *RAV1oe*/WT GO analysis, the GO analysis of the DEX/MOCK transcriptome highlighted the GO terms response to stimulus as one of the most significant GO terms and showing an overall activation (Appendix, Fig. S5; Dataset S4; Tian et al., 2017). The intersection between *RAV1oe*/WT and DEX/MOCK DEGs groups identified 27 common genes that are transcriptionally activated or repressed by RAV1 (Fig. 18E; Appendix, Dataset S4) indicating that these genes are consistent targets of RAV1. Among them, ERF and WRKY transcription factors, associated to abiotic and biotic stress responses (Fujimoto et al., 2000; Gutterson & Reuber, 2004; Ye et al., 2018), genes involved in plant response to dehydration (Hinch & Thalhammer, 2012) and to pathogen attack (Torres et al., 2002; Kadota et al., 2015). Taken together, these results suggest that RAV1 function is related to plant response to environmental stresses.

Figure 18. RAV1 is induced during the transition from growth to dormancy.

(A) *RAV1* annual mRNA expression in poplar stems sampled every month in the field. Gray lines represent normalized gene expression of two biological replicates obtained from microarrays studies and the red line represents the mean. (B) *RAV1* protein annual pattern in poplar stems. (C) Relative *RAV1* daily mRNA expression under LDs (16:8 h, light/dark) or after 1 week under SDs (8:16 h, light/dark). Time is expressed as Zeitgeber time (ZT), being ZT0 when light turned on. Light and dark gray panels represent night duration of SD and LD, respectively. Values represent the mean $\pm$ standard deviation. These experiments were repeated at least three times with the same trend. (D) Confocal microscope image of *35S::GR:eGFP:RAV1* root cells treated with MOCK (Tween20 0.05%, EtOH) or DEX (Tween20 0.05%, dexamethasone 30 μ M) after 4 hours. White bar: 50 μ m. (E) Venn diagram representing the intersection between DEGs of WT vs *p35S::RAV1* (light gray) and DEGs of MOCK vs DEX in *p35S::GR:eGFP:RAV1* background (dark gray). The intersection shows 27 consistent target genes of *RAV1*.

***RAV1*, *ERF72-1* and *RbohD1* could constitute a transcriptional module under autumnal SDs and low ambient temperature**

Because *RAV1* showed a marked autumnal-winter induction, we next investigated the seasonal expression pattern of the 27 *RAV1* target genes using microarray-based gene expression data (from June to December) collected monthly from poplar stem tissue. We identified three different clusters: (cluster 1) genes showing negligible seasonal variation, (cluster 2) genes repressed during autumn-winter, and (cluster 3) genes induced in autumn-winter (Fig. 19; Appendix, Dataset S5).

Figure 19. *RAV1* targets genes show seasonal patterns.

Clustering of *RAV1* target genes based on their annual gene expression in poplar stem tissues obtained from microarrays studies. To compare expression patterns, levels of gene expression for each gene were normalized with the maximum (=1). Gray lines represent the average between two biological replicates for each gene, while the red line represents the total mean of the clusters.

To further understand the role of *RAV1* autumnal upregulation, we focused on two poplar orthologs of Arabidopsis known function genes of cluster 3, Potri.008G210900

and Potri.003G159800, which were coexpressed with RAV1 during the autumn-winter transition (Fig. 19). Phylogenetic analyses identified Potri.008G210900 as an ortholog of *Arabidopsis ERF72*, *ERF72-1*, which belongs to the ERF-VII family (Fig. 20A; Nakano et al., 2006). Members of this family have an oxygen sensitive motif in the N-terminus and their function is regulated via the N-end rule pathway in *Arabidopsis* (Licausi et al., 2011; Gibbs et al., 2011; Gibbs et al., 2014). This N-end motif is conserved in poplar *ERF72-1* (Fig. 20B), suggesting that this post-translational regulation may be widely conserved between plant species.

Figure 20. Maximum likelihood phylogenetic and protein sequence analysis of ERFs-VII members in poplar and Arabidopsis.

(A) The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 100 replicates (Felsenstein et al., 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein et al., 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein et al., 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of

pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 1.2015)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 24.94% sites). The analysis involved 11 amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 132 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). **(B)** Protein sequence analysis showing that ERF72 conserved the amino-terminal amino acid sequence MCGGAI characteristic of ERFs type VII and AP2 DNA-binding domain.

Potri.003G159800 encodes a poplar ortholog of Arabidopsis *RbohD*, *RbohD1*, a plasma membrane NADPH protein recently identified in *Populus* (Fig. 21A; Jakubowicz et al., 2018). This enzyme generates ROS in response to various stimuli and it is considered to be a central node for the integration of a multitude of signal transduction pathways associated with ROS signaling (Torres & Dangl, 2005; Marino et al., 2012; Xia et al., 2015). EF-hand motifs and several serines located at the N-terminus of the Arabidopsis RBOHD have been attributed to stomatal closure regulation under pathogen attack (Li et al., 2014; Chen et al., 2017). Most of these elements are conserved in the N-terminus of the poplar RBOHD1 protein (Fig. 21B) also suggesting a conserved regulation of RBOHD function among these plant species.

Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). **(B)** Protein sequence analysis showing the N-terminal of Arabidopsis RbohD/F compared to poplar RbohD1. Serine targets of phosphorylation (red) and EF hand motifs responsible of Calcium-mediated regulation are conserved in poplar and Arabidopsis.

To confirm transcriptomic results, we carried out qRT-PCR analysis of *ERF72-1* and *RbohD1* genes. We found that *ERF72-1* and *RbohD1* gene expression is activated in *RAV1oe* plants compared with WT (Fig. 22A), confirming *RAV1oe* vs WT RNA-seq results. However, *ERF72-1* was repressed after 4 hours of DEX treatment in *GERoe#9* plants, whereas *RbohD1* was activated (Fig. 22B and C). To evaluate the effect of continuous RAV1 induction on *ERF72-1* and *RbohD1* transcription, constitutive activation of GR:eGFP:RAV1 by daily application of DEX was performed. We found that GR:eGFP:RAV1 continuous induction produced *ERF72-1* and *RbohD1* transcriptional activation compared to MOCK treatment after 5 days (Fig. 22B and C). Thus, RAV1 high levels generate transcriptional activation of *ERF72-1* and *RbohD1*.

Figure 22. Prolonged induction of RAV1 produces *ERF72-1* and *RbohD1* transcriptional activation.

(A) Relative expression of *ERF72-1* and *RbohD1* mRNA obtained by qRT-PCR of in *RAV1oe#3* and WT plants. Values represent the means $\pm$ standard deviation. **(B)** Relative expression of *ERF72-1* and *RbohD1* mRNA obtained by qRT-PCR of samples collected after 4 hours (RNA-seq experiment) and 5 days of DEX induction in *GERoe#9* plants with and without CHX. Values show the mean $\pm$ SEM (n = three biological replicates). Asterisks indicate significant differences (Student's t-test, **P<0.01, *** P<0.001)..

As we showed, the regulation of *ERF72-1* and *RbohD1* is linked to *RAV1*. Thus, these genes may work as a seasonally-controlled transcriptional module and respond similarly to autumnal cues, SD and low ambient temperature conditions. To test this hypothesis, the diurnal gene expression pattern of *RAV1*, *ERF72-1* and *RbohD1* was analyzed in WT poplars grown under SD at 21°C and SD at 15°C. qRT-PCR analyses

showed that *RAV1*, *ERF72-1* and *RbohD1* presented the highest expression at ZT12 during the night period under SD at 21°C (Fig. 23). These genes showed an upregulation at all time points in the 24h time course at 15°C compared with 21°C under SD conditions. Therefore, *RAV1*, *ERF72-1* and *RbohD1* are coexpressed under SD at 21°C and are sensitive to low ambient temperature under SD conditions (Fig. 23). These gene expression analyses support the molecular connection between *RAV1*, *ERF72-1* and *RbohD1* genes acting as a transcriptional module under SD and low ambient temperature conditions.

Figure 23. *RAV1*, *ERF72-1* and *RbohD1* gene expression is induced by low ambient temperature under SDs.

Time course experiment showing the relative expression of *RAV1*, *ERF72-1* and *RbohD1* mRNA obtained by qRT-PCR of WT poplar samples collected after 1 week of SD at 21°C or 15°C (8:16 h, light/dark). Values represent the mean $\pm$ standard deviation. Time is expressed as Zeitgeber time (ZT), being ZT0 when the light was turned on. The gray background represents night duration.

Because *ERF72-1* and *RbohD1* were identified as *RAV1* target genes and coexpressed under SD and low ambient temperature conditions, we investigated whether *RAV1* actually binds to their promoters. The promoter sequences of *ERF72-1* and *RbohD1* were examined for *RAV1* binding elements (Kagaya et al., 1999). We identified ‘CAACA’ boxes at -590bp and -468bp upstream of the transcription start site (TSS) of *ERF72-1* and *RbohD1* promoters, respectively (Fig. 24).

Figure 24. In-silico analysis of putative RAV1-binding motifs in the *ERF72-1* and *RbohD1* promoters. Schematic representation of *ERF72-1* and *RbohD1* promoters showing presence of RAV1 binding elements, ‘CAACA’ boxes, identified by in silico analysis of the upstream 1000 bp sequence from the *ERF72-1* and *RbohD1* TSS.

Next, we assessed the direct binding of purified MBP:RAV1 protein to double strand oligonucleotides designed based on the promoter sequences and containing the ‘CAACA’ motif using electrophoretic mobility shift assay (EMSA). These assays showed the binding of RAV1 to *RbohD1* promoter sequence but not to the *ERF72-1* one (Fig. 25A). Mutation of the ‘CAACA’ sequence eliminates the binding of RAV1 to the *RbohD1* oligonucleotide (Fig. 25B) indicating that the ‘CAACA’ motif is specifically recognized by the RAV1 protein. These analyses indicate that RAV1 transcriptionally could activate *RbohD1* by direct binding to the ‘CAACA’ promoter element.

Figure 25. RAV1 binds ‘CAACA’ element in the *RbohD* promoter.

