The regulation of reproductive competence in the perennial *Arabis alpina*

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ABSTRACT

At the beginning of development many organisms undergo a juvenile phase during which they are unable to reproduce even in favorable conditions. The length of this phase varies significantly among species, reflecting different strategies for energy allocation and contributing to fitness. In plants, the length of the juvenile phase is more pronounced in perennial species and can last from a few weeks to several years.

The polycarpic perennial *Arabis alpina* L. (*A. alpina*) acc. Pajares was used as a model to investigate the molecular basis regulating the acquisition of reproductive competence. *A. alpina* has a well defined juvenile phase and flowers in response to prolonged exposure to cold (vernalization) only if previously grown for more than 4 weeks in long days (LD). Comparison of the transcriptomic profiles of main apices from plants grown for 2 weeks (juvenile or incompetent) and 8 weeks (adult or competent) in LDs identified several members of the gene family encoding miR156-regulated SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) transcription factors to be higher expressed in adult apices. The miR156-SPL pathway is evolutionarily conserved in land plants and known in *Arabidopsis thaliana* to regulate the floral transition as well as contributing to vegetative development.

The results presented in this thesis suggest that miR156 acts to confer the juvenile phase in apices of *A. alpina* by regulating the age at which the plant becomes sensitive to vernalization. This is supported by expression data that show a strong correlation between high miR156 levels and inability to flower. Moreover, flowering in *MIR156b* overexpressing lines is strongly repressed and plants do not undergo reproduction even when grown for several months in LDs before exposure to vernalization. Interestingly, temperature was found to influence the rate of miR156 expression changes during development, suggesting that this might also influence the timing of the transition to adult development.

miR156 targets a conserved group of *SPLs* in *A. alpina*. Aa *SPL9* was chosen as a candidate gene acting to promote the adult phase of *A. alpina* because it shows complementary expression pattern to miR156 and is upregulated as plants grow older. Consistent with this conclusion, preliminary results show that overexpressing Aa *SPL9* strongly accelerates flowering.

Finally, a physiological characterization of maturation in vegetative axillary shoots growing in the second year supports a flowering model in which single shoots acquire competence to reproduce independently. miR156 accumulation in axillary shoots resembles the pattern observed in the main shoot apex suggesting that similar molecular mechanisms might regulate maturation in different shoots and that in perennial species juvenility contributes also to polycarpy.

ZUSAMMENFASSUNG

Am Anfang ihrer Entwicklung durchleben viele Organismen eine Juvenilitätsphase, während der sie nicht in der Lage sind sich fortzupflanzen, selbst wenn die Bedingungen günstig sind. Die Länge dieser Phase ist je nach Art unterschiedlich und spiegelt unterschiedliche Strategien in der Ressourcen Allokation wider. Bei Pflanzen haben insbesondere ausdauernde Arten eine ausgeprägte die Juvenilitätsphase - sie kann wenige Wochen, aber auch mehrere Jahre dauern.

Um die molekularen Grundlagen der Erlangung der Reproduktionsfähigkeit zu untersuchen, wurde als Model die ausdauernde Art *Arabis alpina* L. (Akzession Pajares) gewählt. *A. alpina* hat eine ausgeprägte Juvenilitätsphase und blüht im Anschluss an eine längere Kälteperiode (Vernalisation) nur, wenn sie zuvor mindestens vier Wochen unter Langtagsbedingungen (LD) kultiviert wurde. Durch Vergleich des Transkriptoms von Hauptspross-Apizes zwei Wochen alter Pflanzen (juvenil, bzw. incompetent) mit dem acht Wochen alter Pflanzen (adult, bzw. competent), wurden mehrere Mitglieder einer Gen-Familie identifiziert, die miR156-regulated SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) Transkriptions Factoren kodieren. In adulten Apizes sind diese stärker exprimiert als in jungen. Der miR156-SPL pathway ist in Landpflanzen konserviert und in *Arabidopsis thaliana* dafür bekannt, sowohl beim Übergang zur Blühphase, als auch bei der vegetativen Entwicklung eine Rolle zu spielen. Die Ergebnisse dieser Arbeit deuten darauf hin, dass die miR156 Aktivität in den Apizes von *A. alpina* das Alter bestimmt in dem die Pflanze für eine Vernalisierung empfänglich wird und somit die Juvenilitätsphase reguliert.

Expressionsanalysen zeigen, dass bei hohem miR156 Leveln die Pflanzen nicht zur Blüte gelangen. Zudem wird auch in überexprimierten *MIR156b* Linien die Blühphase unterdrückt, sodass selbst Pflanzen, die viele Monate bei LD kultiviert und dann vernalisiert wurden, nicht zur Blüte gelangten. Interessanterweise hatte auch die Temperatur einen Einfluss auf die Änderungen der miR156 Expression, sie beeinflusst vermutlich den zeitlichen Verlauf der Entwicklung zum Adult-Stadium.

Das Ziel der miR156 in *A. alpina* ist eine Gruppe konservierter SPL Gene. Als „candidate gene“ wurde Aa SPL9 gewählt, da es bei der Entwicklung zur Adult-Phase im Vergleich zu miR156 ein gegensätzliches Expressionsmuster zeigte und mit zunehmendem Alter der Pflanzen hochreguliert wird. Dies wiederum steht im Einklang mit ersten Ergebnissen, die zeigen, dass die Überexpression von Aa SPL9 den Blühbegin beschleunigt.

Eine physiologische Charakterisierung der Reifung von im zweiten Jahr befindlichen vegetativen Seitensprossen deutet zudem darauf hin, dass die einzelnen Sprosse die Reproduktionsfähigkeit unabhängig voneinander erlangen. Die Akkumulation von miR156 scheint dabei in den Seitensprossen auf die gleiche Weise zu erfolgen wie im Hauptspross-Apex, was darauf hin weist, dass für ihre Reifung die gleichen molekularen Mechanismen verantwortlich sind. Die Juvenilitätsphase scheint somit mit dafür verantwortlich zu sein, dass ausdauernde Arten mehrfach blühen können.

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1. INTRODUCTION

1.1 Acquisition of reproductive competence in the context of life history theory in plants and animals

The development of many organisms includes an initial period of growth during which they can not reproduce even in favourable conditions. This phase is commonly called juvenility, whereas when the organism acquires the ability to reproduce it is considered as adult and has reached maturity. Age at maturity is one of the life history traits contributing to fitness and it is linked to fecundity and survival of an organism (Roff, 2007). Variation in life history traits among species can be extreme but in some cases large variation can also be observed within a single species (Ballinger, 1979; Roff, 1992). Evolutionary life history theory deals with the strategic allocation of an organism's energy for growth, maintenance and reproduction (Hochberg, 2009; Stearns, 1992). It predicts that natural selection maximises fitness by promoting efficient allocation strategies that involved trade offs between different aspects of the organism's growth and development. Such a trade-off between two traits is said to occur when an increase in fitness due to a change in one trait is opposed by a decrease in fitness due to a concomitant change in the second trait (Roff, 2007). For example, when an individual matures, energy that could be used for growth will instead be used for reproduction. Although early maturation confers higher chances of survival to maturation and production of progeny, later maturation would permit further growth that is likely to cause higher fecundity and higher quality of offspring (Roff, 2000; Stearns, 1992). Which of these strategies predominates is likely to depend on the environment in which the organism lives.

The processes of growth and development between plant and animal Kingdoms differ greatly. However, in acquisition of reproductive competence some general similarities between the two Kingdoms can be identified. Firstly, maturation in both animals and plants is regulated by endogenous factors (e.g. hormones) and by environmental cues such as nutrition, predation, light and temperature (Hackett, 1985; Kuzawa, 2007; Riddiford, 2008; Zimmerman et al., 1885). In mammals and insects, transitions between different phases of the life cycle are mainly marked by changes in the types and patterns of sexual hormones that are present (Hochberg, 2009; Riddiford, 2008). Phytohormones also regulate development in plants, but their involvement in reproductive maturation is still poorly understood (Hackett, 1985; Zimmerman et al., 1885). Better characterized in plants is the role of two classes of micro

RNAs (miRNAs) and their respective target mRNAs, in the regulation of developmental changes (Fornara and Coupland, 2009; Poethig, 2009). Remarkably, the role of miRNAs in developmental timing was discovered in the worm *Caenorhabditis elegans* and subsequentially shown to be evolutionarily conserved in other animals (Ambros, 2011; Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000). The miRNAs regulating developmental timing in plants and animals are unrelated but similar mechanisms have evolved to regulate comparable processes in different species, even in different Kingdoms. This suggests convergent evolution occurred, generating miRNA-controlled pathways for the regulation of developmental switches.

Another common feature between animals and plants is the general association of juvenility and adulthood with changes in morphological, physiological and behavioural traits that indirectly contribute to fitness (Roff, 2007). Some species exhibiting such divergent juvenile and adult morphological forms were erroneously classified as two independent species (Le Comte and Webb, 1981; Rietschel, 1975). Especially animals can display extremely different colours and patterning during development. In some cases this variation has been clearly shown to confer advantages to the organism in a particular stage of development and thus to its overall fitness (Mahon and Mahon, 1994). In plants the term heteroblasty is used to describe a form of development in which substantial morphological differences between earlier and later stages are observed (Goebel, 1900; Jones, 1999). In general these changes refer to differences in leaf shape and size and although these might appear subtle, they can confer advantages on the juvenile plant under specific environmental conditions (Taulavuori et al., 2010).

1.2 Acquisition of reproductive competence in plants

The decision of when to undergo flowering ultimately determines the reproductive success of a plant. It is also an adaptive trait that enables to respond to different environmental seasonal changes in order to undergo the floral transition at the most appropriate time of the year (Alonso-Blanco et al., 2005; Izawa, 2007). Environmental cues such as photoperiod and temperature are sensed by plants and used to correctly time flowering (Amasino, 2010; Searle and Coupland, 2004). However, in order to initiate flowering in response to these environmental signals, plants must first acquire a reproductively competent state. At early stages of development, plants grow only vegetatively, postponing flowering even in the presence of favorable environmental conditions (Fig.1). During development, they acquire a

reproductively competent state and then undergo the reproductive transition when exposed to the appropriate day length and temperature (Poethig, 2003). Other morphological changes are temporally correlated with maturation, hinting that they are also associated with the ability of the plant to respond to inductive stimuli (Fig.1). However, whether the morphological changes observed in the leaves during this transition and the acquisition of reproductive competence are regulated by similar mechanisms remains unclear. Studies in the model species *Arabidopsis thaliana* L. (*A. thaliana*) reported that the same molecules control the regulation of leaf development by age and age-dependent flowering (Wang et al., 2009a; Yamaguchi et al., 2009). However, there are also examples in which plants undergo the transition to flowering while still producing juvenile leaves and others in which flowering does not occur even if adult vegetative structures are produced and the plants are exposed to inductive conditions (Brunner and Nilsson, 2004; Poethig, 2003, 2010). Nevertheless, the term vegetative phase change has been used to identify both heteroblasty and acquisition of reproductive competence, since the two processes take place during the vegetative growth that precedes reproductive transition (Poethig, 2009).

The length of the juvenile phase varies greatly among species and it is likely to have an adaptive value (Jones, 1999). Annual species are fast growing and proceed quickly from the juvenile incompetent phase to the adult competent phase in order to flower and complete their life cycle within one growing season. All shoots in an annual plant behave similarly and become reproductive at the same time to maximize seed set. This reproductive strategy is also referred to as monocarpy or semelparity and the species following it devote all the energies and resources to maximizing the number of offspring in one reproductive event before dying (Amasino, 2009; Cole, 1954). In contrast, many perennials are iteroparous and reproduce many times in their life (Amasino, 2009; Cole, 1954). Perennial species have a long life cycle with a prolonged juvenile phase and upon flowering, only some shoots are committed to reproductive growth while others remain vegetative to support subsequent years of flowering, a strategy called polycarpy (Amasino, 2009; Thomas et al., 2000). The regulation of reproductive maturation has been investigated in both annual and perennial species although with different approaches, probably due to their different life strategies. Studies in annuals predominantly focused on the heteroblastic changes that occur in leaves and linked the findings to flowering. Studies conducted in perennials used the presence of flowers as an indication of the reproductively adult state. From a developmental point of view these plants have already undergone reproduction, which means that these studies in perennials focused on downstream events after acquisition of reproductive maturation. Overall only few studies

directly tried to address the regulation of reproductive competence in the context of the ability to respond to flower inductive stimuli. In the next sessions the present knowledge about vegetative phase transition in annual and perennial species will be introduced.

