ABA and Chromatin Remodelling Regulate the Activity-Dormancy Cycle in Hybrid Aspen

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Doctoral Thesis
Swedish University of Agricultural Sciences
Umeå 2011

Acta Universitatis agriculturae Sueciae
2011:17

Every day, many things.
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Cover: Home
(photo: M. Petterle)

ISSN 1652-6880
ISBN 978-91-576-7552-1

Print: Arkitektcopia, 2011

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Abstract

One of the most important survival strategies plants have evolved is the cessation of growth and development of dormancy in response to the seasonal changes. Apical meristems of deciduous woody plants living in temperate and boreal zones undergo a yearly cycle between an active and a dormant state, which allows them to protect the meristem and avoid damage provoked by extremes of low temperatures during the winter. The transition between an active and a dormant state includes a complex network of physiological and developmental processes such as acquisition of cold hardiness, bud formation and maturation and metabolic changes, which are underlined by a global change in gene expression.

The studies described in my thesis provide an overview of the transcriptional control underlying the activity-dormancy cycle and are aimed to dissect the regulation of overlapping short days (SD)-induced processes. We investigated the control of SD-induced bud formation and maturation, acquisition of adaptive responses and of endodormancy and identified novel key molecular players regulating these processes. Our results provide evidence for a composite control of the activity-dormancy cycle by the SD signal that involves plant hormone abscisic acid (ABA) and a component of a chromatin remodelling complex, *FERTILISATION INDEPEDENDENT ENDOSPERM* (*FIE*), as key players. Importantly, our work reveals a degree of conservation in the regulatory framework for the control of dormancy in seeds and apical buds.

Keywords: Populus, activity-dormancy cycle, ABA, chromatin remodelling, *FIE*, cold acclimation, apical bud, seed dormancy.

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Dedication

To the Russian. 1-0 for me.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Karlberg A., Englund M., **Petterle A.**, Molnar G., Sjodin A., Bako L., Bhalerao R.P. (2010). Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology 27*(1), 1-16.

II **Petterle A.**, Resman L. and Bhalerao R.P. (2011). ABA acts at multiple stages to control SD-mediated activity-dormancy transition. Manuscript.

III Englund M., Molnar G., **Petterle A.,** Gaboreanu I., Bereczky Z., Karlberg A. and Bhalerao R.P. (2011). Polycomb repression complex component *FIE* regulates dormancy related processes in perennial plants and *Arabidopsis*. Manuscript.

The contribution of Anna Petterle to the papers included in this thesis was as follows:

I Anna Petterle contributed to data analysis and writing of the manuscript. Her contribution is about 25-30%.

II Anna Petterle contributed to generation of data, data analysis and writing of the manuscript. Her contribution is about 80%.

III Anna Petterle contributed to generation of data, data analysis and writing of the manuscript. Her contribution is about 30-35%.

Abbreviations

All abbreviations are explained when they first appear in the text.

1 INTRODUCTION

Plants are sessile, and thus unable to avoid unfavourable conditions by simply moving away from sites with such conditions as animals do. Hence, plants have evolved a variety of mechanisms that enable them to sense and respond to environmental changes and fluctuations in a timely manner, thus maximising their chances of survival. One of the most important survival strategies plants have developed is dormancy, which has been defined by Lang (1987) as "the temporary suspension of visible growth in any plant structure containing a meristem". This definition covers various types of dormancy that were classified by Lang, according to the provenance of the signal controlling the inhibition of growth, as paradormancy, ecodormancy and endodormancy. Signals regulating these three types of dormancy may be present simultaneously in any given meristem. Paradormancy, the most extensively studied, refers to the inhibition of lateral buds' growth from the apical meristem and plays a crucial role in controlling plant architecture and reserving resources for reproduction. Ecodormancy, defined as the inhibition of growth by temporarily unfavourable environmental conditions, can be initiated by a variety of signals including shortening of daylength, cold and drought stress. Endodormancy, in contrast, is an intrinsic state of the meristem in which growth is inhibited by signals originating from within the dormant tissue or organ itself (Horvath *et al.*, 2003; Lang *et al.*, 1987).

Due to the cycling of the seasons, and consequently of growth-permitting conditions, apical meristems of deciduous woody plants living in temperate and boreal zones undergo a yearly cycle between an active and a dormant state (Rohde & Bhalerao, 2007; Welling & Palva, 2006). The definition of endodormancy by Lang (1987), although substantially correct, needs to be refined in order to address the molecular basis of this process,

since it has two major shortcomings. First, endodormancy development requires exposure to short days, signal that originates from the environment, not from inside the dormant tissue. Second, once dormancy is developed, the plant is not able to respond to growth-promoting signals, and regains this sensitivity only after winter. Following this, the meristem actively grows only if the environmental conditions are not adverse, even if dormancy has been released. In addition, the definition does not distinguish between absence of visible growth by cell division or cell expansion, which precedes cell division during reactivation. Moreover, growth can be a visually difficult parameter to record, due to the structure of the apical meristem. Therefore, endodormancy can be more correctly defined as the inability to initiate growth under favourable conditions (Rohde & Bhalerao, 2007).

Figure 1 illustrates the activity-dormancy cycle. During the summer, light and temperature conditions are growth permissive, while in the winter actively growing tissues would suffer fatal damage. When daylength decreases, plants cease growth and set apical buds, reaching first a stage of ecodormancy (Fig. 1). In this stage plants are still able to respond to growth-promoting environmental stimuli, such as exposure to long days (LD), by reinitiating growth. Once endodormancy has developed, the apical meristem loses this capacity and only treatment with prolonged periods of chilling temperatures can break dormancy. Cold treatment does not initiate growth but restores the plant's ability to respond to growth-promoting signals, for example warm temperatures in spring (Fig. 1). Daylength seems not to play a key role in reactivation, as spruce plants were able to flush buds even in complete darkness once subjected to cold treatment (Worrall & Mergen, 1967).

The development of dormancy is a complex process that is essential for the survival of the meristem, as it prevents premature bud break. Understanding of the molecular mechanisms underlying responses to short days (SD), and in particular the development of dormancy, is crucially important because of their impact on crop yield and availability, biomass production, and on the distribution of certain crops and perennials. The development of techniques to manipulate dormancy would allow specific plant varieties to be more readily adapted to specific climates, and trees capable of adapting to changing climate and latitudes to be bred. However, despite their relevance, little is known about the physiological and molecular mechanisms underlying these processes. The focus of this thesis, and the studies it is based upon, is the development of endodormancy that occurs in

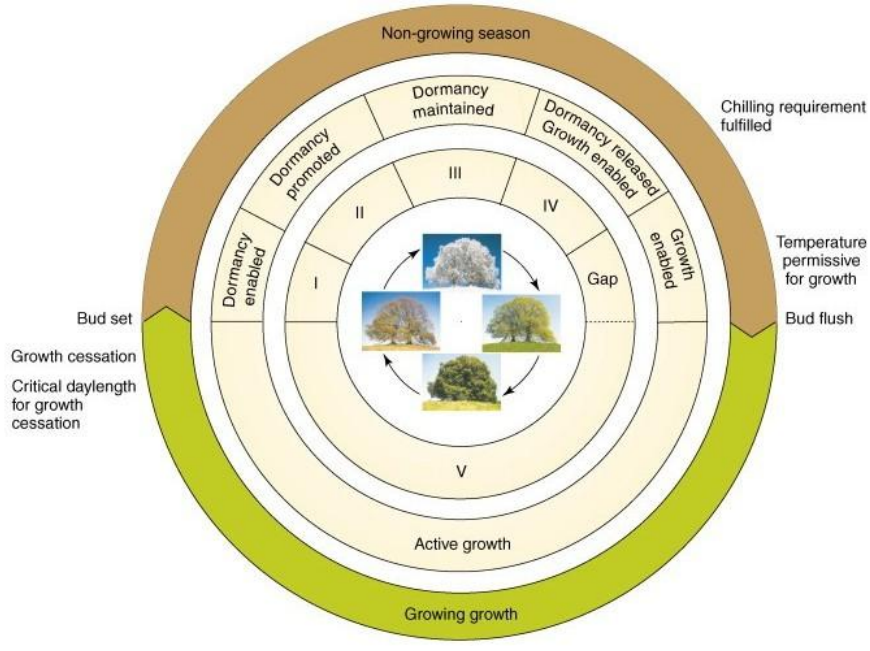

FIGURE 1: Transitions between active growth and dormancy in the apical meristem of *Populus spp.* in response to SD. The inner circles depict the growth-dormancy status and the corresponding meristem stages: I, cessation of cell division; II, establishment of dormancy; III maintenance of dormancy; IV release from dormant state and V, resumption of cell division. Gap=phase when growth does not occur because of environmental restraints.
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response to shortening of the daylength in hybrid aspen apical buds (*Populus tremula x tremuloides*), and the related SD-triggered responses. The following section of this introduction provides a brief review of the mechanisms leading to SD-related responses and processes that accompany dormancy development, paying particular attention to aspects that are of most relevance to the thesis and appended manuscripts.

1.1 Photoperiodism

The molecular bases of the control of endodormancy are poorly understood in comparison to paradormancy. Nevertheless, some knowledge has been obtained on the first signals and part of the signalling cascade that leads to its

establishment. Several environmental factors, including daylength, temperature, water and nutrient availability play major roles in controlling the development of endodormancy in apical buds (Nooden & Weber, 1978). In particular, temperature (for instance, in apple and pear), photoperiod (in *Populus spp.* and silver birch) and a combination of the two have been shown to be the most influential cues for dormancy induction and release in woody plants (Stevenson & Tanino, 1994). Daylength represents, in contrast to temperature changes, a very reliable indicator of both the night and day cycle and the progress of seasons, while other cues, such as temperature, fluctuate too variably each year. During the spring and early summer the daylength increases gradually, while it declines in late summer and autumn. When the length of the day drops below a certain threshold, defined as the critical daylength for the plant, plants start to undergo growth cessation and activate SD-induced responses. Photoperiodism, the capacity to respond to photoperiod, is a highly adaptive trait, which is under strong genetic control. In addition to seasonal growth cycles, photoperiodism controls a number of other processes including flowering in both annual and perennial plants (Bohlenius *et al.*, 2006; Suarez-Lopez *et al.*, 2001; Koornneef *et al.*, 1991), etiolation and seed germination.

1.1.1 Perception of light signals and clock entrainment

Plants live in a rhythmic environment and need to differentiate between the day/night cycle of light (important for photosynthesis) and temperature, in order to anticipate this cycle and optimise their responses. The solution that plants and other organisms have evolved is the circadian clock system, which drives matching metabolic, physiological and behavioural rhythms (Harmer *et al.*, 2001). The evolution of the clock has allowed plants not only to anticipate the day and night cycle but also to measure daylength changes as indicators of the cycling of seasons. The circadian clock consists of a core central oscillator, plus several input and output signals (reviewed in Mas & Yanovsky, 2009; Eriksson & Millar, 2003). The core oscillator generates and maintains an oscillation that guides all other circadian rhythms. The natural period of the clock is often different from 24 hours, so these innate rhythms need to be entrained with the cycling environment in order for plants to be able to properly respond and adapt. The entrainment of the circadian clock requires the light to be perceived and in turn regulate clock components, synchronizing with the period and phase of the external rhythm (reviewed in(Mas & Yanovsk *y*, 2009; Fankhauser & Staiger, 2002).

Day/night cycles, and seasonal changes, are accompanied by changes in the light regime which is monitored by photoreceptors. The best characterised photoreceptors are the phytochromes (PHYs), which mediate red and far red light signals, and cryptochromes (CRYs) and phototropins (phot), which absorb blue light (reviewed by Devlin, 2002; Fankhauser & Staiger, 2002). Phytochromes work as homodimers conjugated with a chromophore that absorbs the light. In *Arabidopsis* there are five phytochromes, designated PHYA to PHYE, two cryptochromes, CRY1 and CRY2, and two phototropins, phot1 and phot2. Phytochromes can be reversibly shifted between active and non-active forms by light pulses of different quality; they are synthesized as inactive forms (Pr) that are activated if they absorb red light (R; λ_{max}, 660 nm). The active forms (Pfr) can enter the nucleus, where they can profoundly affect gene expression, and absorb far red light (FR; λ_{max}, 730 nm), which converts them back to Pr (Nagy & Schäfer, 2002). Phytochromes activate gene expression by interacting with components of the light signalling cascade, PHYTOCHROME INTERACTING FACTORS (PIFs), which are degraded in response to the light stimulus and therefore release their inhibition of photomorphogenesis (Kircher *et al.*, 1999; Sakamoto & Nagatani, 1996). PHYA belongs to the photolabile class of photoreceptors, whereas PHYB, C, D and E are more stable during illumination. In the evening, the ratio of red/far red light increases in direct proportion to shortening of the daylength and consequently the ratio of Pr/Pfr decreases (Smith, 1982). Therefore, it is not surprising that phytochrome action has been implicated in plant responses to daylength (Nooden & Weber, 1978). A *PHYB* gene has been mapped to a linkage group that contains both bud set- and bud break-associated QTLs (Frewen *et al.*, 2000), and Ingvarsson et al., (2006) demonstrated that a two amino acid substitution in PHYB2 is associated with natural variation in bud set in *Populus* (Ingvarsson *et al.,* 2006). In addition, PHYA has been shown to play a prominent role in the photoperiodic regulation of growth cessation (Ruonala *et al.*, 2008; Olsen *et al.*, 1997b). Indeed, modulation of *PHYA* expression significantly affects growth cessation responses. For example, overexpression of oat *PHYA* in hybrid aspen results in plants that are not sensitive to shortening of the daylength and are not able to undergo growth cessation and bud set (Olsen *et al.*, 1997b). Moreover, over-expression of *PHYA* changes (shortens) the critical photoperiod of hybrid aspen (Olsen *et al.*, 1997b). In contrast, aspen plants transformed with an antisense construct for *PHYA* show increased sensitivity to SD (Kozarewa *et al.*, 2010). However, it seems that not only photoperiod but also the quality of the light plays an important role in growth cessation, as FR light appears to

maintain growth more efficiently than R light. High irradiance of FR and R is needed for northern accessions to prevent growth cessation (Olsen, 2010). The relative distribution of FR and R varies not only during the day, but also during the year and with latitude (Nilsen, 1985).

1.1.2 The external coincidence theory

Currently there are two models to explain how plants integrate information about the day and night cycle to measure the photoperiod: the internal and external coincidence models. The more widely accepted is the external coincidence theory proposed by Erwin Bünning based on observations of various plants and insects (Bünning, 1936). In *Arabidopsis*, flowering time is controlled in response to variation in daylength, and flowering occurs in response to LD. According to the external coincidence model the photoreceptors function is rhythmic and it can generate the input signal only at a specific circadian phase. The coincidence between the active form of the receptor, created by the light, and the rhythm of a specific signal given by the circadian clock allows plants to measure daylength. The photoreceptors thus have two functions: entrainment of the circadian clock (enabling the generation of a daily oscillation of a component with peak expression in late afternoon) and generation of the signal that regulates this component. If the signalling phase that needs to be activated by light occurs at the end of the day, SD responses are activated if the daylight has already ended. The key gene in this external coincidence model in *Arabidopsis* is *CONSTANS (CO)*, which positively regulates *FLOWERING LOCUS T (FT)*, a gene that promotes transition to flowering. The signals from light and the circadian clock are integrated through the regulation of *CO*; the clock generates a rhythm in the level of *CO* mRNA, with a peak 14–20 h after dawn and a minimum early in the day, and the light regulates CO protein stability (Valverde *et al.*, 2004; Yanovsky & Kay, 2002). CO in turn is able to activate transcription of *FT*. The rhythm of *CO* expression creates a light-sensitive phase starting from 8 h after dawn. Therefore, if there is still light when *CO* expression peaks, the light will stabilise the CO protein, and *FT* will be expressed. When days become shorter, *CO* expression will peak in the dark, the CO protein will not be stable and *FT* expression will not be induced; therefore, the plant will not flower (Ayre & Turgeon, 2004; Takada & Goto, 2003). The light signal is perceived in the leaves, while FT protein has been proposed to move from the leaves to the apex to promote flowering (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007).

A study of flowering regulation in hybrid aspen revealed that the CO/FT module controls not only flowering time in *Populus,* but also growth

cessation, bud set and dormancy acquisition in response to the annual decrease in daylength, once the night length falls below the critical daylength (Bohlenius *et al.,* 2006). The critical daylength increases with increasing latitude or altitude of origin of the plant, in other words in the northern hemisphere trees from northern latitudes stop growing before trees from southern latitudes (Pauley & Perry, 1954). This is because *CO* expression is initiated earlier in southern accessions; therefore its peak will fall more easily in the light than for northern accessions (Bohlenius *et al.,* 2006). The level of *FT* expression has been found to be a critical determinant of the timing of growth cessation and bud set in woody plants. During seasonal shortening of the daylength, levels of *FT* decrease during the first three days once the critical daylength has been reached, and its expression disappears completely after one week. *FT* downregulation is required for normal growth cessation and transition to dormancy (Bohlenius *et al.,* 2006), as shown in plants overexpressing *FT*, which are not able to respond to SD and thus do not cease growth, set buds and develop dormancy (Bohlenius *et al.,* 2006). In contrast, plants with reduced *CO*2 and *FT*1 levels are hypersensitive to changes in daylength (Bohlenius *et al.,* 2006).

1.2 SHORT DAY-RELATED RESPONSES AND DORMANCY DEVELOPMENT

Populus tremula x tremuloides, the hybrid aspen specie used in the studies presented in this thesis, responds to daylengths shorter than its critical daylength by ceasing growth and developing dormancy. Growth cessation and dormancy induction are accompanied by morphological changes leading to the formation and maturation of a terminal bud (also referred to as bud set), and a series of adaptive responses such as extensive transcriptional and metabolic changes and cold hardiness development. The establishment of dormancy temporally overlaps with some of those processes, hence isolating the regulation of individual processes is challenging. Many of the changes occurring in response to shortening of the daylength are reversed in the spring; for example, cold hardiness is gradually lost, storage reserves are remobilized and cell division is reactivated.

1.2.1 Cessation of elongation growth

The first response to the shortening of the daylength in woody plants is the gradual arrest of stem elongation, which precedes dormancy development. However, these two are genetically separate processes, as demonstrated for

example by the incapacity of grafts of P35S:As*PHYA* scions on wild type stocks to reach dormancy following growth cessation and bud set (Ruonala *et al.*, 2008). Earlier studies on *Arabidopsis* demonstrated that *PHYA* plays a role in stem elongation, since *phya* mutants have elongated hypocotyls whereas strong overexpression of this gene reduces internode length in several species (Eriksson *et al.*, 2000; Olsen *et al.*, 1997b). Inhibition of elongation growth by the shortening of daylength has been correlated with reductions in gibberellin (GA) levels in the elongation zone below the apex (Olsen *et al.*, 1997b; Junttila & Jensen, 1988), and overexpression of an oat *PHYA* gene in hybrid aspen resulted in dwarf trees that were insensitive to photoperiod and showed constant levels of the hormone GA (Olsen *et al.,* 1997b). More information about the role of GAs can be found in section 3.2, describing the hormonal control of the activity-dormancy cycle.

The apical meristem can be divided into two zones: the shoot apical meristem (SAM) and the rib meristem (RM), which are responsible for the production of new leaves and new shoot segments, respectively. The RM constitutes a morphogenetic zone of its own (Esau, 1977; Sachs *et al.*, 1960). A gene that may play a role in stem elongation in *Populus* is a homologue of the *Arabidopsis* gene *TERMINAL FLOWER 1 (TFL1), CENTRORADIALIS-LIKE 1 (CENL1)* (Ruonala *et al.,* 2008). The gene *TFL1* of *Arabidopsis* belongs to the same group of proteins as FT and its expression coincides with floral induction, but has opposite effect compared to FT (Kobayashi *et al.*, 1999). In *Populus tremula x alba CENL1* is expressed in the RM zone and it is normally downregulated at the time of growth cessation (Ruonala *et al.*, 2008; Ruttink *et al.*, 2007). P35S:As*PHYA* plants, which fail to make the transition to dormancy, also fail to downregulate the expression of *CENL1* in the RM zone. However, RNAi-dependent downregulation of *Populus CENL1* and *CENL2* does not reportedly affect the timing of growth cessation (Mohamed *et al.*, 2010), suggesting that they are not involved in growth cessation responses to SD treatment. Furthermore, in P35S:As*PHYA* scions grafted on wild type stocks, which can cease growth and set buds but are unable to maintain dormancy, *FT* expression levels initially decrease under SD, but subsequently increase again and plants show repetitive bud flush (Ruonala *et al.,* 2008).

1.2.2 Arrest of meristematic activity

Establishment of the dormant state follows arrest of cell division in the apex upon SD treatment. A simplified version of the cell cycle is outlined here, and illustrated in Figure 2, to help contextualising the results of studies on the regulation of the cell cycle during the activity-dormancy transition.

Usually, the decision of a cell to enter a new cell cycle is regulated at the G1 phase restriction point by phosphorylation and dephosphorylation cascades triggered by environmental and hormonal signals. Progression through the G1/S and G2/M checkpoints is regulated by distinct classes of CYCLIN DEPENDENT KINASES (CDKs), principally CDKA and CDKB. *CDKAs* are expressed in cells that are dividing or competent to divide (Hemerly *et al.*, 1993), while *CDKB* expression is restricted to dividing cells. The initial commitment to enter a cell cycle requires CDKAs to associate with D-TYPE CYCLIN (CYCDs), forming a complex that is activated by phosphorylation and is responsible for hyperphosphorylation of RETINOBLASTOMA (RB), which in turn permits the release of ELONGATION-2 FACTOR (E2F) and transcription of E2F-regulated genes (Fig. 2). Further progression into the cell cycle is regulated by CDKB associated primarily with CYCA and CYCB in complexes, the activity of which can be controlled by ubiquitination and phosphorylation (Fig. 2). Cytometric data indicate that the majority of cells are arrested in the G1 phase during dormancy in buds (Rohde *et al.*, 1997), although a small proportion may be arrested in the G2 phase.