(A) EMSA showing RAV1 binding to the *RbohD1* and *ERF72-1* *cis*-elements -468 and -590, respectively. The asterisk represents unspecific DNA-protein binding. (B) EMSA showing RAV1 binding to the *RbohD1* wild-type and mutated (*pRbohD1M*) *cis*-elements -468. Asterisks represent non-specific DNA-protein binding.

***RAVI* overexpression increased stomatal conductance and photosynthesis under SD and low ambient temperature**

The activation of *RAVI* by early autumnal cues suggested that RAV1 might participate in poplar acclimation to SDs and low ambient temperature. To get greater insight into the functional role of the *RAVI* seasonal upregulation in these conditions, we performed phenological assays of WT, *RAVIkd* and *RAVIoe* lines (Fig. 26A). After 6 weeks under SDs and 15°C treatment, quantification of aerial dry weight indicated that

RAV1oe lines have 17-26% more biomass than the WT and *RAV1kd* lines (Fig. 26B). Moreover, *RAV1* transgenic lines showed negligible differences in the timing of growth cessation and bud set compared with WT plants, indicating that *RAV1* autumnal induction does not have a function in growth cessation and bud formation (Fig. 26C and D). Moreover, this result confirms that *RAV1* is not required the timing of growth cessation and bud set as we observed in the phenological assays at 21°C after 5 weeks of SDs (Fig. 13).

RAV1oe lines were slightly shorter than WT and *RAV1kd* lines, however these differences were not significant (Fig. 26C).

Figure 26. Overexpression of *RAV1* increased total aerial poplar biomass under low ambient temperature and SDs.

(A) Representative pictures of *RAV1* transgenic lines and WT plants after 2 weeks of SDs at 15°C. White bar: 15 cm. (B) Total aerial dry biomass of *RAV1* transgenic lines and WT plants. Values represent the means $\pm$ SEM (n=6). Asterisks represent significant differences (Student's t-test, *P<0.05). (C) Height increment of *RAV1* transgenic lines and WT plants during 6 weeks under SD 15°C. Values represent the means $\pm$ SEM (n $\geq$ 6). (D) Stage of buds after 6 weeks under SD at 15°C in *RAV1* transgenic lines and WT plants. Box plots show two biological replicates for each genotype (n $\geq$ 6). Picture of representative bud for each genotype is shown. White bar: 1 mm.

We also found that the increased aerial biomass in *RAV1oe* lines resulted from higher total leaf and stem dry weight compared with WT and *RAV1kd* lines, although the differences were not significant between *RAV1oe* and WT when they were analyzed separately (Fig. 27A and B). *RAV1oe* lines showed a significant higher number of leaves than WT and *RAV1kd* lines, however single leaf dry weight and size were similar among genotypes (Fig. 27C, D and E). Thus, total leaf and stem dry weight determine the increased aerial biomass observed in *RAV1oe* lines. Moreover, *RAV1oe* lines could have a higher rate of leaf formation and non-apical stem growth compared respect to WT plants.

Figure 27. Total leaf, stem dry weight and leaf number were higher in *RAV1oe* lines than WT and *RAV1kd* lines.

(A) Total leaf and (B) stem dry weight of WT and *RAV1* transgenic lines expressed in grams. Values show the mean $\pm$ SEM (n=5). (C) Total number of leaves of WT and *RAV1* transgenic lines. Values show the mean $\pm$ SEM (n=5). (D) Single leaf dry weight of WT and *RAV1* transgenic lines expressed in grams. Values show the mean $\pm$ SEM (n=5). Statistical analysis was performed by the Kruskal-Wallis test followed by a pairwise Wilcoxon test (BH method). Groups of significance are indicated with different letters ($P < 0.05$). (E) Representative picture showing the size of youngest fully expanded leaves of WT and *RAV1* transgenic lines. White bar: 15 cm.

To better understand the growth differences observed in *RAV1oe* lines compared with WT and *RAV1kd* (Fig. 27), we quantified several physiological parameters associated with water relations (leaf water potential and water content), chlorophyll content and gas-exchange (photosynthesis, transpiration and stomatal conductance) after two weeks of SD at 15°C under non-limiting water conditions (Fig. 28A-G). Water potential and water content did not show differences in *RAV1oe* compared to WT and *RAV1kd* lines (Fig. 28A and B), indicating a similar water status in every genotype. Chlorophyll content was also similar among genotypes, suggesting an equivalent physiological state of the plants under SD at 15°C (Fig. 28C). We found an increased photosynthetic rate in *RAV1oe* lines compared with the other genotypes (Fig. 28D). Similarly, transpiration was higher in *RAV1oe* lines compared with WT plants, while *RAV1kd22* showed the lowest (Fig. 28E). Finally, measurement of stomatal conductance revealed statistically significant higher values in *RAV1oe* lines compared with WT and *RAV1kd* lines (Fig. 28F).

Figure 28. *RAVI* overexpression increases stomatal conductance under low ambient temperature and SDs.

(A) Water potential measured in poplar leaves of WT and *RAVI* transgenic lines. Values represent the means $\pm$ SEM (n=6). (B) Water content measured in poplar leaves of WT and *RAVI* transgenic lines. Values represent the mean $\pm$ SEM (n=6). (C) Chlorophyll content measured in poplar leaves of WT and *RAVI* transgenic lines. Values represent the mean $\pm$ SEM (n=6). (D) Photosynthetic rate measured in poplar leaves of WT and *RAVI* transgenic lines. Values represent the mean $\pm$ SEM (n=6). (E) Transpiration rate measured in poplar leaves of WT and *RAVI* transgenic lines. Values represent the mean $\pm$ SEM (n=6). (F) Stomatal conductance measured in poplar leaves of WT and *RAVI* transgenic lines. Values represent the mean $\pm$ SEM (n=10). All measurements were performed after 2 weeks of SD at 15°C in 2-month-old plants. Statistical analysis was performed by the Kruskal-Wallis test followed by a pairwise Wilcox test (BH method). Groups of significance are indicated with different letters (P<0.05).

Moreover, WT and *RAVIkd* lines under SD and 15°C showed a 35% reduction of stomatal conductance while *RAVIoe* lines showed only a 20% reduction with respect to 21°C under LDs (Fig. 29). The higher stomatal conductance may be the cause of the higher photosynthetic and transpiration rates in *RAVIoe* and may explain the increased biomass observed in *RAVIoe* lines.

Figure 29. Stomatal conductance in *RAVIoe* lines was less affected by low ambient temperature compared with WT and *RAVIkd* lines.

Stomatal conductance of WT and *RAVI* transgenic lines under LD 21°C and after two weeks under SD 15°C. Values represent the mean $\pm$ SEM (n=10).

The non-significant differences in stomatal conductance observed between WT and *RAVIkd* lines could be due to either insufficient reduction of *RAVI* expression in these RNA interference lines (Moreno-Cortés et al., 2012) or functional complementation by *RAV* homologs (Moreno-Cortés et al., 2012) or/and alternative pathways complementing

RAV1 downregulation. The transcriptomic comparison between *RAV1kd* and WT identified 75 DEGs (Appendix, Dataset S6). Gene ontology analysis identified an overrepresentation of GO terms related to response to a stimulus, biological regulation and cellular and nitrogen metabolic processes (Appendix, Fig. S6; Dataset S6). Among response to stimulus genes, we did not detect the downregulation of *RbohD1* which was upregulated in *RAV1oe* and *GERoe* lines. Surprisingly, we found that *ERF72-1* was upregulated in *RAV1kd* compared to WT. Thus, *ERF72-1* activation could explain the functional complementation in *RAV1kd* plants.

These results show that *RAV1oe* plants may regulate their stomata differently, which could be a physiological advantage for poplar acclimation to temperature decrease under autumnal conditions.

***RAV1* overexpression improves diurnal stomatal movement under SDs and low ambient temperature**

RAV1-dependent activation of *RbohD1* expression could cause the observed physiological differences in stomatal function in *RAV1oe* plants. It has been shown that ROS produced by RBOHD and RBOHF enzymes is required for Ca^{2+} and ABA-induced stomatal closure in Arabidopsis (Pei et al., 2000; Kwak et al., 2003; Wang and Song, 2008; Kim et al., 2010). Furthermore, the RBOHD/F-dependent ROS burst has been functionally associated with the ATP-induced stomatal opening in Arabidopsis (Hao et al., 2012). Therefore, ROS is capable of stimulating both stomatal opening and closure.

Thus, we firstly tested whether WT, *RAV1kd* and *RAV1oe* lines showed different ROS production in guard cells. Using the H2DCFDA ROS sensitive fluorophore, we measured H_2O_2 level produced in guard cells after ABA treatment of WT, *RAV1oe*, and *RAV1kd* leaf discs. Quantification H2DCFDA fluorescent signal revealed that *RAV1oe* guard cells showed higher accumulation of H_2O_2 compared with WT and *RAV1kd* after 20 min of ABA treatment, indicating increased levels of ABA-induced ROS burst in *RAV1oe* stomata (Fig. 30A and B). *RAV1kd* plants also had statistically significant less ROS production compared with WT (Fig. 30B). However, we did not detect any significant differences in physiological measurements related to stomatal function in this knock-down line (Fig. 28F). To test whether the observed differences in ROS burst upon ABA treatment could affect stomatal activity, we carried out stomatal closure assay.

Notably, *RAV1oe* reduced stomatal aperture faster than WT and *RAV1kd* plants (Fig. 30C). Moreover, *RAV1oe* showed a lower rate of leaf water loss than WT and *RAV1kd*, confirming that *RAV1oe* close stomata faster than WT and *RAV1kd* (Fig. 30D). These results showed an increased stomatal activity in *RAV1oe* suggesting a higher sensitivity to ABA.

Figure 30. *RAV1oe* stomata showed higher ABA-induced ROS burst and responsiveness.