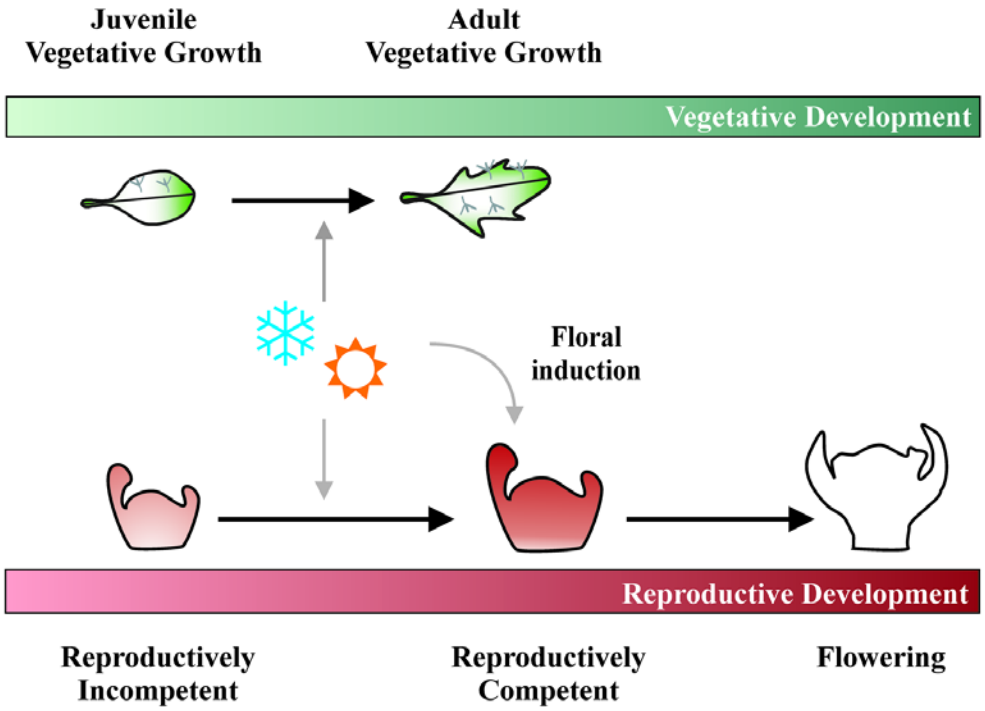

Figure 1. Developmental regulation of leaf morphology and reproductive competence

During development several transitions occur in the morphology of the leaves as well as in the competence of the meristem to undergo reproduction. In *A. thaliana* for example, leaves produced early in development present juvenile traits such as small round shape and absence of trichomes on the abaxial side of the leaf blade. Leaves produced at later stages present adult phenotypes such as a larger size, enhanced margin serration and trichome distribution on both sides of the blade. Changes occurring in the meristem are associated with the acquisition of reproductive competence. In early stages the SAM is unable to undergo the reproductive transition even if exposed to inductive stimuli. Later it acquires competence to flower and to undergo the floral transition if the plant is exposed to inductive photoperiods and/or to vernalization. Both meristem maturation and leaf morphological changes are under endogenous as well as environmental control. Sun and snowflake represent the environmental control that can influence the acquisition of maturation and the appearance of adult leaf traits. These symbols can also represent photoperiod and vernalization that can induce flowering. Black arrows represent developmental transitions. Grey arrows indicate environmental responses.

1.3 Studies in annual species

1.3.1 Environmental and endogenous pathways regulate flowering time in *A. thaliana*

The regulation of flowering time has been extensively investigated in the annual model species *A. thaliana*. Several pathways that regulate the decision to shift from vegetative growth to reproductive development have been characterized. Some of them regulate flowering in response to environmental conditions whereas others are considered plant endogenous factors. Flowering is controlled by the environment in response to changes in day length through the photoperiod pathway, in response to prolonged exposure to chilling temperature by the vernalization pathway and in response to changes in ambient growth temperature by the thermosensory pathway (Imaizumi, 2010; Kim et al., 2009; Penfield, 2008; Turck and Coupland, 2011; Turck et al., 2008). The plant hormone Gibberellin (GA-pathway), epigenetic regulation (autonomous pathway) and age regulate flowering endogenously (Bergonzi and Albani, 2011; Farrona et al., 2008; Fornara and Coupland, 2009; Mutasa-Gottgens and Hedden, 2009). All these different pathways converge at the shoot apical meristem (SAM) to regulate transcription of floral integrator genes that mark the beginning of the floral transition. The role of these genes is to integrate the environmental and developmental state of the plant and in turn to regulate floral meristem identity genes that directly specify floral meristem differentiation (Albani and Coupland, 2010).

A. thaliana is a facultative long day (LD) plant that flowers earlier when exposed to light for more than 16 hours each day than when exposed to 10 hours light. Inductive conditions are therefore similar to spring or early summer. At the molecular level, photoperiod promotes the transcription in the leaves of the floral promoter *FLOWERING LOCUS T* (*FT*), a member of the *CETS* family, that encodes proteins related to phosphatidylethanolamine binding proteins (Pnueli et al., 2001). *FT* is activated under long days because of both transcriptional and post-transcriptional regulation of the zinc finger protein CONSTANS (CO) by day length (Jang et al., 2008; Kardailsky et al., 1999; Kobayashi et al., 1999; Putterill et al., 1995). CO accumulates in LDs and activates *FT* transcription. FT protein moves through the phloem from the leaves to the SAM where it interacts with the bZIP transcription factor FLOWERING LOCUS D (FD) to activate transcription of the MADS-box transcription factors *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FRUITFUL*

(FUL) (Abe et al., 2005; Borner et al., 2000; Corbesier et al., 2007; Jaeger and Wigge, 2007; Lee et al., 2000; Lin et al., 2007; Mathieu et al., 2007; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005). Transcription of *SOC1* and *FUL* in the meristem is closely associated with flowering.

Winter accessions of *A. thaliana* require vernalization to flower in spring. In these accessions flowering is repressed before vernalization by the MADS-box transcription factor *FLOWERING LOCUS C* (*FLC*) that directly represses *FT* and *SOC1* in leaves and apices preventing premature flowering in autumn (Helliwell et al., 2006; Hepworth et al., 2002; Lee et al., 2000; Searle et al., 2006). *FLC* transcription is promoted before vernalization by active alleles of the plant specific nuclear protein FRIGIDA (FRI) (Michaels and Amasino, 1999; Sheldon et al., 1999). Upon vernalization, *FLC*, but not *FRI*, transcription is repressed. Winter temperatures trigger enrichment of tri-methylated histone H3 Lys^{27} (H3K27me3) at the *FLC* chromatin, which results in epigenetic stable repression of *FLC* once plants are returned to warmer temperatures (Bastow et al., 2004; Finnegan and Dennis, 2007; Gendall et al., 2001; Sheldon et al., 2000; Sung and Amasino, 2004). Two non coding RNAs (ncRNA) are involved in *FLC* repression. Early *FLC* silencing is mediated by *COOLAIR* (cold induced long antisense intragenic RNA), a cold-induced *FLC* antisense transcript expressed early during vernalization (Swiezewski et al., 2009). The transient expression of *COOLAIR* is followed by the expression of a long intronic sense ncRNA *COLDAIR* (cold assisted intronic noncoding RNA) which is required for the epigenetic repression by physically associating with components of the polycomb repressive complex 2 (PRC2) (Heo and Sung, 2011). The transcriptional repression of *FLC* during vernalization is one of the best studied epigenetic mechanisms in plants, but other flowering genes are also targets of chromatin regulation (Farrona et al., 2008; Kotake et al., 2003; Turck et al., 2008). Moreover, a recent genome-wide identification of FLC targets by chromatin immune precipitation followed by high-throughput sequencing (ChIP-seq) reported that the range of FLC targets is very broad (Deng et al., 2011). The ChIP-seq results implicate that FLC acts at different levels of reproductive development, from vegetative phase change to floral morphogenesis.

Vernalization is not the only way by which temperature influences flowering. Moderate changes in ambient temperature during plant growth also significantly affect many aspects of plant physiology including the reproductive transition (Penfield, 2008). H2A.Z-containing nucleosomes mediate ambient temperature in plants by influencing the ability of RNA polymerase II to transcribe genes in response to temperature (Kumar and Wigge, 2010). Genetic screens identified mutants whose flowering time is not affected by changes in

ambient temperature. Genes belonging to the autonomous pathway such as *FCA* and *FVE* and the floral repressors *SHORT VEGETATIVE PHASE* (*SVP*) and *FLOWERING LOCUS M* (*FLM*) encoding for MADS-box transcription factors are required for the acceleration of flowering observed in *A. thaliana* plants growing at higher temperatures (Balasubramanian et al., 2006; Blazquez et al., 2003; Lee et al., 2007). Six classes of miRNAs were also identified to be differently expressed at different temperatures (Lee et al., 2010). Among them, the floral promoter miRNA 172 (miR172) was shown to mediate SVP regulation of *FT* mRNA, which represents the integrator genes of the thermosensory pathway (Balasubramanian et al., 2006; Blazquez et al., 2003; Lee et al., 2010; Lee et al., 2007).

The GA-pathway also promotes flowering. Exogenous application of GAs leads to an increase in transcription of *SOC1* by a yet unknown mechanism (Lee and Lee, 2010; Moon et al., 2003b). In *A. thaliana*, gibberellins are considered to play a major role in flowering under short day (SD) since under these non inductive conditions, bioactive GA_4 accumulates in the meristem prior to the transition to flowering (Eriksson et al., 2006; Wilson et al., 1992).

Environmental stimuli regulating flowering can be perceived at the leaves but the reproductive transition occurs at the meristem. SOC1 represents one of the earliest floral promoters that integrates in the SAM inputs from photoperiod, autonomous, vernalization, and GA pathways (Lee and Lee, 2010). SOC1 functions with two other MADS-box transcription factors expressed in the meristem at an early stage of floral induction: FUL and AGAMOUS LIKE 24 (AGL24). Mutations in these genes also delay flowering (Gu et al., 1998; Yu et al., 2002). The double mutant *ful soc1* flowers significantly later than the two single mutants, suggesting that the two genes act redundantly (Melzer et al., 2008). Interestingly, in the *ful soc1* double mutant the floral transition is unstable and after flowering the meristems revert to vegetative growth, strongly resembling reversion of inflorescence meristem observed in other species (Melzer et al., 2008; Tooke et al., 2005). Thus, SOC1 and FUL play a role also in maintenance of inflorescence development. AGL24 and SOC1 are involved in a positive feedback loop to increase each other´s transcription during floral induction. The two proteins interact and are co-localized in the nucleus (Liu et al., 2008; Michaels et al., 2005). SOC1, FUL and AGL24 act together in the SAM to integrate floral inducing signals and to promote the transcription of floral identity genes such as *LEAFY* (*LFY)*, which encodes a plant specific transcription factor, and *APETALA 1* (*AP1)*, which encodes a MADS-box transcription factor (Weigel et al., 1992). Transcription of *LFY* or *AP1* in the floral primordia marks the end of flowering time and beginning of flower development (Hempel et al., 1997). Mutations in both genes strongly repress floral identity partially

converting flowers into shoots (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Moreover, LFY and AP1 enhance each other's expression, making the floral transition irreversible (Bowman et al., 1993).

Flowering is also regulated in the meristem by floral repressors. One of them, *TERMINAL FLOWER 1* (*TFL1*), encodes a member of the CETS family like FT but with opposite effects on flowering. Mutations in *tfl1* accelerate flowering whereas *TFL1* overexpression delays flowering (Bradley et al., 1997; Ratcliffe et al., 1998). Based on gene sequence comparisons and interaction studies, it has been proposed that TFL1 and FT compete in the meristem for binding to FD to repress or promote flowering (Abe et al., 2005; Ahn et al., 2006; Hanzawa et al., 2005; Wigge et al., 2005). Moreover, TFL1 also plays a role in the control of inflorescence development preventing *AP1* and *LFY* mRNA expression in the centre of the inflorescence meristem (Bradley et al., 1997; Ratcliffe et al., 1999). Additional roles for *TFL1* have been proposed in perennial species and will be discussed in the next sections.

1.3.2 The age pathway in *A. thaliana*

Environmental signals accelerate flowering in *A. thaliana*. Nevertheless, photoperiodic shifts showed that LDs can induce early flowering only when plants were previously grown for at least 4 days in SDs (Mozley and Thomas, 1995). This indicates that the SAM must first acquire reproductive competence to undergo the transition to flowering. In annual plants only a few studies directly addressed how reproductive competence is gained and regulated. Studies conducted in *A. thaliana* mainly focused on the juvenile to adult transition for leaf morphology and then related the discoveries to changes in flowering time (Wang et al., 2009a; Yamaguchi et al., 2009).

1.3.2.1 Regulation of heteroblastic changes

The shoot of *A. thaliana* produces different types of leaves during vegetative development (Telfer et al., 1997; Telfer and Poethig, 1994; Usami et al., 2009). Leaves produced at an early stage are small, round, have long petioles and present trichomes only on the adaxial (upper) side of the blade. These leaves are considered juvenile. Adult leaves are produced later in development and are bigger in size, consist of more cells, have elongated blades with serrated margins and develop trichomes on both adaxial and abaxial sides. While progressing

from the juvenile to the adult several transition leaves are produced, suggesting a gradual dose-dependent regulation of these traits (Willmann and Poethig, 2011).

Gibberellins influence the appearance of age-related leaf traits in both Arabidopsis and Maize. Mutants with reduced levels of bioactive GAs or GA–insensitive mutants delay the appearance of abaxial trichomes whereas application of exogenous GAs accelerates the formation of adult leaf traits (Chien and Sussex, 1996; Evans and Poethig, 1995; Telfer et al., 1997). Thus GA promotes the shift to adult leaf development.

Environmental growing conditions can also influence the expression of adult traits. For example, *A. thaliana* plants grown in SD conditions develop leaves with abaxial trichomes at later nodes than plants grown in continuous light (Chien and Sussex, 1996; Telfer et al., 1997). Also several late flowering mutants that impair the environmental response delay abaxial trichome formation (Telfer et al., 1997). Recently abaxial trichome formation was also associated with the floral repressor *FLC (Mentzer et al., 2010)*. Modulation of *FLC* mRNA levels, through mutations in its regulators or exposure to vernalization, can delay or accelerate the timing of adult leaf development as it does with flowering time.