In a study of cambium dormancy, Espinoza-Ruiz et al., (2004) highlighted two important features of the cell cycle response to the transition from active growth to dormancy. First, it induces changes in the activity of key cell cycle genes at multiple levels, since not all cell cycle genes are sensitive at the transcriptional level to the shortening of the daylength. Second, ecodormancy and endodormancy involve different, stage-specific regulation of the cell cycle machinery (Espinosa-Ruiz *et al.*, 2004). The cited study focused, in particular, on the differential regulation of cyclin-dependent kinases (CDKs) during establishment of eco and endodormancy. The findings indicate that CDKA and CDKB transcript and protein levels do not decline when cell division arrests and the plant enters ecodormancy, while their capacity to phosphorylate histone 1 (H1) decreases. Therefore, the decline in cell division in ecodormancy seems to depend on post-transcriptional regulation of CDKs, via increased activity of CDK inhibitors and/or enhanced inhibitory phosphorylation of CDKs, and on transcriptional or post-transcriptional regulation of CDK-associated cyclins (CYCs). Other relevant findings in this context are that SD treatment can reduce transcription in buds from an *Arabidopsis* B-type cyclin promoter and transcriptionally downregulate *CYC1A* expression (Rohde *et al.,* 1997), and that in cambium cells D-type cyclins are downregulated as cell division terminates (Druart *et al.*, 2007). The latter authors also found that establishment of an endodormant state appears to be preceded by a

transient increase in CDK-dependent phosphorylation of the plant homologue of RB, RETINOBLASTOMA RELATED (RBR), and a peak of E2F phosphorylation (which has a negative effect on its DNA binding capacity), while its establishment correlates with a reduction in CDKA phosphorylation of RBR, transcriptional downregulation of *CDKB* and disappearance of both CDKA and CDKB proteins.

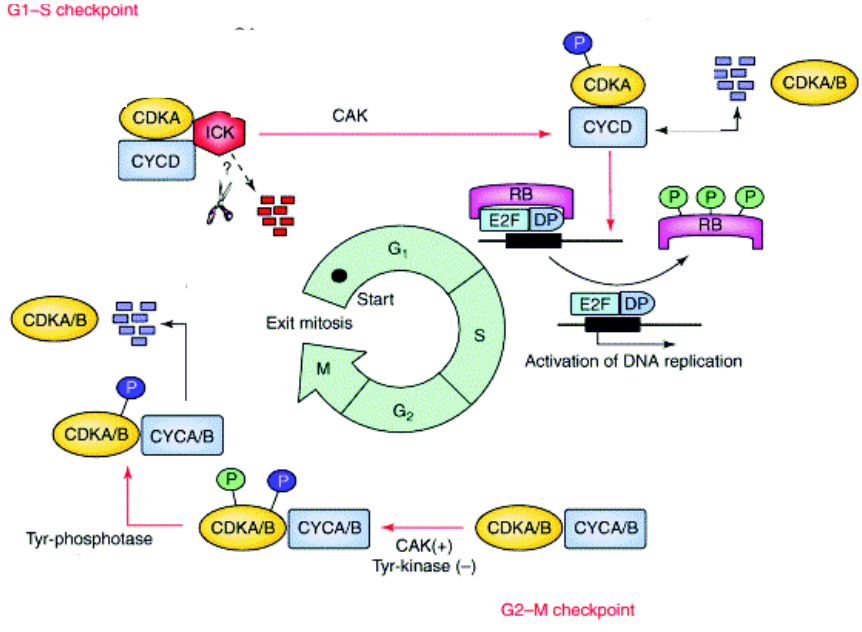

FIGURE 2: Schematic representation of the G1-S and G2-M transitions in plants.
Reprinted and modified from Horvath *et al.*, 2003; Gutierrez *et al.*, 2002; Anderson *et al.*, 2001; Stals & Inze, 2001 with permission from Elsevier. http://www.sciencedirect.com/science/article/B6TD1-49S6WJ9-3/2/fcf07e1db1f10864a149d62b76951d77; doi:10.1614/0043-1745(2001)049[0581:rcrotr]2.0.co;2; doi:10.1016/S1360-1385(01)02016-7

In addition, a microarray analysis of the activity-dormancy cycle in apices of *Populus tremula x alba* by Ruttink et al., (2007) showed that a substantial number of cell proliferation genes are downregulated quite early following the onset of SD and during bud development. This set of genes includes not only core cell cycle genes but also genes involved in DNA replication, nucleosome assembly, marker genes associated with dividing cells and cell cycle regulatory genes such as *AINTEGUMENTA (ANT)*. *ANT*, which is also

downregulated in the cambium during SD treatment (Schrader *et al.*, 2004), plays a crucial role in retention of cells' capacity to divide in *Arabidopsis* (Mizukami & Fischer, 2000). In cambium, *CDKA* downregulation has been found to be milder than *CDKB* downregulation, prompting the hypothesis that some cells may retain competence to divide (Acosta *et al.*, 2004). In buds, *CDK2A* has been found to be not transcriptionally SD-regulated (Rohde *et al.,* 1997). Further information on changes related to the transition to dormancy was obtained in an analysis of a full-genome array of *Populus*, presented in Paper I appended to this thesis, including genes not present in cDNA arrays previously described (Druart *et al.,* 2007; Ruttink *et al.,* 2007) and homologues of recently described cell cycle genes in *Arabidopsis* (Menges *et al.*, 2005). The results show that a coordinated transcriptional downregulation of *CDKBs* and several classes of *CYCs* occurs in response to SD.

Once dormancy is established, the plants need to experience a prolonged cold period to regain responsiveness to growth-promoting stimuli. When dormancy breaks, leaf primordia emerge from the bud in a process that requires cell elongation prior to cell division, while cambium cells will simply resume cell proliferation. The effects of cold and dormancy release on cell cycle genes have been investigated in vascular cambium, where there appears to be little if any correlation between the activation of cell division and the induction of cell cycle genes, leading to the hypothesis that post-transcriptional control of cell cycle genes plays an important role during the early phase of cambium reactivation (Schrader *et al.,* 2004; Druart *et al.,* 2007). As observed in seeds, in which the translation of stored transcripts is important for dormancy release (Nakabayashi *et al.*, 2005), in the cambium transcripts of core cell cycle genes such as *CDKA* remain at low levels and their translation is suppressed during dormancy. Translation of these retained transcripts in spring could be sufficient to initiate early cell division (Druart *et al.,* 2007). In addition to information on the transition to dormancy, Paper I also provides data on the regulation of cell cycle machinery upon chilling treatment and dormancy release, which had not been previously investigated in apical buds.

1.2.3 Morphological changes during short days: bud formation and closure of plasmodesmata

Bud formation and development

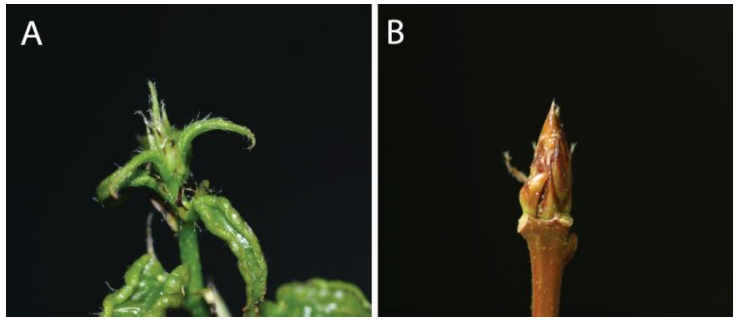

FIGURE 3: A) actively growing apex and (B) bud.

Once stem elongation ceases, the apex undergoes several morphological modifications resulting in the formation of a bud, a structure that protects the meristem and contains pre-formed organs (described below) in preparation for the following growing season. Bud formation and development are a gradual and complex process that includes the essential dehydration and hardening of the bud too. Although bud formation starts immediately after the onset of SD, it takes several weeks for a visible terminal to develop (Fig. 3). Goffinet and Larson (1981) investigated anatomical changes that occur during bud development in *Populus deltoides*, discovering that all leaf primordia that are already formed by the time the plant senses SD will develop into new leaves, while the last primordium initiated before the onset of SD will instead subtend the bud, and often not mature to full size (Goffinet & Larson, 1981). In contrast, the fate of the first primordium initiated after the onset of SD will be redirected; in this case, the leaf lamina will prematurely abort and the stipules enlarge to form bud scales that will mature, becoming thick, sclerified, and highly resinous. The side of the scales exposed to the external environment presents a thick cuticle and one or more layers of cork and cork cambium, giving a brown coloration (Curtis & Lersten, 1974). Bud scales and a few inner stipules can produce resin, which fills the spaces of the bud and often extrudes onto the bud surface (Curtis & Lersten, 1974).

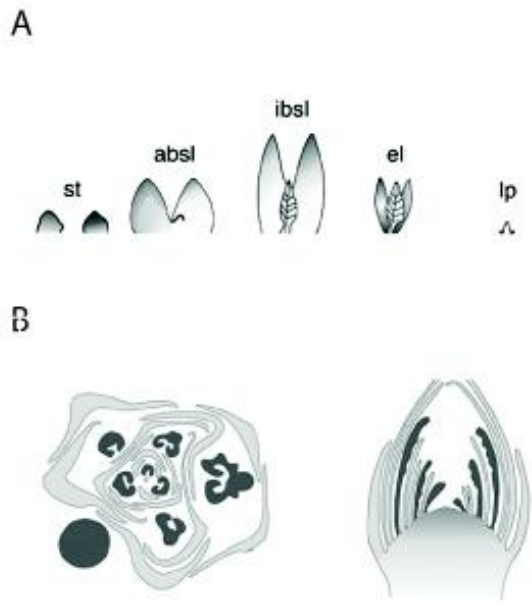

FIGURE 4: (A) from left to right, schematic representation of the organs present from the outside to the inside of an apical bud of *Populus tremula x alba*: st= stipules, absl= abortive bud scale leaf, ibsl= incipient bud scale leaf, el= embryonic leaf, lp= leaf primordium. (B) Schematic representation of a birch apical bud in transversal and longitudinal sections.
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As illustrated in Figure 4, a bud contains a densely packed series of embryonic leaves and leaf primordia, enclosed by two or more pairs of bud scales. Immediately inside the scales there are incipient embryonic leaves that have short, broad lamina. Proceeding further towards the inside of the bud, there are embryonic leaves *sensu stricto*, each with two stipules, and leaf primordia that do not yet have distinct stipules (Rohde *et al.*, 2002). For simplicity, all organs inside the scales are here referred to as embryonic leaves. All the tissues present in the bud undergo dehydration and cold acclimation, but only the embryonic leaves develop dormancy, being the organs that will grow in the next season. Hence, embryonic leaves need to reach a certain stage of maturity, in which they will remain until bud flush in spring.

One gene that has been found to play a key role in this process is *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), a transcription factor that has been extensively studied in *Arabidopsis*, and plays a role in late seed development

and maturation. Interestingly, ectopically expressed *ABI3* is also functional in vegetative tissues (Parcy *et al.*, 1994) and *ABI3* expression is not restricted to seeds in *Arabidopsis*, since it has also been found to be expressed in stipules, abscission zones of siliques, and the vegetative meristem during dark-induced quiescence (Rohde *et al.*, 2000). This last indication of a role of *ABI3* in meristems during rest periods prompted Rohde et al., (1998; 2002) to isolate a homologue of *ABI3* in *Populus trichocarpa* and investigate its possible roles in the regulation of bud dormancy and related processes (Rohde *et al.*, 2002; Rohde *et al.*, 1998). *ABI3* was found to be expressed in buds of *Populus tremula x alba* plants during natural bud set, in organs that are actively growing but will undergo growth arrest (e.g., young embryonic leaves). *ABI3* overexpression and downregulation phenotypes in the same species highlighted a role in the control of the relative growth rate and differentiation of embryonic leaves and bud scales/stipules differentiating from primordia under SD conditions; if *ABI3* was ectopically expressed, the buds showed an open conformation with enlarged embryonic leaves and reduced stipules/scales, while downregulation of *ABI3* resulted in larger bud scales and smaller leaves (Rohde *et al.,* 2002). In its target tissues, wild type expression of *ABI3* is required for adequate growth and differentiation of the embryonic leaves, which is crucial as leaves must not elongate during winter, but must be sufficiently differentiated to allow growth to be initiated quickly in spring. *ABI3* seems not to control entry into dormancy, as both *ABI3*-overexpressing and -downregulated lines present normal dormancy development (Ruttink *et al.,* 2007) and *ABI3* is not expressed during the time of dormancy development (Rohde *et al.,* 2002). However, as overexpression of *ABI3* leads to constitutive expression in tissues that are not normally its target, as bud scales, the effects of ABI3 in these tissues remains difficult to evaluate. Moreover, the possibility that this gene plays a role in dormancy cannot be completely excluded, as the phenotype of lines generated to date in which it is downregulated is quite subtle, possibly because its downregulation in these lines is insufficient to affect dormancy. On the other hand, if ABI3 is involved in dormancy establishment, its overexpression should increase plant dormancy. It should also be noted that the test currently used to assess if a plant is dormant involves shifting plants from SD to LD conditions without prior chilling, which does not allow the *degree* of dormancy in the apical bud to be assessed.

Even if acquisition of dormancy temporally overlaps with the final stages of bud formation, the regulation of these two processes may be genetically distinct, as demonstrated by studies in *Betula pendula*. Ruonala et al., (2006) expressed the dominant mutation of the ethylene receptor *ETHYLENE*

RESPONSE 1 (ETR1) in this species, generating hormone-insensitive plants that were able to develop dormancy, although delayed, in the absence of formation of a clear terminal bud (Ruonala *et al.*, 2006). The same conclusion can also be inferred from an experiment in which P35S:As*PHYA* scions, grafted on wild type stocks, were able to develop buds, but not able to maintain dormancy, as they flushed several times in SD (Ruonala, 2008).

Closure of plasmodesmata connections

Another SD-induced morphological change is the formation of sphincters at plasmodesmata (PD) connections, which impair symplastic communication. Maintaining symplasmic connections is important during morphogenesis, since they allow the exchange of signalling molecules, formation of gradients of various substances, and metabolic coupling of cells. Rinne and van der Schoot (1998) and Rinne et al., (2001) investigated this phenomenon, and its involvement in dormancy maintenance, in *Betula pubescens* (Rinne *et al.,* 2001; Rinne & van der Schoot, 1998). They found that plasmodesmata connections in the shoot apical meristem (SAM) are gradually closed by deposition of 1,3 β-D glucan very early after the onset of SD, and there is a temporal coincidence between the onset of dormancy and the appearance of plasmodesmatal sphincters, formed by an extracellular ring of proteins and callose that associates with the plasmodesmata channel, compressing it into a plug. At this point, symplasmic communication is completely blocked (Rinne *et al.,* 2001; Rinne & van der Schoot, 1998). Chilling treatment after SD has been seen to promote movement of vacuoles containing 1,3 β-D glucanase to the cell membrane, and to reconstitute in this way plasmodesmata connections (Rinne *et al.,* 2001; Rinne & van der Schoot, 1998).

To date, only plants that are unable to maintain an endodormant state have been found to be unable to close plasmodesmata connections definitively. For example, P35S:As*PHYA* plants, which are not able to undergo growth cessation and dormancy, do not suspend plasmodesmatal communication in SD (Ruonala *et al.,* 2008). Furthermore, the formation of temporary, reversible and narrow plasmodesmata has been observed in P35S:As*PHYA* scions grafted on wild type stocks, which can undergo growth cessation but cannot develop endodormancy. It has therefore been proposed that *PHYA* overexpression in the rib meristem (RM) could interfere with the process that leads to sphincter formation (Ruonala *et al.,* 2008). However, the role of plasmodesmata connections in dormancy development remains to be proven. For example, following symplasmic isolation growth in the apical meristem continues, as embryonic leaves and

bud scales are formed. In this respect, it is also important to consider that plasmodesmatal connections are not required for the movement of some growth regulators (e.g. auxin) in the plant. In addition, there is as yet no direct evidence that opening of plasmodesmata is essential for dormancy release, although it temporally coincides with chilling treatment.

1.2.4 Cold hardiness development

Low temperatures may irreparably damage the vital organs contained in buds, thereby compromising the potential of the plant to resume growth in the following growth season. Therefore, the ability to withstand low temperatures is critical for all plants that are exposed to them, and the development of cold hardiness (hereafter also referred to as cold acclimation) is a key annual process. Cold acclimation is an adaptive mechanism, initiated in response to sub-optimal temperatures, which allows woody plants, and their essential meristems, to withstand severe cold stress. The process involves alterations in several cellular features and metabolic functions, such as membrane composition and fluidity, carbohydrate and protein contents, and levels of antioxidant enzymes and compounds, through remodelling of the transcriptome (reviewed in Ruelland *et al.*, 2009). One of the most important aspects of cold acclimation is the ability of the plant to handle tissue dehydration caused by the formation of extracellular ice upon exposure to freezing temperatures, a key aspect of which is the accumulation of various solutes and proteins that protect cell structures. Particularly interesting proteins that accumulate in response to low temperatures (LT) are dehydrins, which are believed to function as cryoprotectants, protecting the membranes and cellular proteins from damage (Puhakainen *et al.*, 2004; Bravo *et al.*, 2003). Another important step for cold acclimation is the accumulation of soluble sugars, partly due to starch breakdown, which act as osmolytes and membrane stabilizers. The process of cold acclimation is better understood in herbaceous annual plants than in woody plants. One of the most important and best characterized genetic controls of cold acclimation in *Arabidopsis* is the C-REPEAT BINDING FACTOR/DEHYDRATION RESPONSIVE ELEMENT BINDING 1 (CBF/DREB1) response pathway (Yamaguchi-Shinozaki & Shinozaki, 2006; Thomashow, 1999). In *Arabidopsis* there are six CBF paralogues, of which three (*CBF1*, *CBF2* and *CBF3*) are rapidly but transiently induced by LT (Liu *et al.*, 1998; Stockinger *et al.*, 1997) through the action of INDUCER OF CBF EXPRESSION1 (ICE1) (Chinnusamy *et al.*, 2003). The CBF/DREB1 proteins regulate transcription of a set of genes by binding to a cis-regulating element called the DEHYDRATION RESPONSE ELEMENT/C-

REPEAT/LOW TEMPERATURE RESPONSIVE ELEMENT (DRE/CRT/LTRE) sequence. Cold stress is followed by upregulation of a number of cold-regulated genes (CORs), some of which are under the CBF control and constitute what is called the CBF regulon. Constitutive expression of *Arabidopsis CBFs* has been shown to result in increased freezing tolerance, in the absence of a LT stimulus, via upregulation of the CBF regulon (Fowler & Thomashow, 2002; Jaglo-Ottosen *et al.*, 1998).

In herbaceous plants the development of cold hardiness is largely driven by LT, while cold acclimation in woody plants may occur either during the growing season or during dormancy development (Puhakainen *et al.*, 2004; Weiser, 1970). Furthermore, while annual plants at high latitudes/altitudes may survive the winter by being covered by an insulating layer of snow, and thus need to develop only limited cold acclimation, woody plants need to be able to face extremes of temperatures, and thus require the development of much higher levels of cold hardiness. Thus, woody plants need to sense the onset of winter and anticipate its arrival, developing not only dormancy but also cold hardiness well ahead of the time when actual freezing temperatures are experienced. In this respect, measuring the shortening of the daylength provides a reliable cue that is correlated to the arrival of the cold season and allows the development of resistance to extreme temperatures before the onset of winter. Cold hardiness during winter in woody plants has been found to be reached through three sequential stages: a first level of cold acclimation is achieved in response to shortening of the photoperiod, a second level by the combined action of SD and LT, and a maximal level following exposure to freezing temperatures (Weiser, 1970). Studies on *Populus tremula x tremuloides* have shown that while both SD and LT can induce cold acclimation, they seem to operate through independent mechanisms. For instance, plants overexpressing *PHYA* are not able to sense SD, and thus are unable to develop SD-induced cold hardiness like wild type plants, while they can cold acclimate in response to LT treatment (Welling *et al.*, 2002; Olsen *et al.*, 1997b). Members of the *CBF* family have been recently identified in a number of woody species, including *Populus spp.* (Benedict *et al.,* 2006) and Betula *pendula* (Welling & Palva, 2008). The aspen and birch *CBF* genes have been shown to have similar structures to the *Arabidopsis CBFs*, which indicates that they may have conserved functions. The CBF pathway has also been found to operate in trees in response to LT, since overexpression of *AtCBF1* in aspen activates a similar set of genes as in *Arabidopsis* (Benedict *et al.,* 2006) and ectopic expression of birch *CBFs* increases freezing tolerance in non-acclimated *Arabidopsis* plants (Welling & Palva, 2008). Both of the cited studies indicate that these

transcription factors play important roles not only in cold acclimation during the growing season, but also in the development of winter hardiness. Hence, it appears that *CBFs* could be involved in regulation (at least) of the second and third phases of cold acclimation, in response to LT. SD alone has been found, in several microarray analyses, to be able to stimulate the transcription of cold-responsive genes, several of which belong to the *CBF* regulon of *Arabidopsis*. This induction seems to occur in different waves, which could explain the sequential acquisition of cold tolerance (Ruttink *et al.,* 2007; Druart *et al.,* 2007). A large proportion of the induced genes encode proteins that contribute to desiccation tolerance, including dehydrins, bark storage proteins, enzymes involved in phospholipid biosynthesis, lipid desaturation, oxidative stress responses or cryoprotection, and several transcription factors (Schrader *et al.,* 2004; Druart *et al.,* 2007; Ruttink *et al.,* 2007). Genes whose products counter osmotic stress may also be important as dormancy development is characterised by the disappearance of free water in the cells, a phenomenon connected to the increase of freezing tolerance (Faust *et al.*, 1991; McKenzie *et al.*, 1974). However, neither *CBFs* nor *ICE1* are upregulated in response to SD in the apex (Ruttink *et al.,* 2007), while only a *CBF-like* gene has been found to be upregulated in vascular cambium in response to SD treatment (Schrader *et al.,* 2004). The transcriptional regulation of the development of cold hardiness remains largely unknown, and undescribed transcription factors, other than *CBF*, may be involved due to the complexity of the phenomenon. The results of studies described above are based on analyses of cDNA microarrays that did not contain all the *CBF/DREBs* genes described in *Populus*, hence they did not elucidate whether *CBFs* also mediate the first step of winter hardiness development. This issue is addressed in a full genome array analysis presented in Paper I.