(A) Pictures of representative stomata of WT, *RAV1kd* and *RAV1oe* showing ROS production as indicated by the fluorescent dye H2DCF-DA taken with confocal microscopy. Leaf discs were incubated with H2DCF-DA for 20 min before 50 μ M ABA was added. After 20 min, leaf discs were photographed. (B) Quantification of ROS production in guard cells of the wild type and *RAV1kd#1* and *RAV1oe#3* after 20 min of ABA treatment (50 μ M). ROS production in each pair of guard cells was quantified based on pixel intensity. Values are means $\pm$ SEM from three independent experiments (** $P < 0.01$; *** $P < 0.001$). 100 stomata were measured for each genotype per experiment. (C) Stomatal closure in the WT and *RAV1* transgenic lines after 2 hours of ABA treatment (5 μ M). Values represent the means $\pm$ SEM ($n > 100$). This experiment was repeated at least three times with the same trend. Statistical analysis was performed using a Kruskal-Wallis test followed by pairwise Wilcoxon test (BH method). Groups of significance are indicated with different letters ($P < 0.05$). (D) Water loss rate measured by the fresh weight loss of detached and fully expanded leaves per genotype. Values show the mean $\pm$ SEM ($n = 5$).

The observed higher stomatal movement in *RAV1oe* could be attributed to higher expression levels of *RbohD1*. To test this hypothesis, we performed ABA-induced

stomatal closure assay in the presence of diphenyleneiodonium chloride (DPI), an inhibitor of RBOH protein activity (Pei et al., 2000; Hao et al., 2012). Stomatal closure is partially inhibited by DPI in *RAV1oe* and WT leaf discs suggesting that RBOH-dependent ROS production may contribute to ABA-induced stomatal closure (Fig. 31). Similarly, a partial inhibition of ABA-induced stomatal closure upon DPI application was shown in *Arabidopsis* (Xie et al., 2016).

Figure 31. DPI application partially inhibits stomatal closure in WT as well as *RAV1oe* plants.

Stomatal closing in the wild type and *RAV1oe#3* line after 2 hours of ABA treatment alone or in combination with DPI. Values represent the means $\pm$ SEM ($n > 100$). This experiment was repeated at least three times with the same trend. Statistical analysis was performed using a Kruskal-Wallis test followed by pairwise Wilcoxon test (BH method). Groups of significance are indicated with different letters ($P < 0.05$).

The higher stomatal conductance in *RAV1oe* could be also explained by an increase in stomatal density or larger stomatal size. Our measurements showed that stomatal density and size were not significantly different among genotypes (Fig. 32), indicating that the physiological differences were due to higher stomatal responsiveness in *RAV1oe* plants.

Figure 32. RAV1 did not affect stomatal density and size.

Stomatal density and size in poplar WT, RAV1 knock down and overexpressing lines. Values represent the mean $\pm$ standard deviation. To calculate stomatal density, different pictures were taken using optical microscope using 20X magnitude from different leaves (n=5). To calculate stomatal density, number of stomata from different leaves were measured (n>100). Statistical analysis was performed using a Kruskal-Wallis test followed by pairwise Wilcox test (BH method).

To evaluate stomatal performance in WT, *RAV1oe*, and *RAV1kd* lines after two weeks of SD and low ambient temperature conditions, we performed a time course of stomatal conductance in WT and *RAV1* poplar transgenic lines by doing measurement at the time points close to night/day and day/night transitions. The results showed that *RAV1oe* reached the highest conductance levels earlier than WT and *RAV1kd* plants during daytime indicating that the stomata of *RAV1oe* bear the capacity to reach a higher maximal aperture without affecting their diurnal rhythm of stomatal aperture-closure under SD at 15°C conditions (Fig. 33A). Moreover, stomatal opening experiment showed a faster *RAV1oe* stomatal aperture than WT and *RAV1kd* line after 2h of light treatment (Fig. 33B).

All together, these results suggest that *RAV1* overexpression intensifies diurnal stomatal movements through a mechanism that could activation of *ERF72-1* and *RbohD1* gene expression.

Figure 33. RAV1 overexpression improves stomatal conductance during daytime under SDs and low ambient temperature.

(A) Stomatal conductance of fully expanded leaves was determined in WT and RAV1 transgenic lines under SDs at 15°C. The gray panels represent the night. Values represent the means $\pm$ SEM (n=15). (B) Stomatal opening assays in WT, *RAV1kd* and *RAV1oe* leaf discs. Values represent the mean $\pm$ SEM (n>100). Statistical analysis was performed using a Kruskal-Wallis test followed by pairwise Wilcox test (BH method). Letters represent different groups of significance (P<0.01).

Chapter 3

A brief introduction

In the previous chapter, we found that RAV1 contribute to the diurnal stomatal movements and this function could be mediated in part by the transcriptional activation of *RbohD1*. To date, the temporal regulation of *RbohD* transcription remains unexplored in poplar and Arabidopsis. In this chapter, we explore *RbohD* diurnal expression in both poplar and Arabidopsis showing some conserved features. Moreover, we show that diurnal regulation of *RbohD* transcription contributes to generate a different magnitude of response against pathogen attack.

***RAV1* and *RBOHD1* show a non-circadian diurnal pattern in poplar**

As we showed in the chapter 1, we found that *RAV1* showed a marked cyclic diurnal pattern (Fig. 34A). Because *RbohD1* is transcriptionally controlled by *RAV1*, we investigated *RbohD1* transcription during 48 h time course experiment under LDs. We found that *RbohD1* also showed a marked cyclic diurnal pattern with a night peak of expression showing a maximum at ZT18, similar to *RAV1* night expression peak (Fig. 34B).

Figure 34. *RAV1* and *RbohD1* mRNA expression show a marked diurnal pattern under LD conditions.

(A, B) 48h time course showing the relative mRNA expression of (A) *RAV1* and (B) *RbohD1* obtained by qRT-PCR of poplar WT samples collected under LD conditions. Values show the mean $\pm$ standard deviation. White and black panels represent day and night duration, respectively. Time is expressed as Zeitgeber time (ZT), being ZT0 when the light was turned on.

RAV1 displayed two peaks of mRNA accumulation during the day, the smaller one during the day at ZT9 and the bigger one at night, showing maximum expression between ZT18 and ZT21. Similarly, *RbohD1* showed a strong induction in the night at ZT18. Thus, *RAV1* and *RbohD1* are coexpressed under LD conditions. Because circadian clock is the main regulator of diurnal rhythms in plants, we investigated if *RAV1* and *RbohD1* gene expression is modulated by the clock. We performed 48h time course experiment under continuous light (LL) conditions because clock-dependent gene expression keeps oscillations with a period of 24h under constant light conditions. As control, we analyzed the expression of the clock gene *LHY2*. Our qRT-PCR analyses showed a robust oscillation of *LHY2* under LD as well as LL with a period of 24h and the

peak maximum of gene expression at ZT0 (Fig. 35A and B). This pattern is consistent with the one observed in our previous report (Ramos-Sánchez et al., 2017). Mutations that affect clock period and amplitude show relative amplitude error (RAE) values around 0.5 in reporter gene assays (Herrero et al., 2012). In our experiments, RAE analysis revealed the robustness of *LHY2* rhythms with a RAE value less than 0.05 and 24h period (Fig. 35C and D).

Figure 35. *LHY2* gene expression shows a circadian period under LD and LL conditions.

(A) 48h time course showing the relative mRNA expression of *LHY2* obtained by qRT-PCR of poplar WT samples collected under LD conditions. Values show the mean $\pm$ SEM ($n =$ two biological replicates). White and black panels represent day and night duration, respectively. Time is expressed as Zeitgeber time (ZT), being ZT0 when the light was turned on. (B) Period versus RAE analysis for *LHY2* under LDs. Values show the mean $\pm$ standard deviation ($n =$ two biological replicates). (C) 48h time course showing the relative mRNA expression of *LHY2* obtained by qRT-PCR of poplar WT samples collected under LL conditions. Values show the mean $\pm$ SEM ($n =$ two biological replicates). White and gray panels represent subjective day and subjective night duration, respectively. Time is expressed in circadian time (CT), being CT0 when the continuous light condition was set on. (D) Period versus RAE analysis for *LHY2* under LL conditions. Values show the mean $\pm$ standard deviation ($n =$ two biological replicates).

Contrarily to *LHY2*, *RAV1* and *RbohD1* lost the LD cyclic diurnal pattern under LL conditions. Moreover, *RAV1* and *RbohD1* expression showed a stochastic pattern along

48h time course in LL (Fig. 36A and B). The period of gene expression was 8 and 12h for *RAV1* and *RbohD1*, respectively, indicating that *RAV1* and *RbohD1* expression is not controlled by the clock, therefore, the cyclic diurnal pattern observed in LD conditions should be promoted by a different cyclic stimulus (Fig. 36C and D). The analysis of *RAV1* and *RbohD1* diurnal patterns in LD and LL conditions revealed that the night period is necessary to promote the transcriptional activation and the cyclic diurnal oscillation of *RAV1* and *RbohD1*. Therefore, a night dependent stimulus must be responsible of *RAV1* and *RbohD1* cyclic diurnal expression.

Figure 36. *RAV1* and *RbohD1* gene expression show non-circadian rhythms under LL conditions.

(A) 48h time course showing the relative mRNA expression of *RAV1* obtained by qRT-PCR of poplar WT samples collected under LL conditions. Values show the mean $\pm$ SEM ($n =$ two biological replicates). White and gray panels represent subjective day and subjective night duration, respectively. Time is expressed in circadian time (CT), being CT0 when the continuous light condition was set on. (B) Period versus RAE analysis for *RAV1* under LL conditions. Values show the mean $\pm$ standard deviation ($n =$ two biological replicates). (C) 48h time course showing the relative mRNA expression of *RbohD1* obtained by qRT-PCR of poplar WT samples collected under LL conditions. Values show the mean $\pm$ SEM ($n =$ two biological replicates). White and gray panels represent subjective day and subjective night duration, respectively. Time is expressed in circadian time (CT), being CT0 when the continuous light condition was set on. (D) Period versus RAE analysis for *RbohD1* under LL conditions. Values show the mean $\pm$ standard deviation ($n =$ two biological replicates).

***RbohD* shows a tight transcriptional regulation in Arabidopsis**

To gain further insight of the cyclic diurnal transcriptional regulation of *RbohD*, we asked if the observed cyclic diurnal pattern of poplar *RbohD1* is also conserved in its Arabidopsis homolog. There are not previously report of diurnal transcriptional regulation of *RbohD* gene in Arabidopsis. To check if *RbohD* diurnal oscillations exist in Arabidopsis, we analysed *RbohD* gene expression using the database Diurnal (Mockler et al., 2007). We found that *RbohD* gene displays a strong mRNA accumulation during the night period showing a peak of expression with a maximum at ZT20 under LDHC conditions (12:12 h light/dark and 22:12 °C day/night; Fig. 37) similar to poplar *RbohD1* diurnal pattern of gene expression (Fig. 34B). This result suggests that Arabidopsis and Poplar *RbohD* expression share a night-dependent induction.