The presence of abaxial trichomes is the most frequently used marker in *A. thaliana* to screen for regulators of vegetative phase change. Interestingly, these screens gave also insights on the regulation of the small RNA machinery since they resulted in the identification of a number of genes involved in the biogenesis or activity of miRNAs. A large class of genes such as *SERRATE*, *SUPPRESSOR OF GENE SILENCING3* and *SUPPRESSOR OF GENE SILENCING 2* / *SILENCING DEFECTIVE 1* / *RNA- DEPENDENT POLYMERASE 6* regulate microRNA (miRNA) and small interfering RNA (siRNA) biogenesis (Clarke et al., 1999; Peragine et al., 2004). Moreover, *ARGONAUTE1*, *ZIPPY*, *SQUINT* and *HASTY* are responsible for miRNA activity by promoting miRNA target cleavage or repress translation of target mRNAs (Berardini et al., 2001; Bohmert et al., 1998; Hunter et al., 2006; Park et al., 2005; Peragine et al., 2004; Smith et al., 2009; Telfer and Poethig, 1998; Yang et al., 2006). Mutants in these genes have reduced accumulation of several miRNAs resulting in highly pleiotropic developmental phenotypes (Lobbes et al., 2006; Park et al., 2005; Smith et al., 2009; Wu and Poethig, 2006). Furthermore, one of these miRNAs, miR156, plays a key role in regulating several leaf characters and related phenotypes such as plastochron length. Overexpression of *MIR156* delays the appearance of abaxial trichomes whereas the overexpressor of the miR156 target mimicry, that reduces the activity of mature miR156, produces only adult leaves (Franco-Zorrilla et al., 2007; Wu et al., 2009; Wu and Poethig, 2006). In *A. thaliana* 8 *MIR156* genes, and 4 from the closely related *MIR157*, encode the

mature form of miR156/157 (Reinhart et al., 2002). The precise function of each of these loci is not characterized but they seem to have some expression specificity (Yang et al., 2011).

miR156, targets a family of genes encoding plant specific transcription factors named SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPLs). In *A. thaliana* there are 17 members of the family of which 11 are targets of miR156 (Gandikota et al., 2007; Rhoades et al., 2002; Schwab et al., 2005; Wang et al., 2008; Wu and Poethig, 2006). miR156 and *SPLs* are expressed in several organs including leaves and apices (Fig.2). The levels of miR156 are high at the beginning of vegetative development and decrease with time whereas the levels of the miR156-regulated SPLs are initially low and increase as the plant ages (Cardon et al., 1997; Schmid et al., 2003; Schwab et al., 2005; Wang et al., 2009a; Wu and Poethig, 2006). Single mutations in members of the *SPL* family do not cause phenotypes, suggesting a high degree of redundancy among the family members (Schwarz et al., 2008). Combinations of different mutants or overexpression of different *SPLs* show overlapping but also specialized functions in the regulation of leaf traits (Fig.2A). For example, overexpression of *SPL3* accelerates the production of trichomes on the abaxial side of the leaf and increases cell number. Overexpression of *SPL9* increases leaf size and reduces the rate of leaf initiation. These results indicate that *SPL3* is involved in the regulation of abaxial trichome formation and number of the cells in the leaf whereas *SPL9* regulates leaf shape and plastochron (Schwarz et al., 2008; Usami et al., 2009; Wang et al., 2008; Wu and Poethig, 2006). Although *SPL15* is considered to be the paralog of *SPL9*, mutations in the miR156 cleavage site of *SPL15* caused a different phenotype than the overexpressor of *SPL9*, such as production of leaves with an increased number of small cells at an earlier stage (Usami et al., 2009). To date, the downstream mechanisms by which SPLs control leaf morphology are unknown.

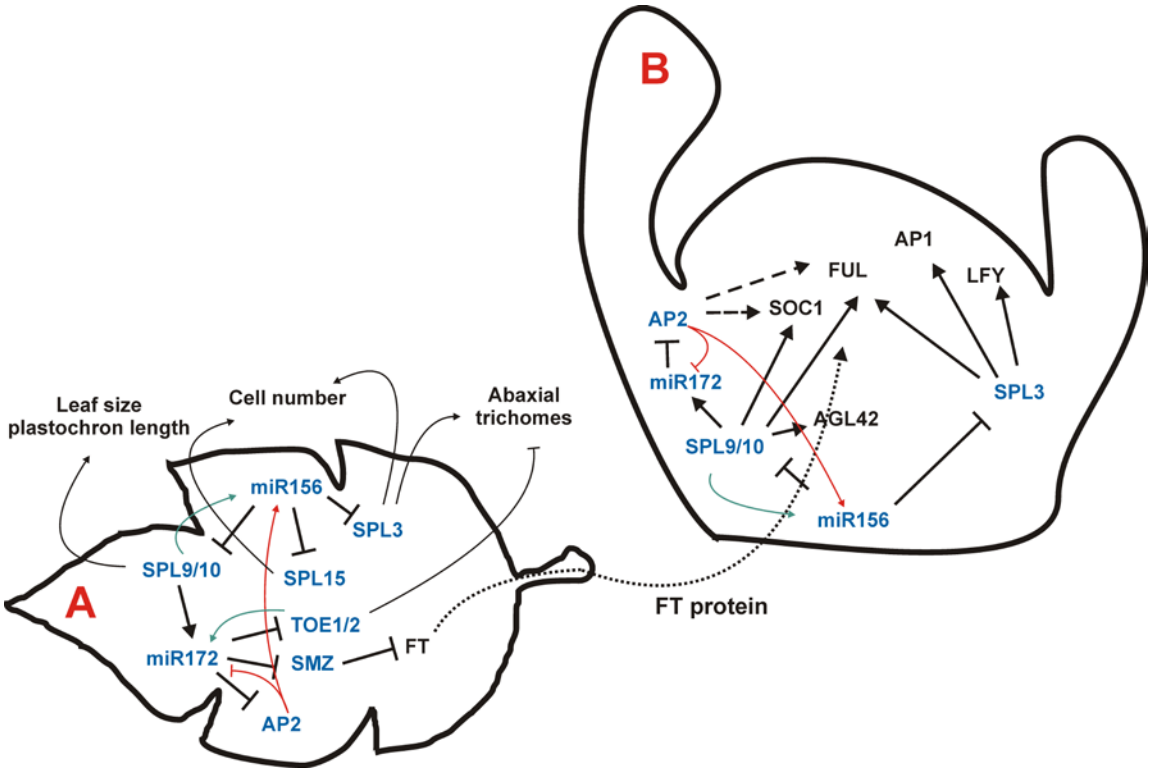

Figure 2. Molecular regulation of juvenile to adult transition in leaves and apices of *A. thaliana*

Juvenile to adult transition in *A. thaliana* is regulated by the complementary expression of two classes of miRNAs, miR156 and miR172. miR156 expression decreases in both leaves (A) and apices (B) during development allowing the upregulation of the SPL class of transcription factors and of miR172. miR156 represses mRNA accumulation in early stages of development of 11 of the 17 members of the *SPL* gene family. The transcription factors encoded by these genes promote adult leaf development and flowering. For example in the leaves SPL3 regulates abaxial trichome formation and together with SPL15 cell number, whereas SPL9 regulates plastochron length and leaf size (A). In the SAM these transcription factors promote flowering by directly binding to flower integrator and floral meristem identity genes. In particular SPL9 binds to *SOC1*, *FUL* and *AGL42* whereas SPL3 binds to *FUL*, *AP1* and *LFY* (B). The complementary increase in expression of miR172 to miR156 decrease is mediated by SPL9/10 that directly bind to *MIR172b*. miR172 represses the accumulation of mRNA encoding the AP2-like family of transcription factors that repress flowering and expression of adult leaf traits. For example, TOE1/2 in the leaves delay abaxial trichome production (A). In the SAM, AP2 is well characterized for its role in floral organ development and it regulates flowering time perhaps by binding to the floral integrator genes *SOC1* and *FUL* (B). Additionally in the leaves SMZ regulates *FT* expression (A). The FT protein travels through the vasculature to induce flowering in the apex (B). Dashed lines represent ChIP-seq identified targets; solid black lines represent tested interactions or regulations. Red lines represent positive feedback loops whereas green arrows show negative feedback loops. The dotted line represents the movement of FT protein from leaves to the SAM. The regulators of the juvenile to adult phase transition are shown in blue.

1.3.2.2 Relationship of factors that control changes in leaf morphology with flowering and reproductive competence

In the SAM, SPL transcription factors promote flowering. The family was initially identified in *Antirrhinum majus* (*A. majus*) where two members were found to bind to the promoter of

SQUAMOSA, the *A. majus* ortholog of *AP1 (Klein et al., 1996)*. Despite this early discovery, their contribution to flowering was difficult to determine due to the lack of mutations in the genes. It is now becoming clear that *SPLs* act as integrators of several flowering pathways including age, photoperiod and vernalization (Deng et al., 2011; Wang et al., 2009a; Wu et al., 2009). The mRNAs of several *SPLs* are upregulated upon floral induction and their overexpression can accelerate flowering (Cardon et al., 1999; Cardon et al., 1997; Schmid et al., 2003; Schwarz et al., 2008; Wu and Poethig, 2006). Together with the idea that *SPLs* are targets of miR156, plants overexpressing precursors of miR156 flower later whereas the overexpression of the miR156 target mimicry accelerates flowering (Franco-Zorrilla et al., 2007; Schwab et al., 2005; Wu and Poethig, 2006). Interestingly, *MIR156* overexpressor lines are extremely late flowering in SDs whereas in LDs flowering is only mildly delayed compared to wild-type, suggesting that the effect of miR156 can be overcome by long photoperiod (Schwab et al., 2005). When tested for competence to flower *35S:MIR156b* plants showed less responsiveness to inductive photoperiod than wild-type control plants, indicating that miR156 might delay flowering by regulating the reproductive maturity of plants (Schwarz et al., 2008). Among the miR156 targets, *SPL9* is one of the earliest expressed genes, already detectable in the vegetative shoot apex of SD-growing plants suggesting a possible role in the regulation of reproductive competence (Cardon et al., 1999; Schmid et al., 2003). However, *spl9* mutant does not have a clear flowering phenotype (Schwarz et al., 2008). *SPL15* is the paralog gene to *SPL9* and play redundant role with *SPL9*. *spl9 spl15* double mutants have reduced response to photoperiodic shifts similar to miR156 overexpressor plants (Schwarz et al., 2008). These results indicate that miR156 through *SPL9/SPL15* and possibly other *SPLs*, regulates competence to flower in *A. thaliana*.

Members of the *SPL* gene family were chosen as representatives of different phylogenetic clades and studied in more detail. Specific target genes of different SPLs have been identified in the SAM. These studies showed that SPLs affect flowering by regulating the expression of floral integrator genes and floral identity genes (Fig.2B) (Wang et al., 2009a; Yamaguchi et al., 2009). SPL9 binds to the promoters of *SOC1, FUL* and the closely related gene *AGAMOUS LIKE 42* (*AGL42*) whereas SPL3 plays a role in the regulation of *FUL, LFY* and *AP1* (Wang et al., 2009a; Yamaguchi et al., 2009). Differences in the timing and pattern of expression of *SPL9* and *SPL3* are reflected in the different genes they regulate (Wang et al., 2009a). *SPL9* is expressed during development already in the vegetative apex in leaf primordia and it is quickly and strongly upregulates in the meristem flanks upon induction. It targets *FUL* and *SOC1*, which are among the earliest genes expressed in the SAM when

plants are shifted to LDs. In contrast *SPL3* is not expressed in SDs and a first *in situ* hybridization signal is visible after 5 days in LDs throughout the shoot apex. SPL3 regulates the meristem identity genes *AP1* which is visible in the floral buds.

miR156 is linked to another miRNA, miR172. In *A. thaliana*, miR172 increases during development in an opposite way to the decrease of miR156 (Aukerman and Sakai, 2003; Jung et al., 2007; Wu et al., 2009). SPL9 and SPL10 bind to the promoter of a *MIR172* locus providing a direct link between the two miRNAs in the regulation of phase transition (Wu et al., 2009) (Fig.2A and 2B). miR172 promotes flowering by targeting the euAP2 lineage of the floral repressors *APETALA 2-like* family (*AP2-like*) of transcription factors (Aukerman and Sakai, 2003; Chen, 2004; Kim et al., 2006; Schmid et al., 2003; Schwab et al., 2005; Yant et al., 2010). Additionally to AP2 itself, this family comprise five members named *TARGET OF EAT 1, 2* and *3* (*TOE1, TOE2, TOE3*), *SCHLAFMUTZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*). Similarly to the *SPL* transcription factor encoding genes, the *AP2-like* genes are expressed in several tissues, show redundant roles as well as pleiotropic effects. AP2 plays a well characterized role in flower development and together with the other five AP2-like members represses flowering (Bowman et al., 1989; Yant et al., 2010). SMZ represses flowering time by directly binding to *FT* whereas AP2 appears to regulate among others the floral integrative genes *SOC1* and *FUL* (Mathieu et al., 2009; Yant et al., 2010).
miR172 and *AP2*-like genes also regulate leaf traits. Overexpression of miR172 accelerates the appearance of leaves with abaxial trichomes, which is similar to the *toe1 toe2* double mutant and to the *AP2*-like hexuple mutant phenotypes (Wu et al., 2009; Yant et al., 2010).