1.2.5 Changes in metabolism

The shortening of daylength and downstream processes (such as bud formation, cold acclimation and dehydration) induce massive changes in the transcriptome of woody plants. These massive changes in gene expression are mirrored by substantial changes in the metabolome of the plant, which reflect the plant's need to accumulate storage reserves and to produce cryoprotectants to prevent tissues from freeze damage. During cold stress the lipid composition of plant membranes changes and solutes are produced that help to maintain turgor in cells that need to stand dehydration stress. Thus, transcriptional and metabolic changes are key adaptive responses of plants to the cycling of the seasons.

Starch, an important storage carbohydrate in plants, shows diurnal turnover. During the day, light is used for photosynthesis in the leaves, which produces sucrose that is exported to the rest of the plant. The overflow of newly assimilated carbon is stored as starch. During the night the plant relies on the accumulated starch to support leaf respiration and sucrose production. Normally, the rate of starch accumulation in daylight is balanced by its utilization during the night. However, in a transcriptomic and metabolomic study on the onset of dormancy, Ruttink et al., (2007) showed that the shift to a shorter photoperiod creates an imbalance in the starch diurnal cycle. Starch granules disappear and there is a transient shortage of sugars such as maltose, fructose, and glucose, while the photosynthetic rate decreases. To support gluconeogenesis and meet energy requirements in this phase of SD, the plants upregulate enzymes of the glyoxylate cycle (Schrader *et al.,* 2004; Ruttink *et al.,* 2007; Druart *et al.,* 2007) and glycolysis (Druart *et al.,* 2007).

Later on, during the onset of dormancy, the plant switches its metabolism to a storage mode, accumulating storage compounds (proteins, lipids and carbohydrates). Starch biosynthetic genes are upregulated, concordantly with the appearance of amyloplasts in the subapical domain (Ruttink *et al.,* 2007). An important set of proteins that are accumulated in response to short photoperiods are the BARK STORAGE PROTEINS (BSP). These proteins function as nitrogen stores that accumulate during dormancy induction and are reutilized in spring when growth is reinitiated (Coleman *et al.*, 1992; Coleman *et al.*, 1991). In the cambium, SD exposure stimulates the accumulation of amino acids that are necessary for the biosynthesis of storage proteins. During the reactivation of growth in spring, storage proteins are degraded, increasing the abundance of amino acids (Druart *et al.,* 2007).

When winter arrives and cold acclimation is established, starch breakdown enzymes, such as *β-amylase* and *starch phosphorylase*, are upregulated, and the consequent degradation of starch results in the accumulation of sugars such as sucrose, raffinose, stachyose and galactinol in stem tissues of *Betula* and *Populus* (Regier *et al.*, 2010; Druart *et al.*, 2007). These compounds are used as cryoprotectants, for lipid synthesis and energy supply. Increases in raffinose levels have been correlated to low temperature treatment in *Populus tremuloides* (Cox & Stushnoff, 2001).

Synthesis of lipids is also very important in this phase, because during dormancy induction the central vacuole breaks into several small vacuoles, requiring the synthesis of new membranes. Lipid biosynthesis and elongation seem to be supported by starch degradation and glycolysis, and

several genes encoding proteins involved in fatty acid biosynthesis are upregulated in SD (Druart *et al.,* 2007). In spring, when growth is reactivated, catabolism of sucrose and fatty acids (the latter through β-oxidation and activation of the glyoxylate cycle) occurs, providing energy and carbon skeletons (Druart *et al.,* 2007).

1.3 HORMONAL CONTROL OF DORMANCY AND SD-RELATED RESPONSES IN APICAL BUDS

The development of dormancy in apical buds is an elaborate process, regulated by both internal and external signals. This section describes current knowledge regarding hormonal control of SD-related responses and of development of apical dormancy. Abscisic acid (ABA), a water stress-responsive hormone and known growth inhibitor, has received a great deal of attention with respect to bud dormancy, since it also plays key roles in the induction and maintenance of seed dormancy (Kucera *et al.,* 2005). Other interesting hormones for the control of some SD-related responses are gibberellins (GA) and ethylene.

1.3.1 Abscisic acid (ABA)

ABA signalling

ABA is a known stress response hormone, both in herbaceous and woody plants (Finkelstein *et al.*, 2002; Li *et al.*, 2002). ABA and ABA responsiveness have also been proposed to play a role in the control of dormancy and SD-related responses (Eagles & Wareing, 1964). Molecular players of the ABA signalling pathway have been extensively investigated in herbaceous plants, and studies on ABA signalling, especially in dormant seeds, have been used as a guide in attempts to elucidate the functions of ABA in woody plants. In *Arabidopsis*, ABA is sensed through a receptor that is able to bind to the hormone and to the ABA INSENSITIVE 1 (ABI1) protein (Ma *et al.*, 2009), a negative regulator of the ABA-signalling pathway (Gosti *et al.*, 1999). ABI1 and the closely related ABA INSENSITIVE 2 (ABI2) protein are members of the serine/threonine protein phosphatase 2C (PP2C) family (Leung & Giraudat, 1998). The binding of ABA to the receptor blocks their phosphatase activity, releasing repression of downstream effectors (Fig. 5). The gene *ABI1* is required for proper ABA responsiveness not only in seeds, but also in vegetative tissues (Leung & Giraudat, 1998; Rock & Quatrano,

1994). In addition, indication that the *Arabidopsis* ABI1 protein is functional in the transduction of ABA signals in woody plants has been obtained from observations that grey poplar plants overexpressing a dominant negative mutant of the gene show decreased sensitivity to the hormone (Arend *et al.*, 2009).

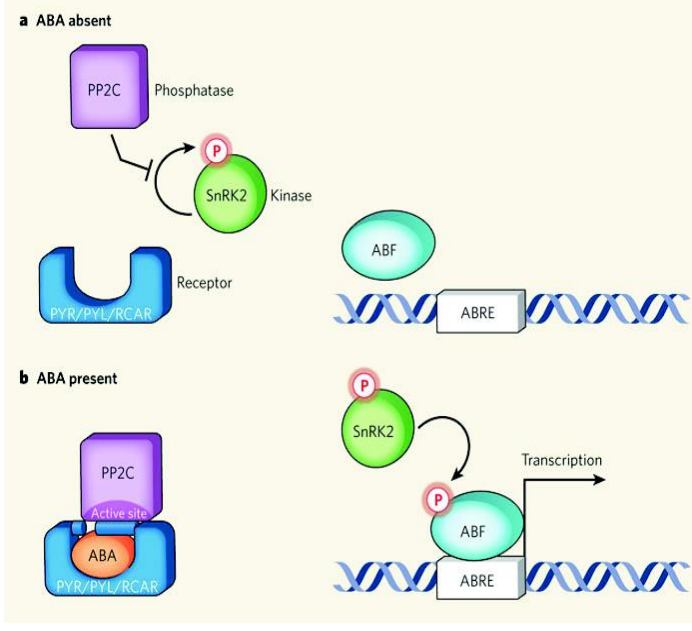

FIGURE 5: A) in the absence of the plant hormone ABA, the phosphatase PP2C is free to inhibit autophosphorylation of a family of SnRK kinases. B) ABA enables the PYR/PYL/RCAR family of proteins to bind to and sequester PP2C. This relieves inhibition on the kinase, which becomes auto-activated and can subsequently phosphorylate and activate downstream transcription factors (AREBs/ABFs) to initiate transcription at ABA-responsive promoter elements (ABREs).
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http://dx.doi.org/10.1038/462575a

Role of ABA in growth cessation and apical bud dormancy

The role of ABA in the regulation of both growth cessation and dormancy is controversial. In some studies ABA concentrations have been found to be higher under LD than SD, or no changes have been detected (Barros & Neill, 1986; Johansen *et al.*, 1986; Lenton *et al.*, 1972), while ABA levels

have increased during SD treatment in several species (Druart *et al.*, 2007; Ruttink *et al.*, 2007; Li *et al.*, 2005; Li *et al.*, 2003; Rohde *et al.*, 2002; Rinne *et al.*, 1998; Welling *et al.*, 1997; Rinne *et al.*, 1994b; Rinne *et al.*, 1994a). Some authors have found correlations between high concentrations of ABA and the onset of dormancy (Li *et al.*, 2005; Rinne *et al.*, 1994a; Alvim *et al.*, 1978). In addition, Welling et al., (2002) demonstrated that an SD-induced ABA peak is under the control of phytochrome, since *PHYA* overexpressing plants did not transiently increase ABA levels during SD treatment (Welling *et al.*, 2002).

A recent microarray analysis have also shown that ABA biosynthetic genes such as *NINE-CIS EPOXYCAROTENOID DYOXYGENASE 3* (*NCED3*) and *ABA-DEFICIENT 2 ALCOHOL DEHYDROGENASE/OXIDOREDUCTASE /XANTHOXIN DEHYDROGENASE* (*ABA2*) are upregulated in response to SD, concurrently with the downregulation of many cell proliferation genes (Ruttink *et al.,* 2007). The microarray analysis reported in Paper I corroborates the upregulation of these (and other) biosynthetic genes during SD treatment, but also highlightes the importance of downregulation of catabolic enzymes. Rinne et al., (1994a, b) found that application of exogenous ABA to chilled buds of *Betula pubescens* delayed bud burst, and Fladung et al., (1997) found that a transgenic *Populus* clone with reduced ABA levels resumed growth earlier than untransformed plants (Fladung *et al.*, 1997). However, no clear role for ABA was proven, as external applications may not necessarily result in effective penetration of the hormone in bud tissues, or in physiologically active concentrations even if uptake occurs. Moreover, delayed bud burst in plants treated with ABA, and faster resumption of growth of plants with reduced levels of the hormone may be due to inhibitory effects of ABA on cell elongation after dormancy release rather than effects on dormancy *per se*, and chilling treatment after SD reportedly has no clear effect on ABA concentrations in *Betula pubescens* (Rinne *et al.,* 1994b). This finding prompted the cited authors to speculate that ABA responsiveness, rather than actual changes in ABA levels, could be important in the regulation of dormancy onset and release. Other evidence of a possible role of changes in sensitivity to the hormone in the control of bud dormancy has been provided by Barros and Neill (1986), who reported that the ability of applied ABA to delay or prevent bud flush in *Salix viminalis* increased the longer the shoots were exposed to SD. ABA had no effect in LD treatment, and chilling treatment removed sensitivity to the hormone. Finally, Borkowska and Powell (1982) found that the response of apple's bud tissue to ABA in the culture medium depended on the stage of bud growth, and Rinne et al., (1998) reported that woody plants show a

seasonal variation in ABA sensitivity, which peaks in autumn and is lowest in spring (Rinne *et al.*, 1998; Borkowska & Powell, 1982). Taken together, these findings suggest that changes in dormancy status could be more closely related to changes in ABA receptivity than to changes in ABA concentration. Accordingly, Ruttink et al., (2007) recently showed that genes involved in ABA signalling, such as *ABI1*, are also upregulated along with the ABA biosynthetic genes, and this upregulation occurs concomitantly with the transition of the apex to a closed bud, but before the cessation of meristematic activity. This finding provides evidence of a potential role for the *ABI* genes in transduction of the environmental signal and regulation of dormancy via alteration of the capacity of the apical meristem to respond to ABA. In support to this hypothesis, negative regulators of ABA signal transduction, such as *ABI1B*, *ABI1D* and *ABI3*, have been found to map on QTLs involved in the regulation of endodormancy (Frewen *et al.,* 2000).

Role of ABA in bud formation

Due to the correspondence of the peak of ABA production and the expression of the *ABI3* gene in *Populus trichocarpa,* a possible role of ABA in bud formation was proposed by Rohde et al., (2002), who suggested that ABA and ABI3 may cooperate in the process of bud formation; ABA counteracting the effect of ABI3 to promote proper formation of bud scales. In the absence of strong phenotypes in ABI3 antisense plants and of plants in which interactions between ABI3 and ABA could be evaluated, this suggestion remained untested so far. The relationship between these actors is explored in Paper II appended to this thesis.

Role of ABA in the control of bud dehydration and development of freezing tolerance

In *Arabidopsis* ABA plays a role in cold stress resistance and many CBF-regulated promoters contain ABA RESPONSIVE ELEMENTS (ABREs). A role for ABA in regulating cold hardiness development in woody plants has also been indicated by several studies; notably alteration of ABA levels through exogenous application or by blocking ABA biosynthesis has been shown to affect freezing tolerance (Welling *et al.,* 1997; Li *et al.,* 2003). In addition, an ABA-deficient birch reportedly showed delayed or reduced cold acclimation in SD (Rinne *et al.,* 1998), and in the cambium of *Populus tremula* plants a transient increase in ABA levels seems to correlate with a late stage of cold hardiness development (Druart *et al.,* 2007). In birch, the increase in freezing tolerance appears to be accompanied by tissue desiccation and the accumulation of dehydrins (Rinne *et al.,* 1998). Furthermore, an ABA-

deficient mutant examined by the cited authors not only lacked cold acclimation, but also showed reduced water loss, defective osmoregulation and no upregulation of a set of dehydrins. A correlation between the increase of ABA levels during SD and the decrease of water content in the bud has also been observed by Rinne et al., (1994a, b).

Conclusive proof of ABA's role in growth cessation and dormancy development is still lacking

In the previously described studies, the photoperiod and experimentally induced alterations of ABA content did not apparently affect growth cessation and dormancy (Welling *et al.,* 1997), and an ABA-defective mutant of birch developed dormancy and responded to ABA applications in the same way as the wild type (Rinne *et al.,* 1998), leading the cited authors to propose that ABA plays a more important role in cold acclimation than in dormancy regulation. Involvement of ABA in the control of SD-induced growth cessation and dormancy development/maintenance has not yet been either proven or refuted. Furthermore, several studies indicate that changes in sensitivity to the hormone may be more important in the control of these processes than the hormone level. Paper II addresses this (previously untested) hypothesis.

1.3.2 Gibberellins (GAs)

GAs have well-known roles in internode elongation and growth control (Eriksson *et al.*, 2000), hence the possibility that they may be involved in SD-induced growth cessation has also been investigated. Several studies have indicated roles for GAs in the control of photoperiodic-related growth cessation and growth initiation after dormancy release (Eriksson & Moritz, 2002; Eriksson *et al.*, 2000; Olsen *et al.*, 1997a; Olsen *et al.*, 1995). Interest in gibberellins is also due to their antagonistic role to ABA action in seeds, in which the balance between these hormones is one of the most important determinants of dormancy versus germination. More details about ABA- and GA-mediated control of seed dormancy and its release are given in this and the following section.

The subapical meristem, the site of formation and elongation of most of the cells forming the stem (Sachs, 1965), is thought to be the target tissue for active GAs. Accordingly, visible signs of growth cessation are preceded by a decrease of GA_1 content in this region in *Salix pentandra* (Olsen *et al.,* 1995), accompanied by a decrease of cell division frequency (Hansen *et al.*, 1999). Furthermore, plants moved back to LD conditions after early SD treatment show bud break and growth reinitiation, correlated with increases

in levels of GA_1 and its precursors (Olsen *et al.,* 1997a), and application of GA to apical buds of such plants can substitute for LD treatment (Hansen *et al.*, 1999; Olsen *et al.*, 1997a). Active Gas are known to influence cell cycle control by promoting histone H1 kinase activity and the expression of *CDKs* and *CYCs* in *Arabidopsis* (Sauter, 1997; Sauter *et al.*, 1995). Thus, GA_1 affects stem elongation, in connection with bud set and bud break, primarily by affecting cell division in subapical tissues. Subsequent studies in several species revealed that the process under photoperiodic control is GA_{19} metabolism. In fact, application of GA_{20} and GA_1 (but not GA_{19}) to SD-treated plants of *Salix pentandra* induces shoot elongation, mimicking LD treatment (Mølmann *et al.*, 2003; Junttila, 1993; Junttila & Jensen, 1988). The enzyme catalyzing the conversion of GA_{19} to GA_{20} is encoded by the *GA_{20}-oxidase* gene. Shortening of the daylength has been found to modulate the transcription level of this key GA biosynthesis enzyme, causing downregulation of both its mRNA levels and enzymatic activity in shoots, as demonstrated by an accumulation of the substrate GA_{53} in *Salix pentandra*, hybrid aspen and silver birch (Mølmann *et al.*, 2003; Eriksson *et al.*, 2000). In addition, SD-induced growth cessation is reportedly delayed in plants overexpressing a *GA_{20}-oxidase* gene (Eriksson & Moritz, 2002). In cambium of *Populus tremula*, *GA_{20}-oxidase* levels did not appear to be influenced by SD, while an upregulation of this biosynthetic enzyme was seen in spring, during cambium reactivation (Druart *et al.,* 2007). However, shortening of the daylength not only affects active GA levels, but also seems to affect sensitivity to the hormone. Sensitivity of *Salix pentandra* plants to exogenously applied GA_{19} decreases in SD, while plants that are transferred back to LD after increasing treatment with SD show reduced responses to GA_1 and GA_3 (Junttila & Jensen, 1988; Junttila, 1976). Consistently with a decrease in GA sensitivity, Ruttink et al., (2007) observed upregulation by SD of two genes encoding GIBBERELLIC ACID INSENSITIVE (GAI) in the apex of *Populus tremula x alba* and Druart et al., (2007) detected upregulation of a gene encoding REPRESSOR OF GA1-3 1 (RGA1) in the cambium of *Populus tremula.* Both GAI and RGA1 are members of the DELLA domain protein family, acting as negative regulators of GA signalling (reviewed in (Sun & Gubler, 2004). The observation that plants overexpressing oat *PHYA* do not present SD-induced downregulation of GA_1 biosynthesis (Mølmann *et al.*, 2003; Olsen & Junttila, 2002; Olsen *et al.*, 1997a) suggested that GA reduction in SD is under the control of the phytochrome system. Such plants are unable to stop growth and develop cold hardiness and dormancy (Olsen *et al.,* 1997a). Experiments in which growth cessation was induced by a combination of SD, low night temperatures and an

inhibitor of GA biosynthesis in oat *PHYA*-overexpressing hybrid aspen plants, demonstrated that the reduction of GA following SD is involved only in control of growth cessation, cold acclimation and bud set, since the plants were not able to develop dormancy. The experiments performed by Olsen et al., (1997a) also indicated that GA does not play a role in dormancy release as GA_1 levels increased after dormancy was released, when growth was reinitiated. However, this does not exclude a possible role for changes in GA sensitivity in dormancy release.

1.3.3 Ethylene

In *Arabidopsis* ethylene is involved in the control of diverse processes during growth and development, ranging from germination to senescence. Senescence is often associated with the induction of endodormancy in woody plants, and it is regulated both by ABA and ethylene. However, the role of ethylene in dormancy and related processes remains largely unexplored in woody plants and to date little evidence of its possible involvement has been presented. A microarray analysis has shown that *Populus tremula x alba* genes involved in ethylene biosynthesis and signalling are transiently upregulated in an early phase of SD exposure, before formation of the apical bud (Ruttink *et al.,* 2007). The authors suggested therefore a possible control of bud development by ethylene (Ruttink *et al.,* 2007). In addition, *Betula pendula* plants carrying the dominant negative version of an *Arabidopsis* receptor for ethylene signalling, ETR1, are reportedly unable to form a closed apical bud upon SD induction (Ruonala *et al.,* 2006). Scale formation did not occur in these plants, and while the youngest leaves continued to grow, they did not expand to full size. Growth cessation was also delayed in these plants in a controlled environment, while ethylene insensitivity had no effect on growth cessation in natural outdoor conditions. Furthermore, birch plants expressing the *etr1* mutation showed delayed endodormancy development, and possibly impairment or delay of SD-induced ABA accumulation. In this case, ethylene was also found to control paradormancy. The cited authors hypothesized that ethylene plays a role in the regulation of growth cessation, bud formation, and entry into dormancy, through the control of a *β-xylosidase* gene involved in cell wall modification, which is not upregulated in *etr1* plants (Ruonala *et al.,* 2006).

1.4 DIFFERENCES AND SIMILARITIES BETWEEN DORMANCY IN APICAL BUDS AND VASCULAR CAMBIUM

The transition to dormancy is a complex phenomenon, involving multiple processes, and affecting diverse plant structures such as seeds, buds and the vascular cambium. The most visibly dramatic changes during activity-dormancy transitions occur in the apical meristem, but a considerable number of studies have been conducted on another meristem, the vascular cambium of woody plants (Schrader *et al.,* 2004; Espinoza-Ruiz *et al.,* 2004; Druart *et al.,* 2007). This meristem offers several advantages as an experimental system compared to the apex because of its size, relatively simpler cellular organization, and opportunities to study dormancy at a higher resolution using appropriate cell-type isolating techniques (Schrader *et al.*, 2004; Hertzberg *et al.*, 2001). In contrast to the apex, where extensive morphological reorganization occurs during bud formation, the vascular cambium shows only minor modifications during the transition to dormancy, such as thickening of the cell walls and alteration of the vacuolar structure (Ermel *et al.*, 2000). Auxin has been implicated as a key signal regulating cambial cell proliferation, and polar auxin transport is readily reduced, leading to insensitivity to exogenous auxin, during the transition to dormancy (Little & Bonga, 1974). In contrast, auxin has not been heavily implicated in apical dormancy, in which other hormones seem to have more important roles. Nevertheless, there are several similarities between dormancy in the two organs: notably in both the apex (Ruttink *et al.,* 2007) and the cambium (Druart *et al,* 2007; Schrader *et al.,* 2004) the establishment of a dormant state involves massive transcriptional changes, accompanied by metabolic and physiological modifications such as induction of cold hardiness, desiccation tolerance and accumulation of storage compounds (Welling *et al.,* 1997). Cell division also ceases in both organs, although some cell cycle regulators seem to be kept in a skeletal state in the cambium (Schrader *et al.,* 2004) while cell cycle activity completely ceases in the apex (Espinoza-Ruiz *et al.,* 2004; Ruttink *et al.,* 2007). This may account for the differences in behaviour between the apex and the cambium after chilling requirements are fulfilled during winter and warm temperatures in the spring promote growth. While buds generally flush via a process involving cell expansion prior to cell division, cell division is simply reinitiated in the cambial meristem in spring. Accordingly, warm temperatures can directly upregulate *CDKBs* and *CYCBs* in the cambium (Li *et al.*, 2009).