Figure 37. Arabidopsis *RbohD* transcript levels show a strong accumulation during the night.

RbohD mRNA expression analysis obtained from WT (Col-0) samples collected every 4h during 44h under 12:12h light/dark conditions. Gene expression data corresponds to microarrays analysis obtained from the Diurnal database (LDHC conditions). White and black panels represent day and night duration, respectively.

For a deeper understanding of the Arabidopsis *RbohD* transcriptional regulation, we used the Arabidopsis *pRbohD::LUC* transcriptional reporter line previously characterized and available in Dr. MA Torres Laboratory, CBGP, Madrid. (Morales et al., 2016). This reporter was created with 2000bp of *RbohD* upstream sequence from the TSS fused to luciferase gene reporter (LUC) (Morales et al., 2016). To analyze the *pRbohD::LUC* line, we used a microplate reader system for monitoring luciferase activity in a temporal manner (Perales et al., 2006; Ramos-Sanchez et al., 2017). Leaf discs of

pRbohD::LUC line obtained from 4-weeks-old plants were analyzed during 3 days under LDs. We found that *pRbohD::LUC* showed daily oscillation under LDs as well as SDs with highest transcriptional activation during the daytime (Fig. 38A and B). Moreover, when leaf discs grown under SDs were shifted to LL conditions, *pRbohD::LUC* kept oscillating in a similar manner as they did under LD and SD conditions (Fig. 38C). Moreover, period analysis revealed approximately a 24h period, which suggests a circadian regulation of *RbohD* promoter in Arabidopsis (Fig. 38D). Remarkably, this diurnal pattern was different from one observed for Arabidopsis and poplar *RbohD* transcript levels, which presented a cyclic expression peak with a maximal expression during the night period. This paradox could be caused by a different perception of *pRbohD::LUC* reporter in the conditions assayed.

Figure 38. Arabidopsis *pRbohD::LUC* reporter shows diurnal rhythms under LD, SD and LL conditions.

(A) *pRbohD::LUC* activity detected by luminescence assay every 2h during three days under LDs. (B) *pRbohD::LUC* activity detected by luminescence assay every 2h during three days under SDs. (C) *pRbohD::LUC* activity detected by luminescence assay every 2 h for one day under SD and two days under LL conditions. (D) Period versus RAE analysis for *pRbohD::LUC* under LD, SD and LL conditions. Values show the mean $\pm$ standard deviation ($n =$ three independent assays). White and black panels represent day and night duration under LD and SD conditions, respectively. Gray panel represents subjective night under LL conditions.

Thus, we hypothesized that *pRbohD::LUC* transcription could be modulated by systemic signals and/or whole plant-dependent mechanisms that were activated or missed by measuring *pRbohD::LUC* activity in leaf discs. To test the hypothesis that leaf disc *pRbohD::LUC* lack system input, we used 2-weeks-old seedlings grown with 1% sucrose (~30 mM) and measured luciferase reporter activity for several days under LDs. We found a strong induction of *pRbohD::LUC* during the night period as observed for *Arabidopsis* and poplar *RbohD* gene expression (Fig. 39).

Figure 39. *Arabidopsis pRbohD::LUC* reporter shows a similar diurnal pattern of *RbohD* transcript under LDs in seedlings.

pRbohD::LUC activity detected by luminescence assay every 2 h during three days under LDs. Values show the mean $\pm$ standard deviation ($n =$ three independent assays). White and black panels represent day and night duration, respectively.

Because seedling were grown in a medium containing a supplement of 1% of sucrose, to exclude the potential effect of sucrose on *pRbohD::LUC* activity, we performed an experiment with *pRbohD::LUC* using leaf discs with or without sucrose. Surprisingly, leaf discs supplemented with 1% of sucrose resembled the diurnal pattern observed in *pRbohD::LUC* seedlings, indicating that sucrose may be the signal that induces *RbohD* transcription during the night period regardless of the type of plant material used (Fig. 40). Sucrose is a mobile signal that have been shown to modulate the diurnal regulation of rhythmically expressed transcripts and the circadian clock (Bläsing et al., 2005; Haydon et al., 2013). Our results indicate that sucrose or sucrose-dependent signal is required to induce *pRbohD::LUC* transcription during the night period.

Figure 40. Exogenous addition of sucrose resembles seedling-dependent *pRbohD::LUC* diurnal pattern under LDs in leaf discs.

pRbohD::LUC activity detected by luminescence assay every 2 h during three days under LDs. Values show the mean $\pm$ standard deviation ($n =$ three independent assays). White and black panels represent day and night duration, respectively.

Since the induction of *pRbohD::LUC* activity by sucrose occurred during the night period, we tested whether *pRbohD::LUC* activity in presence of sucrose shows cyclic expression in LL conditions. We measured *pRbohD::LUC* activity in seedlings grown under LDs in sucrose supplemented growth medium and they were shifted to LL conditions. We found a loss of sucrose-dependent oscillations of *pRbohD::LUC* under LL conditions, indicating that sucrose activation of *RbohD* gene expression required day/night cycles (Fig. 41).

Figure 41. Sucrose activation of *pRbohD::LUC* activity is night-dependent.

pRbohD::LUC activity detected by luminescence assay during two days every 2 h under LDs and two days under LL conditions. Values show the mean $\pm$ standard deviation ($n =$ three independent assays). White and black panels represent day and night duration, respectively. Gray panel represents subjective night under LL conditions.

Moreover, to exclude the osmotic effect of sucrose on *pRbohD::LUC* activation, we tested the effect of mannitol on the activity of *pRbohD::LUC*. The result of luminescence assays showed that 1% mannitol (~55 mM) did not induce *pRbohD::LUC* activity, presenting similar pattern in mannitol and mock treated samples (Fig. 42). This result suggests that *pRbohD::LUC* activation is due to sucrose signalling.

Because starch and derived sugars are hydrolyzed during the night to sustain plant cell respiration and growth (Pal et al., 2013), we checked if the night-dependent activation of *pRbohD::LUC* is due to sucrose *per se* or sucrose breakdown. We found that *pRbohD::LUC* seedlings supplemented with 1% glucose (~55 mM) showed the same pattern of those with 1% sucrose during two days, indicating that sucrose-derived glucose is the responsible of the sucrose-dependent activation of *pRbohD::LUC* in the night (Fig. 42). These results indicate that the activation of *pRbohD::LUC* during the night depends on the sucrose-derived glucose suggesting the potential regulation of *RbohD* transcription by sucrose breakdown-dependent signaling.

Figure 42. Sucrose and glucose produce *pRbohD::LUC* activation in the night.

pRbohD::LUC activity detected by luminescence assay every 2h during two days under LDs. MOCK, 1% Man, 1% Suc, 1%Glu represent control, mannitol, sucrose and glucose treatments, respectively. Values show the mean $\pm$ standard deviation ($n =$ three independent assays). White and black panels represent day and night duration, respectively.

The biological relevance of *RbohD* transcriptional regulation in *Arabidopsis*

As arises from the studies carried out in this work, *RbohD* gene expression shows a tight regulation which integrates different environmental and endogenous signals in *Arabidopsis* and poplar. As we hypothesized, RAV1-dependent *RbohD1* induction by SDs and low ambient temperature could contribute to the diurnal regulation of stomatal activity to cope with environmental cues in autumn contributing to poplar acclimation. However, the contribution of *RbohD* diurnal transcriptional regulation to plant physiology remains still unexplored in *Arabidopsis* and poplar.

RBOHD is a plasma membrane NADPH oxidase involved in the generation of ROS in the apoplast mainly involved in the defense response to pathogen attack (Kadota et al., 2015). Thus, to test if *RbohD* diurnal pattern could influence the magnitude of the plant response to pathogen attack, we performed an experiment using Col-0 leaf discs treated with the bacterial PAMP flagellin (flg22), an inducer of RBOHD-dependent ROS production in *Arabidopsis* (Torres et al., 2013). We measured ROS production induced by addition of flg22 in WT leaf discs in the day (ZT4) and in the night (ZT22). We found a higher ROS production in the night-WT leaf discs compared to day-WT leaf discs, indicating that the transcriptional activation of *RbohD* in the night increases the magnitude of the plant defense signaling and the sensitivity to pathogen attack (Fig 43).

Figure 43. RBOHD-dependent ROS production is higher in the night compared to the day in response to flg22.

(A) H_2O_2 production measured in a luminol-based assay using leaf discs from 4-weeks-old Col-0 plants after treatment with flg22 (500 nM). Values are means $\pm$ SEM ($n = 8$). The experiment was repeated at least three times showing similar results. (B) Quantification of the total H_2O_2 production from a luminol-based assay on leaf discs from 4-weeks-old plants of Col-0 triggered by flg22 (500 nM) over a 40 minutes period. Values represent the mean $\pm$ SEM ($n = 8$) of the areas under the curve (AUC) of each leaf disc and treatment. Asterisk indicates significant difference (Student's t -test, * $P < 0.05$).

DISCUSSION

1. Diurnal patterns and functional characterization of poplar TEMPRANILLO suggest an alternative mechanism for photoperiodic regulation in Arabidopsis and poplar.

Seasonal changes in day length are perceived in leaves. The photoperiodic information is integrated through the transcriptional regulation of the *FT*, which acts as a long-distance molecule to induce Arabidopsis shoot transition from vegetative growth to flowering (Corbesier et al., 2007). When diurnal rhythms of *FT* gene expression match with the external permissive photoperiod, flowering is induced as it was earlier proposed by the external coincidence model (Pittendrigh and Minis, 1964). The diurnal oscillation of *FT* transcription is modulated by activators and repressors (Song et al., 2015). Among them, the main activator is CONSTANS transcription factor, while well-known repressors are TEMPRANILLO genes (Suárez-López et al., 2001; Castillejo and Pelaz, 2008).