The regulation of miR156, miR172 and their targets is also linked by a series of positive and negative feedback loops. Feedback loops usually stabilize developmental transitions either by buffering small changes in expression (negative) or by amplifying a certain effect (positive) (Martinez et al., 2008). Negative feedback loops are observed between SPL9/10 and miR156a but also between TOE1/TOE2 and miR172b (Wu et al., 2009) (Fig.2, green arrows). Positive feedback loops are observed in the negative binding of AP2 to miR172b and in the positive binding of AP2 to miR156e, both supporting the final upregulation of AP2 (Schwab et al., 2005; Yant et al., 2010) (Fig.2, red lines). The importance of these tight regulatory mechanisms during development is supported by other systems in which miRNAs and their targets are under similar control mechanisms (Ambros, 2011; Herranz and Cohen, 2010; Kim et al., 2007).

1.3.2.3 Regulation of miR156

Further understanding of the age pathway requires a description of the mechanisms by which the developmental decrease of miR156 levels is controlled. Recently, several publications suggested how the regulation of miR156 in the shoot apex may be controlled, but the general picture is still unclear. Ambient temperature, for example, has an effect on miR156 expression but the increase in miR156 accumulation is not mediated by genes known to act in the ambient temperature pathway (Lee et al., 2010).

Long photoperiod promotes transcription of *SPL* genes but does not influence miR156 accumulation, indicating that in long days the effect of miR156 can be bypassed to accelerate flowering (Lal et al., 2011; Schmid et al., 2003; Wang et al., 2009a). Recently the photoperiodic regulation of several *SPL* genes was shown to involve the BELL1-like homeodomain transcription factors PENNYWISE (PNY) and POUND-FOOLISH (PNF) (Lal et al., 2011). *PNY* and *PNF* mRNAs are expressed in vegetative and reproductive shoot meristems to specify meristem identity and mediate meristem maintenance (Kanrar et al., 2008; Smith et al., 2004; Ung et al., 2011). The double mutant *pny pnf* completely fails to initiate flowers (Rutjens et al., 2009; Smith et al., 2004). Interestingly, the decrease of miR156 during development is blocked in *pny pnf (Lal et al., 2011)*. However, it needs still to be tested if this regulation is direct or indirect.

Finally, the hormone GA does not affect miR156 accumulation although its effects on flowering are similar. As for the age pathway, gibberellins in the SAM are considered an endogenous pathway that promotes flowering in the absence of inductive stimuli. Application of exogenous gibberellins can bypass all environmental pathways for flower induction and can activate the expression of major flowering regulators in the shoot apical meristem (Moon et al., 2003a). Despite these suggestive similarities, the application of exogenous GAs, as well as other plant hormones, did not influence miR156 accumulation (Schwarz et al., 2008; Wang et al., 2009a).

1.4 Evolutionary conservation of the age pathway

The model discussed so far in Arabidopsis involving miR156, miR172 and their respective *SPL* and *AP2-like* target gene families is well conserved in other plant species (Poethig, 2009). The *SPL* gene family has been identified in all the main green plant lineages (Guo et al., 2008; Riese et al., 2007; Yang et al., 2008). Moreover, the miR156 target site in the *SPL* mRNAs is

conserved in land plants, from mosses to lycophytes and angiosperms, suggesting that the regulatory module miR156-SPL is evolutionarily old and that it plays important roles in development (Guo et al., 2008). The involvement of miR156 in vegetative development and flowering is demonstrated in maize and rice (Chuck et al., 2007; Poethig, 2009; Xie et al., 2006). In maize, already at the beginning of the 1950s a dominant mutation with a striking effect on vegetative and reproductive morphology was isolated. This mutant was named *Corngrass* (*Cg*) as it was thought that it may represent the grass-like ancestors of maize (Singleton, 1951). Similar phenotypes were observed for the dominant gain of function mutations *teopod1 (tp1)* and *teopod2 (tp2)* (Poethig, 1988). Recently, all these mutations were shown to cause overexpression of *MIR156* genes in maize (Chuck et al., 2007; Poethig, 2009). In rice, overexpression of miR156 causes a phenotype that strongly resembles *Cg, Tp1* or *Tp2* mutations (Xie et al., 2006).

miR156 and *SPL* genes have been identified also in *Physcomitrella patens* (*P. patens*) (Arazi et al., 2005; Riese et al., 2007). However, a different moss specific miRNA, Pp-miR534a, regulates the juvenile to adult gametophyte transition (Saleh et al., 2011). Pp-miR534 controls the timing of development by spatially and temporally regulating the expression of *BLADE-ON-PETIOLE 1* and *2* (Pp *BOP1* and Pp *BOP2*) encoding for transcriptional coactivator in protonema cells. Additionally, the downregulation of miR534 during development was shown to be controlled by cytokinin, the phytohormone that regulates the phase transition in mosses (Decker et al., 2006; Saleh et al., 2011).

MIR172 genes are also identified in maize and rice but seem to be missing in mosses (Arazi et al., 2005; Aukerman and Sakai, 2003; Chuck et al., 2007; Tanaka et al., 2011). As observed in Arabidopsis, miR172 shows complementary temporal pattern of expression with miR156 (Aukerman and Sakai, 2003; Chuck et al., 2007; Jung et al., 2007; Tanaka et al., 2011; Wu et al., 2009). Moreover, as in Arabidopsis also in maize miR156 regulates miR172 through the SPLs but which members of the family mediate miR172 expression are still unknown (Chuck et al., 2007; Wu et al., 2009). In all these species, overexpressors of *MIR172* show very pleiotropic phenotypes indicating roles in flowering time, floral organ identity and vegetative development (Phipps, 1928; Zhu et al., 2009). This suggests that, as has been shown in more details in Arabidopisis, the different targets of miR172 play very different roles in plant development (Zhu and Helliwell, 2011).

1.5 Studies of phase transition in perennial plants

Perennials live for many years and follow the polycarpic life strategy where they are able to flower consecutively for many years (Albani and Coupland, 2010; Amasino, 2009; Battey and Tooke, 2002; Martin-Trillo and Martinez-Zapater, 2002; Townsend et al., 2006). However, there are also perennials, such as bamboo and agave, that die after flowering following a monocarpic life strategy (Battey and Tooke, 2002). In polycarpic perennials maintenance of vegetative buds after flowering can be achieved either by keeping some buds in a dormant state or by maintaining some shoots vegetative (Thomas et al., 2000).

Different species can follow different flowering strategies but generally they all include in the early stages of development a juvenile phase during which plants are not competent to flower. The length of juvenility is pronounced in perennials, in comparison to annuals, lasting from a few weeks in herbaceous species to many years in trees. Understanding the regulation of flowering and especially of shoot maturation is of major interest in perennials since the length of the juvenile phase is one of the main constraints to breeding programs in these species (Townsend et al., 2006). Therefore, shortening the duration of the juvenile phase could accelerate the genetic improvement of economically important species. Many morphological and physiological studies have been conducted in diverse crops in the past although in more recent years, the focus on model annual plants as genetic systems has tended to reduce these studies, especially at the level of understanding biological mechanisms. Studies in perennial plants are often difficult to interpret because several non model species have been used and different non standard conditions applied. Moreover, the long generation times of many perennials makes genetic approaches difficult. Therefore, it is quite challenging to compare the results obtained in different perennial species and draw general conclusions. Additionally, most of the studies focused on the whole vegetative phase of development, but called it the juvenile phase, without testing the moment in which maturity to respond to environmental conditions was attained.

1.5.1 Physiological studies

In many perennials, environmental conditions or treatments that cause continuous and vigorous growth shortens the chronological age of flowering. The conditions applied vary according to the species but generally light intensity and temperature that accelerate photosynthesis also affect the length of vegetative growth (Hackett, 1985). These results

indicate that plants must reach a certain size before they are able to respond to flower inductive signals. Among the phytohormones known to regulate growth and development, GAs have the most evident effect on phase change. Contrary to *A. thaliana*, GAs do not always promote flowering in perennials. In conifers, exogenous GA applications can accelerate flowering in young trees whereas in many woody angiosperms GA inhibits flowering (Goldschmidt and Samach, 2004; Hackett, 1985; Zimmerman et al., 1885). In some cases, application of exogenous gibberellins can even cause reversion from reproductive to vegetative development, including formation of leaves with juvenile traits (Hackett, 1985; Zimmerman et al., 1885). In addition, measurements of endogenous levels of GAs showed that juvenile shoot apices contain higher levels of the hormone than adult shoot apices (Hackett, 1985).

1.5.2 Molecular studies

Although many attempts to identify environmental and cultural conditions that accelerate flowering have been made, the molecular mechanisms regulating reproductive competence in perennials are still poorly understood. Crosses in several woody species demonstrated that the length of the juvenile phase is inherited genetically (Hackett, 1985). The molecular approaches used so far in perennial studies took advantage of the knowledge obtained in *A. thaliana* to identify candidate genes. Overexpression of downstream genes in the floral pathways can shorten vegetative growth by several years in many woody plants. The overexpression of *A. thaliana* floral identity genes *LFY* and *AP1* in citrus shortened the length of the vegetative phase (Pena et al., 2001). In these transgenic lines flowering was observed after 1-2 years compared to the 6 years in control plants. Interestingly, when *AP1* was constitutively overexpressed in citrus, additional vegetative phenotypes were observed such as accelerated production of adult leaves. This suggests the existence of a relationship between vegetative growth and meristem maturation, as discussed for annual species. Several studies also reported the effect of the expression of the floral promoter *FT* on juvenility. *FT* expression levels increase progressively in higher nodes in poplar (Bohlenius et al., 2006; Hättasch et al., 2009; Hsu et al., 2006). Thus adult leaves express higher levels of *FT* mRNA than juvenile leaves, suggesting a possible role for the capacity to express *FT*, in the acquisition of shoot competence to flower. Moreover, overexpression of *FT* homologues induces premature flowering in many perennials (Bohlenius et al., 2006; Endo et al., 2005; Hsu et al., 2006; Kotoda et al., 2010; Trankner et al., 2010; Zhang et al., 2010).

The floral repressor *TFL1* provided major contributions to the understanding of the regulation of the juvenile phase in perennials. Constitutive expression of several *TFL1* homologues in *A. thaliana* delays flowering similarly to At *TFL1* expression (Jensen et al., 2001; Pillitteri et al., 2004; Ratcliffe et al., 1998). This indicates that a functionally conserved protein is present in evolutionarily distant species. Downregulation of *TFL1* in poplar and apple accelerated flowering by shortening the length of vegetative growth before the first flowering event (Kotoda et al., 2006; Mohamed et al., 2010). Recently the role of *TFL1* in the perennial herbaceous *Arabis alpina* (*A. alpina*) was reported. Transgenic lines with reduced expression of Aa *TFL1* showed a shorter juvenile phase than wild-type plants (Wang et al., 2011b). The juvenile phase of *A. alpina* and its regulation will be the focus of the next sections and of this thesis.

1.5.3 Relationship between juvenility and polycapy

Many perennials follow a polycarpic growth habit in which they undergo several reproductive cycles during their life. In order to sustain such a life strategy they must also grow new vegetative axillary shoots every year. How on the same plant reproduction and vegetative growth are contemporaneously maintained is still largely unknown. A recent study in poplar show that repeated cycles of reproduction and vegetative growth in adult plants are conferred by two FT paralogs that functionally diverged after whole genome duplication (Hsu et al., 2011). Their yearly transient expression patterns evolved so that *FT1* is expressed exclusively in winter contributing to reproductive onset whereas *FT2* is expressed in spring regulating vegetative growth.

In *A. alpina*, yearly fluctuation of *PERPETUAL FLOWERING 1* (*PEP1*) transcription controls seasonal transitions between reproduction and vegetative growth (Wang et al., 2009b). Moreover, PEP1 plays a redundant role with the *A. alpina* Aa TFL1 to regulate flowering in axillary shoot branches (Wang et al., 2011b). More axillary meristems were induced to flower in *A. alpina* plants that had reduced levels of Aa *TFL1* mRNA. Combining the *pep1* mutant with the downregulation of Aa *TFL1* by Double Stranded RNA Interference (dsRNAi) further enhanced the number of flowering branching. This additional role for *TFL1* in the flowering fate of axillary meristems was previously suggested by studies of *TFL1* orthologs in other perennial species. For example, the Perennial Ryegrass Lp *TFL1* was reported to be expressed in axillary shoots (Jensen et al., 2001). In Poplar, reduction of Pop *CEN1*/Pop *CEN2* expression by dsRNAi promoted early flowering only in axillary meristems

but did not affect the vegetative state of the terminal shoot apex that in poplar stays vegetative throughout the life of the individual (Mohamed et al., 2010). As described in the previous section TFL1 also contribute to the length of the juvenile phase in several perennial species. Therefore, the observation that the same gene contributes both to juvenile phase and to the fate of axillary meristems in polycarpic perennials indicates that juvenility might play a more general role in polycarpy.

1.6 *A. alpina* as a model plant to study perennial flowering

A. alpina belongs to the Brassicaceae, a family that includes 44 tribes, 338 genera and approximately 3700 species (Franzke et al., 2011). Included in the family is the annual plant model *A. thaliana* and several important crops species. Therefore, comparative studies between *A. thaliana* and other species are used to address evolutionary questions in plant biology. For example, comparisons with *Arabidopsis lyrata* are used to understand the evolutionary forces driving self incompatibility, with *Arabidopsis halleri* and *Thlaspi caerulescens* to understand tolerance to heavy metal and with *Cardamine hirsuta* to understand the evolution of plant architectures and leaves morphologies (Canales et al., 2010; Hanikenne et al., 2008; Leinonen et al., 2011; Meyer et al., 2009; Milner and Kochian, 2008; Nasrallah et al., 2007; Willi and Maattanen, 2010).