1.5 SEED DORMANCY

The seed is a survival structure that is believed to have evolved independently around 100-400 million years later than the bud. Seed and bud dormancy share a number of similarities, including similar physiological features such as chilling requirements to release dormancy (in some species and ecotypes), changes in water availability and acquisition of desiccation tolerance, accumulation of storage compounds, and similar effects of ABA and GA on dormancy development and release (Dennis, 1996; Powell, 1987; Saure, 1985). Furthermore, massive changes in the transcriptomes of both buds and seeds occur during the transition to dormancy, and the transcriptomes of the two dormant organs have been seen to partially overlap (Ruttink *et al.,* 2007). These similarities may derive from, at least partly, shared molecular mechanisms underlying the processes (Rohde & Bhalerao, 2007; Wareing, 1956). Therefore, studies on seed dormancy, even if conducted on herbaceous plants such as *Arabidopsis*, may provide insights into events taking place in the apical meristem during dormancy and highlight possible common regulators. This section will therefore briefly introduce seed development and concentrate on the aspects that may have most relevance to the studies presented in this thesis.

The *Arabidopsis* seed is formed by a mature embryo surrounded by a single layer of endosperm cells (the aleurone) and the testa, a dead tissue at maturation. Seed development can be divided in two main phases: embryo development and seed maturation. Embryo development starts after pollination, when one male gamete fuses with the egg cell to form a diploid zygote that will develop into the embryo, and another fuses with the diploid central cell to give the endosperm. Embryogenesis then proceeds via a first, morphogenetic phase, ending at the heart stage, when all embryonic tissues have been differentiated, and a second phase in which the embryo grows from torpedo stage to a mature embryo filling the embryo sac (Fig. 6). In parallel with embryo development, the endosperm initially proliferates, then undergoes cellularization and maturation, eventually being reduced to a single cell layer. At the end of this phase, cell division is terminated (Raz *et al.*, 2001). When the maturation phase is subsequently initiated the seed accumulates storage compounds, such as proteins and lipids, which will support the growth of the seedling, and acquires desiccation tolerance. Primary dormancy is initiated early during seed maturation, increases until the seed is fully developed, and is maintained after the seed is shed (Karssen *et al.*, 1983)(Fig. 6).

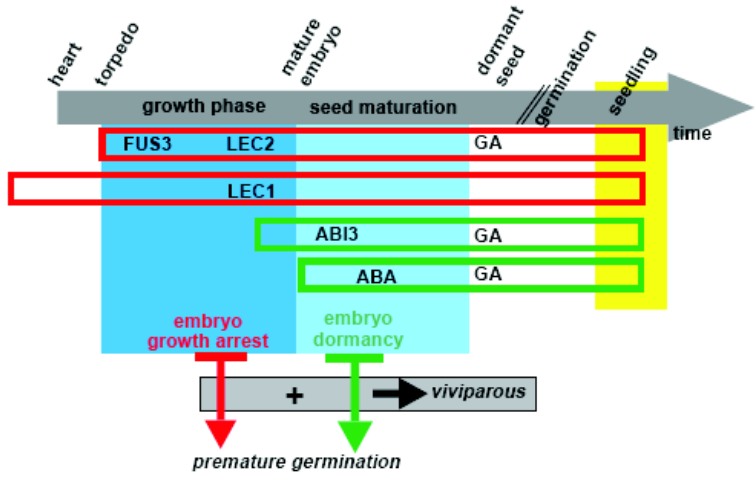

FIGURE 6: Genetic and hormonal control of developmental arrest phases in *Arabidopsis* seeds.
Reproduced from Raz *et al.*, 2001 with permission from the publisher.
http://dev.biologists.org/content/128/2/243.full.pdf

Seed dormancy has been defined as the incapacity of a viable seed to germinate under a specified period of time under favourable conditions (Finch-Savage & Leubner-Metzger, 2006). It is an essential stage for plants generally, due to the necessity of ensuring that germination does not occur while the seed is still attached to the mother plant or when conditions are unfavourable for survival of the next generation. According to the classification of Baskin and Baskin (2004), dormancy in *Arabidopsis* is physiological (PD), nondeep, has embryo and coat components, and can be broken by GA treatment, scarification, ripening in dry storage, and cold or warm stratification (Baskin & Baskin, 2004). In PD dormancy, extension growth of excised embryos is inhibited, while non-dormant embryos excised from coat-dormant seeds will be able to extend and grow. Dormancy can be seen as a continuum of stages of varying dormancy degrees, for which the width of windows of germination-permissive conditions varies correspondingly. This cycling of dormancy leads to the emergence of seedlings in specific periods of the year and reflects the seeds' responsiveness to environmental factors such as temperature, light and nitrate availability. Seeds that have lost primary dormancy but are exposed to unfavourable conditions for germination may enter a state of secondary dormancy. The capacity to germinate is therefore the result of a balance between the degree of dormancy and the growth potential of the embryo.

Dormancy is controlled by environmental, hormonal and genetic factors, with both synergistic and antagonistic effects. Once the embryo's growth potential surpasses the dormancy state, the seed can germinate. Germination starts with the uptake of water by the quiescent seed, followed by cell elongation (Kucera *et al.,* 2005) and, once the radicle emerges, cell division. Seed development has been extensively studied using *Arabidopsis* mutants that are defective in various aspects of the process, for example mutants defective in testa pigmentation or structure, embryo growth and maturation mutants, and hormone-deficient and -insensitive mutants. The following sections describe the most relevant mutants for the studies included in this thesis.

1.5.1 Embryo growth arrest and maturation mutants

Embryo growth arrest mutants

Genes controlling the embryo growth arrest phase include those encoding for the transcription factors FUSCA 3 (FUS3), LEAFY COTYLEDON 1 AND 2 (LEC1 and LEC2) (Raz *et al.,* 2001) (Fig. 6). Loss of function mutations of those genes cause a continuation of growth in excised immature embryos, and premature seed germination due to the abnormal continuation of cell division through the phase of seed maturation (Raz *et al.,* 2001). *Lec* mutants display premature expression of seedling-associated traits accompanied by substantial defects in the maturation phase of the seed developmental program; when overexpressed, *LEC* genes are sufficient to promote seed developmental programs in adult plant tissues (Gazzarrini *et al.*, 2004; Stone *et al.*, 2001; Lotan *et al.*, 1998). Seed maturation is also severely affected in *fus3* mutants (Bentsink & Koornneef, 2008).

abi3, a maturation mutant

ABI3 is a transcription factor that controls specific aspects of the seed maturation program, including appropriate accumulation of storage compounds, the acquisition of desiccation tolerance, ABA sensitivity, and dormancy development. While some aspects such as absence of chlorophyll degradation and decreased ABA sensitivity are specific for these mutants (Gutierrez *et al.*, 2007; Raz *et al.*, 2001), *abi3* mutants share some common phenotypic traits (defective accumulation of storage proteins and dormancy at maturation) with embryo growth mutants. ABI3, FUS3, LEC1 and LEC2 all interact in a network that regulates various aspects of seed maturation (reviewed in Holdsworth *et al.,* 2008). However, compared to *LEC* and *FUS3* genes, *ABI3* controls a later stage of seed development, since excised

immature embryos of *abi3* mutants do not germinate, while seeds in early maturation stages show premature germination and mature seeds are not dormant. Therefore ABI3 acts downstream of termination of cell division (Raz *et al.,* 2001). Moreover, ABI3 acts in ABA signalling, as *abi3* seeds are ABA insensitive (Koornneef *et al.*, 1984), increases in ABI3 protein levels are induced by exogenous ABA (Lopez-Molina *et al.*, 2002), and ectopic expression of *ABI3* leads to ectopic expression of seed transcriptional programs and some ABA transcriptional programs (Leung & Giraudat, 1998; Parcy *et al.*, 1994). However, as ABI3 influences processes that are not affected in ABA-deficient mutants, it has been proposed to have a broader function than simply ABA signalling. ABI3 functions are not restricted to embryogenesis and seed development in *Arabidopsis*. *ABI3* is also expressed in vegetative tissues (e.g. the meristem, stipules and abscission zones of siliques), and controls processes such as termination of cellular differentiation, vegetative quiescence, plastid and vascular differentiation and phase transition (Kurup *et al.*, 2000; Rohde *et al.*, 2000; Rohde *et al.*, 1999). It has therefore been proposed that ABI3 acts as a general regulator of the timing of developmental transitions throughout the plant life cycle (Holdsworth *et al.*, 2001; Rohde *et al.*, 2000).

1.5.2 Hormonal control of seed dormancy

Physiological and genetic studies have shown that the outcome between seed dormancy and germination is determined by a balance between ABA and GA pathways, environmental signals and internal developmental signals (reviewed in Bentsink & Koornneef, 2008; Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008; Finch-Savage & Leubner-Metzger, 2006; Kucera *et al.*, 2005). ABA induces primary dormancy and is involved in its maintenance. The dormant seed is characterized by a high ABA:GA ratio, high ABA sensitivity and low GA sensitivity. GA controls dormancy release and the progress through germination; embryo dormancy release requires a low ABA:GA ratio, a decrease in ABA sensitivity and an increase in GA sensitivity. Release from dormancy is promoted by several environmental signals, such as light, temperature and nutrient availability, which affect the ABA:GA balance by modifying the expression of biosynthetic and catabolic enzymes and hormone signalling pathways (reviewed in(Finkelstein *et al.*, 2008; Cadman *et al.*, 2006; Finch-Savage & Leubner-Metzger, 2006; Ali-Rachedi *et al.*, 2004; Nambara & Marion-Poll, 2003). In turn, the changing ABA:GA ratio affects signalling pathways that alter the dormancy status and thus the sensitivity of the seed to environmental factors. When the fulfilled

requirements for germination overlap with the changing environmental conditions, germination can proceed.

ABA and seed dormancy

ABA is responsible for the induction of seed desiccation and primary dormancy during late phases of seed development. Two peaks of ABA levels can be seen in the developing seed: a peak of maternal ABA during early maturation, needed to prevent viviparism (Karssen *et al.,* 1983), and a second peak of ABA produced by the seed itself during dormancy induction. ABA produced by the seed, but not maternal or exogenously applied ABA, is able to induce lasting dormancy (Kucera *et al.,* 2005). ABA levels decrease, via ABA 8'-hydroxylation, at the end of seed maturation and during imbibitions (Okamoto *et al.*, 2006). Catabolism of ABA, catalyzed by the CYTOCHROME p450s (CYP707A) gene product, is a key step in regulation of the germination potential of the seed. Dormancy is then actively maintained in imbibed seeds by *de novo* production of ABA (Ali-Rachedi *et al.,* 2004). Dormancy is reduced in seeds that are ABA deficient, whereas overexpression of genes for ABA biosynthesis or mutation of ABA catabolic genes enhances dormancy or delays germination (Kushiro *et al.*, 2004; Nambara & Marion-Poll, 2003). Furthermore, mutants with ABA signalling defects also show seed dormancy defects. *abi1* mutations, for example, result in ABA-insensitive plants, which produce non-dormant seeds that can germinate and grow in the presence of ABA (Koornneef *et al.*, 1984). Overexpression of the ABA binding protein REGULATORY COMPONENT OF ABA RECEPTOR 1 (RCAR1) results in plants that are hypersensitive to ABA with respect to seed germination (Ma *et al.*, 2009; Park *et al.*, 2009). Knockdown lines of another ABA receptor, ABAR/CHLC/GENOMES UNCOUPLED 5 (GUN5), show upregulation of the ABI1 protein, ABA-resistant germination, and reduced expression of LATE EMBRYOGENESIS ABUNDANT (LEA) proteins (Shen *et al.*, 2006). Plants carrying mutations of other downstream components of ABA signalling pathways, such as *abi3, abi4* and *abi5* mutants, show reduced expression of seed maturation genes, but only *abi3* mutants are nondormant (Finkelstein & Rock, 2008; Nambara *et al.*, 1992).

GAs and seed dormancy

GAs accumulation is necessary to promote embryogenesis. Subsequently, the GAs content needs to decrease as dormancy is initiated. The gene encoding the enzyme *GA_2oxidase1*, responsible for catabolism of biologically active GAs, is expressed at high levels in seeds of the highly dormant

accession of *Arabidopsis* Cape Verde Island (Cvi-0), and in response to environmental signals that prevent germination. Active GAs accumulation is instead correlated both with dormancy release and germination promotion (Penfield *et al.*, 2006a; Penfield *et al.*, 2006b; Koornneef *et al.*, 1982). This hormone acts in two different ways: by stimulating expansion of the embryo and inducing mobilisation of storage compounds, and by inducing expression of cell wall hydrolases that weaken the mechanical constrains imposed by the endosperm and testa (reviewed in(Yamauchi *et al.*, 2004; Bewley & Black, 1994). The action of these hydrolytic enzymes has been shown to be inhibited by ABA (Leubner-Metzger, 2003). In particular, environmental signals promoting germination, such as light and temperature, can affect GA contents of seeds by promoting GA synthesis, increasing GA sensitivity (Debeaujon & Koornneef, 2000), and inhibiting GA degradation (Yamauchi *et al.*, 2004; Ogawa *et al.*, 2003). Stratification leads to increased expression of genes encoding the enzymes that catalyze the last steps in the biosynthesis of active GAs (*GA_{20}oxidase1, GA_{20}oxidase2 and GA_3oxidase1*) and decreased expression of the gene *GA_2oxidase2*, encoding a catabolic enzyme (Penfield *et al.*, 2005; Yamauchi *et al.*, 2004; Ogawa *et al.*, 2003). Light eliminates the transcriptional repression on biosynthetic genes such as *GA_3oxidase* (Yamaguchi *et al.*, 2001), while decreasing DELLA protein levels and GAs catabolism. The DELLA proteins, GAI, RGA1, and REPRESSOR OF GA1-3-LIKE 1, 2 and 3 (RGL1, 2 and 3) are negative regulators of diverse GA responses (reviewed by(Dill & Sun, 2001); in the presence of GAs, DELLA protein levels are reduced by proteasomal degradation (Yamauchi *et al.*, 2007). In contrast, DELLA proteins are stabilized by ABA and phosphorylation, and in turn ABA levels seem to be positively regulated by DELLA proteins (Zentella *et al.*, 2007). RGL2 appears to be the major DELLA protein acting as a repressor of seed germination (Cao *et al.*, 2005; Tyler *et al.*, 2004; Lee *et al.*, 2002). Seeds of triple mutant plants for the GAs receptors GIBBERELLIN INSENSITIVE DWARF 1 (GID1A, B and C) fail to germinate (Griffiths *et al.*, 2006). DELLA mutants display low primary dormancy, indicating that GAs play a role in dormancy control as well as germination (Penfield *et al.*, 2006a; Penfield *et al.*, 2006b). In general, mutations leading to defects in GAs biosynthesis or GAs signalling are associated with germination-defective phenotypes (Steber, 2007; Griffiths *et al.*, 2006; Mitchum *et al.*, 2006).

Hormonal cross talk

Physiological data and genetic studies suggest that the overall sensitivity of a plant cell to a hormone is established, at least partially, by the interplay of

several hormones (reviewed in Kucera *et al.,* 2005; Holdsworth *et al.,* 2008). Hormone signalling cascades can interact in elaborate networks, and the nature of the interactions can vary depending on the tissue and the developmental stage of the plant. Cross-talk between phytohormones can also occur at the metabolic level.

Apart from ABA and GAs, other hormones are also involved in the regulation of germination potential. One of these is ethylene, which has been shown to promote germination by antagonising ABA signalling (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). Antagonistic interactions have also been seen between ABA and both cytokinins and brassinosteroids (reviewed in Finkelstein & Rock, 2008; Bentsink, 2002; Finkelstein *et al.*, 2002). Although drawing direct parallels between the hormonal control of seed and apical dormancy could be incorrect, it is still possible that some interactions and key players are shared between the two organs. Not much is known about hormonal cross-talk in the control of apical dormancy in woody plants. Some evidence of interactions between ABA and ethylene has been obtained from studies of ethylene-insensitive birch lines, which reportedly show no SD-induced ABA accumulation and impaired ABA responsiveness after release from dormancy by chilling treatment (Ruonala *et al.,* 2006). These findings suggest that ethylene may be required for ABA accumulation in birch apices during SD-induced growth cessation.

1.6 CHROMATIN REMODELLING AND REGULATION OF GENE EXPRESSION IN RELATION TO DORMANCY

Transcriptional control plays a key role in regulating various aspects of plant development. Chromatin remodelling provides a convenient way to coordinately regulate transcriptional patterns at global scale and this type of regulation also allows for transcriptional states to be heritable. Therefore, chromatin remodelling is developmentally significant and epigenetic mechanisms maintain the transcriptional status of genes as either active or repressed. Recent studies have provided genetic evidence for transcriptional control via chromatin remodelling in processes that share similarities to bud dormancy and dormancy release, e.g. vernalization and seed dormancy. As highlighted in several microarray studies, the transition to dormancy in seeds involves extensive remodelling of the transcriptome. In seeds, there is evidence of a requirement for an epigenetic mechanism to fine-tune such massive changes in gene expression. For example, seeds carrying a mutation in the *REDUCED DORMANCY locus 4* (*RDO4*, subsequently renamed *HUB1*, and its homologue *HUB2*), encoding a histone monoubiquitination

enzyme, show reduced dormancy. Furthermore, several studies have highlighted the importance of chromatin-based mechanisms in limiting to seeds the expression of both seed regulators and their targets to prevent ectopic expression of seed-associated traits (Zhang & Ogas, 2009; Kohler & Makarevich, 2006; Tai *et al.*, 2005; Takada & Goto, 2003). Due to the similarities between bud and seed dormancy, it is possible that chromatin remodelling may be involved in the establishment of dormancy in woody plants as this process also involves a highly coordinated and extensive rearrangement of the transcriptome in both buds and vascular cambium (Ruttink *et al.,* 2007; Schrader *et al.,* 2004; Rohde & Bhalerao, 2007). Moreover, the similarities between vernalization and dormancy release prompted us to hypothesize that there may be a common regulatory mechanism, and thus that chromatin remodelling could control dormancy release in woody plants (Rohde & Bhalerao, 2007). Papers I and III appended to this thesis investigate this possibility. The following section presents a brief introduction to chromatin and mechanisms controlling genes' transcriptional status.

1.6.1 Chromatin organization and remodelling

Genomic DNA of eukaryotic cells is organized inside the nucleus as chromatin, which consists of basic repetitive units, the nucleosomes (Kornberg & Klug, 1981). Nucleosomes consist of 145-147 bp of DNA wrapped around a histone octamer (two copies of each histones H2A, H2B, H3 and H4). A region of DNA linker of around 20 bp, to which histone H1 binds, links successive nucleosomes. Chromatin remodelling is a biochemical mechanism that influences gene activities without altering the DNA sequence, and involves modifications of histones or DNA. Importantly, this mechanism can also ensure that the transcriptional state (active or repressed) of genes required at any moment of a plant life cycle is maintained through the lifecycle and can be inherited by the subsequent generation. Several developmental transitions initiated by the perception of promoting environmental cues such as daylength and temperature, e.g. flowering, involve chromatin remodelling. Indeed, chromatin remodelling has been shown to occur in responses to temperature (Gendall *et al.*, 2001; Stockinger *et al.*, 2001), light (Chua *et al.*, 2001), and photoperiod (Wagner & Meyerowitz, 2002; Chua *et al.*, 2001). Chromatin can be found in two forms in the nucleus: heterochromatin and euchromatin. While heterochromatin is associated with low gene content, high content of repetitive sequences and low transcriptional activity, euchromatin is decondensed during interphase, rich in genes, and correlates to an active

state of transcription. The term "constitutive heterochromatin" is related to the permanently inactive regions of telomeres and pericentromeres, while "facultative heterochromatin" can exist within euchromatin, and indicates regions that may be silenced, for example in one developmental stage and not in another, providing a way to register transient signals (e.g., winter) and establish a cellular memory (Grewal & Elgin, 2002).

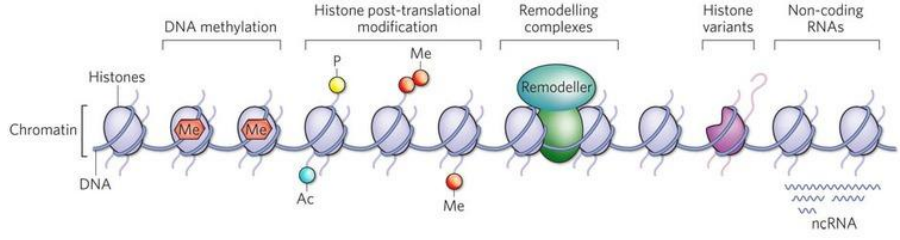

FIGURE 7: Examples of possible mechanisms of alteration of chromatin structure: from left to right, DNA methylation, histone modification, remodelling by chromatin-remodelling complexes and insertion of histone variants.
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Chromatin remodelling (reviewed in Clapier & Cairns, 2009; Lafos & Schubert, 2009; Hsieh & Fischer, 2005; Wagner, 2003; Goodrich & Tweedie, 2002) is therefore an epigenetic process that results in a heritable, but reversible, modification of gene expression. Chromatin structure can be modulated in several ways, described below and illustrated in Figure 7:

1) ATP-dependent remodelling complexes can change and reconfigure the interaction between DNA and nucleosomes, temporarily opening and closing access to DNA (reviewed in Langst & Becker, 2004). In animals these enzymes are divided into several classes based on the presence of other protein motifs in addition to the ATPase domain: SWITCH 2/SUCROSE NONFERMENTING 2 (SWI2/SFN2), chromodomain/helicase/DNA-binding domain (CHD) and ISWI. The gene *PICKLE (PKL)*, which has a zinc-finger domain, is an orthologue in *Arabidopsis* of the CHD3/Mi-2 proteins in humans and *Drosophila* (Ogas *et al.*, 1999). *PKL* regulates the transition between embryonic and vegetative development by repressing the expression of seed-associated genes during germination (Zhang *et al.*, 2008).