Similar to Arabidopsis, poplar photoperiodic information is integrated at *FT2* transcription level and is responsible of the shoot apical meristem transition to growth cessation (Böhlenius et al., 2006). However, transcriptional regulation of *FT* in Arabidopsis and poplar shows important differences. For instance, overexpression of Arabidopsis *CO* causes high expression levels of *FT* and promotes early flowering irrespective of the photoperiod (Putterill et al., 1995, Onouchi et al., 2000). On the other hand, the overexpression of poplar *CO1* or *CO2* was found neither to upregulate *FT2* nor to delay growth cessation under SDs in poplar (Hsu et al., 2012). This difference indicates an alternative regulation of poplar CO activity respect to Arabidopsis. In addition, we found that poplar *CO1* and *CO2* showed a different diurnal gene expression pattern respect to the Arabidopsis *CO* indicating that regulation of *CO* expression in poplar is different respect to Arabidopsis. Thus, poplar *CO1* and *CO2* diurnal gene expression patterns and their functional characterization suggest that *CO1* and *CO2* do not act as major activators of diurnal *FT2* gene expression in poplar. Recently, it has been described *GIGANTEA* gene as the main direct activator of poplar *FT2* transcription under both LDs and SDs (Ding et al, 2018), while in Arabidopsis *GI* promotes both *CO* and *FT* transcription. (Sawa et al., 2007; Sawa et al., 2011). In this thesis, it is shown that a poplar ortholog of TEM floral repressor, *RAV1* gene, showed a different diurnal pattern of gene expression respect to Arabidopsis. Our studies indicate that *RAV1* does not repress *FT2*

in poplar. Indeed, *FT2* is activated by RAV1 in poplar transactivation assays and in *RAV1oe* lines under LDs. Even more, *RAV1oe* lines did not show higher levels of *FT2* under SDs compared to WT plants, indicating that the SD-induced downregulation of *FT2* is dominant over RAV1-dependent activation of *FT2* under LDs. Even more, our phenological assays showed that RAV1 is not involved in the photoperiodic control of poplar shoot apical growth. Although regulation of Arabidopsis flowering and poplar growth to dormancy transition have FT as principal photoperiodic integrator, the regulation and the function of main Arabidopsis flowering time regulators show remarkable differences. These differences strongly suggest that alternative mechanisms, likely posttranslational regulatory layers, are operating in the core of photoperiodic control of growth to dormancy transition.

2. *FT2* could promote poplar axillary bud outgrowth.

Functional studies performed in our laboratory indicated that the overexpression of chestnut *RAV1* increased axillary branching under LDs, however the molecular mechanism involved has yet to be determined (Moreno-Cortés et al., 2012; Moreno-Cortés et al., 2017). Similarly, in this thesis, it is shown that overexpression of the poplar RAV1 promoted both higher levels of *FT2* gene expression and axillary bud outgrowth under LDs. It has been reported that FT together with TSF are required for shoot axillary outgrowth in Arabidopsis (Hiraoka et al., 2013). QTLs, transgenic complementation and gene expression analyses revealed that natural variation at *FT* promoter level determines variation of flowering time and reduced stem branching (Huang et al., 2013). It has been also shown that BRANCHED1 (BRC1), a known positive regulator of axillary bud dormancy in Arabidopsis, tomato and potato (Martín-Trillo et al., 2011; González-Grandío et al., 2013; Nicolas et al., 2015), interacts with FT and TSF in Arabidopsis (Niwa et al., 2013). It has been proposed that BRC1 could prevent premature flowering in axillary buds by interacting with FT and TSF (Niwa et al., 2013). Moreover, overexpression of Arabidopsis *FT* in cassava showed increased shoot branching (Adeyemo et al., 2017). In tomato, overexpression of a zinc finger protein *SIZFP2*, which produced higher level of *FT* expression in leaves, showed increased shoot branching (Weng et al., 2016).

Therefore, one plausible hypothesis is that the high levels of *FT2* expression under LDs may be the responsible of the increased branching observed in *RAV1oe* lines.

Supporting our hypothesis, poplar overexpressing *FT2* showed augmented shoot branching (Böhlenius et al., 2006). Even more, poplar overexpressing *GI*, the major activator of *FT2*, produced more branches respect to control plants under LDs (Ding et al., 2018). All together, these results suggest that axillary bud outgrowth in poplar could be also induced by an FT-dependent pathway under LD conditions.

3. Mining photoperiodic regulators of poplar shoot apical growth

A tight regulation of *FT2* diurnal gene expression it is necessary to promote shoot apical growth under LD conditions defining the annual elongation rate of the tree (Böhlenius et al., 2006; Hsu et al., 2011; Ding et al., 2018). Impaired regulation of *FT2* expression would change the timing of shoot growth affecting tree height. As we demonstrated in the case of TEM/RAV1, a common phylogenetic origin and protein sequence homology is not sufficient to assume a conserved function of Arabidopsis flowering regulators into poplar shoot apical growth. Therefore, we identified new potential regulator of poplar shoot apical growth seeking photoperiodic controlled genes associated genetically to tree height. By combining diurnal expression data with poplar height-associated SNPs, we found that 17% of height-associated genes showed robust diurnal gene expression patterns. Among them, we found 14 poplar orthologs of known *FT* and flowering time regulators in Arabidopsis, such as *GI*, *FKF1*, *PRR7*, *RVE8*, *CO*-like proteins, *TFL*, *CDF* and *AG*-like transcription factors. The presence of *GI* and *CDF2* genes, which are *FT2* regulators in poplar (Ding et al., 2018), reinforces our *modus operandi* to identify putative regulators of *FT2* in poplar. Moreover, the overexpression of poplar *CDF3* causes repression of *FT2* and earlier growth cessation. *CDF3* directly binds *FT2* genomic region and repress its transcription (Ding et al., 2018). *CDF* proteins repress *CO* and *FT* transcription in Arabidopsis (Fornara et al., 2009; Song et al., 2012). Thus, *CDF* transcription factors show a conserved function in Arabidopsis and poplar. Further, *CDF2* was found to physically interact with *GI* and *FKF1* in yeast two-hybrid assays similar to Arabidopsis, though in future work it remains to be elucidated if this interaction is meaningful (Ding et al., 2018).

Some of the identified genes form part of circadian clock core, such as *PRR7* and *RVE8*, or are clock-controlled genes, such as *GI*, *FKF1* and *CDF2* in Arabidopsis, supporting the idea that the circadian clock participates in the poplar shoot apical growth

regulation (Ibañez et al., 2010). However, how the circadian clock controls *FT2* expression to promote growth has yet to be determined. The roles of *PRR7*, and *RVE6* in the photoperiodic regulation of poplar shoot apical growth requires further investigation. Moreover, *VIP4*, *VIP5*, *AG-like*, and *TFL2* have been shown to participate in the epigenetic regulation of *FLC* and *FT* (Kotake et al., 2003; He et al., 2004; Oh et al., 2004; Yoo et al., 2011), suggesting a potential role of epigenetic in poplar the shoot apical growth. Recently, it has been reported the importance of epigenetic dynamics in the regulation of growth-dormancy cycles in poplar (Conde et al., 2017a; Conde et al., 2017b). Moreover, these poplar orthologs to *VIP4*, *VIP5*, *AG-like*, and *TFL2* showed robust diurnal rhythms of gene expression which are absent in *Arabidopsis*. It would be interesting to determine whether poplar orthologs of *VIP4*, *VIP5*, *AG-like*, and *TFL2* participate in the regulation of shoot apical growth via diurnal rhythmic deposition and recognition of epigenetic marks in poplar.

To summarize, we identified several genes required for tree height elongation that could participate in the photoperiodic control of *FT2* transcription. Some of the genes identified have been already demonstrated to be genetically linked to *FT2*, but future functional characterization is required to validate their function as regulators of *FT2* transcription and poplar shoot apical growth. Although *FT* is a hub for photoperiodic signal integration in *Arabidopsis* and poplar, the speciation process imposes particular regulatory layers on the control of shoot apical growth as it was shown for *CO* and *TEM*. The conserved and diverged features in photoperiodic regulation of *FT* transcription between poplar and *Arabidopsis*, likely reflecting the different habits of herbaceous and woody perennial plants.

4. Predicting temperature-signalling components involved in poplar bud set

In addition to photoperiodic changes, decrease of temperature during autumnal season impact the timing of growth cessation and bud set (Rohde et al., 2011). In this thesis, it is shown that low ambient temperature delayed bud set, with a delayed of approximatively two weeks comparing 21°C to 15°C (Maurya et al., 2018). Despite in the last years our knowledges of photoperiodic control of shoot apical growth has considerably increased, very little is known about the molecular components acting in the

temperature signalling which participate in the control of growth cessation and bud formation. In poplar, overexpression of Arabidopsis *CBF1*, transcription factor induced by low temperature, confers freezing tolerance through the activation of a conserved CBF regulon (Benedict et al., 2006). Moreover, as we described in the introduction, components of the light signalling and circadian clock, such as PHYA and LHY, have been demonstrated to contribute to cold acclimation in poplar (Olsen et al., 1997; Welling et al., 2002; Ibañez et al., 2010; Johansson et al., 2015). In Arabidopsis, phytochromes and circadian clock transmit the cold signaling information to CBF transcription, therefore, the interaction of these components could modulate cold temperature responses in Arabidopsis and poplar (Shi et al., 2018).