A. alpina is a perennial. Thus, comparative analysis with the annual *A. thaliana* can give insights into the evolutionary forces driving life history traits such as perennialism. *A. alpina* diverged from *A. thaliana* at about the same time as the Brassica lineage which is estimated about 30 mya (Bell et al., 2010; Franzke et al., 2009; Koch et al., 2001; Wikstrom et al., 2001). *A. alpina* is an arctic-alpine herbaceous species distributed throughout the alpine habitats in Europe, Greenland and North America, high mountains of northern and eastern Africa, Anatolia and central Asia (Ansell et al., 2011; Koch et al., 2006). *A. alpina* originated in western Anatolia from where around 2 Mya the different haplogroups started to diverge. Two groups migrated independently to the East African high mountains of Ethiopia via the Arabian Peninsula and southwards (Ansell et al., 2011; Koch et al., 2006). All the European and north populations originated from a third group and served as a source for the northwest African Population (Koch et al., 2006). *A. alpina* grows in alpine and sub-alpine zones with a preference for calcareous screes and moist bedrock (Hegi et al., 1986). In the Alps and Scandinavia it behaves as a pioneer plant colonizer of glacier foreland (Whittaker, 1993).

The relatively small diploid genome (current estimation of the genome size is 371 Mbp, Nördstrom K. unpublished), self fertilizing habit and the possibility for genetic transformation by *Agrobacterium tumefaciens* (*A. tumefaciens*) make *A. alpina* a good model system for molecular studies. In addition, many resources have been recently developed to facilitate the study of this species. These include two BAC libraries, two mutagenized populations, a collection of several *A. alpina* accessions and populations from different European habitats as well as collections of different species belonging to the *Arabis* genus. Recently, the genome of *A. alpina* Pajares has been sequenced and assembled.

The simple traceability of flowering and vegetative development in physiological studies favoured the establishment of *A. alpina* as a model system to study perennial flowering (Wang et al., 2009b). Comparative studies showed that perennialism is correlated with the ability to maintain vegetative development after flowering. *A. thaliana* is an annual monocarpic species that quickly proceeds from germination to reproduction in one growing season (Amasino, 2009). By contrast, *A. alpina* is polycarpic perennial and therefore able to reproduce and survive for several years (Fig.3). In temperate climates as in Europe, the cycles between vegetative growth and reproduction are synchronized to the changing seasons (Battey, 2000). Many *A. alpina* accessions have an obligate requirement for vernalization to flower and flowering is restricted to spring (Fig.3) (Wang et al., 2009b). *A. alpina* Pajares has been mainly characterized at the physiological and molecular levels. *A. alpina* Pajares was collected in the north of Spain (Pajares pass 1378 m), where *A. alpina* is found in the Cantabrian Mountains mainly in calcareous north facing slopes (Joerg Wunder, unpublished). Studies in *A. alpina* Pajares showed that the vernalization requirement is conferred by the gene *PERPETUAL FLOWERING 1* (*PEP1*). *pep1* mutant plants flower without vernalization (Wang et al., 2009). *PEP1* is the ortholog of the *A. thaliana FLC* (Wang et al., 2009b). Comparative analysis between *A. alpina* and *A. thaliana* showed that differences in *PEP1/FLC* expression pattern contribute to different life strategies. Vernalization triggers chromatin modifications that stably repress *FLC* transcription resulting in a full flowering response when plants are returned to warm temperatures (Bastow et al., 2004; Finnegan and Dennis, 2007; Gendall et al., 2001; Heo and Sung, 2011; Sung and Amasino, 2004). In *A. alpina* flowering is also repressed before vernalization by PEP1. In contrast to *A. thaliana*, *PEP1* is only transiently repressed during vernalization and its expression increases again when plants experience warm temperatures after the cold winter (Wang et al., 2009b). Unstable repression of *PEP1* mRNA correlates with the unstable modification of H3K27me3 chromatin mark at the *PEP1* locus. Cycling of *PEP1* expression is a major part of the

molecular basis of polycarpy in this species. In *A. alpina* flowering is initiated during vernalization. Only the shoots that establish flower buds during vernalization will form flowers when returned to warm temperatures. In all the other shoots the increase of *PEP1* mRNA that occurs after return to warm temperature will repress flowering, therefore maintaining vegetative growth. Flowering in these shoots will take place the following year after a second exposure to winter temperatures. The *pep1* mutant does not need vernalization to flower and flowers perpetually, indicating that *PEP1* contributes to seasonal control of flowering in *A. alpina* (Wang et al., 2009b). Nevertheless, *pep1* plants can still live for many years because new shoots are continuously produced, indicating that more factors contribute to perennialism.

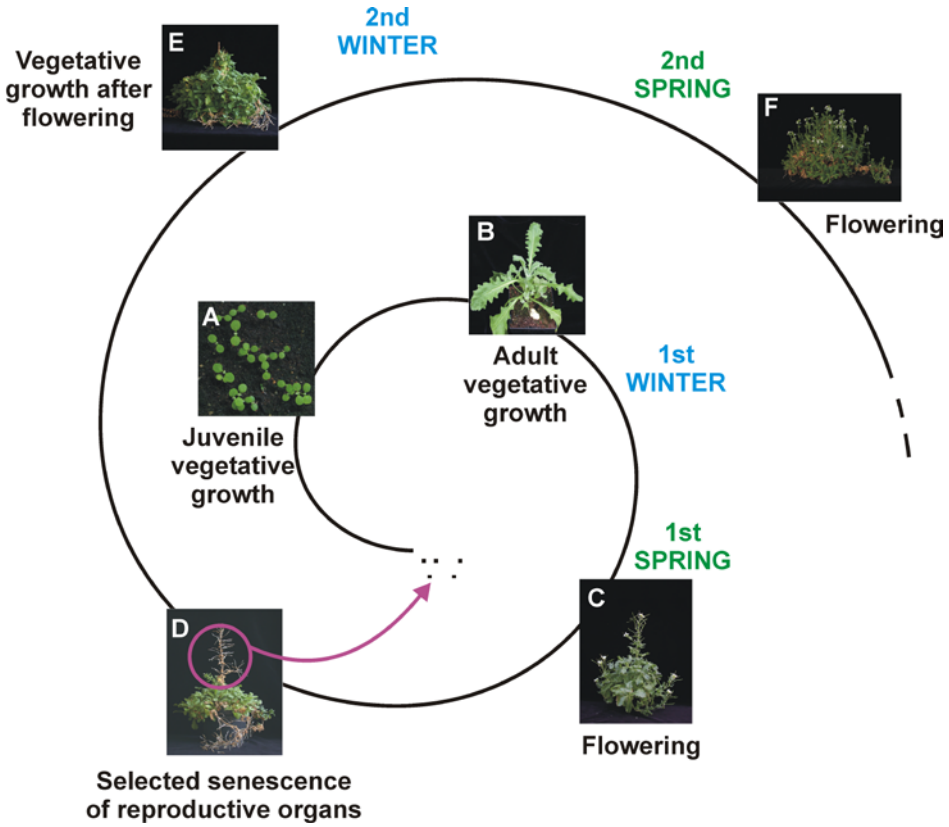

Figure 3. Perennial life cycle of *A. alpina* Pajares

A. alpina follows a perennial polycarpic life cycle. After germination the seedlings grow vegetatively for several weeks developing through a juvenile phase (A) and then gaining the adult phase of development when they become competent to flower (B). Long exposure to cold treatment during the first winter triggers adult plants to flower in spring (C). Only some shoots undergo reproduction, flower, set seeds and senescence (D) while others continue to grow vegetatively (E). After a second winter these vegetative shoots grown the year before will be induced to reproduce (F) and this cycle will continue for several years.

1.6.1 The juvenile phase of *A. alpina* Pajares

A.alpina Pajares has an obligate requirement for vernalization to flower (Wang et al., 2009b). In addition, the flowering response only occurs if plants have been grown for more than 4 weeks in normal growing conditions (LDs and 20°C) prior to exposure to cold (Wang et al., 2011b). The inability of young plants to flower in response to floral inductive stimuli indicates that *A. alpina* has a juvenile phase of 4 weeks and that only plants older than 4 weeks are competent to flower. The molecular mechanisms regulating the age dependency of the plant to respond to vernalization have been previously studied. It is known that differences in flowering response of juvenile and adult plants are not due to differences in sensing and responding to vernalization because *PEP1* is repressed upon vernalization treatment in both juvenile and adult apices (Wang et al., 2011b). As a consequence of *PEP1* downregulation, the *A. alpina* homolog Aa *SOC1* is transcriptionally upregulated in both juvenile and adult apices. This suggests that the block on flowering in juvenile plants occurs downstream of *PEP1* and Aa *SOC1*, or in a parallel pathway. Moreover, reduced expression of Aa *TFL1* through dsRNAi construct shortened the length of the juvenile phase to 1-2 weeks indicating a role for this gene in regulating the maturation process (Wang et al., 2011b). However, Aa *TFL1* has similar expression pattern in vegetative apices that are able or unable to respond to prolong exposure to cold. Upon vernalization, Aa *TFL1* mRNA expression changes only in adult apices. Aa *TFL1* expression decreases in the external part of the meristem and it is restricted to the centre where, based on the terminal flower phenotype of *35S:AaTFL1 dsRNAi* lines and on the similarity with *A. thaliana,* it likely plays a role in inflorescence indeterminacy. In adult plants Aa *TFL1* mRNA accumulation is restricted at the time Aa *LFY* mRNA levels increase and their patterns of expression are largely complementary. In the *35S:AaTFL1 dsRNAi*, Aa *LFY* expression is upregulated earlier but it is not ectopically expressed. In addition, in Arabidopsis TFL1 and LFY act in the meristems to antagonize each other (Ratcliffe et al., 1999). Based on these observations a role for Aa *TFL1* in setting a threshold of expression for *LFY* in order to promote flowering was proposed. The expression of *LFY* would be then promoted by a still unknown factor that increases with the plant ageing. In young plants the expression of the age pathway would be low favouring the repression of Aa *LFY* via Aa TFL1 while in older plants Aa *LFY* upregulation would repress Aa *TFL1* and promote flowering.

Aa *TFL1* also regulates the fate of axillary shoots together with *PEP1 (Wang et al., 2011b)*. Small axillary shoots exposed to vernalization do not flower after cold treatment. In these shoots, the mRNA levels of the floral repressor Aa *TFL1* do not change during

vernalization, similarly to what is observed for juvenile apical meristems. Aa *TFL1* expression is instead remodelled during vernalization in axillary shoots longer than 1 cm. By the end of vernalization these shoots undergo the floral transition. In Aa *TFL1* dsRNAi lines more shoots undergo the floral transition. Moreover, when the downregulation of Aa *TFL1* is combined with *pep1* the number of flowering shoots is increased further, suggesting that Aa TFL1 and PEP1 redundantly regulate polycarpy.

2. AIM OF THE PROJECT

Previous studies in *A. thaliana* and other annual species demonstrated the importance of the non coding RNA miR156 and its targets, SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) transcription factors, in the regulation of vegetative phase change and flowering time. However, there are limitations to the use of annual species to understand the acquisition of competence to respond to environmental signals to induce flowering. Most importantly, a clear distinction between a competent and incompetent meristem state is difficult to define due to the rapid progression of annual species from one developmental phase to another. Moreover, studies in *A. thaliana* showed that long photoperiod not only represents one of the major flowering signals but is also involved in the regulation of *SPL* transcription, making Arabidopsis a difficult system to test reproductive competence.

The aim of this study was to understand the molecular control of reproductive competence using the model species for perennial flowering *A. alpina*. The suitability of this species for reproductive competence studies was recently described (Wang et al., 2011b). Here, we combine genome wide expression analyses and candidate gene approaches that exploit the close relationship between *A. alpina* and *A. thaliana*. In particular, the role of miR156 and Aa *SPL9* transcription in the regulation of reproductive competence acquisition is investigated and a possible role for temperature in the regulation of the developmental decrease of miR156 is presented. Finally, preliminary experiments to extend the observations made in the main shoot apex to the axillary shoots of the plant aim to characterize the contribution of juvenility to the polycarpic life history strategy of *A. alpina.*

3. MATERIALS AND METHODS

Plant material and growth conditions

The *A. alpine* acc. Pajares was used for all the studies presented in this thesis. *A. alpina* seeds were cold–imbibed on wet filter paper for 2-4 days at 4°C and darkness and then transferred to soil at growing temperature conditions to germinate. *A. alpina* plants were routinely grown in control conditions in LD of 16 h light and 8 h dark, unless differently indicated. Light was provided by fluorescent tubes complemented by incandescent bulbs to increase the proportion of far red light. The temperatures were ranging from 20°C during the day to 18°C during the night. Vernalization was performed at 4°C and in SD conditions with 8 h light and 16 h dark to mimic the natural winter conditions. *A. thaliana* acc. Colombia was grown using the same conditions.

For the shift experiment at ambient temperature *A. alpina* plants were grown in the same cabinets but at 15°C LD for different lengths of time and then shifted to vernalization for 3 months before being returned to LD 20°C and scored for flowering.

The shift experiment for the characterization of reproductive competence of the axillary shoots growing in the second year was performed as following. *A. alpina* plants were grown in LDs (20°C) for 8 weeks, vernalized for 12 weeks and returned to LDs (20°C). At this moment, 12 individuals for each group were grown in LDs (20°C) for different numbers of weeks (0, 2, 4, 5, 6, 7, 8) and then vernalized a second time for 12 weeks. When returned to LDs (20°C), the axillary shoots that grew vegetatively the year before were scored for flowering. Data are reported for the scoring after 12 weeks in LDs (20°C).