2) Covalent post-translational modifications of the N-terminal tails of histones, which are rich in highly charged lysine, can directly alter chromatin structure or recruit other chromatin remodelling complexes. Such modifications include: acetylation, methylation, phosphorylation, ubiquitination, ribosylation and SUMOylation. The pattern of these alterations is proposed to constitute a histone code (Strahl & Allis, 2000) to which the nature, position and order of the modifications all seem to contribute. All post-translational modifications (PTMs) are reversible.

In general, acetylation of lysine residues of histone tails is strongly correlated with active transcription of DNA (Narlikar *et al.*, 2002; Chua *et al.*, 2001). Acetyl groups are added by HISTONE ACETYLTRANSFERASES (HATs), which act as part of multi-subunit complexes and are targeted to DNA by other components of the complex that may possess DNA binding activity. Distinct HAT families can be classified according to the presence and position of other conserved protein domains (Chen *et al.*, 2001; Marmorstein & Roth, 2001). For example, GCN5/PCAF HATs have a C-terminal bromodomain and in *Arabidopsis* play a role in temperature-regulated development (Stockinger *et al.*, 2001) and in regulating floral meristem activity (Bertrand *et al.*, 2003). The acetylation state of histones is reversible and dynamically balanced by the contrasting activity of HISTONE DEACETYLASES (HDACs). Like HATs, HDACs are found in large protein complexes and their activity is generally related to transcriptional repression and silencing. Plants contain a novel class of HDACs, HD2, in addition to homologues of the Rpd3, HDA1 and SIRTUIN2 families found in other eukaryotes (Wu *et al.*, 2000). HDACs often act in concert with histone methyltransferases (HMTs) and DNA methyltransferases (DNMTs) (Richards & Elgin, 2002). Methylation of histones is linked, depending on the type and position of the modification, to both activation and repression of transcription; H3K27, H3K9 and H4K20 methylation leads to transcriptional repression, while H3K4 and H3K36 methylation leads to gene activation (Fuchs *et al.*, 2006). Histone methyltransferase activity (HMTase) depends on the presence of a SET domain in the protein and requires protein-protein interaction for it to be recruited to the region of interest. Methylation of histone tails is another reversible modification, as several histone demethylases, which belong to the KDM1/LSD1 (Shi *et al.*, 2004) or jumonji C-domain containing protein family (Tsukada *et al.*, 2006), have been identified in plants. Another type of histone modification, histone tail ubiquitination, may lead to different effects depending on the residues that are modified and their context; for example, two ubiquitination sites in the C-terminal end of H2B and H2A correlate,

respectively, with active and repressed transcription (Bergher, 2007). In *Arabidopsis,* homologues of two components of the yeast Rad6/BRE1 complex (UBIQUITIN CONJUGATING ENZYME 1 and 2, UBQ1 and UBQ2; HISTONE MONOUBIQUITINATION 1 and 2, HUB1 and HUB2, respectively) mediate histone H2B ubiquitination, which is required for upregulation of the expression of *FLOWERING LOCUS C (FLC)* (Gu *et al.*, 2009). Interestingly, *hub1-2* mutants in *Arabidopsis* display reduced expression of dormancy-related genes, and *hub1* and *hub2* mutants show reduced seed dormancy (Liu *et al.*, 2007).

3) Insertion of histones variants in the protein core of nucleosomes is a mechanism that can help to quickly reactivate chromatin that has been marked as inactive through, for instance, displacement of H3, marked with H3K9me2, with the variant H3.3, marked with H3K4me2/3 (Henikoff *et al.*, 2004).

4) Another important modification is DNA methylation. Not only histone tails but also DNA can be subjected to methylation, a modification that is generally associated with transcriptional repression. Methylation of the 5' position of cytosine, catalysed by DNA methyltransferases, can occur either symmetrically or asymmetrically within the sequence CpG, or CpNpGp in plants. This modification provides stable chromatin marks that can be used to recruit other protein complexes capable of binding to methylated DNA. Several classes of DNA methyltransferases are known in plants, representative members of which include: METHYLTRANSFERASE 1 (MET1), responsible for maintenance of methylation at CG sites; CHROMOMETHYLASE 3 (CMT3), unique to plants, responsible for maintenance of methylation at CNG sites; and the DOMAIN REARRANGED METHYLTRANSFERASES DMT1 and DMT2, responsible for *de novo* methylation of DNA (Cao & Jacobsen, 2002). Plant genomes are extensively methylated, and overall DNA methylation patterns can change in response to environmental signals (Steward & Sano, 2002). In this respect, four enzymes responsible for the removal of DNA methylation on cytosine have been found in plants: DEMETER (DME), a DNA glycosylase required for the parent of origin expression of MEDEA (MEA) (Gehring *et al.*, 2006; Kinoshita *et al.*, 2004); DEMETER LIKE 2 (DML2) and DEMETER LIKE 3 (DML3); and REPRESSOR OF SILENCE 1 (ROS1) (Morales-Ruiz *et al.*, 2006).

Interplay between all the mechanisms described above can also occur. In animals, for example, the effect of DNA methylation on chromatin structure is linked to histone deacetylation, as proteins that are able to bind to methylated DNA are able to recruit HDACs (Bird & Wolffe, 1999). In addition, DNA methylation and histone methylation are linked in plants, animals and fungi.

PcG complexes and TrxG complexes in plants

Polycomb group (PcG) complexes are a set of chromatin remodelling proteins that act as repressors of both homeotic and non-homeotic genes (reviewed in Alvarez-Venegas, 2010; Hennig & Derkacheva, 2009; Kohler & Villar, 2008; Pien & Grossniklaus, 2007; Pirrotta *et al.*, 2003; Brock & van Lohuizen, 2001). PcG proteins form an epigenetic memory system, conserved in plants and animals, which controls gene expression during development. Its function is ensuring that all alternative genetic programs that are not required in a particular tissue or time are silenced. Polycomb group complexes assemble at their target sites and silence neighbouring genes when they are not actively transcribed; they have the ability to distinguish between active and inactive genes. First discovered in *Drosophila melanogaster*, in which they prevent inappropriate expression of homeotic genes (*HOX*), which would cause posterior homeotic transformation in embryos and adults (Simon, 1995), PcG complexes have also been identified and studied in other animals and plants. Three PcG complexes have been described in *Drosophila*: PcG repressive complex 1 (PRC1), involved in stable maintenance of gene repression; PcG repressive complex 2 (PRC2), implicated in initiation of gene repression; and Pleiohomeotic repressive complex (Pho-RC), containing Pho and dSfmbt, which selectively interacts with methylated histones to maintain a repressed chromatin state. Homologues of Pho-RC components have not as yet been found in plants. The POLYCOMB REPRESSIVE COMPLEX 1 of *Drosophila* is formed by a core of five proteins - POLYCOMB (PC), POLYHOMEOTIC (Ph), POSTERIOR SEX COMBS (PSC), dRING3 (ligase/ubiquitinase), and SEX COMB ON MIDLEG (SCM) - with which additional proteins such as ZESTE, and general transcription factors, can interact. In *Drosophila* and mammals, PRC1 can bind to H3K27me3 marks via the PC domain, providing a stable and long term means of silencing genes through dRING ubiquitination of H2A lysine 119. Plant genomes appear to lack a homologue for the PRC1 core component PC. However, a functional PC analogue, two *Arabidopsis* RING1 homologues, and other proteins, such as VRN1 and EMF1, involved in stabilization of PcG-mediated repression, have been identified. The product of the gene *LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)*, a chromodomain protein, may perform in plants the same role as the PC protein (Zhang *et al.*, 2007). Although functional analogues of PRC1 have

been found in plants, the complex may function differently from the corresponding complex in animals. The PcG complex PRC2 of *Drosophila* is composed of four core subunits: ENHANCER OF ZESTE (E(Z)), SUPPRESSOR OF ZESTE 12 (S(Z)12), EXTRA SEX COMB (ESC), and p55 (Ringrose & Paro, 2004). This PcG complex acts via its E(Z) subunit, which displays SET activity and can trimethylate lysine 27 on histone 3, imposing a transcriptionally repressive mark. The model plant *Arabidopsis thaliana* has 12 homologues of Drosophila PRC2 subunits: 3 E(Z) homologues, CURLY LEAF (CLF), MEDEA (MEA) and SWINGER (SWN); three Suppressor of Zeste (Su(z)12) homologues, EMBRYONIC FLOWER 2 (EMF2), FERTILIZATION INDEPENDENT SEED 2 (FIS2) and VERNALIZATION 2 (VRN2); the single Extra Sex Comb (Esc) homologue FERTILIZATION INDEPENDENT ENDOSPERM (FIE); and five p55 homologues, MULTICOPY SUPPRESSOR OF IRA 1-5 (MSI1-5) (Henning & Derkacheva, 2009). Molecular and genetic evidence indicates that in *Arabidopsis* those subunits are combined in at least three different PRC2 complexes, each with specific functions during development, which can share target genes (Makarevich *et al.*, 2006).

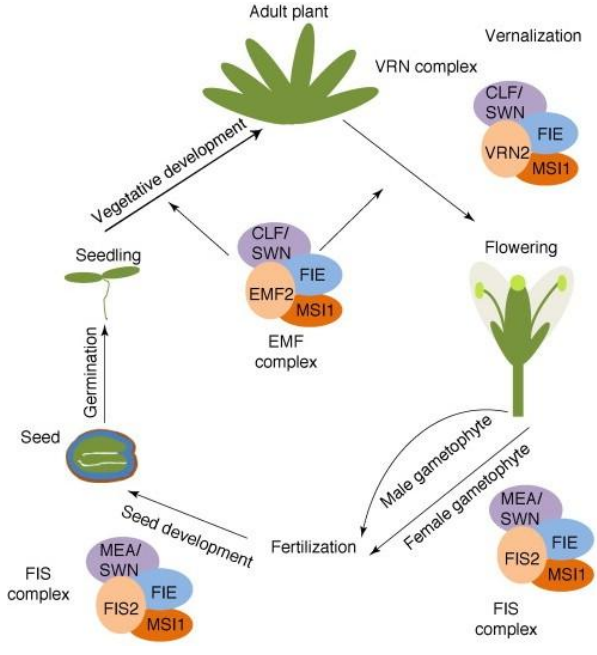

FIGURE 8: PRC2 complexes that act at different stages of the *Arabidopsis* life cycle.

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doi:10.1016/j.tig.2009.07.002

These three complexes control cell and organ specification, cell proliferation, differentiation, and developmental phase transition (Fig. 8). The EMBRYONIC FLOWER (EMF-PRC2) complex suppresses precocious flowering and promotes vegetative development; the VERNALIZATION (VRN-PRC2) complex controls flowering, and the FERTILIZATION INDEPENDENT SEED (FIS-PRC2) complex, the only one to have been biochemically investigated in detail, is involved in gametogenesis and early seed development. Figure 8 shows how varying components and the accessory proteins that can interact with them give specificity to those complexes. The genes *FIE* and *MSI1* (the only one of the *MSI* genes for which functional data are available) are present in all complexes, while other players are variable. Mutations of both *FIE* and *MSI1* genes in *Arabidopsis* lead to proliferation of an endosperm-like structure in the absence of fertilization, and abortion of the embryo (Ohad *et al.*, 1999).

Plants present more variability of core components than animals, probably due to the necessity to fine-tune the expression of different sets of target genes during their lifecycle. PRC2 complexes in animals and plants need to associate with plant homeodomain (PHD)-finger proteins for efficient deposition of H3K27me3 and transcriptional repression (De Lucia *et al.*, 2008). Subsequently, H3K27me3 marks on target genes recruit additional PcG complexes (PRC1 in animals, potentially LIKE HETEROCHROMATIN PROTEIN 1, LHP1, and homologues of RING1 in plants). The proteins recognising the marks left by PRC2 complexes are not evolutionarily conserved, and the mechanisms of repression differ between kingdoms. Different effects are caused by loss of PRC2 function in mammals and plants. While *cfl/swn* double mutants show cell-dedifferentiation, which results in formation of callus-like structures lacking organization (Chanvivattana *et al.*, 2004; Kinoshita *et al.*, 2001), and loss of *Physcomitrella patens FIE* causes meristems to overproliferate and prevents differentiation of leafy gametophytes (Mosquna *et al.*, 2009), loss of the mammalian PRC2 subunit *EED* (ESC homologue) causes embryonic stem cells to differentiate into different cell types, and overexpression of PcG proteins leads to loss of cell differentiation and overproliferation, as observed in cancer (reviewed in Kohler & Villar 2008). Therefore, plant PcG proteins seem to be essential for maintaining cells in a dedifferentiated state in addition to repressing genetic programs (thus establishing specific cellular identity). Chromatin remodelling is a flexible mechanism for fine-tuning the transcriptional status of genes, so in order to control development it is important to balance the action of PcG complexes.

In addition to other mechanisms that regulate dynamics at the chromatin level, trithorax group (TrxG) complexes act antagonistically to PcG complexes. In *Drosophila*, both PcG and TrxG complexes act by binding to PcG and TrxG RESPONSE ELEMENTS (PREs and TREs), regulatory regions that range from several hundred bp to several kb (Pirrotta, 1997). The elements required for TrxG activation and PcG repression are separable, but situated within 30–40 bp from each other, and are found in these regions. However, consensus sequences of PREs and TREs have not yet been identified in plants. Many genes of the TrxG complexes were initially identified as suppressors of Polycomb mutations, suggesting that TrxG proteins work antagonistically to PcG complexes, and PcG target genes are positively regulated by TrxG proteins (Francis & Kingston, 2001). Proteins of the TrxG group are required for maintenance, but not initiation, of activation of homeotic loci (Breen *et al.*, 1995). In *Drosophila* and mammals, TrxG complexes contain proteins with SET domains, whose main activity is to methylate lysine 4 of histone 3, resulting in activation of transcription. At least three TrxG complexes have been identified in *Drosophila*, and they are all involved in the formation of an open chromatin configuration by promoting an active epigenetic modification (methylation, acetylation and ATPase-dependent activity) (Breiling *et al.*, 2007). While TrxG complexes have not yet been isolated in plants, homologues of TrxG proteins that regulate floral homeotic genes have been identified: in *Arabidopsis* there are at least five TrxG-like proteins, of which *ARABIDOPSIS* HOMOLOG OF TRITHORAX 1 AND 2 (ATX1 and ATX2) have been further studied. Both proteins contain SET domains with H3K4 methylation activity and are required for normal flower development and floral organ development (Pien *et al.*, 2008; Saleh *et al.*, 2008; Alvarez-Venegas *et al.*, 2003). Recently, products of the genes *PKL* and *PICKLE RELATED 2 (PKR2)* in *Arabidopsis* have been shown to have trithorax-like activity. Both of these products display ATP-dependent chromatin remodelling activity and are able to counteract the action of PcG complexes on a set of their target genes, acting as transcriptional activators and antagonistically determining cell identity (Aichinger *et al.*, 2009).

1.6.2 Vernalization, an epigenetic phenomenon sharing similarities with dormancy release

Vernalization presumably evolved in annual flowering plants due to selective pressures to develop the ability to memorise a transient environmental signal, winter, to synchronise the timing of reproduction with the most favourable season, spring. Vernalization has all the hallmarks of an epigenetic

mechanism, as the vernalized state is reset at each generation, is cell autonomous and mitotically inheritable. This process promotes competence to flower via a two-step process. Initially, expression of the repressor of flowering *FLOWERING LOCUS C (FLC)* (Michaels & Amasino, 1999; Sheldon *et al.*, 1999) is downregulated, a step that requires expression of *VERNALIZATION INSENSITIVE 3 (VIN3)*, which is involved in histone deacetylation and is gradually induced by cold (Bastow *et al.*, 2004; Sung & Amasino, 2004). This repressive mark is probably required for *FLC* transcription to be maintained inactive by a PcG-like complex (VRN-PRC2 complex) that promotes H3K27 methylation, and by VRN1 which promotes H3K9 methylation. This last chromatin mark also seems to be required to recruit LHP1, an analogue of *Drosophila* PRC1 in *Arabidopsis*, on the *FLC* gene. *FLC* repression is then maintained after plants are returned to warm temperatures, allowing activation of *FT* and other floral activator genes (De Lucia *et al.*, 2008). There is also evidence of another vernalization pathway, *FLC* independent (Schönrock *et al.*, 2006), acting on the MADS-box gene *AGL19*. This pathway again controls flowering through an epigenetic mechanism that requires the action of *VIN3* and the EMF-PRC2 complex.

Vernalization shares some similarities with the process of apical dormancy release. In both cases, the plants need to coordinate reproduction or growth with favourable environmental conditions to avoid damage; the meristem needs to experience a prolonged period of low, non-freezing temperatures to re-gain competence to flower (Michaels & Amasino, 1999) or resume growth (Rohde & Bhalerao, 2007); and cold is necessary but not sufficient for the subsequent state transition. Vernalization and dormancy release also present some differences, for example the fact that *FLC* repression is reset meiotically in *Arabidopsis*, while the action of a possible chromatin remodelling mechanism in apical buds would have to be reset in another way. Importantly, as mentioned above, all PTMs are reversible, and thus this could be the mechanism whereby repression of key genes is counteracted. In addition, vernalization is thought to occur only in actively dividing cells, while in the cambium no cell division occurs during dormancy, although there are reports of growth occurring at very low rates in the apical meristem (Chouard, 1960; Samish, 1954).

2 RESULTS AND DISCUSSION

2.1 *Populus*, A MODEL TREE

Not so long ago, the *Populus* genome project was finalized (Tuskan *et al.*, 2006). *Populus* had been chosen as a model woody plant for several reasons, including its relatively easy transformation and regeneration, small genome (compared to, for example, pine genome), and wide distribution. The scientific advantages of using a tree as a model plant derive from the fact that some processes, for instance the development of winter dormancy, do not occur in herbaceous model plants like *Arabidopsis,* and therefore cannot be studied in such plants. However, working with a tree has some downsides, mostly connected to time and space issues. For instance, it is not practically feasible to generate mutant *Populus* plants by crossing, due to the extended juvenile time before floral transition. In addition, careful planning of experiments is important, as the regeneration of cuttings is time consuming, and the space in which experiments are performed is often limited.

2.2 OUR APPROACH

The studies in this thesis were undertaken to deepen the understanding of the molecular control of the SD-induced shift to dormancy in woody plants, and of dormancy release. Paper I includes an analysis of the activity-dormancy cycle, comprehensive therefore not only of SD treatment, but also of cold treatment after SD and budburst. We performed a full genome array analysis, thus more informative than previous cDNA array analyses of the activity-dormancy cycle (Druart *et al.,* 2007; Ruttink *et al.,* 2007). Paper

I provides in essence a broad overview of the process, from which emerged the idea that ABA and chromatin remodelling may play important roles in the control of dormancy induction and related processes. These results prompted us to further investigate the role of ABA in dormancy, bud formation and other SD-induced processes (Paper II) and to study how chromatin remodelling could be involved in the control of dormancy establishment and release (Paper III). The results presented in Papers II and III also highlight the presence of conserved molecular players between seed and bud dormancy. Therefore, I will also discuss similarities and differences between the control of dormancy in these organs. The following sections report and discuss the most significant results presented in the papers appended to the thesis.

2.3 CONTROL OF SD-INDUCED CELL CYCLE ARREST AND GROWTH CESSATION (PAPERS I, II)

2.3.1 Cell cycle arrest in SD depends on transcriptional downregulation of CYCs

SD-induced growth cessation has been correlated with a reduction of mitotic activity, coinciding with a decline in the activity of various CDKAs and CDKBs in the cambium (Espinosa-Ruiz *et al.,* 2004). We investigated the effect of SD on the core cell cycle genes, including some recently described genes (Menges *et al.*, 2005) and their regulators, in apex. According to our analyses, in partial contrast with previous studies in cambium, all CDKs except CDKBs appeared to be post-transcriptionally regulated following SD treatment (Paper I). Espinosa-Ruiz had hypothesized that the reduction in mitotic activity at the time of growth cessation could be due, for example, to an increase in expression of CDK inhibitors or a decrease in cyclin expression. We showed that SD control of cell cycle arrest derives from transcriptional regulation of several classes of CYCs. Interestingly, only some CYCDs showed transcriptional regulation, with unusual patterns. We therefore speculated that CYCDs may fulfil other roles in the activity-dormancy cycle rather than merely being switches for activation of CDKs (Paper I).

2.3.2 Hormonal control of SD-induced growth cessation- SD controls GA production at the hydroxylation and active GA production levels. It does not affect growth cessation and cell cycle arrest through ABA

We investigated the effects of SD treatment on the expression of biosynthetic and catabolic genes for the hormones ABA and GA during the transition to dormancy (0-10 weeks SD). Gibberellins have been previously linked to the control of growth cessation (Olsen *et al.,* 1997a; Eriksson *et al.,* 2000). Our analysis confirms this, and reveals the role of transcriptional control of GA biosynthesis by SD, as it shows a SD-induced downregulation of the expression of GA biosynthetic genes in early stages of SD treatment. In particular, SD seems to act through transcriptional downregulation of the active GA production steps catalyzed by the enzyme GA_{20}oxidase. At the same time, genes encoding enzymes involved in GA catabolism are upregulated and subsequent SD treatment further lowers expression levels of GA biosynthetic genes (Paper I).

ABA, in contrast, has been proposed to control SD-induced cell cycle arrest, due to the correspondence of the timing of the peak of ABA production and growth cessation during SD treatment (Rohde *et al.,* 2002; Ruttink *et al.,* 2007). We registered an upregulation of ABA biosynthetic genes during SD treatment, and a downregulation of catabolic genes. Since genes encoding components of the ABA signalling pathway, such as *RCAR* and *ABI1*, were also upregulated early during SD treatment, one could hypothesize that changes in ABA sensitivity may also be important in the control of growth cessation. However, analysis of *Populus* plants that overexpress a dominant mutated version of the *abi1-1* gene and have reduced ABA sensitivity showed that they are able to cease growth at the same time as wild type plants upon SD treatment (Paper II). Moreover, microarray analysis of *abi1-1* plants during SD treatment demonstrated that changes in ABA sensitivity do not impair SD-induced downregulation of cell cycle genes (Paper II). These results indicate that SD control of the cell cycle and growth cessation does not depend on changes in ABA sensitivity or ABA levels.