In this thesis it is shown that the timing of bud set was highly sensitive to a decrease of 3°C. To infer new candidate genes required for the thermoregulation of this process, we identified SNPs-associated to bud set showing diurnal gene expression sensitive to thermocycles. Among them, we found a poplar ortholog of Arabidopsis *PLDδ* gene, a phospholipase which produces phosphatidic acid (PA), a signal molecule involved in ABA-induced stomatal closure. In presence of thermocycles, *PLDδ* diurnal oscillations were reinforced, indicating a potential role of this enzyme in the regulation of diurnal stomatal aperture in response to daily temperature variations. However, NO production, which modulates the activity of PLD enzymes during ABA-induced stomatal closure and is accumulated under low temperature, did not induce PLD-dependent PA accumulation in response to cold (Zhao et al., 2009; Cantrel et al., 2011). Thus, the seasonal function of *PLDδ* in poplar bud formation and cold acclimation might involve a PA-independent stomatal closure or a stomatal closure-independent mechanism. We also found a poplar ortholog to *CBF* gene, *CBF4*, which showed a strong activation in response to low night temperature, suggesting its putative role in the temperature signalling. In Arabidopsis, overexpression of *CBF4* in Arabidopsis results in the activation of C-repeat/dehydration-responsive element containing downstream genes that are involved in cold acclimation and drought tolerance, suggesting that poplar *CBF4* might participate in the response to both cold and drought stresses occurring during bud formation in autumn (Haake et al., 2002). Moreover, *CBF4* is induced by low ambient temperature and not by cold in Arabidopsis as well as in poplar (Wang & Hua, 2009; Fig. 8E), suggesting that a moderate decrease in temperature may invoke an adaptive response to prepare plants to cope with

a more drastic decrease in temperature, a typical situation occurring during the autumn-winter transition.

Conversely, we found 174 SNPs-associated to bud set genes which diurnal rhythms were lost under thermocycles. Between them, we found a poplar ortholog to *CCR1*, a circadian- and cold-controlled gene involved in the alternative splicing regulation in *Arabidopsis*. The disruption of clock rhythms by cold has been reported in tree and it has been suggested that this arrhythmia could function in cold acclimation (Ramos et al., 2005; Ibañez et al., 2008; Johansson et al., 2015). Moreover, the splice variants of the clock gene *CCA1* have been reported to participate in freezing tolerance in *Arabidopsis* (Seo et al., 2012). Thus, poplar *CCR1* diurnal rhythmicity loss by thermocycles supports the idea that circadian clock mediates in the transmission of temperature information to gene expression for cold acclimation and this mechanism might involve alternative splicing. Moreover, a similar diurnal rhythmicity loss was found for the poplar ortholog *PUR7*, involved in nucleic acid metabolism (Senecoff et al., 1996), indicating that daily temperature oscillations affect primary metabolism.

These analyses revealed that seasonal temperature variations could control the timing of bud set through the perturbation of diurnal expression of genes involved in many aspects of plant growth. Further functional analyses will provide evidences whether these candidates play a major role in thermoregulation of bud set.

5. *RAV1* induction modulates expression of plant stress response-related genes and improves stomatal conductance under SDs and low ambient temperature

In this thesis, we have shown that *RAV1* overexpression did not sustain *FT2* gene expression under SDs, therefore, *RAV1* up-regulation by early autumnal environmental cues may have a role which does not affect the timing of growth cessation and bud formation. *RAV1* homologs have been previously associated to response to biotic and abiotic stress in several herbaceous plants and seasonal regulation of growth in *Arabidopsis* and trees (Gutiérrez et al., 2002; Fowler et al., 2005; Lee et al., 2005; Sohn et al., 2006; Castillejo & Pelaz, 2008; Kagaya & Hattori, 2009; Li et al., 2011; Moreno-Cortés et al., 2012). In perennials, autumnal environmental cues and continuous SD up-regulate the expression of several family of transcription factors that have been proposed

to play a role in seasonal acclimation to cold and dehydration, and progression of bud set (Ruttink et al., 2007; Karlberg et al., 2010; Resman et al., 2010). The functional analysis of these transcriptional regulators is very scarce in poplar. The effect of low ambient temperature on physiological and developmental processes occurring during dormancy entrance has been poorly investigated at the molecular level. We dissected the seasonal activation of *RAV1* in poplar, which results to be induced by SDs and low ambient temperature. Through a reverse genetic approach, we identified *RAV1* transcriptionally regulated candidate genes involved in plant response to environmental stresses. Among them, 3 of 27 *RAV1* target genes showed similar seasonal patterns to *RAV1* transcript, *ERF72-1*, *RbohD1* and a *LEA* protein-like. We also found that *RAV1*, *ERF72-1* and *RbohD1* are tightly coexpressed under SDs at 21°C and upregulated at 15°C compared with 21°C under SDs. Interestingly, *TEM* genes, homologs to *RAV1*, are also induced by low ambient temperature suggesting that their temperature sensitivity is conserved in *Arabidopsis* and poplar (Marín-Gonzalez et al., 2015). EMSA assays identified that *RAV1* binds *in vitro* to *RbohD1* promoter but not to *ERF72-1* one, indicating that only *RbohD1* is a direct target of *RAV1*. Moreover, *ERF72* was repressed after 4h of DEX induction compared with MOCK treatment in *GERoe* plants under SDs but its transcription was activated by continuous daily induction of *RAV1*.

All together, these results suggest that *RAV1-RbohD1* is a responsive transcriptional module to the early autumnal cues. *ERF72-1* activation by *RAV1* could require activation or repression of intermediary players in a time-dependent manner.

Our phenological assays using poplar overexpressing *RAV1* and knockdown lines grown under SDs and low ambient temperature revealed that *RAV1oe* lines showed 17-26 % increased biomass at the time of bud set under SDs at 15°C. Moreover, we found higher photosynthesis and transpiration rates in *RAV1oe* lines when compared to WT and *RAV1kd* lines after two weeks of SDs at 15°C. Part of this increased biomass was attributed to higher leaf number and non-apical stem growth. Stems are one of the sink tissues for the storage of sugars, proteins and fats during poplar acclimation (Rinne, Welling & van der Schoot, 2010). Thus, part of the increased biomass in *RAV1oe* plants might be attributed to higher synthesis of storage compounds. *RAV1oe* plants may have more storage due to increased photosynthesis and better acclimation than WT during autumnal season.

A plausible functional complementation of *RAV1kd* by the activation of *RbohD1* independent pathways may explain the non-significant phenotypic differences observed in these plants, compared with WT. Transcriptomic comparison between *RAV1kd* plants and WT did not reveal the downregulation of *RbohD1*. However, *ERF72-1* was upregulated in *RAV1kd* plants compared with WT, suggesting that the functional complementation of *RAV1kd* plants could occur through *ERF72-1* activation. RAV1 and ERF72-1 might be interdependent elements which tend to compensate each other's levels.

6. RAV1 induction may enhance diurnal stomatal movements through the activation of *RbohD1* and *ERF72-1* transcription under SDs and low ambient temperature

Because we found that RAV1 directly binds to the *RbohD1* promoter in EMSA assays and *RAV1oe* led to higher expression levels of *RbohD1*, we studied if RBOHD1 activity causes the higher stomatal responsiveness observed in *RAV1oe*. We observed that an increased ROS burst in poplar *RAV1oe* guard cells is associated with faster stomatal closure after ABA treatment. Moreover, DPI treatment partially reduced ABA-dependent stomatal closure in *RAV1oe* as well as in WT, pointing again to the implication of NADPH oxidases in the regulation of poplar stomatal movement.

Regulation of stomatal movement has been widely studied in Arabidopsis, however only few transcription factors have been implicated in their regulation (Cominelli et al., 2010). MYB60 transcription factor has been described as integrator of multiple signal-transduction processes by modulating the expression of genes involved in different guard-cell responses (Cominelli et al., 2005). Similarly, the function of RAV1 as a transcription factor is related to plant response to environmental stimuli indicating that RAV1 could be a modulator of gene expression for guard cell responses to environmental changes. In Arabidopsis, NADPH oxidases RBOHD/F have been functionally associated with the regulation of ROS production in guard cells, that are necessary for stomatal opening and closure (Kwak et al., 2003; Hao et al., 2012). Structure-function of RBOHD has been extensively explored in Arabidopsis. (Boudsocq et al., 2010; Dubiella et al., 2013; Li et al., 2014; Kadota et al., 2014; Chen et al., 2017). However, transcriptional regulation of *RbohD* is less understood. In tomato, brassinosteroids induced *RBOH* transcription and increased NADPH oxidase activity

with the concomitant increase in apoplastic H₂O₂ leading to stress tolerance (Nie et al., 2013). Similar effects of ABA on *RBOH* expression and the production of H₂O₂ have been reported (Xia et al., 2015). Recently, it was reported that ERF74, a member of ERFs-VII group, activates *RbohD* transcription in response to different abiotic stresses conferring tolerance in Arabidopsis (Yao et al., 2017a). We found that *ERF72-1* is coexpressed with *RbohD1* under SDs and low ambient temperature suggesting that ERF72-1 could act in parallel with RAV1 to activate *RbohD1* transcription similar to what observed in Arabidopsis (Yao et al., 2017a; Yao et al., 2017b). However, whether transcriptional regulation of *RbohD* modulates diurnal or seasonal stomatal responsiveness was not explored.

In poplar, regulation of molecular mechanisms underlying stomatal movements remains less well understood. It has been recently reported that overexpression of a ubiquitin E3 ligase, showing higher expression levels of *RbohD/F*, conferred drought tolerance via ABA-induced stomatal closure by hydrogen peroxide production (He et al., 2018). This suggests that *RBOH* genes could play a conserved role in the regulation of ROS signalling and stomatal movements in Arabidopsis and poplar. Although, molecular mechanisms controlling stomatal movements which involve transcriptional regulation has not been discovered in poplar yet.

Our time course measurements of stomatal conductance of poplar grown under SD and low ambient temperature conditions suggested that *RAV1oe* plants have faster stomatal aperture without compromising the closure in response to light to dark transition in comparison with WT and *RAV1kd* plants. Similarly, stomatal aperture assay showed faster aperture of stomatal guard cells in *RAV1oe* than WT and *RAV1kd* when leaf discs were shifted from darkness to light conditions for 2 hours. These experiments indicated that constitutive high levels of *RAV1* increased stomatal responsiveness and consequently produced faster stomatal movement in response to daily dark/light transitions and this effect could be mediated by upregulation of *RbohD1* and *ERF72-1*.

Higher levels of RBOHD1 cellular sensor may produce hypersensitive stomata to the environmental and endogenous signals that govern diurnal stomatal movements. The endogenous signals that regulate RBOH-dependent ROS production have been shown to display diurnal oscillatory patterns, such as ABA and Ca²⁺ (Love et al., 2004; Legnaioli et al., 2009; Pokhilko et al., 2013). Both ABA and Ca²⁺ regulate the activity of RBOH protein and the generation of ROS signalling in guard cells (Sagi and Fluhr, 2006). Moreover, the stability of ERF72 protein, Arabidopsis ortholog of ERF72-1, has been

functionally associated to stomatal opening in *Arabidopsis* (Gibbs et al., 2014). Thus, a plant showing higher levels of *RbohD1* and *ERF72-1* could improve its stomatal movements.