In all the experiments where apices were used for expression profile analysis (Microarray, Next Generation Sequencing and qRT-PCR), harvesting was performed by hand with the use of forceps. Therefore the material used was apex-enriched partially contaminated with the last developing leaves. Apices of plants growing in the same conditions were pulled in order to obtain enough material for further studies. Depending on the stage 10 to 30 apices were pulled for each biological sample. Harvesting of every sample was conducted at ZT8 to avoid the effect of the Circadian Clock on gene expression.

The quantification of miR156 in leaves was done on single leaves or on a few (up to 10) pulled leaves growing at the same node in different plants in order to obtain enough material for further quantitative experiments.

Microarray development and hybridizations

A common reference model was used in the microarray experiment in which a pool of the RNA of the 16 samples was used as the common reference (Yang and Speed, 2002). Every slide was therefore always hybridized with the common reference sample, labelled with the green fluorescent dye Cyanine 3 (Cy3), and with one of the biological samples, labelled with the red fluorescent dye Cyanine 5 (Cy5). Main apices were collected as described previously in "Plant Material and growth conditions". The apices collected at different stages of development and in different conditions were pooled and homogenized in liquid nitrogen. Pooled apices from plants grown for 2 weeks in LDs formed the juvenile sample (J). Pooled apices from plants grown for 8 weeks in LDs formed the adult sample (A). Pooled apices from plants grown for 2 weeks in LDs and then shifted for 4 weeks in vernalization formed the juvenile vernalized sample (JV). Pooled apices from plants grown for 8 weeks in LDs and then shifted for 4 weeks in vernalization formed the adult vernalized sample (AV). Each pool was considered as a single biological replicate. Four biological replicates were collected for each condition. Total RNA was isolated using the RNeasy^{TM} Mini Kit (Qiagen). At this stage, double column purification was performed using the same Kit and following the manufactures' instructions. Approximately 100 mg of tissues were used for the RNA extraction of every biological replicate. Genomic DNA was afterwards digested using the DNA-*free*^{TM} Kit (Ambion). RNA quality and integrity were measured with the Bioanalyzer (Agilent). The Agilent Quick Amp Labeling kit was used to synthesise Cy3 and Cy5 labeled cRNA. For each RNA sample, 500 ng were used for the labelling. Afterwards the concentration and incorporation of the cRNA and dyes were measured with the Nanodrop. For the hybridization, 2000 ng of Cy3 and Cy5 labeled cRNA were used for further fragmentation and hybridization to a customized Agilent Arabidopsis Oligo Microarray whose features are described in the result chapter. In brief, it is an *in situ* synthesised array that includes 244K probes. Every Arabidopsis gene is represented by up to 10, 60-mer long oligos. Oligos have been designed using the eArray application based on the Arabidopsis DNA sequence starting from the less conserved 3´ end and going in the direction of the 5´ end. The distance between two oligos on the same gene varied based on optimization protocol of the eArray that accounts for low complexity and repetitive regions. The calculated median distance between the end and the start of two consecutive oligos was 27 nt. Oligos specific for 21 known genes of *A. alpina* were also included in the array. Hybridization was conducted at 60°C, whereas hybridization times, washing and scanning of the arrays condition were performed according to the Agilent protocols.

For the genomic DNA hybridization test, DNA from *A. alpina* and *A. thaliana* was isolated using the DNeasy™ Kit (Qiagen). About 6 μg of labelled DNA were hybridized to the array. A dual colour dye swap has been performed, hybridizing the samples for 40 hours and following the Comparative Genomic Hybridization (CGH) protocol. For the RNA hybridization tests, a commercially available 22K Agilent array has been used. As for the custom array, the oligos on this array are *in situ* synthesised and 60-mer long. Two hybridization temperatures (50°C and 60°C) were tested for both *A. alpina* and *A. thaliana*. After hybridization the slides were scanned and the data extracted with the Agilent Features Extraction Software version 9.5.

The array was designed by ImaGenes GmbH (Berlin, DE). DNA hybridization test was also performed at ImaGenes GmbH (Berlin, DE). RNA hybridization tests and hybridizations to compare juvenile and adult apices were performed at ServiceXS (Leiden, NL).

Microarray data analysis

To obtain a representative gene expression value for each gene in each array we corrected for the background signal, normalized the intensity distribution and summarized the different probes into one single value. To normalize the data the log-transformed intensity distribution within each array was first normalized using intensity dependent normalization (function "normalizeWithinArrays", method "loess", R package "limma"). To make the values comparable between arrays a quantile normalization was applied taking advantage of the common reference channel (function "normalizeBetweenArrays", method "Gquantile", R package "limma"). The probes with lowest intensity were then excluded ensuring at least 5 probes in the final probeset for each gene. This filtering step was motivated by the interspecific hybridization and because some oligos were designed on intronic regions. The summarization step was performed using the Robust Multichip Average (RMA) (Irizarry et al., 2003) on the normalized log-transformed red channel intensities. In brief, the RMA procedure uses the median-polishing algorithm to find a robust median expression value for each gene on each array. To find genes differentially expressed between two conditions a moderated t-test was employed (Smyth, 2004) and the resulting p-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg procedure.

Roche 454 Transcriptome Sequencing

Total RNA was isolated and purified from apex-enriched material as described for the microarray development and hybridizations. Full-length enriched cDNA was synthesised with

the CloneTech SMART™ cDNA synthesis kit using 454-ready adapter oligos. The frequency of abundant cDNA species was reduced using Duplex-Specific Nuclease (Zhulidov et al., 2004). Normalized cDNA was fragmented by sonication, blunt end repaired, and ligated to 454 sequencing adapters. Normalization of the samples were performed by Evrogen (Moscow, Russia). Sequencing was performed using the GS FLX Titanium from Roche 454 Life Sciences Technology. Two samples were sequenced with a full run for a total of ~1 million reads for each sample whereas two samples were sequenced with half a run each. Sequencing was performed at the Genome Centre of the University of Liverpool (UK). In total 3.045.967 row sequence reads were generated and deposited in an internal Blast Server from the MPI for Plant Breeding Research. A summary of the number of reads and average read length for each run is reported in Table I of the Appendix.

Identification of SBP-box genes in *A. alpina*

The Arabidopsis SBP-box gene list was obtained from the DATF SBP-box gene family database http://datf.cbi.pku.edu.cn/ (Guo et al., 2005). HMMER search was performed against the *A. alpina* predicted proteome using the extracted Pfam SBP domain (http://hmmer.janelia.org/). All hits with E-values below $1e^{-30}$ were extracted.

Sequence alignments and phylogenetic analysis

Multiple alignment of the amino acid sequences were generated using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree has been inferred from 10000 replicates (Felsenstein, 1985). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Visualization of synteny conservation

The comparison between the genomic regions containing *Aa SPL9* and *At SPL9* was performed with the use of the GATA plotter (Nix and Eisen, 2005).

Identification of *MIR156* genes in *A. alpina*

To identify the *A. alpina MIR156* genes we follow an approach previously described by Bonnet and colleagues (Bonnet et al., 2004). The sequences of the mature miR156s from *A. thaliana* and several other species obtained from the miRBase database (www.miRBase.org) were used to BLAST against the genome of *A. alpina*. In total, 3,029 hits were recovered using a cut off of 90% similarity. Based on synteny conservation the putative ortholog genes

to the *A. thaliana MIR156* genes were identified among the obtained hits. The sequences were extended on both sides of the miR156 with few hundreds bases in order to obtain the full length of the *A .alpina MIR156* genes. The presence of the reverse and complement miR156 sequence was assessed for each candidate gene. Estimations of the lengths of the *A. alpina* precursors were based on the alignment with the *A. thaliana* pre-miRNAs. Secondary structures and minimum free energy values were generated for both *A. thaliana* and *A. alpina* genes using the webserver RNA fold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Analysis of gene transcript levels (quantitative RT-PCR)

Apex enriched or leaf materials were harvested and total RNA extracted using miRNeasy^{TM} Mini Kit (Qiagen). The kit allows purification of total RNA including RNA from approximately 18 nt in length. The same RNA was then used for miRNA and mRNA quantifications. For mRNA, following purification a DNA digestion was performed using the DNA-*free*^{TM} Kit (Ambion). cDNA synthesis was carried out using OligodT primer and the Superscript II reverse transcriptase enzyme (Invitrogen). cDNA was diluted to 150 µl with water and 3 µl were used as a template for qRT-PCR using a BioRad iQ5 apparatus and SYBR-green detection. A dilution series of one of the samples was used to build the standard curve for each primer pair. *PHOSPHATASE 2A* (*PP2A*) or *RAN GTPASE 3* (*RAN3*) were used as reference genes to normalize the varying amounts of cDNA between samples. Two or more biological replicates were performed for every experiment (with the exception of the extended vernalization experiments described in session 4.14 that has been performed only once) and when consistent one replicate was shown. Error bars represent technical replicates. Primer sequences are listed in Table III of the Appendix.

Analysis of mature miRNA levels

Total RNA including, small RNAs longer than 18 nt, was extracted using miRNeasy^{TM} Mini Kit (Qiagen). After DNAse treatment (DNA-*free*^{TM} Kit, Ambion), 1st strand synthesis of the mature form of miRNAs was performed as previously described in Yang H et al. (Yang et al., 2009) with some modifications from the Huijser laboratory (MPI for Plant Breeding Research, Cologne). The mature form of miR156 and miR172 were reverse transcribed starting from 200 ng of total RNA and using two different Key-primers each composed of an universal sequence of 35 nucleotides at the 5´ end and by 8 nucleotides complementary to the miR156 or the miR172 at the 3´ end (B163 and F408). The reverse primer for snoR101 (B149 for *A.*

*alpin*a and B147 for *A. thaliana*), which is the reference gene used to normalize the qRT-PCR, was also included in the reverse transcription.

1st strand synthesis:

Total RNA (0.2 μg)	1 μl
Key-primer miR156 (4 μM)	1.5 μl
Key-primer miR172 (4 μM)	1.5 μl
snoR101/reference gene (R) (2 μM)	0.5 μl
dNTPs (10 mM)	0.5 μl
H2O to 6.5 μl	

Incubate 5´ at 65°C, cool on ice and then add:

5X 1st strand buffer (Invitrogen)	2 μl
DTT (Invitrogen)	0.5 μl
RNase OUT (Invitrogen)	0.5 μl
Superscript II (Invitrogen)	0.5 μl

Incubate 30´ at 16°C, 50´ at 42°C, 15´ at 75°C.

The product from the 1st strand synthesis was dilute to 200 μl. In a second step, 3 μl of the reverse transcription products were quantified by SYBR-green based qRT-PCR using a combination of a miRNA-specific forward primers (B164 for miR156 and B166 for miR172) and a generic universal primer designed on the Key-primers (B165 for miR156 and F409 for miR172).

iQ SYBR Green Supermix (Bio-Rad)	10 μl
F primer (2.5 μM)	1 μl
R primer (2.5 μM)	1 μl
1st strand synthesis	3 μl
H20 to 20 μl	

Since the mature forms of miR156 and miR172 are likely highly conserved between *A. alpina* and *A. thaliana*, the same primer sets were used for the detection of the two miRNAs in both species. snoR101 was amplified using the primer B148 and B149 for *A. alpina* samples or B146 and B147 for *A. thaliana* samples. Two or more biological replicates were performed for every experiment (with the exception of the extended vernalization experiments) and when consistent one replicate was shown. Error bars represent technical replicates. Primer sequences are listed in Table III of the Appendix.

In-situ hybridization

Longitudinal sections of plant apices were probed with digoxigenin-labelled Aa *SPL9* antisense RNA. Probes to detect Aa *SPL9* transcript were PCR- amplified from cDNA using primer B183, containing the tail for T3 RNA polymerase binding site (5´- ATTAACCCTCACTAAAGGGA -3´) and B184, containing the tail for T7 RNA polymerase binding site (5´- TAATACGACTCACTATAGGG -3´). RNA *in situ* hybridization was carried out as described by Jackson (Jackson, 1992) with small modifications described in Searle et al. (Searle et al., 2006). Primers sequences are listed in Table III of the Appendix.

Aa *SPL9* and Aa *rSPL9* overexpressor plasmid constructs

For the overexpression of Aa *SPL9*, the coding sequence was amplified by PCR on *A. alpina* cDNA using the Expand High Fidelity PCR system (Roche) and ligated into the GATEWAY™ cloning vectors. The forward primer (B187) contained the attB1 extension (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'); reverse primer (B188) contained the attB2 tail (5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'). For the overexpression of the miR156-resistant form of Aa *SPL9* (*rSPL9*) the miRNA target site has been modified at the nucleotide level, preventing the annealing from miR156, but not at the amino acid level, to preserve the protein function. To do so, amplification from cDNA has been performed using an adaptation of the Splicing by Overlapping Extension by PCR (SOE by PCR) (Warrens et al., 1997). In brief, this technique involves three PCRs: in the first two PCRs, two DNA fragments are produced in order to overlap in their flanks and to form the template for the third PCR. The flanking primers were the same used for the overexpression of Aa *SPL9* (B187 and B188), whereas the central primers were designed to modified the nucleotides sequence at the site of miR156 annealing and to overlap with each other in order to perform the third PCR (B194 and B195). The amplicons were inserted into the GATEWAY™ cloning vector pDONR221 via BP reaction, according to the manufacture's instruction of the BP

clonase, resulting in entry vectors. LR reactions were performed to transfer the CDSs from the entry vector to the destination vector pLeela for overexpression in plant. Primers sequences are listed in Table III of the Appendix

A. alpina transformation

The plasmids based on the vector pLeela, for overexpression of Aa *SPL9* and Aa *rSPL9,* were introduced into *A. tumefaciens* strain GV3101 (pPMRK) (Koncz and Schell, 1986). Plasmids containing *35S:MIR156b* and *35S:MIM156* (Franco-Zorrilla et al., 2007; Schwab et al., 2005) were obtained from the Weigel Lab (MPI for Developmental Biology, Tübingen). *35S:MIR156b* is in the pMLBART (Gleave, 1992) and was introduced into the *A. tumefaciens* strain GV3101 (pMKRK). *35S:MIM156* construct is into the pGREEN vector and was introduced into the *A. tumefaciens* strain GV3101 (pSOUP). Plasmids were introduced into *A. alpina* by floral dip method (Clough and Bent, 1998). T1 transformants were identified on the basis of BASTA resistance.