2.4 ADAPTIVE RESPONSES (PAPERS I, II)

2.4.1 CBFs are not involved in development of SD-induced cold hardiness

Development of full cold hardiness in woody plants depends on sequential steps, respectively controlled by SD, SD plus LT and LT alone (Weiser,

1970). Interestingly, our microarray analysis of wild type plants during SD treatment detected two distinct cold adaptive events, in the form of two waves of induction of cold-related genes, an early wave and a late one. Since the plants were not chilled to low temperatures during the experiment, both adaptive waves appear to be under SD control (Paper I). We decided to investigate the possibility that CBFs, key transcription factors in herbaceous plants' cold response, may be involved in SD-induced cold acclimation in woody plants and, potentially, in the induction of the expression of those two observed sets of genes. Previous cDNA array analyses had not been able to rule out or confirm involvement of *CBFs* genes, because those described in *Populus* are not present on the arrays that were used. Our results indicate that CBFs, and their regulator ICE, are not involved, at least at the transcriptional level, in the SD-induced phase of cold acclimation, as they are not upregulated during SD treatment (Paper I). However, it is possible that they control the late phase of cold acclimation, dependent solely on low freezing temperatures, as they are cold-inducible (Benedict *et al.*, 2006). Moreover, it is possible that a novel, as yet undescribed, class of TFs regulates SD-dependent cold acclimation.

2.4.2 SD-induced changes in ABA sensitivity control the induction of a set of adaptive response genes

ABA has long been correlated with stress responses, for example responses to cold and drought (dehydration) (Mantyla *et al.*, 1995). In a way, SD-induced adaptive responses (cold acclimation, dehydration and metabolic changes) may be seen as responses to stress, thus an involvement of ABA would not be surprising. In our array analysis we recorded an upregulation of ABA biosynthetic genes in the apical meristem (Paper I), and a transient increase in ABA levels during SD treatment had already been reported (Rohde *et al.,* 2002). Furthermore, defects in cold acclimation and induction of a class of dehydrins in birch plants unable to raise ABA levels had been demonstrated (Rinne *et al.,* 1998). Our array analysis of ABA insensitive *abi1-1* plants during SD treatment indicated that changes in ABA sensitivity also play a key role in the transcriptional control of several genes underlying those processes. Notably, several genes associated with adaptive responses were differentially regulated between *abi1-1* and wild type plants. Interestingly, changes in ABA sensitivity affected adaptive responses even in the first phase following SD treatment, well before ABA production peak (Paper II). This indicates that ABA plays a role in the control of adaptive responses that is mediated not only through changes in ABA levels but also,

importantly, through changes in sensitivity to the hormone, probably mediated by SD.

In addition, we analyzed the proportion of genes that have ABREs (ABA responsive elements) and/or DREs (dehydration response elements, which are under control of CBFs in *Arabidopsis*) in their promoters and are transcriptionally regulated by SD treatment in wild type plants. A significant number of genes under SD control showed presence of these *cis* elements, and a large number of them were differentially regulated between wild type and *abi1-1* plants. Interestingly, the transcription factors (TFs) that are thought to control those genes in *Arabidopsis* (e.g. *NAC*, *DREB*, *HB12*) also showed the same elements in their promoters and were differentially regulated between wild type and *abi1-1* plants. These findings suggest that ABA may be able to both directly and indirectly control the expression of the adaptive response genes. We can formulate several hypotheses to explain how these genes may be regulated, and I propose a model for ABA regulation of adaptive responses (Fig. 9). According to this model, SD may act directly to regulate the expression of adaptive response genes via changes in ABA sensitivity in a first wave of induction, during which TFs may be activated. These would then reinforce the response, generating a second wave of induction of adaptive genes (Paper II) (Fig. 9). This is consistent with the observation, mentioned above, that in wild type plants there are two waves of induction of genes responsible for cold acclimation, an early and a late response (Paper I). Changes in ABA sensitivity may be responsible for the early adaptive wave, whereas an increase in ABA levels could be responsible for the later adaptive wave, as previously proposed by Druart et al., (2007), since the peak of ABA production correlates temporally with the second wave of induction (Paper II). Interestingly, ABRE elements were also found in the promoters of ABA biosynthetic genes. Thus, it is also possible that SD enhances ABA sensitivity, which in turn activates ABA biosynthesis and leads to the peak in ABA production reported during SD treatment, which occurs after changes in ABA sensitivity have occurred (Fig. 9).

2.5 INTERACTIONS BETWEEN ABA AND ETHYLENE

To date, the role of ethylene in the control of the activity-dormancy cycle has received little attention. However, ethylene sensitivity has been primarily shown to be required for bud formation, and to affect the timing of endodormancy development (Ruonala *et al.,* 2006). Ethylene sensitivity

may also be required for the peak in ABA production to occur during SD treatment (Ruonala *et al.,* 2006). Recently, Arend et al., (2010) showed that expression of ethylene biosynthetic genes is enhanced in ABA insensitive *abi1-1* grey poplar plants.

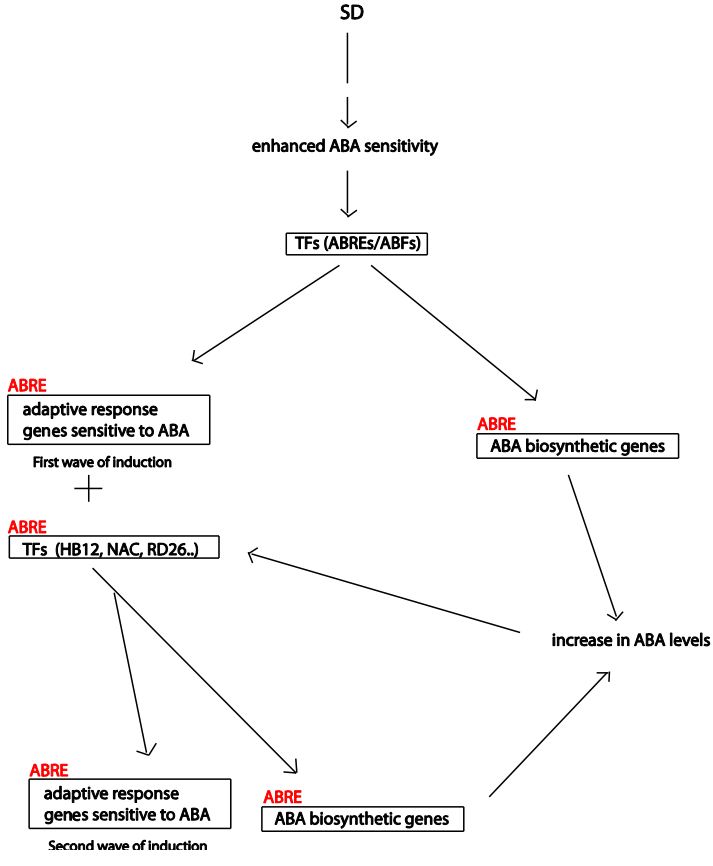

Figure 9: Proposed model of ABA-dependent induction of adaptive responses during SD treatment.

Therefore, ABA and ethylene may also interact at some level during the activity-dormancy cycle. Interestingly, our microarray analysis of *abi1-1* plants showed that expression of the ethylene biosynthetic gene *ACC-oxidase* in *abi1-1* plants is prolonged during SD treatment compared to wild type plants, in which its expression is only transiently activated. A question that remains to be addressed is if the prolonged production of ethylene may be responsible for some of the defective characteristics of the *abi1-1* plants in SD, e.g. their adaptive responses. So far, it is only possible to speculate about

the relationship between these two hormones. Ethylene may be necessary for plants to produce a normal bud and to generate a peak of ABA production. On the other hand, sensitivity to ABA is probably necessary to subsequently lower ethylene production and restrain it in a particular period in SD, possibly because it could negatively influence some of the SD-induced responses. However, we could conclude that defective ethylene sensitivity does not impair changes in ABA sensitivity, since ethylene-insensitive plants show delayed but not a lack of dormancy (Ruonala *et al.,* 2006).

2.6 BUD DEVELOPMENT

2.6.1 ABA is required for complete scale development and bud maturation

The process of bud development can be divided into bud formation and maturation (complete scale development and production of phenolics and resin). Previous studies had suggested that ABA is involved in bud development due to the timing of the peak of ABA production and its correlation with the expression of *ABI3*, a transcription factor involved in ABA signaling in *Arabidopsis* and in bud formation (Rohde *et al.,* 2002; Ruttink *et al.,* 2007). To evaluate the possibility that ABA plays a role in this process, we analysed bud development in plants that show reduced ABA sensitivity. These (*abi1-1*) plants were able to form a normal bud during SD treatment. However, changes in ABA sensitivity were responsible for several phenotypic anomalies: the buds were smaller and greener, the scales did not completely mature, had fewer hairs (which have a cold protective function), and the external cuticular layer of the scales was missing. In particular, the scales presented fewer and less developed bundles of fibres (Fig. 2 in Paper II). The results of the anatomical characterisation of *abi1-1* plants are reflected in the results obtained in the array analysis of the same plants. Genes encoding enzymes of the phenolic biosynthetic pathway were differentially regulated in *abi1-1*, being expressed at a lower level compared to wild type plants during SD treatment. Therefore, we can conclude that ABA is not involved in the formation of buds *per se*, but seems to be involved in the maturation of organs comprised in the bud e.g. bud scales (Paper II).

2.6.2 *ABI3* and ABA display complex interactions during bud development

Rohde et al., (2002) suggested that ABA and the gene *ABI3* could act antagonistically to determine bud formation. In *Arabidopsis*, *ABI3* is part of the ABA signalling pathway and is ABA inducible, but has also been

proposed to serve additional ABA-independent functions (Rohde *et al.*, 2000; Nambara *et al.*, 1992). To obtain further understanding of the nature of the interaction between *ABI3* and the hormone ABA, we analysed bud development in *abi1-1 ABI3* plants (details on production of those lines can be found in Appendix B). Regarding the process of bud formation, we observed an enhancement of the *ABI3* phenotype described by Rohde et al., (2002) when *ABI3* was overexpressed at various levels in the *abi1-1* background (Fig. 10). The developmental program that leads to formation of bud scales, i.e. abortion of the lamina of the first leaf primordia formed at the beginning of the SD treatment, was not initiated and the plants were not able to form a bud (Fig. 11). Nevertheless, the apex stopped to grow actively, since new plastocrons were not produced and plants stopped elongation growth in SD at the same time as wild type plants (Fig. 12).

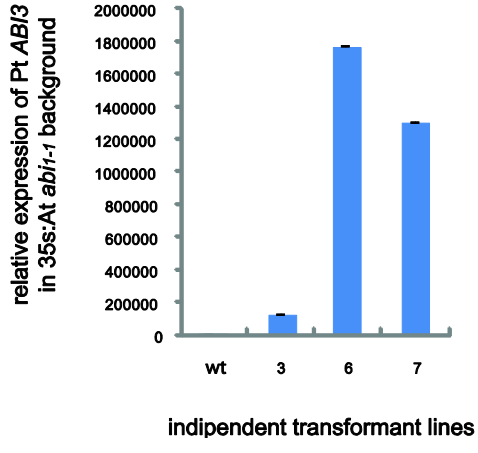

Figure 10: Relative expression levels of the *ABI3* gene in *abi1-1 ABI3* lines. Data are means of independent biological replicates. Error bars represent standard deviation (n=3). Y axis, relative expression (fold change). Materials and methods for the qRT-PCR analysis can be found in Appendix B. wt=wild type.

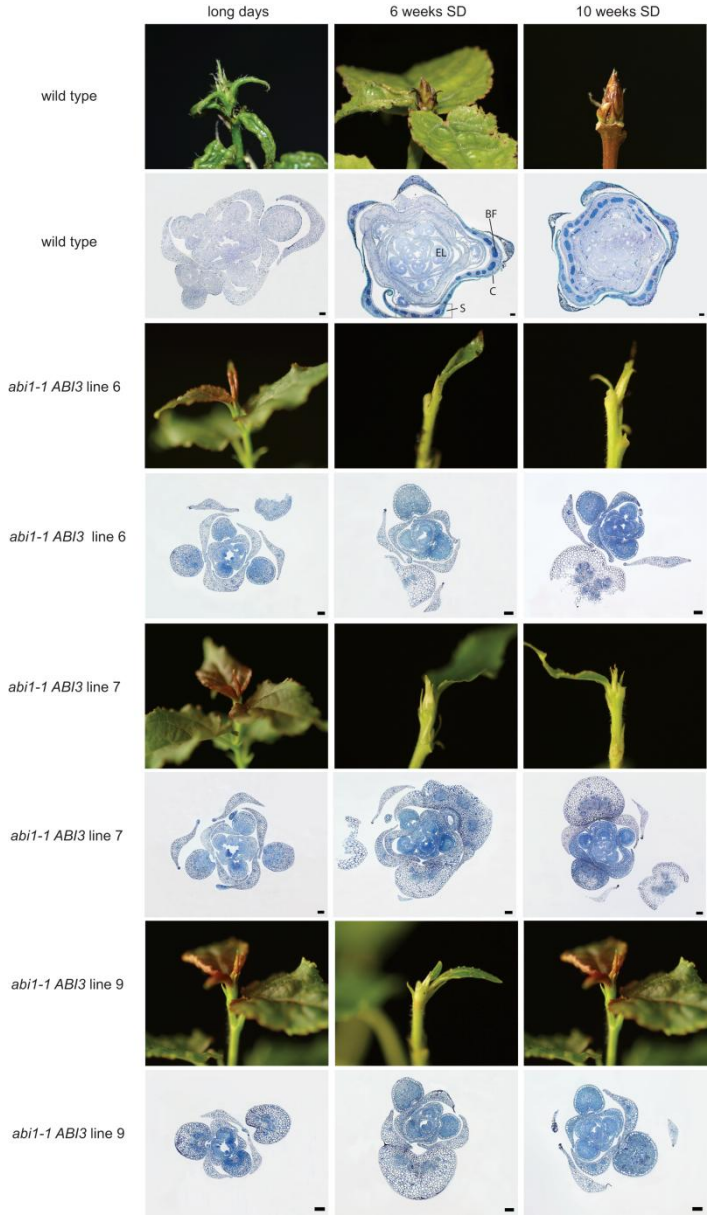

Figure 11: Close-up pictures and anatomical characterisation of the process of bud formation in wild type and 3 independent lines of *abi1-1 ABI3* plants. S=scales, EL=embryonic leaves, C=cuticular layer, BF=bundles of fibers. Bars= 100 micrometers. For comparison with *abi1-1* lines buds, please see Fig. 2 in Paper II.

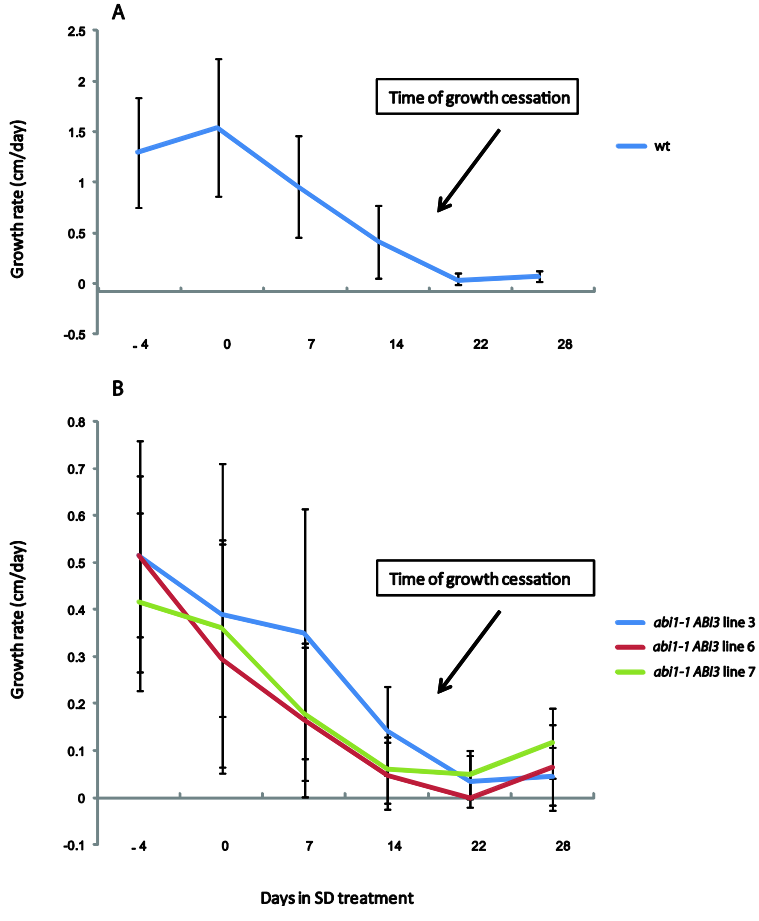

Figure 12: Growth rate during LD and SD treatment of A) wild type (wt) and B) 3 independent lines of *abi1-1 ABI3* plants. Data are means of independent biological replicates. Error bars represent standard deviation (n=6).

Based on the anatomical characterisation of apical buds of *abi1-1* and *abi1-1 ABI3* plants in SD, we can conclude that ABA and *ABI3* interact during bud development. As we observed an enhancement of the bud phenotype in the double mutant plants, ABA and *ABI3* probably do not act in the same pathway. Rohde et al., (2002) proposed the nature of the interaction to be negative, with ABA promoting growth cessation and *ABI3* retarding it in embryonic leaves, thereby allowing their full development and maturation. However, unlike *ABI3* overexpressing plants, *abi1-1* plants did not develop embryonic leaves larger than wild type plants, as would be

expected if the proposed model was true. Interestingly, ABA seems to plays a role in the accumulation of phenolics in bud scales, as *abi1-1* plants did not develop fully mature scales, as reflected in reduced phenolics accumulation in these plants. Similarly, array analysis of *ABI3* overexpressing plants showed that anthocyanin production genes are not upregulated in them to the same extent as in wild type plants (Ruttink *et al.,* 2007). *ABI3* is normally expressed in embryonic leaves and not in bud scales and thus, when overexpressed, is found outside its normal expression domain, where it would normally have no role (e.g. in the scales). If overexpressed, *ABI3* counteracts phenolics production in the scales and this reveals the potential role of *ABI3* as a negative regulator of phenolics accumulation in embryonic leaves, where it is normally expressed.

ABA also plays a role in the activation of adaptive responses of both scales and embryonic leaves, as genes that participate in these responses, such as dehydrins and cold acclimation genes, were not activated in *abi1-1* plants. Interestingly, some of the *ABI3* ectopic targets (e.g. genes associated with seed maturation and adaptive responses) described by Ruttink et al., (2007) were also differentially regulated in *abi1-1* plants (Paper II). Therefore, it seems likely that both ABA and *ABI3* positively regulate these targets and participate in this phase of maturation of embryonic leaves and scales. Obviously, the contribution of *ABI3* to the maturation of scales may simply be a result of the overexpression phenotype. Our findings suggest that the interaction between ABA and *ABI3* is complex, negative or positive depending on the regulated process and tissue type analysed (Fig. 13). Therefore, based on our microarray and anatomical results, and on the fact that *ABI3* is not ABA-inducible in *Populus* (Rohde *et al.,* 2002), the most likely relationship between ABA and *ABI3* in the process of bud development is the one summarized in Fig. 13.

2.7 DORMANCY DEVELOPMENT (PAPERS I, II, III)

In contrast to previous results of Ruttink et al., (2007), we detected massive changes in the transcriptome of the *Populus* apical meristem not only in the first phase of SD response, but also during the transition to dormancy (6-10 weeks SD) (Paper I). Therefore, transcriptional control may also play an important role in the latter phase. To gain further knowledge about the molecular control of dormancy development, we focused on two interesting aspects that emerged from our array analysis.

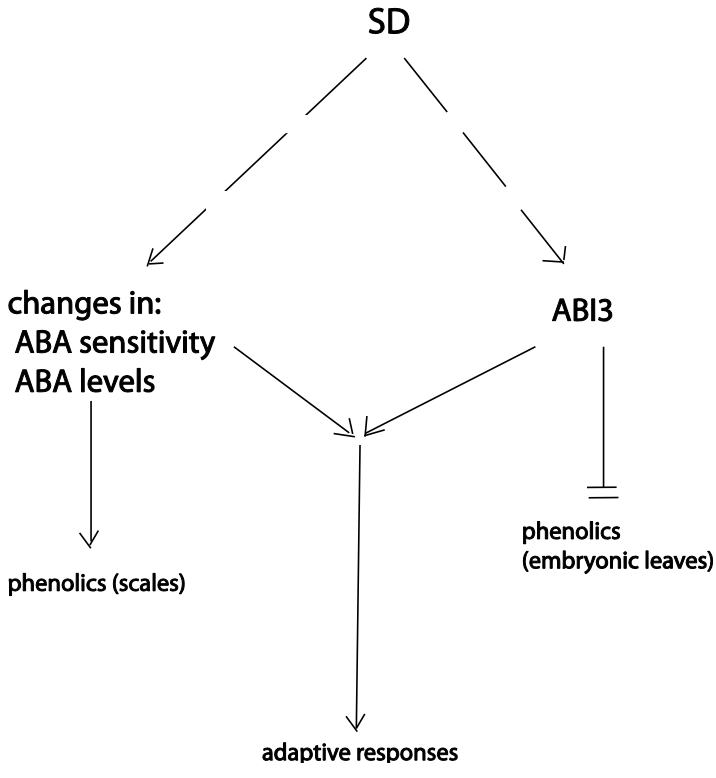

Figure 13: Proposed model of interactions between *ABI3* and ABA in the control of diverse processes during bud development.

Our full genome array analysis showed that ABA biosynthetic genes are upregulated before the onset of dormancy, and that enzymes belonging to the chromatin remodelling machinery are targets of transcriptional regulation during SD treatment (Paper I). In particular, prolonged SD treatment leads to upregulation of genes involved in histone deacetylation, ubiquitination and methylation; modifications that are mainly linked to transcriptional silencing. On the other hand, enzymes belonging to the trithorax groups and a DNA glycosylase, both of which deposit transcriptional activating marks, are downregulated upon SD treatment (Paper I). Therefore, we decided to examine the roles of both ABA and chromatin remodelling in the control of dormancy establishment.