7. The biological relevance of the stomatal movement seasonal control

Regulation of diurnal stomatal movements is a key factor in the control of plant water use efficiency, photosynthesis and growth (Jones et al., 1998; Schroeder et al., 2001; Lawson and Blatt, 2014; Isaac-Renton et al., 2018). Manipulation of the stomatal conductance has been one of the target strategies to improve photosynthesis (Evans, 2013). Genetically-engineered crops showing increased stomatal conductance also display augmented net assimilation rate under non-limiting water conditions (Roche, 2015). Our data indicated that increased responsiveness of stomata in *RAV1oe* lines resulted in faster diurnal stomatal aperture. Consequently, there is higher CO₂ intake and transpiration during the day. In fact, *RAV1oe* lines showed higher photosynthesis and transpiration rates.

It is unknown whether diurnal regulation of stomatal movement participates in the acclimation of perennial plants in the autumnal season. However, temperature modulates stomatal opening in poplar (Urban et al., 2017). Thus, a seasonal drop of temperature might impact the diurnal regulation of stomatal movement and plant physiology. According to this, our stomatal conductance measurements indicated WT and *RAV1kd* plants grown under SD and 15°C showed 35% reduction of stomatal conductance while *RAV1oe* lines showed only 20% reduction respect to 21°C under LDs. This result indicated that poplars showing constitutive high levels of *RAV1* exhibited less decreased stomatal conductance respect to WT and *RAV1kd* lines under SD and low ambient temperature compared to LD at 21°C. This mitigation might be mediated by the transcriptional activation of *RbohD1* and *ERF72-1*. Interestingly, it has recently shown that stomatal conductance participates in the thermal acclimation of photosynthetic rate in poplar (Benomar et al., 2019). Therefore, the less decrease of stomatal conductance displayed by *RAV1oe* plants under SDs and low ambient temperature which resulted in the higher photosynthetic rate compared to WT suggests that *RAV1* activation could contribute to the thermal acclimation of photosynthesis during autumnal season. This also

suggests that seasonal induction of *RAV1* might be relevant to readjust stomatal movement to daily dark/light transitions under autumnal cues.

We propose that the seasonal induction of *RAV1* modulates stomatal responsiveness and consequently improves diurnal stomatal movements and photosynthesis contributing to poplar acclimation during autumn (Fig. 44).

Figure 44. RAV1 induction-dependent diurnal stomatal movement mechanism for poplar autumnal acclimation.

Filled arrows indicate observations that have been experimentally demonstrated. Filled and dashed arrow shows indirect relationship, while empty and dashed arrows indicate hypothetical relationships.

8. *RbohD* diurnal pattern integrates environmental and endogenous signals in poplar and *Arabidopsis*

Daily and seasonally environmental cycles are perceived and integrated by endogenous timekeepers, such as the circadian clock, to fine-tune plant physiology with the changing environment and to perform biological processes at the proper time of the day and year (Nohales & Kay, 2016). We found that *RAV1* and *RbohD* showed diurnal rhythmic gene expression in poplar. The diurnal rhythms of *RAV1* and *RbohD* under LDs support their transcriptional module role acting in the control of diurnal stomatal movements. Because circadian clock is the master regulator of endogenous rhythms, we addressed the hypothesis that diurnal oscillations of *RAV1* and *RbohD* could be dictated

by the clock. However, under continuous light where rhythms clock-controlled genes are maintained, we did not detect consistent diurnal rhythms of *RAVI* and *RbohD*. These results suggest that *RAVI* and *RbohD* diurnal patterns do not follow a circadian rhythm in poplar and strongly depend on day/night cycles.

Similar to the poplar ortholog, diurnal rhythms of *RbohD* gene expression also exists in *Arabidopsis*, with transcript accumulation during the night. We used a luciferase-based *RbohD* transcriptional reporter, *pRbohD::LUC* *Arabidopsis* line to monitor rhythms of *RbohD* transcription. We found diurnal rhythms of *pRbohD::LUC*, with higher luciferase activity during the daytime under both LDs and SDs in leaf discs of 4-week-old plants. Moreover, under continuous light, *RbohD* promoter maintained its oscillations with a period of about 24h, suggesting a circadian regulation. The lack of match between the pattern of *pRbohD::LUC* expression and *RbohD* mRNA diurnal oscillations suggested a potential role of systemic signals for *RbohD* transcriptional regulation that should be present in the entire plant but missing in *pRbohD::LUC* leaf disc assay. We found that exogenous addition of sucrose promoted *pRbohD::LUC* activity in a similar manner to *RbohD* mRNA levels, with marked increment of luciferase activity during the night. This result indicates that sucrose might be the systemic signal acting on *RbohD* promoter. Moreover, we found that the sucrose-promoted oscillations of *pRbohD::LUC* activity strongly depended on day-night cycles, similar to what observed for poplar *RbohD* transcription. These led us to hypothesized that sucrose- and night-dependent activation *RbohD* transcription should be somehow linked to the sucrose breakdown which occurs during the night to sustains ATP production through plant respiration. Indeed, we found that glucose, the product of sucrose degradation, induced *pRbohD::LUC* activity in a similar manner to sucrose, indicating that sucrose breakdown may be the process responsible of *RbohD* activation in the night.

It has been shown that starch degradation during the night is controlled by the circadian clock, thus free sucrose is pumped to the vascular system (Graf et al., 2010). Cell wall invertases (CWIs), sucrose-splitting enzymes, capture sucrose from the vascular system generating free glucose and fructose which enter into the cytosol. Therefore sucrose-dependent plant cell activities, including transcriptional regulation of *RbohD*, are clock downstream processes. Indeed, a tomato cell wall invertase (LIN6) showed diurnal rhythm and its promoter was induced by LHY and CCA1, suggesting a clock contribution in the modulation of invertase diurnal pattern (Proels and Roitsch, 2009). All together,

these results suggest the timing of *RbohD* transcription is directly dependent of sucrose breakdown during the night and indirectly controlled by the circadian clock.

Sucrose-derived hexoses are targets of hexokinases (HXKs) which produce hexoses phosphate, such as glucose-6-phosphate, affecting gene expression through glucose metabolism (Aguilera-Alvarado & Sánchez-Nieto, 2017). Regardless of its catalytic function, HXK1 is also a sensor of glucose which participates in nutrient, light, and hormonal signalling in Arabidopsis (Moore et al., 2003). Thus, HXK could be play a role in a plausible mechanism involving intracellular glucose sensing and/or signalling upstream *RbohD* transcription in Arabidopsis. Future genetics analysis using *pRbohD::LUC* in *hvk* mutant background could support this regulation.

9. The relevance of *RbohD* diurnal rhythms in the immune response of Arabidopsis

The role of *RbohD* during plant immunity has been extensively studied in Arabidopsis (Kadota et al., 2015). The perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) leads to a rapid, specific and strong production of ROS, which depends on RBOHD activity. RBOHD is mainly controlled by Ca^{2+} via direct binding to EF-hand motifs and phosphorylation by Ca^{2+} -dependent protein kinases. Moreover, the connections between the clock, sugars and the plant immunity have been previously presented (Wang et al., 2011; Haydon et al., 2013; Zhou et al., 2015).

The functional link between clock and plant immunity has been recently revealed (Wang et al., 2011). Genes involved in R-gene-mediated resistance against downy mildew in Arabidopsis are under the control of the clock gene *CCA1*, allowing plants to ‘anticipate’ infection at dawn when the pathogen normally disperses the spores. Moreover, NADPH, a substrate of RBOH enzymes to generate ROS, follows a circadian rhythm and this redox rhythm is sensed by the master immune regulator NPR1 which reinforces circadian clock gene oscillations after perception of immune signal salicylic acid (Zhou et al., 2015). Interestingly, NADPH showed a circadian rhythm that perfectly matched with the Arabidopsis *RbohD* diurnal pattern. The rhythmic expression of the defence genes in the absence of pathogen indicates that plants are programmed to ‘anticipate’ infection according to a circadian schedule.

The functional link between photosynthetic sugar and clock oscillations has been identified (Haydon et al., 2013). Moreover, sucrose reinforces circadian rhythm of clock reporter genes such as *CCA1*, *TOC1*, *CAB2* and *GI* (Dalchau et al., 2011).

Thus, *RbohD* diurnal rhythms could be the result of the clock function and sugar metabolism combined action which synchronize the plant basal alert system against possible daily pathogen attack. The regulation of *RbohD* transcription by sucrose-dependent signalling, which might be gated by the clock, sets *RbohD* in a proper phase to cope with the biotic challenges occurring daily. In agreement with this idea, we found that the bacterial peptide flg22, which triggers RBOHD-dependent ROS production, induced higher ROS production during the night than the day in *Arabidopsis*. This result indicates that the magnitude of the immune response is dependent of diurnal pattern of *RbohD* transcription. The capacity to produce a time-dependent magnitude of the response is commonly attributed to clock. Recently, the link between RBOHD and clock function has been identified. After *Pseudomonas syringae* infection, RBOHD mediates in the systemic clock responses which result in period and amplitude changes of circadian rhythms (Li et al., 2018). This work also reveals the participation of RBOHD as a regulator of circadian rhythms in basal conditions. However, the participation of circadian clock in the modulation of *RbohD* diurnal transcription has yet to be determined in *Arabidopsis*. Moreover, if sucrose signalling participates in the generation of high sensitivity to flg22, through RBOHD-dependent ROS burst, has also to be genetically tested.