Application of exogenous gibberellins

GA_3 100 μM was applied by spraying the aerial part of the plants. The applications were performed twice a week during the last 6 weeks of vernalization treatment.

4. RESULTS

4.1 Development and optimization of an Arabidopsis custom array for *A. alpina* heterologous hybridizations

A. alpina is closely related to *A. thaliana* and their divergence is estimated to have taken place about 25-30 mya (Koch et al., 2001). To estimate the sequence similarity between *A. thaliana* and *A. alpina*, genes identified and sequenced in the laboratory at the time the project began were compared. Based on these data, *A. alpina* showed an average of 78% DNA identity in coding regions and higher than 90% in the nuclear internal transcribed spacer regions (Koch et al., 2006). Currently, the whole genome of *A. alpina* has been sequenced. Although the assembly is still in progress, early estimations showed that 75% of *A. alpina* genes are homologues to *A. thaliana* genes across at least 90% of their length. In addition, more than 70% of the predicted proteins of *A. alpina* find a homologue in the *A. thaliana* protein set (Blastp with e-value < e^{-5}; Nordström and Velikkakam, unpublished). Therefore, as the sequence similarity between the two species is high, an Arabidopsis microarray was used for expression analysis of *A. alpina* transcripts. The Agilent microarray technology includes 60-mer probes and was chosen as it offers higher possibilities that *A. alpina* RNA will hybridise on the *A. thaliana* array. The Agilent 3 array was already successfully used for expression studies for other members of the Brassicaceae family (van de Mortel et al., 2006). It includes around 40,000 putative genes representing 27,000 annotated genes and around 10,000 non annotated Arabidopsis genomic regions which are known to have transcriptional activity. The probes in the Agilent 3 array are designed against the 3' UTRs which are generally less conserved among species. To increase the probability of a successful heterologous hybridization a customised Agilent array was designed (see methods). In the customized array every gene was represented with an oligo set including a total of 244,000 oligos, in comparison to the Agilent 3 array which has a total of 44K oligos. Oligos were designed starting from the less conserved 3´ UTRs towards the 5' end of every gene. In addition, probes designed on sequences of *A. alpina* genes already isolated in the laboratory were also included in the array. These oligos allowed comparison of hybridizations to heterologous and homologous genes.

To test the suitability of the customised array for cross species hybridization *A. alpina* genomic DNA and *A. thaliana* genomic DNA were hybridised to the array and the hybridization intensities compared. This approach allowed an estimation of the number of

genes that could be expected to be detected in the expression analysis. DNAs from the two species were hybridized separately on two different arrays. For the majority of the genes on the array, 5 or more oligos hybridized when *A. alpina* DNA was used as probe (Fig.4A). Around 250 *A .thaliana* genes did not hybridize with any oligos to *A. alpina* DNA (Fig.4A, red histogram). When *A. thaliana* DNA was used as probe most of the genes hybridized to all the oligos (Fig.4B). Most of the genes were represented on the array by either 10 or 7 probes. In addition, the oligos designed at the 3' end of the genes were generally those that did not hybridize to *A. alpina*, whereas for *A. thaliana* the position of the oligo in the gene did not influence the hybridization (Fig.4C and 4D). These results were expected because the 3' ends are normally less conserved among species. Moreover, they support the choice of the custom array design in which every gene is represented by an oligo set instead of a single oligo.

To identify the optimal hybridization conditions for the heterologous hybridization two different hybridization temperatures were tested. The standard Agilent array protocol uses 65°C as hybridization temperature for homologous RNA hybridization. Two lower temperatures were tested for both homologous and heterologous hybridizations using *A. thaliana* and *A. alpina* mRNAs. The same biological sample was used for *A. thaliana*, hybridized to all the arrays and used as technical replicate. For *A. alpina* two different biological samples were hybridized as duplicates. A summary of the hybridizations is shown in Fig.4E. The signal intensity for *A. alpina* was in general lower than the signal intensity for *A. thaliana* but the differences were not striking (Fig.4E). The medians of intensities for *A. alpina* hybridizations at 50°C and at 60°C were slightly higher at 50°C compared to 60°C (Fig.4E, left side). In addition, plotting the two hybridizations in a scatter plot showed that additional oligos hybridized at 50°C compared to 60°C (Fig.4F). However, this increase in hybridizing oligos seemed to be not specific because it was also observed in *A. thaliana* DNA hybridizations (Fig.4G). Moreover, since the same biological sample of *A. thaliana* was used for all the hybridizations at both temperatures and in duplicate, reproducibility could be tested at different temperatures (Fig.4H and 4I). Reproducibility was higher at 60°C compared to 50°C.

Taken together the test hybridization results show that temperature slightly influences the intensity of hybridization of *A. alpina* probes (Fig.4E). Additional signals are gained when hybridizing at 50°C compared to 60°C, but these likely reflect at least in part non specific hybridizations since they can also be observed in *A. thaliana* (Fig.4F and 4G). Therefore, the

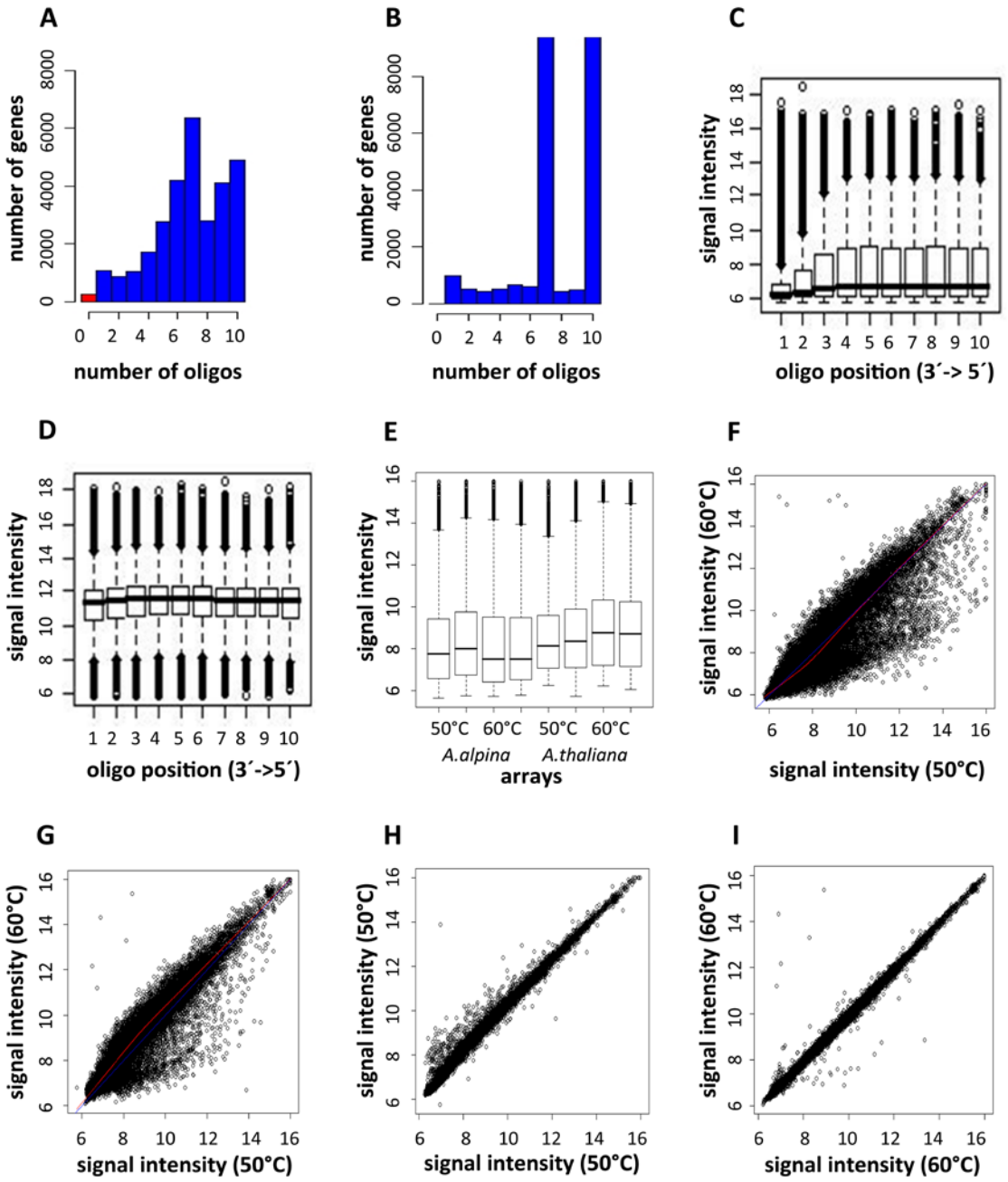

Figure 4. Tests of heterologous microarray hybridization

(A to D) DNA hybridizations. (A and C) *A. alpina* DNA hybridization. (B and D) *A. thaliana* DNA hybridization. (A and B) Every column represents the number of genes hybridizing with 1 oligo of the oligo-set, 2 oligos, 3 oligos etc. x-axis, number of oligos hybridizing with intensity higher than 6 in log2 (the background signal intensity in log2 was equal to 6). y-axis, number of genes hybridizing with intensity higher than 6 in log2. In red are the *A. alpina* genes that did not hybridize with any oligo. (C and D) Box plots representing the hybridization intensities according to the position of the oligos along the gene. x-axis, position of the oligos from the most 3` end (number 1) to the most 5´ end (number 10). y-axis, signal intensity in log2. (E to I) RNA hybridizations. (E) Box Plot of *A. alpina* and *A. thaliana* hybridization at the two temperature tested, 50°C and 60°C. x-axis, arrays. y-axis, signal intensities in log2. (F) Scatter plot representing the *A. alpina* RNA hybridizations at 50°C (x-axis) and 60°C (y-axis). Red line, average depending on the intensities; blue line, ideal correlation. (G) Scatter plot representing the *A. thaliana* RNA hybridization, the rest as in (F). (H and I) Scatter plots representing the technical duplicate of *A. thaliana* hybridization at 50°C (I) and at 60°C (I).

subsequent experiments were performed at 60°C in order to gain in reproducibility and possibly in specificity.

4.2 Experimental design to compare the transcriptome of reproductively competent and incompetent apices

In order to identify candidate genes involved in the regulation of reproductive competence, the transcriptomes of the main shoot apices of juvenile and adult plants were compared using the custom Arabidopsis microarray. *A. alpina* Pajares has a juvenile phase that is 4-5 weeks long (Wang et al., 2011b). Therefore, apices collected from plants grown in LDs for 2 weeks (juvenile apices, J) were compared to apices collected from plants grown in LDs for 8 weeks (adult apices, A). Flowering in adult plants occur during prolonged exposure to cold (Wang et al., 2011b; Wang et al., 2009b). Previous experiments showed that the transcription of the floral repressor *Aa TFL1* is restricted in the SAM of adult plants at around 5 weeks in vernalization and correlates with the progression of flowering (Wang et al., 2011b). Moreover, the floral identity gene *Aa LFY* is also transcribed only in adult apices during vernalization at about the time Aa *TFL1* mRNA is restricted (Wang et al., 2011b). To identify genes that are differently expressed during vernalization, main shoot apices that will initiate flowering (adult vernalized, AV) and main shoot apices that will remain vegetative (juvenile vernalized, JV) were compared after 4 weeks in vernalization. Four biological replicates were hybridized for each of the four time points. A common reference design was used to hybridize every sample on the same slide together with a reference composed of a pool of the 16 samples (Fig.5). This common reference was used to normalize the hybridization signal among different arrays. Data analyses were performed taking account of all oligos representing a single gene and the interspecific hybridization conditions (see methods).

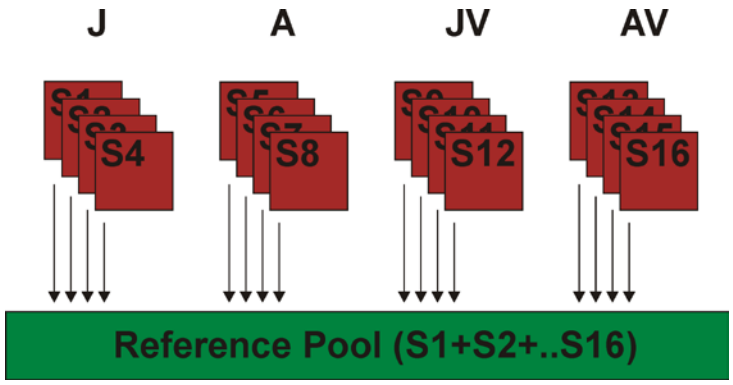

Figure 5. Microarray common reference design

Four developmental conditions were compared in the microarray experiment (J, A, JV and AV). For each condition, four biological replicates were hybridized (red). A common reference design was used in which every sample was hybridized to the same array together with a reference. The same reference, a pool of the 16 samples, was used for each array (green). In total 16 arrays were used. Samples were always labelled with the Cy5 dye (red), reference with the Cy3 dye (green). J, apices from plants grown for 2 weeks in LDs; A, apices from plants grown for 8 weeks in LDs; JV, apices from plants grown for 2 weeks in LDs and then shifted in vernalization for 4 weeks; AJ, apices from plants grown for 8 weeks in LDs and then shifted in vernalization for 4 weeks.