To elucidate the role of changes in ABA sensitivity, we performed a reactivation test on *abi1-1* plants, which showed impaired dormancy development (Paper II) (Fig. 14). As we already know, birch plants unable to change ABA levels show normal dormancy development (Rinne *et al.*,

1998). Therefore, we can conclude that changes in ABA sensitivity, but not in levels of the hormone, control dormancy establishment.

Previous investigations reported that potato dormancy release is associated with an increase in histone H3 and H4 level of acetylation (Law & Suttle, 2004). To assess the possibility that transcriptional silencing through chromatin remodelling may act during dormancy transition at the full genome level, we investigated changes in the level of acetylation of histone H3 in hybrid aspen apices. Surprisingly, the level of H3 acetylation did not decrease dramatically during SD in these apices. This could depend on an overall balance between genes that are up- and down-regulated in the period under analysis, but may also mean that silencing could be targeted to specific loci rather than being executed globally (Paper I). We investigated this hypothesis by downregulating the gene *FIE* by RNAi technology. The gene *FIE* is a member of the PRC2 complexes, which act in herbaceous plants as transcriptional repressors in various processes targeting specific genes (Makarevich *et al.*, 2006). Interestingly, *FIE* was previously found to be upregulated in SD, in both the cambium and apex (Druart *et al.,* 2007; Ruttink *et al.,* 2007). Thus, it seemed to be a good candidate to confirm both our finding that chromatin remodelling may be involved in activity-dormancy cycle control and our hypothesis that silencing may be targeted to specific loci during dormancy transition. Interestingly, RNAi*FIE* lines of *Populus* were not able to establish endodormancy (Paper III) (Fig. 14). We can thus conclude that chromatin remodelling, and in particular the gene *FIE*, a component of PRC2 complexes is part of the mechanism for regulation of dormancy, and that this control is probably targeted to specific loci as opposed to being a full genome-level event.

Prompted by the interaction we detected between ABA and the gene *ABI3* in bud development, and by the role of *ABI3* in dormancy in seeds, we investigated the possibility that *ABI3* may positively influence dormancy development. Rohde et al., (2002) had previously found that both *ABI3* overexpressing and antisense plants can become dormant. However, this did not completely exclude the possible involvement of *ABI3* in the process since as yet it is not possible to test if a plant is more dormant than another, which may have been the phenotype of the *ABI3*OE lines. Moreover, the antisense lines phenotype was subtle, as also demonstrated by the small proportion of genes differentially expressed compared to wild type plants (Ruttink *et al.,* 2007). Hence, the level of downregulation of *ABI3* may have been insufficient to cause detectable effects on dormancy. Therefore, we generated plants that overexpressed the gene *ABI3* in the non-dormant background of *abi1-1* plants. Unfortunately, these plants were difficult to

cultivate in soil and tended to die towards the end of the SD treatment. Hence, we were only able to collect data from the line that was overexpressing *ABI3* most weakly amongst the lines generated (Fig. 10). However, this line expressed *ABI3* at much higher levels than the physiological *ABI3* levels found in wild type plants. Therefore, as these plants were not able to develop dormancy, we can conclude that, unlike ABA, *ABI3* does not have a conserved role in bud dormancy development like in seeds.

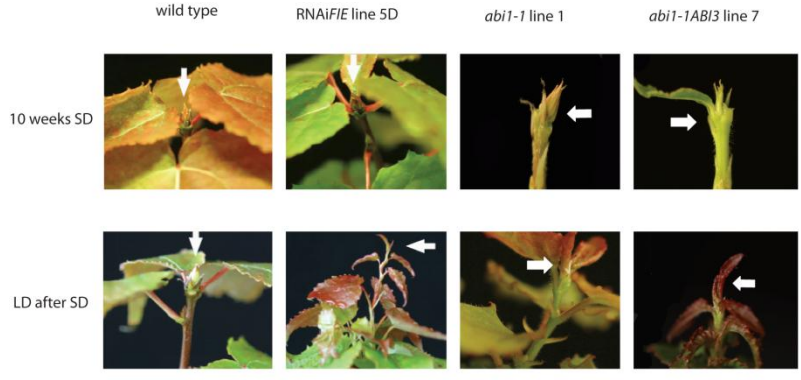

Figure 14: Results of the reactivation test for wild type, RNAi*FIE*, *abi1-1 ABI3* and *abi1-1* plants.

To summarize our findings, dormancy is a complex process, control of which involves multiple processes acting in concert, with a vast array of players interacting via a wide range of mechanisms. Indeed, reliance of dormancy establishment and release on a single component or mechanism would probably be non-viable, since both events must be close to optimally timed for plants to avoid freezing damage to vital organs, but maintain growth while it is safe to do so (and thus maintain competitive advantage). Therefore, possession of complex controls presumably provides plants with the essential ability to respond adequately and safely to their changing environments.

2.7.1 ABA and *FIE* target different processes

Our array analysis of the *abi1-1* plants highlighted the involvement of ABA in the regulation of not only dormancy, but also various other SD-induced processes, such as bud maturation, cold acclimation and metabolic changes (Paper II). We also investigated this possibility for the gene *FIE*. From our

results, it appears that *FIE* is not involved in growth cessation, bud formation, and other adaptive responses, and that its action may be specific to dormancy (Paper III). This conclusion came from an analysis of adaptive response markers during SD treatment in RNAi*FIE* plants, for which we designed primers to amplify several genes belonging to a particular adaptive response class (for example, genes encoding storage proteins or cold acclimation proteins) at the same time. These marker genes were upregulated in wild type plants during SD treatment. When tested on previously described mutants as *PHYA* overexpressing (Olsen *et al.*, 1997b) and *FT* overexpressing plants (Bohlenius *et al.*, 2006), which are not able to respond to SD treatment, these classes of genes were not upregulated during SD treatment (Fig. 15).

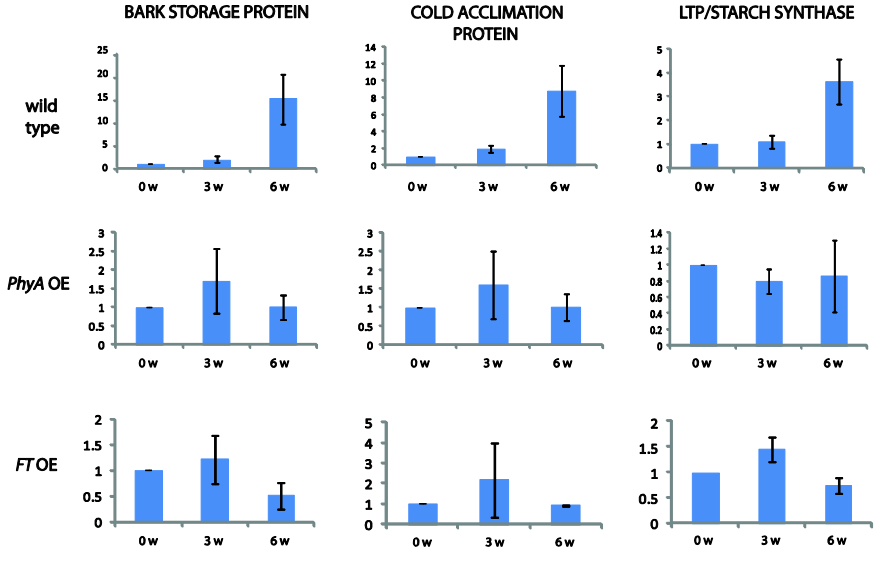

Figure 15: Relative expression levels of three set of marker genes for adaptive responses in wild type, *PHYA* OE and *FT* OE plants. Data are means of independent biological replicates. Error bars represent standard deviation (n=3). X axis, duration of SD treatment; Y axis, relative expression (fold change). Materials and methods for the qRT-PCR analysis can be found in Appendix A.

qRT-PCR analysis of RNAi*FIE* plants showed that, even if the amplitude of gene regulation may not be the same as in wild type plants, the processes for which the genes are representative participants are nevertheless

initiated. Therefore, *FIE* may specifically act in dormancy development, and its effects on adaptive response genes may be minor than those of ABA. However, as our analysis included a small proportion of all adaptive response genes, we cannot completely exclude the possibility that downregulation of *FIE*, and/or of other chromatin remodelling enzymes, may not affect other SD-induced processes other than dormancy.

2.7.2 How do ABA and *FIE* control dormancy?

An interesting question is how ABA and chromatin remodelling, in particular the gene *FIE*, control the transition to dormancy. In an attempt to address this issue, we searched for potential targets of ABA and *FIE*. Array analysis of *abi1-1* plants, as already described, showed that ABA does not control dormancy establishment through the regulation of cell cycle genes and/or cell cycle regulators such as *ANT*, as those genes were not differentially regulated during SD treatment in *abi1-1* plants compared to wild type plants (Paper II). The results also indicated that poplar orthologues of *DOG* and *KEG* (reduction or loss of expression of which results in lack of dormancy in *Arabidopsis* seeds) are not involved in ABA-mediated control of apical dormancy, as they are actually expressed at higher levels in *abi1-1* plants than in wild type during SD treatment. A possible candidate, whose function in hybrid aspen has not yet been elucidated, is the gene *FUS3*, which participates in the control of embryo dormancy in *Arabidopsis* (Raz *et al.*, 2001). *FUS3* was found to be expressed at lower levels in *abi1-1* plants than in wild type (Paper II).

We then tried to identify possible targets of *FIE* in the regulation of dormancy establishment. Phenotypic traits of the *Arabidopsis* PRC2-component mutants *swn* and *mea*, such as dedifferentiation and callus formation, and the fertilization-independent endosperm proliferation in *fie* mutants, indicated that one possibility was that the control of dormancy development though *FIE* is mediated via effects on cell cycle genes. Therefore, we hypothesized that in RNAi*FIE* lines core cell cycle genes and/or their regulators might be sufficiently downregulated to induce growth cessation, but not sufficiently to induce dormancy development, or that they may even be upregulated, after an initial downregulation. However, qRT-PCR analysis showed that genes encoding CYCs and ANT are downregulated in the same manner in wild type plants and RNAi*FIE* plants during SD treatment (Paper III). Therefore, *FIE* targets in the control of the activity-dormancy cycle remain to be discovered.

Based on the above results, we can conclude that the cell cycle machinery is not the target, at least at the transcriptional level, for dormancy

establishment by either ABA or *FIE*. These regulators may act at a downstream level, via mechanisms that could, for example, be key determinants of whether the cell cycle is sensitive or insensitive to specific growth-promoting signals. For example, it would be interesting to investigate the post-transcriptional control of cell cycle genes, since CDKA and CDKB proteins are known to disappear once plants enter endodormancy (Espinosa-Ruiz *et al.,* 2004).

2.7.3 Could there be a correlation between ABA and chromatin remodelling action?

Could it be possible that ABA controls dormancy through chromatin remodelling, or *vice versa*? A hypothesis about a link between the two factors arised from the array analysis of *abi1-1* plants. These plants, which are non-dormant, expressed the gene *PKL* at a higher level than wild type plants during the last phase of SD treatment. This gene has recently been found to antagonistically act on some targets of PcG complexes in *Arabidopsis* (Aichinger *et al.,* 2009). In this case, we do not know what the PKL targets are in *Populus*, but as the genes *PKL* and *FIE* are both normally upregulated to the same extent in the apex (Ruttink *et al.,* 2007), we may speculate that differential expression of one may create imbalance in their antagonistic actions. PKL could act simultaneously as a repressor for some genes and as an activator for some FIE targets. It may therefore need to be upregulated together with *FIE* during SD treatment to counteract its action. This mechanism may be necessary because, in contrast to vernalization, the silenced state is not reset by meiosis during dormancy release, thus there may be a need for the simultaneous presence of two antagonistic factors, one of which prevails on the other during the dormancy-releasing cold treatment. However, cold treatment of wild type plants does not seem to have an effect on transcriptional levels of *PKL*, according to our analysis (Paper I). To test the hypothesis that higher PKL levels in *abi1-1* plants may be responsible for their lack of dormancy, double mutant 35S:*abi1-1* mi*PKL* plants could be generated. In addition, it would be interesting to investigate the effects of *PKL* overexpression and downregulation in *Populus* during SD treatment, and possible effects of chilling treatment on PKL and FIE protein levels. In addition, identifying common targets of PKL and FIE in *Populus* (which are known in *Arabidopsis*) and examining how they are affected during dormancy development could provide further insights.

2.8 CHILLING TREATMENT AND PLANT REACTIVATION

Prolonged chilling treatment is needed to release dormancy. For instance, in *Populus tremula x tremuloides* a period of approximately four weeks at low non-freezing temperatures (4-8 °C) is needed to meet chilling requirements for complete dormancy release (Paper I). Accordingly, our array data show that fewer genes are transcriptionally regulated after two weeks than after four weeks of cold treatment (Paper I). An interesting aspect that remains to be elucidated is the mechanism whereby cold acts on reactivation. One possibility we investigated is that cold may release dormancy by acting on the chromatin remodelling machinery (Papers I and III). However, qRT-PCR results showed that cold treatment does not downregulate the gene *FIE* (Paper III), and only some genes of the chromatin remodelling machinery, which are transcriptionally regulated during SD, are affected by cold treatment (Paper I). Since *FIE* encodes a component of a protein complex that includes several other proteins, cold may act on a partner of *FIE* to release dormancy; possibly *SWN* (which also encodes a component of PRC2 complexes) since our array analysis showed that this gene is downregulated by cold treatment (Paper I). It is also possible that cold may induce another set of genes, which in turn repress positive regulators of dormancy or activate negative regulators of dormancy, thereby leading to dormancy release. In this respect, we found evidence for upregulation during cold treatment of *HDACs* and for a *DNA glycosylase,* which may be attractive candidates for dormancy release (Paper I).

We also investigated direct effects of cold treatment on cell cycle genes. Surprisingly, our results show that cold treatment further downregulates the expression of cell cycle genes that had already been downregulated during SD treatment. As these genes are subsequently upregulated at the time of bud flush, it is possible that cold both releases dormancy and establishes further repression of cell cycle genes (Paper I). This repression may be needed to ensure that plants are not able to reinitiate growth when dormancy has been released but temperatures are still not growth-permissive. In support of this hypothesis, warmth seems to be an important signal for plant reactivation, while daylength plays a minor role in the process since plants that have fulfilled chilling requirements can reactivate even in complete darkness (Worrall & Mergen, 1967). Interestingly, warm temperatures can also directly stimulate cell division in stems (Gričar *et al.*, 2007).

Finally, we investigated the effects of cold treatment on ABA and GA metabolism. ABA biosynthesis seems not to increase during cold treatment, while GA biosynthesis seems to be reactivated during the transition between

cold treatment and plant reactivation. In addition, bud flush appears to be associated with both downregulation of GA catabolic genes and activation of ABA catabolic genes (Paper I). Furthermore, genes involved in the transduction of ABA signals were downregulated during cold treatment. Borowska and Powell (1982) found that while levels of ABA declined in both chilled and unchilled buds, only buds subjected to chilling treatment were able to release dormancy, and ABA application to chilled buds had no effect. Therefore, loss of ABA sensitivity may be the critical event in dormancy release, a hypothesis supported by the lack of dormancy of *abi1-1* plants (Paper II).

In summary, cold restores the sensitivity of plants to growth-promoting signals, possibly by acting on ABA signal transduction, and dormancy release does not seem to rely on cold-induced transcriptional control of either cell cycle genes or chromatin remodelling genes as *FIE*. However, we cannot exclude the possibility that post-transcriptional control of these classes of genes is involved. Figure 16 summarises our findings. Cold seems to be the main dormancy-releasing signal, but it is not sufficient for plants to reactivate and start growing, for which they need another signal or signals. Warm temperatures may be one of the important signals for reinitiating growth.

2.9 THE SEED

2.9.1 *FIE* controls dormancy in both seeds and apical buds

In the light of the physiological similarities between seed and apical dormancy, together with our findings that some molecular players, such as ABA, are conserved between the corresponding processes in the two organs (Paper II), we also investigated the possible role of *FIE* in seed dormancy. For this purpose, a germination test (Raz *et al.,* 2001) was performed with siliques of *Arabidopsis* miRNA lines of medium strength isolated at various stages of maturation; from 6 days after pollination (DAP) to 15 DAP, containing therefore immature to fully mature seeds. Our results indicated a possible role of *FIE* in seed dormancy, since the seeds showed premature germination at 6-8 DAP compared to wild type. As the background of these plants was Columbia-0, an *Arabidopsis* accession that has weak dormancy, we cannot exclude the possibility that the premature seed germination of the miRNA*FIE* seeds was due to accelerated growth after dormancy release rather than impaired dormancy. On the other hand, the Col-0 accession has been routinely used in published dormancy studies, and their premature

germination indicates that one of the processes between embryo or seed dormancy may be impaired in the miRNA*FIE* seeds (Raz *et al.*, 2001).

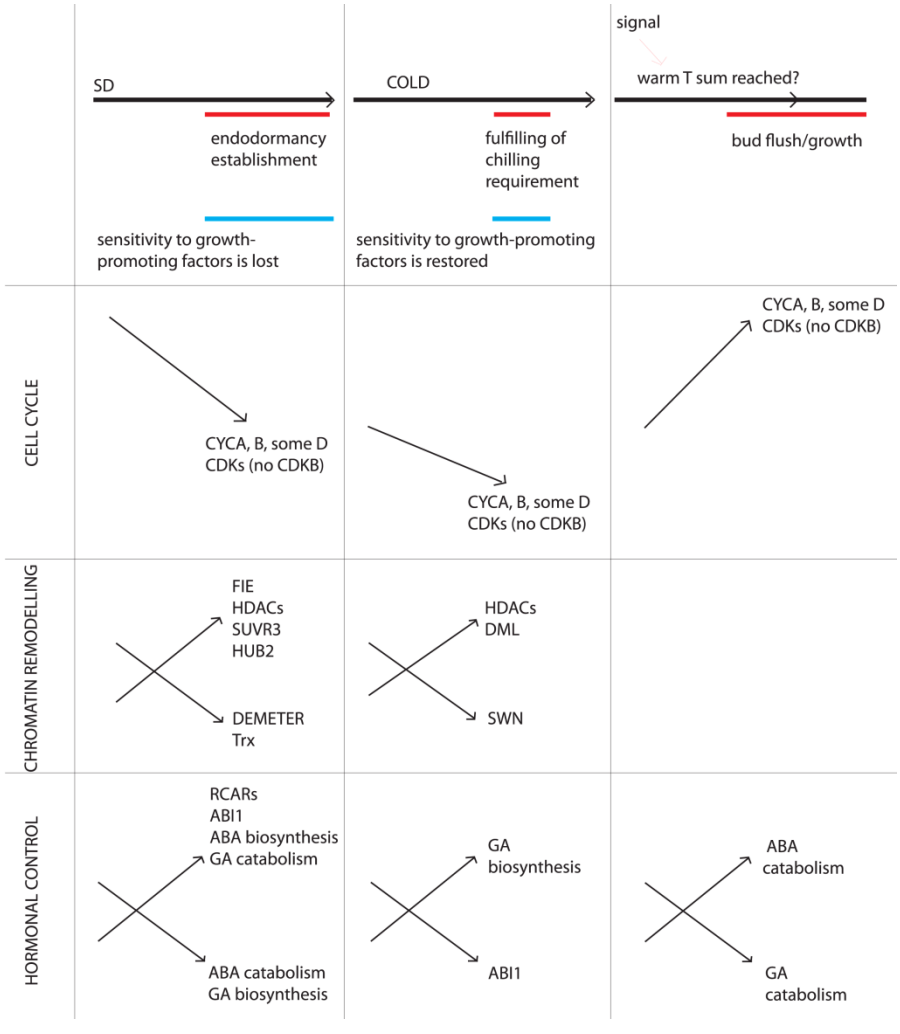

Figure 16: Proposed model for the mechanism of dormancy release in apical buds. For details, see Papers I, II and III.

According to the timeline described by Raz et al., (2001), miRNA*FIE* seeds seemed to germinate before they reach maturity, specifically at the embryo stage of bent cotyledons. Since the premature germination of miRNA*FIE* occurred in a similar timeframe to that of *fus3*, *lec1* and *lec2* mutants, for example, but not *abi3* and *aba1* mutants (Raz *et al.,* 2001), we could speculate that *FIE* may act at a similar level as the former group of genes. *FUS3* and *LEC* genes play a role in embryo dormancy development and their mutation prolongs cell division through the maturation phase of the seed. However, we did not perform a germination test with excised embryos, which could clarify if embryo dormancy is affected in miRNA*FIE* seeds, and as yet we have no data on the mitotic activity of our plants.

2.9.2 Seed and bud dormancy: similarities and differences

Hormonal control

Results of the microarray analysis on the activity-dormancy cycle showed that both ABA and GA metabolic genes, as well as key genes for ABA sensitivity, are under transcriptional control of SD (Papers I, II). In particular, ABA levels and sensitivity appear to increase during SD treatment, while GA levels tend to decrease. In contrast, during dormancy release, GA levels are upregulated after cold treatment and ABA levels decrease due to the activation of catabolism. Therefore, it appears that the balance between those two hormones may be important not only in seed dormancy, but also in bud dormancy.

Our results suggest that ABA biosynthesis may occur *in loco* in the bud (Paper I). However, as we were not able to exclude the possibility that ABA is transported from the leaves to the apex, it may be interesting to investigate whether such transport occurs. In this respect, it should be noted that seed dormancy can be effectively established only by ABA synthesized *in loco* and not by maternal or externally applied ABA. To address this question, one could design an experiment in which leaves are detached once they extend but before they become source leaves, during SD treatment.

Furthermore, ABA sensitivity plays a role in both bud and seed dormancy (Papers I, II). Our analysis of the *abi1-1* plants showed that plants that are insensitive to ABA are not able to reach dormancy (Paper II). SD treatment enhances ABA sensitivity, as genes that are known to encode components of the ABA receptor in *Arabidopsis*, *RCARs* and *ABI1*, are upregulated during SD treatment. These genes are then downregulated by cold treatment (Paper I). On the other hand, no effect of cold on ABA content or sensitivity in seeds has been reported.

Regardless of the mechanisms involved, more experiments are needed to elucidate in further detail the roles and possible interactions between the hormones ABA and GA in apical bud dormancy. A possible experiment, as we now know that ABA hypersensitivity can increase the degree of a plant's dormancy, would be to overexpress GA biosynthetic enzymes and genes involved in GA signal transduction in an ABA-hypersensitive background obtained via constitutive expression of the RCAR protein, and test dormancy development of the resulting plants in SD.