CONCLUSIONS

1. We show that RAV1 neither represses *FT2* nor plays a role in the regulation of growth cessation timing, therefore photoperiodic regulation of shoot apical growth in poplar shows conserved and particular features respect to Arabidopsis flowering.
2. We predict that known regulators of Arabidopsis flowering and *FT* transcription could play a role in the photoperiodic regulation of poplar shoot and apical height growth.
3. We show that progression of bud set is sensitive to a little decrease of ambient temperature and unravel thermosensitive candidate genes acting in the temperature-dependent regulation of bud formation.
4. We show that RAV1 autumnal induction during the transition from growth to dormancy is promoted by SDs and low ambient temperature.
5. We identify the gene regulatory network controlled by RAV1, indicating that its molecular function is related with plant response to environmental stresses.
6. We find that RAV1 target genes, *RbohD1* and *ERF72-1*, are co-expressed with *RAV1* under SDs and low ambient temperature.
7. *RAV1* overexpression improves diurnal stomatal movement, compared with wild-type and *RAV1* knock down lines, under SDs and low ambient temperature. The lack of differences between *RAV1kd* and WT could be due to a functional complementation.
8. The higher stomatal responsiveness in *RAV1oe* lines could be explained by the *RbohD1* and *ERF72-1* transcriptional activation observed under SDs and low ambient temperature.
9. *RAV1* and *RbohD1* are also coexpressed under LDs and show non-circadian diurnal rhythms of transcription in poplar.

10. Diurnal rhythms of *RbohD* transcription are conserved in Arabidopsis and poplar.
11. The strong activation during the night of Arabidopsis *RbohD* transcription is induced by sucrose-dependent signalling.
12. The daily rhythms of *RbohD* transcription contributes to create a diurnal period of maximal response against pathogen attack.

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APPENDIX

Supplementary Figure 1. Quantification of *p35S::3xHA:RAV1* transgene expression determined by qRT-PCR in 12 poplar *RAV1oe* lines.

qRT-PCR analysis of *p35S::3xHA:RAV1* transgene expression in 12 independent transgenic lines. Values represent mean $\pm$ standard deviation of three technical replicates. Gene expression values were normalized with the transgenic line showing the lower expression of the transgene (=1). Transgene expression was evaluated using HA FW qPCR/RAV1 RV qPCR primers.

Supplementary Figure 2. Heatmap showing poplar transcriptomic changes during the year.

The expression values were normalized with the maximum of gene expression for each gene (=1). Heatmap represents the mean of two biological replicate.

Supplementary Figure 3. Gene ontology enrichment analysis of DEGs in *RAV1oe* vs WT.

To identify enriched groups of gene ontology, Arabidopsis homologs to poplar were analysed in agriGO database (Tian et al., 2017) with advanced settings (selected reference: TAIR9 genome locus; statistical test method: Hypergeometric; multi-test method: Bonferroni; P adjusted value<0.01; minimum mapping entries:5; gene ontology type: Complete GO). GO plot package in R software was used to combine the most representative enriched groups of gene ontology with gene expression from the RNAseq results. The overlapping between GO terms were reduced using GPlot function “reduce_overlap” (=0.8). z-score represents the overall tendency of each biological process to be repressed or activated and is calculated as described in (Wencke et al., 2015). Size and colour of semi-inner circles represent the p adjusted value and the overall regulation, respectively. Blue and red points represent logarithmic fold-change (logFC) of expression for each gene.

Supplementary Figure 4. Characterization of poplar *p35S::GR:eGFP:RAV1* transgenic lines.

(A) qRT-PCR analysis of *p35S::GR:eGFP:RAV1* transgene expression in 10 independent transgenic lines. Values represent mean $\pm$ standard deviation of three technical replicates. Gene expression values were normalized with the transgenic line showing the lower expression of the transgene (=1). Transgene expression was evaluated using EGFP FW qPCR/EGFP RV qPCR primers. (B) Schematic representation GR system to control nuclear localization of a transcription factor after DEX treatment. (C) Confocal microscope root cell images of *35S::GR:eGFP:RAV1* transgenic lines treated with MOCK (Tween20 0.05%, absolute EtOH) or DEX (Tween20 0.05%, dexamethasone 30 μ M) after 4 hours. White bar: 50 μ m.

Supplementary Figure 5. Gene ontology enrichment analysis of DEGs DEX vs MOCK after 4 hrs in *GERoe* line.

To identify enriched groups of gene ontology, Arabidopsis homologs to poplar were analysed in agriGO database (Tian et al., 2017) with advanced settings (selected reference: TAIR9 genome locus; statistical test method: Hypergeometric; multi-test method: Bonferroni; P adjusted value<0.01; minimum mapping entries:5; gene ontology type: Complete GO). GO plot package in R software was used to combine the most representative enriched groups of gene ontology with gene expression from the RNAseq results. The overlapping between GO terms were reduced using GOpilot function “reduce_overlap” (=0.8). z-score represents the overall tendency of each biological process to be repressed or activated and is calculated as described in (Wencke et al., 2015). Size and colour of semi-inner circles represent the p adjusted value and the overall regulation, respectively. Blue and red points represent logarithmic fold-change (logFC) of expression for each gene.

Supplementary Figure 6. Gene ontology enrichment analysis of DEGs in *RAV1kd* vs WT.

To identify enriched groups of gene ontology, Arabidopsis homologs to poplar were analysed in agriGO database (Tian et al., 2017) with advanced settings (selected reference: TAIR9 genome locus; statistical test method: Hypergeometric; multi-test method: Bonferroni; P adjusted value <0.01; minimum mapping entries:5; gene ontology type: Complete GO). GO plot package in R software was used to combine the most representative enriched groups of gene ontology with gene expression from the RNAseq results. The overlapping between GO terms were reduced using GOpot function “reduce_overlap” (=0.9). z-score represents the overall tendency of each biological process to be repressed or activated and is calculated as described in (Wencke et al., 2015). Size and colour of semi-inner circles represent the p adjusted value and the overall regulation, respectively. Blue and red points represent logarithmic fold-change (logFC) of expression for each gene.

Primer.name	Sequence
M001 GR FW	ATGGCTAGTGAAGCTCGAAAAACAAAG
M002 GR RV	AGGCCTGCTAGTAGATTTTGTATGAAACAG
M003 eGFP FW StuI	CAGGCCTATGGTGAGCAAGGGCGAGGAGC
M004 eGFP RV NotI	TGCGGCCGCTTGTACAGCTCGTCCATGCCGAG
M005 RAV1 FW NotI	GGCGGCCGCAATGGATGGAAGCTGCATAGATGAAAG
M007 RAV1 RV	TCTAGATTACAAGGCTCCAACGATCCTTTGTTTCTTAC
GR BP FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTAGTGAAGCTCGAAAAAC
RAV1 BP RV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACAAGGCTCCAACGATCCTTTG
RAV1 CDS BP FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGGAAGCTGCATAGATGAA
RAV1 CDS BP RV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACAAGGCTCCAACGATCCTTTG
EGFP FW qPCR	AACCACTACCTGAGCACCCAG
EGFP RV qPCR	TCGTCCATGCCGAGAGTGATC
HA FW qPCR	AATTAACATCTTTTACCCATACGA
RAV1 FW qPCR	CCTCATCTCTCTGTCTCTCTTGC
RAV1 RV qPCR	TGGTAGCAGGTGGTGGGAAGTGA
18 S FW qPCR	TCAACTTTCGATGGTAGGATAGTG
18 S RV qPCR	CCGTGTCAGGATTGGGTAATTT
ERF72 qPCR FW	ATGAAGATTATGAGCTGGAAGAG
ERF72 qPCR RV	CGATACTGATGATGTGTCACAAG
RbohD qPCR FW	GCAGGATAAGTGAAGCTAATC
RbohD qPCR RV	ACTAAAGGCTGTTCTGTCTCGC
pRBOHD probe FW	AAAGACATCAGTTGTTGCAAAGTCCATGGA
pRBOHD probe RV	TCCATGGACTTTTGCAACAAGTATGTCTTT
pRBOHD probeM FW	AAAGACATCAGTAAAAACAAAGTCCATGGA
pRBOHD probeM RV	TCCATGGACTTTGTTTTTACTGATGTCTTT
pERF72 probe FW	TGCCAACCCGCTTGTGATCCAACCAATAT
pERF72 probe RV	ATATTGGTTGGATCAACAAGCGGGTTGGCA
pUPD RAV1 FW1	GCGCCGTCTCGCTCGAATGATGGATGGAAGCTGCATAGAT
puPD RAV1 RV1	GCGCCGTCTCGCTGTCTCATTAACTTGCTTGAAG
pUPD RAV1 FW2	GCGCCGTCTCGACAGAAGATGATGAAATAGAGGC
puPD RAV1 RV2	GCGCCGTCTCGCTCGCTGCTTACAAGGCTCCAACGATCC

Table 2. List of primers used in this work.

List of Annexes:

Annex I: Maurya JP, Triozzi PM, Bhalerao RP, Perales M. 2018. Environmentally Sensitive Molecular Switches Drive Poplar Phenology. *Frontiers in Plant Science* 9:1873. Indication of Quality for UPM Thesis.

Annex II: Triozzi PM, Ramos-Sánchez JM, Hernández-Verdeja T, Moreno-Cortés A, Allona I, and Perales M. 2018. Photoperiodic Regulation of Shoot Apical Growth in Poplar. *Frontiers in Plant Science* 9: 1030. Indication of Quality for UPM Thesis.

Dataset S1: Diurnal oscillations of poplar genes showing height-associated SNPs.

Dataset S2: Diurnal oscillations of poplar genes showing bud set-associated SNPs.

Dataset S3: Analysis of global gene expression in 2-year-old branches of *Populus alba* during the whole year. *RAV1* annual gene expression pattern.

Dataset S4: DEGs in *RAV1oe#3* compared to WT after 10 days of short days at ZT12. GO enrichment analysis of DEGs in *RAV1oe#3* line compared to WT. DEGs in *GERoe#9* after 4 hours of DEX compared to MOCK treatment after 10 days of short days at ZT12. GO enrichment analysis of DEGs in *GERoe#9* line in DEX VS MOCK treatment. *RAV1* target genes resulting from the intersection between DEGs of *RAV1oe#3* VS WT and DEGs of *GERoe#9* DEX VS MOCK.

Dataset S5: Seasonal gene expression patterns of *RAV1* consistent target genes.

Dataset S6: DEGs in *RAV1kd#1* compared to WT after 10 days of short days at ZT12. GO enrichment analysis of DEGs in *RAV1kd#1* compared to WT.