Some of the molecular players are conserved between bud and seed dormancy, but their molecular functions may not be the same

Even if seeds and buds evolved separately, they share many physiological similarities and some of the molecular mechanisms underlying their dormancy are thought to be conserved. Our results showed that other molecular players, such as *ABI1*, *FUS3* and *FIE*, in addition to the previously described *ABI3* (Rohde *et al.,* 2002; Ruttink *et al.,* 2007), are shared between control of dormancy and/or related processes in seeds and buds (Papers II, III). We also demonstrated that there are differences between the role of *ABI3* in buds and seeds; while *ABI3* is involved in both dormancy and maturation in seeds, in buds its function has been redirected to control of embryonic leaves' differentiation and maturation, and it has lost its role in dormancy control (Paper II).

The cell cycle does not seem to be a direct target of *FIE* at the transcriptional level, at least in *Populus*. On the other hand, data on the miRNA*FIE* lines of *Arabidopsis* suggested that it may participate in cell cycle control, as previously discussed (Paper III). The cell cycle is also not a target of changes in ABA sensitivity, either in seeds or apical buds, as cell cycle genes were unaffected in *abi1-1* plants maintained in SD (Paper II), and *abi1-1* lines of *Arabidopsis* do not present premature germination (Raz *et al.,* 2001).

2.9.3 How much can we depend on results from the seed for studying the bud?

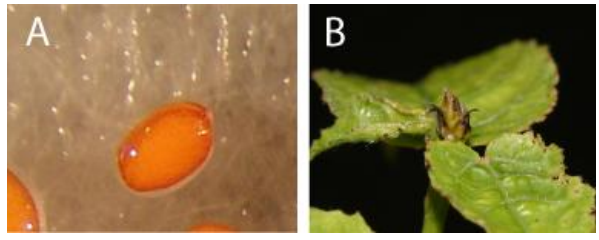

Figure 17: A) *Arabidopsis* seed and B) *Populus tremula x tremuloides* apical bud.

As dormancy of seeds and buds share several physiological characteristics, and seed dormancy has been extensively studied in herbaceous plants such as *Arabidopsis*, it is tempting to think that it should be possible to draw inferences about apical dormancy from these studies (Fig. 17). However, based on our results, we can conclude that even if individual molecular players may be conserved between the processes in the two organs, in some cases (e.g. *ABI3* and perhaps *FIE*) their molecular mechanisms of action and targets may not necessarily be the same. These differences may derive from differences, both anatomical and functional, between the two organs and from their different requirement for dormancy. In fact, even if they are similar, buds and seed are also quite different in several respects. Notably, unlike the bud, the seed has a maternal component and an embryonic component. Moreover, seed dormancy is not acquired in response to SD, and can be lost in responses to diverse signals, while apical bud dormancy in hybrid aspen can be released only by chilling treatment. Thus, it is very likely that the evolution of the seed may have required some genes to specialise and acquire new functions.

For all these reasons, direct extrapolation of information from seed and direct drawing of parallelisms with the bud should be treated with caution. On the other hand, studies on *Arabidopsis* seeds will surely continue to provide molecular markers to investigate in the context of dormancy control in woody plants.

2.10 ANALYSIS OF DORMANCY-NEED FOR STANDARDISATION OF PROTOCOLS

Due to the complexity of activity-dormancy cycle control, it is very important to establish common protocols to facilitate the comparison of results between studies from different laboratories. Even if different species of *Populus* may acquire eco and endodormancy at different times, it is necessary for authors to clearly define the boundaries of these and other processes (timing of growth cessation, timing of bud set etc.). Moreover, it is important to test dormancy release and the involvement of specific transcription factors in dormancy establishment and/or release properly. The only procedure currently available for determining whether or not a plant can reach a dormant state is the reactivation test, which involves subjecting plants to LD conditions after SD treatment, without prior chilling treatment. Recently, a paper has been published in which a role for *CEN1* downregulation in dormancy release is proposed, based on the finding that RNAi*CEN1* lines reactivate more rapidly than *CEN1* overexpressing lines and wild type plants (Mohamed *et al.,* 2010). However, the experiment performed included cold treatment after SD, which itself releases dormancy. Therefore, it is probably not correct to infer that *CEN1* is involved in dormancy release, as it may only affect the speed of reactivation growth once dormancy has already been released. Thus, appropriate experimental design is essential in order to draw robust conclusions from the results. Another important factor in the design of relevant experiments is appropriate choice of mutants to address the posed scientific questions.

3 SUMMARY AND FUTURE PERSPECTIVES

The studies this thesis is based upon provide not only an overview of the transcriptional control underlying the activity-dormancy cycle, but also identify novel key molecular players that regulate dormancy development and SD-related responses. We have shown that the transition to dormancy involves massive transcriptional regulation (Paper I). Interestingly, our results provide evidence for a composite control of the activity-dormancy cycle (Papers I, II and III). In particular, ABA and a component of a chromatin remodelling complex, *FIE*, play key roles in the control of dormancy development and/or SD-induced responses (Papers II, III). Moreover, our results suggest that some regulators of dormancy have been conserved between seeds and apical buds (Papers II and III). Importantly, we also generated new mutants with impaired dormancy and defective adaptive responses and maturation processes, which will provide useful material for addressing challenges associated with elucidating the molecular basis of activity-dormancy cycle controls.

Many questions remain open or unanswered. For example, it would be very interesting to identify the targets of *FIE* regulation. Chromatin immunoprecipitation (ChIP) analysis may be highly informative for identifying these targets during SD treatment, and the generation of lines over- and under-expressing identified targets in *Populus* should provide important information on their roles. A microarray analysis of RNAi*FIE* plants may also help to elucidate its impact on adaptive responses during SD.

In addition, we would like to investigate the effect of downregulation of components of the PRC2 complexes other than FIE, in particular of both MSI1 and MSI2 at the same time, as we found no effect on dormancy establishment downregulating either one, singularly (Paper III).

Another interesting aspect that is still open is the link between SD and associated changes in ABA sensitivity. Although we know SD acts on genes encoding products such as ABI1 and RCAR, we do not know in detail how daylength shortening correlates with the transcriptional regulation of those genes. In order to identify these regulatory upstream components of the pathway, use of light signalling mutants and clock mutants may be necessary. Moreover, it could also be very interesting to use inducible promoters to switch on *abi1-1* expression after plants have reached dormancy, and thus determine if ABA sensitivity is solely involved in dormancy establishment or also in its maintenance.

Importantly, our results indicate a role for both ABA and chromatin remodelling in bud dormancy. Intriguingly, a link between epigenetic mechanism and ABA regulation has been reported in *Arabidopsis* for processes such as seed maturation, seed dormancy and germination, which share similarities with the activity-dormancy cycle of apices of woody plants. In particular, these results indicate that ABA could be acting for example on the accumulation of seed maturation-related proteins through epigenetic processes and that chromatin remodelling may modulate ABA levels and ABA responses (reviewed in Chinnusamy *et al.*, 2008). Therefore, it could be very interesting to continue investigating the nature of the link between these two components of bud dormancy regulation in woody plants.

My personal interest, during the last phase of my PhD studies, has been caught by the parallels between dormancy in seeds and apical buds. I would like to continue the investigation of shared molecular players and physiological similarities between the two organs.

3.1 APPENDIX A — SUPPLEMENTARY MATERIAL AND METHODS

Plant material and growth conditions:

Clonal wild-type hybrid aspen T89 (*Populus tremula x tremuloides*), *PHYA* overexpressing plants (previously described by Olsen *et al.,* 1997b) and *FT* overexpressing plants (described by Bohlenius *et al.,* 2006) were grown in sterile MS medium (Duchefa). *In vitro* cuttings were planted in soil and grown in greenhouse conditions (20 h photoperiod, 60% minimum relative humidity and ca. 22 ˚C) for six weeks, then transferred to a growth chamber. At this point fertilization was interrupted. Plants were kept under LD conditions (16 h day/8 h night, 20-15 ˚C, 80% humidity) for one week to acclimate to the growth chamber, then shifted to SD conditions (8 h day/16 h night, 20-15 °C, 80% humidity) for a total of six weeks.

Sampling for RT-PCR analysis:

Samples consisting of apices and buds were harvested after 0, 3 and 6 weeks of SD treatment, 90 minutes before dark. Leaves and leaf primordia were removed if visible. Three apices were pooled for each genotype/time point and immediately frozen in liquid nitrogen.

RNA extraction and cDNA synthesis:

Total RNA was extracted using an Aurum™ Total RNA mini kit (Bio-Rad) according to the manufacturer's recommendations. 1 μg of RNA was DNAse-treated. Portions (100 ng) of the RNA samples were checked for integrity by gel electrophoresis, and they were subjected to PCR amplification to check for DNA contamination. DNAse treatment was performed using a DNA-free™ kit (Ambion), then RNA integrity and DNA were checked using a Bioanalyser 2100 (Agilent). First-strand cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad).

Quantitative Real-time PCR (qRT-PCR) analysis:

qRT-PCR was applied to analyse the relative expression of three cold markers in SD-treated plants, in triplicate in a 96-well transparent plate with LightCycler® 480 II (Roche) in 20 μl mixtures containing 10 μl 5X SYBRGreen™ I Master (Roche), forward and reverse primers, sterile

milliQ water and 5 µl of cDNA (1/10 diluted). For each experiment, the best reference gene was chosen using GeNorm software as described by Vandesompele *et al.* (2002). Primers used are described in detail in the next section. Additional information about the primers' amplification efficiency and annealing temperature can be found in Table 1, and the methods are described in detail in Appendix 1.

The expression data presented in the figures represent means for biological replicates expressed as fold changes relative to the measured expression at the first time point, which was set at 1. Error bars indicate ±SD (n=3).

Primer details:

Table 1: Description of primers for qRT-PCR analysis.

Primer name	Gene model amplified	Annealing temperature	Amplification efficiency
Bark storage protein	POPTR_0013s07800 POPTR_0013s07810 POPTR_0013s07840	58 ˚C	1.9
Cold acclimation protein	POPTR_0009s11290 POPTR_0004s15610	57 ˚C	1.91
LTP/starch synthase	POPTR_0016s01720 POPTR_0006s01020	57 ˚C	1.97
ubiquitin	(Brunner *et al.*, 2004)	57 ˚C	1.97

Appendix 1:

Four reference genes were tested: estExt_fgenesh4_pg.C_LG_II1155 (Gutierrez *et al.,* 2008), *CYP* (Brunner *et al.,* 2004), estExt_fgenesh4_pm.C_LG_IX0344 (Gutierrez *et al.*, 2008) and *UBIQUITIN* (Brunner *et al.*, 2004).

Ubiquitin was found to be the most stably expressed (Vandesompele *et al.*, 2002) of the reference genes tested and was used to normalize expression levels measured in the experiments using the ratio between $(1+E_R)^{CtA}{}_{ref}/(1+E_T)^{CtA}{}_{tar}$ and $(1+E_R)^{CtB}{}_{ref}/(1+E_T)^{CtB}{}_{tar}$, where A and B are the two samples being compared, while E_R and E_T are the amplification efficiencies for the reference gene and target gene, respectively. The

following qRT-PCR program was applied using the Lightcycler® 480 (Roche): initial denaturation at 95°C for 10 s, followed by 40 cycles of 95 °C for 10 s, 57-58°C for 30 s and 72 °C for 15 s. Melting curves were obtained by increasing the temperature by 0.5°C increments from 70 °C to 95 °C, and Ct values for each sample were acquired using the fit points algorithm; the specificity of the amplification was assessed for each gene by dissociation curve analysis, and a unique peak on the dissociation curve was confirmed for each gene (allowance, 1 °C). For the LTP/starch synthase, as there are small differences in the sequence of the amplicons obtained with the primer pair used, a maximum difference of 1.5 °C was allowed for the melting curve. For each primer pair, amplification efficiency was calculated using four serial 2-fold dilutions of cDNA.

3.2 APPENDIX B-SUPPLEMENTARY MATERIALS AND METHODS

Plant material and growth conditions:

Hybrid aspen clone T89 (*Populus tremula x tremuloides*), and double mutants 35s:Pt *ABI3* At abi_{1-1} plants were grown in sterile MS medium (Duchefa). In vitro cuttings were planted in soil and grown in greenhouse conditions (20h light photoperiod, 60% minimum relative humidity and approximately 22 ˚C) for 6 weeks with weekly fertlisation, then transferred to growth chamber at which point fertilisation was discontinued. Plants were grown under LD conditions (16h photoperiod, 20-15 ˚C, 80% humidity) for one week followed by further growth in SD conditions (8h photoperiod, 20-15 °C, 80% humidity) for 10 weeks. After 10 weeks of SD treatment plants were shifted back to greenhouse conditions and examined for bud burst. 35s:Pt *ABI3* At abi_{1-1} lines were sprayed with water on the leaves daily, from beginning to end of the experiment.

Cloning of *PtABI3* cDNA and transformation of At *abi1-1* and Pt *ABI3* in hybrid aspen

Agrobacterium tumefaciens strain GV3101 containing the 35S:At $abi_{1\text{-}1}$ coding sequence in pBI121 vector (kindly contributed by Dr. Alexander Christmann) (Meyer *et al.*, 1994), was transformed into hybrid aspen as described in (Nilsson *et al.*, 1992). A fragment corresponding to full length coding sequence of *PtABI3* obtained from cDNA from *Populus tremula* seeds was cloned in the plasmid pENTR/D-TOPO (Invitrogen). Using a Gateway LR Clonase Enzyme Mix (Invitrogen) the full length *PtABI3* coding sequence was transferred into the binary vector pPH2GW7 (Karimi *et al.*, 2002). *Agrobacterium tumefaciens* strain GV3101 containing the 35S:Pt *ABI3* construct pPH2GW7 vector was then transformed into 35s:At $abi_{1\text{-}1}$ background.

RNA extraction and cDNA synthesis:

Total RNA was extracted using Aurum™ Total RNA mini kit (Biorad) according to manufacturer's descriptions from in 35s:Pt *ABI3* At $abi_{1\text{-}1}$ in vitro cuttings deprived of the roots. 100 ng of RNA were checked for integrity on a gel. DNAse treatment was performed on 500 ng of total RNA with DNA-free™ kit (Ambion). The RNA quality after DNAse was again checked on gel. First strand cDNA was synthesized using the qscript™ cDNA synthesis kit (Quanta).

qRT-PCR analysis:

qRT-PCR was applied to detect the expression levels of the 35S:Pt *ABI3* construct in in vitro cuttings. qRT-PCR was performed in triplicates in a 96 wells white plate with LightCycler® 480 II (Roche) in 20 µl mixture containing 10 µl LightCycler 480 SYBR Green I Master (Roche), forward and reverse primers, sterile milliq water and 5 µl of cDNA 1/5 diluted. The best reference gene was chosen using the GeNorm software as described in Vandesompele *et al.*, (2002). For the *ABI3* transformant lines selection four different reference genes were tested: 1) estExt_fgenesh4_pg.C_LG_II1155 (Gutierrez *et al.*, 2008), 2) *CYP* (Brunner *et al.*, 2004) and 3) estExt_fgenesh4_pm.C_LG_IX0344 (Gutierrez *et al.*, 2008), 4) *UBIQUITIN* (Brunner *et al.*, 2004).

estExt_fgenesh4_pm.C_LG_IX0344 was found to be the most stably expressed (Vandesompele *et al.*, 2002) among the reference genes tested and was used to normalize the expression in the experiment using the ratio between the formulas: $(1+E_R)^{CtA}{}_{ref}/(1+E_T)^{CtA}{}_{tar}$ and $(1+E_R)^{CtB}{}_{ref}/(1+E_T)^{CtB}{}_{tar}$, being A and B the two samples in comparison, with E_R and E_T being the

amplification efficiency for the reference gene and the target gene respectively. Pt *ABI3* was below the limit of detection in T89 samples, therefore the Ct value was arbitrarily set to 35 for those cDNA. Details for the primers used are given in supplementary table 1.

The following qRT-PCR program was applied on Lightcycler ® 480 (Roche) instruments: initial denaturation at 95°C for 10 sec, followed by forty cycles of 95 °C for 10 sec, 57, 58, 60 or 51.2 °C for 30 sec, 72 °C for 15 sec. Melting curves were performed increasing the temperature with 0.5°C increments from 70 °C to 95 °C. The Ct values for each sample were acquired by using the appropriate software (fit points algorithm); the specificity of the amplification was assessed for each gene by dissociation curve analysis, and a unique peak on the dissociation curve was confirmed for each gene. For each primer pair amplification efficiency was calculated using 4 serial 2-fold dilution of cDNA. The expression data presented in the figures are the means of biological replicates (±SD), n=3.

Supplementary table 1: qRT-PCR primers

gene name	Primer sequence	Annealing T	Concentration in final mix	Amplification efficiency
ABI3	**F** AGCAGAGTGATGTGGGGAG **R** AGTGTTTTCGAGGAGATACATC	58 °C	350 nM	1.91
estExt_fgenesh4_pm.C_LG_IX0344	Gutierrez *et al.*, (2008)	60 °C	300 nM	2

Anatomical characterization

For morphological observation with light microscope, leaves, but not primordia, were removed from apical shoots. Actively growing apices and hybrid aspen buds collected at 6 weeks and 10 weeks of short days treatment were fixed overnight in 2% glutaraldehyde, 2% paraformaldehyde in 0.02M cacodylate buffer (pH 7.2); post fixed (w/v) OsO_4 in water, sequentially dehydrated, gradually infiltrated and embedded in Spurr's resin (TAAB) in polypropylene capsules (TAAB). Transversal sections of 1 µm were obtained with a Microm HM350 microtome (Microm International) using a glass or diamond knife, hot stained with Methylene blue-Azure II and mounted in Entellan neu (Merck) to be investigated with an Axioplan 2 (Zeiss) light

microscope. Additional sections were stained with a saturated solution of phloroglucinol in 18% HCl for 1 minute, and then investigated with an Axioplan 2 (Zeiss) light microscope.

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Acknowledgment

It has been quite a ride. I did enjoy some of it; I did hate some of it. Balance, they say, is a good thing in life. I'm happy I reached this goal, and the crystal clear certainty that I would decline the offer to repeat the experience...
I'm quite bad in writing down on paper how much I appreciate people, especially because I tend to become emotional about it. Therefore, I'll try to be concise (by my standards). I apologize if anyone feels left out; it was not done on purpose.

Thank you to my supervisor Rishi, for giving me the opportunity to pursuit a PhD here at UPSC. I think I did really benefit from being part of the ADONIS network, both as on a personal and scientific level. Thank you for your support during all this time and for designing such an interesting project. I think we found a good way to interact, discuss and work together and I hope to leave your lab leaving a good impression of me both as a person and a student.
To my opponent and members of the committee, for they very kind comments, which I am not sure I fully deserve, on my thesis. I'm very glad you appreciated my work and the way I decided to summarize and present it. It was a real shot of self-confidence to receive your evaluations.

Thank you to my office mates members, past and present, for the nice scientific discussion, but most of all for always being there for support, a joke, scientific and non-scientific help. Irene Pucci, smettila di guardare siti di mutande…per il mio e tuo bene!

Thanks to Poplar-Marie and Britt-Marie, not only for how lovingly they took care of my plants (and there were many and many and many), but also for the friendship, their endless availability and sweet personalities. To all others that make sure UPSC can run as smoothly as possible, and of whom I used the services, thank you.

To Gergo, thank you for being…you. You have no idea how much you helped me to come out of my shell and make display of my, although special, logic. You are a truly funny guy, and I absolutely love the way you speak Italian. Grazie Gennarino, ti voglio bene. Oh Soleeee mioooo…
Krisztina, you are truly a breath of fresh air and one of the most transparent and pleasant people to be around. Honey-bunny, thank you for your friendship.
Rozenn, thank you for everything, tiamotiadorocomelasalsadipomodoro. You are a great person, do not forget that.

Nat, Tiggy, I'm very glad I got to know you during my time here. Thank you for being among the nicest people I know.
To everyone that had a part, small or big, in this almost 5 years I spent in Umea …. I truly appreciate the time spent together.

Grazie Gigio, per esser venuto fino a qui e per essere cosi' come sei, un po' primaverile forse, ma… ☺
Grazie a Mara, per essere rimasta una buona amica da cosi' tanto tempo. Peccato che tu non ce l'abbia fatta a esserci, avremmo potuto sfogare una volta per tutte le nostre arti danzatorie di conquiline padovane!

To S., that is always there to help me when I fall, who makes me laugh and certifies I have a brain. I want to get that in writing.

Alla mia famiglia, che mi ha supportata in tutti questi anni di studio, e soprattutto di lontananza. Grazie per essermi venuti a trovare, nonostante Umea sia in c…aehm, non sia proprio dietro l'angolo… Alla mia mamma, che quando la chiamo al telefono e sente la mia voce si anima d'improvviso e esclama "Ciao stella!". Al mio papa', che intraprende conversazioni skype ad ore tarde e di lunghezza faraonica, e mi tiene aggiornata sui progressi della casa, facendomi sentire parte delle cose che succedono 2000 km piu' giu' (sono felice che tu non abbia ancora affittato il mio letto a qualcuno). A Lucia, che ha intrapreso la mia stessa strada. Sono qui che ti aspetto, facciamo un pezzetto insieme? Ps: grazie di essere venuta fin qui per la difesa.

To Stefano, for everyth…ops, no ☺. Grazie per i sorrisi, per le discussioni, per essere sempre disponibile al dialogo, per la tua pazzia, per come mi rendi ogni giorno una persona migliore, per come spupazzi Matilda, per essere un bambino ordinato, per avere gusti di gelato completamente opposti ai miei, ma adorare il sushi quasi quanto me, per non avermi definitivamente sostituita con il tuo nuovo videogioco, per avere sempre fame, perche'cosi' vicino a te non faccio mai la figura della puccettona, per la tua razionalita' e il modo in cui collide con la mia impulsivita', per esserti congelato ai mercatini con me, per il tuo appoggio e la tua indipendenza, per.., per…per. Grazie di cuore. Sei proprio una persona a posto. Manco ti avessi cercato con la lanternina.