4.3 Analysis of known flowering gene expression by array hybridization

The transcriptome information obtained from the microarray hybridizations was used to characterize the stage of floral development reached by juvenile and adult plants at 4 weeks in vernalization. To do so, the expressions of floral integrator genes and floral meristem identity genes in the samples before and during vernalization were compared (Fig.6A and 6B). Relative expression levels (y-axes in Fig.6) around 200 are comparable to background signal, whereas levels above this value denote gene expression. Nevertheless, mRNAs consistently showing background expression levels in all samples might denote true absence of expression, sequence divergence or even absence of that gene from the *A. alpina* genome. Consistent with previous reports, *SOC1* expression levels increase in response to vernalization in both juvenile and adult apices (Wang et al., 2011b) (Fig.6A). In contrast, *FUL* is expressed only in adult apices induced to flower, whereas *AGL42, AGL24*, and the floral meristem identity genes *AP1* and *CAULIFLOWER (CAL)* were not detected at any time point (Fig.6A and 6B). *LFY* shows an increase in expression, although not statistically significant, in adult apices exposed to vernalization (Fig.6B). Taken together these data show that 4 weeks in vernalization are sufficient to induce expression of the first floral integrator genes *SOC1* and

FUL. Interestingly, while as previously reported Aa *SOC1* expression is induced in both juvenile and adult apices in vernalization, *FUL* expression seems to be exclusive to adult apices that will undergo the flower transition. The increase in *LFY* expression observed in the microarray could also be meaningful since a clear Aa *LFY* signal was detectable by *in situ* hybridization in lateral floral primordia at 5 weeks in vernalization (Wang et al., 2011b). Plants exposed to vernalization for longer period would probably give a stronger signal.

To further validate the sensitivity of the array we compared homologous and heterologous hybridizations of few characterized genes for which *A. alpina* oligo sequences were synthesized on the array (Fig.6C and 6D). Aa *TFL1* is expressed in both juvenile and adult apices before vernalization but its expression is restricted during vernalization only in adult apices (Wang et al., 2011b). This pattern of expression was confirmed by both homologous and heterologous hybridizations (Fig.6C and 6D). Aa *SOC1* mRNA increases in both juvenile and adult samples upon vernalization as a consequence of *PEP1* decrease and this could be observed in both heterologous and homologous hybridization (Fig.6A and 6D) (Wang et al., 2011b). *In situ* hybridization demonstrated that Aa *FD* expression increases only in adult apices during vernalization (Bergonzi, unpublished). Similarly, *FD* expression in microarray was slightly increased in adult apices in both *A. thaliana* and *A. alpina* oligos (Fig.6C and 6D). Unlike the above examples, *FLC* expression was only detected in the homologous hybridizations where, as expected, it was repressed in both juvenile and adult apices upon vernalization (Fig.6C and 6D) (Wang et al., 2009b). This showed that expression of *A. alpina* genes is not always successfully detected by the Arabidopsis oligos on the custom microarray. For the tested genes, the levels of expression in the homologous hybridizations were very much higher when compared to the heterologous hybridizations indicating that miss matches can influence the hybridization intensities. However, for our analysis we did not need to take into account these differences since the array was used to compare only *A. alpina* samples at different developmental stages.

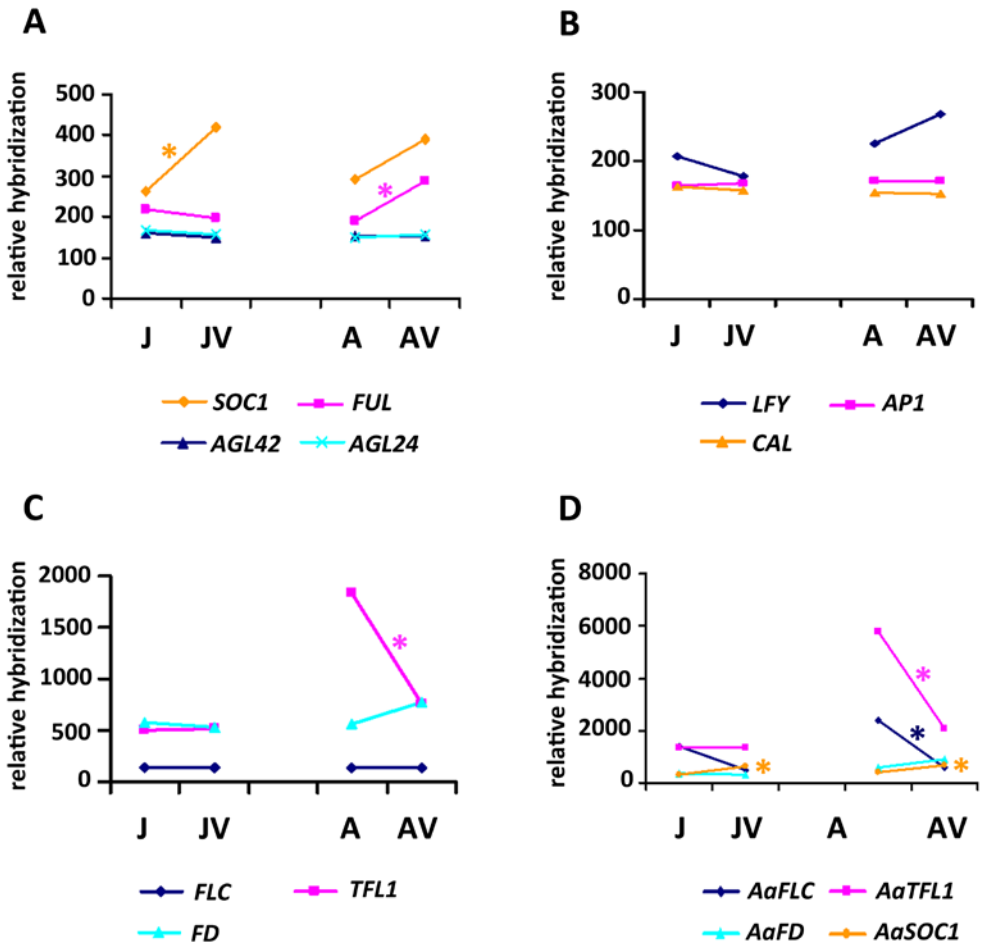

Figure 6. Microarray expression of selected flowering genes

(A to D) Microarray expression levels. x-axis, the four samples tested; y-axis, relative hybridization levels. (A) Expression of floral integrator genes *SOC1*, *FUL*, *AGL24*, *AGL42*. (B) Expression of floral meristem identity genes, *LFY*, *AP1*, *CAL*. (C and D) Comparison between heterologous (C) and homologous (D) oligo hybridizations of selected genes (*FLC/PEP1*, *TFL*/Aa *TFL1*, *FD*/Aa *FD*, *SOC1*(6A)/Aa *SOC1*). Asterisks indicate a statistically significant difference (Adj. p-value < 0.05). J, JV, A and AV as in Fig.5.

4.4 Members of the *SPL* gene family are differently expressed between juvenile and adult apices

The comparison of the transcriptomes of juvenile and adult apices collected before vernalization shows 5.716 genes to be differently regulated (Adj. p-value < 0.05). Included in this group were 2.901 genes that showed higher expression in adult apices and 2.815 genes with higher expression in juvenile apices. These two groups of differently regulated genes could include candidate promoters of the competent state and candidate repressors respectively. Among the differentially expressed genes were several members of the *SPL* gene family (Fig.7A, Table II of the Appendix). Members of this family have been intensively

characterized in *A. thaliana* and other species where they were mainly shown to promote flowering and adult leaf traits (Bergonzi and Albani, 2011; Fornara and Coupland, 2009). Especially Aa *SPL9* and Aa *SPL13* were higher expressed in adult apices compared to juvenile already before vernalization and stayed highly expressed during vernalization (Fig.7A, left side). Aa *SPL5*, and probably Aa *SPL4* and Aa *SPL15*, showed higher expression in adult apices only upon vernalization which coincided with floral induction (Fig.7B, right side). Interestingly, all the *SPLs* that showed differences in expression are targets of miR156 (Rhoades et al., 2002; Schwab et al., 2005). The six members known in *A. thaliana* not to be miR156 targets did not show any expression change in the microarray in response to age or vernalization, although some of them were expressed in the samples (Table II of the Appendix, *SPL*$^{\#}$).

The mRNA made from similar plant samples was used to generate cDNA that was sequenced using 454 Roche technologies. The main shoot apices were collected from 2 and 8 week old plant grown in LDs, similarly to the microarray experiment, whereas for the vernalization time points plants were shifted to cold temperatures for 5 weeks. The samples were normalized before being sequenced to ensure representation of lowly expressed genes, but expression differences could still be detected. The numbers of reads obtained for Aa *SPL9*, Aa *SPL13* and Aa *SPL5* in the different samples confirmed the expression trends observed in the microarrays (Fig.7B). In addition, the expression profiles of Aa *SPL9*, Aa *SPL13* and Aa *SPL5* were also confirmed by qRT-PCR using different biological samples (Fig.7C).

To conclude, the custom Arabidopsis microarray can be used to detect expression differences of *A. alpina* genes. A large number of genes were identified as differently expressed between juvenile and adult apices before vernalization (~1/6 of the whole transcriptome). Among them, genes known in other species to regulate flowering and vegetative phase change were also detected. *SPL* homologues represent good candidates for the regulation of reproductive competence in *A. alpina.*

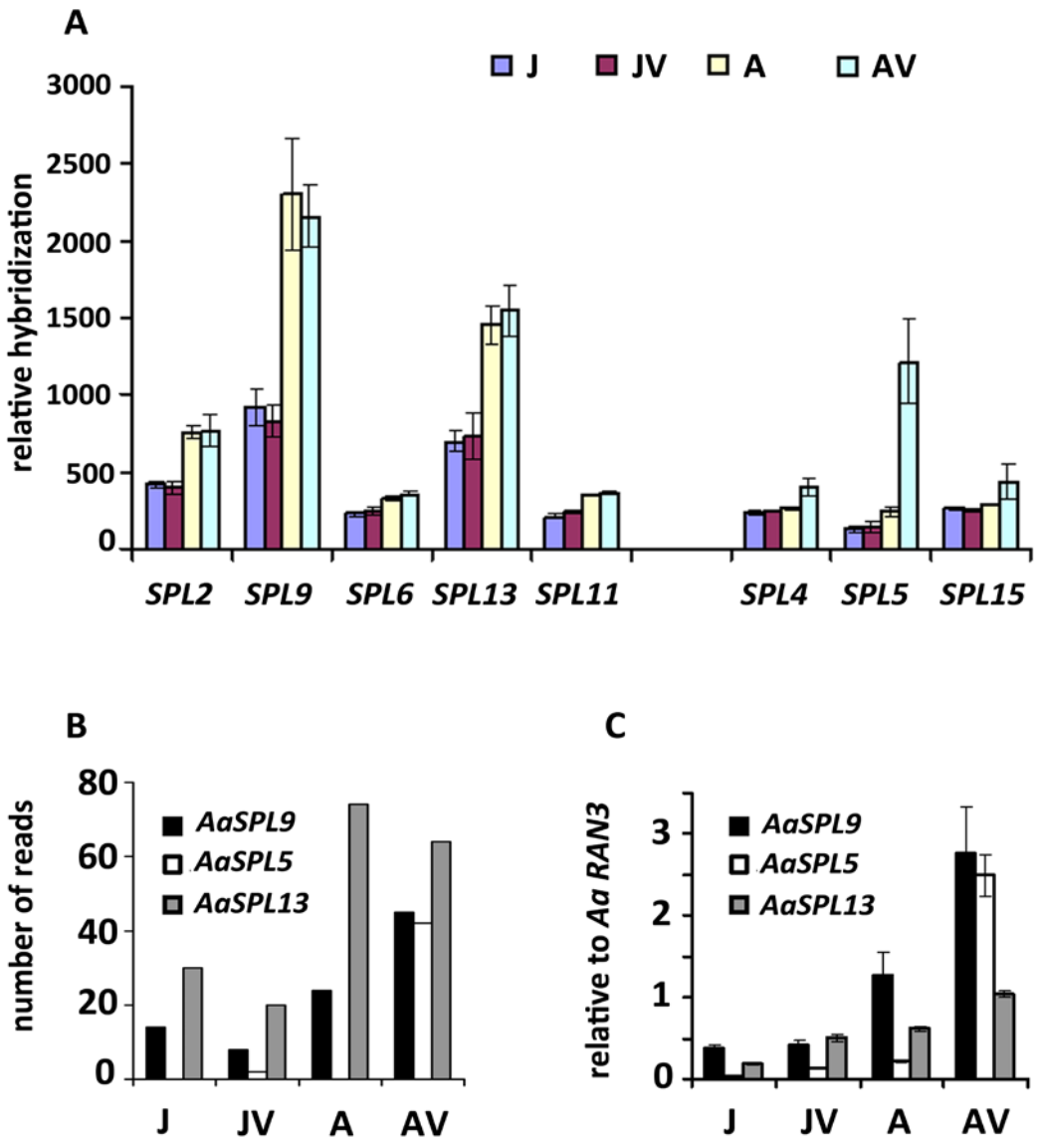

Figure 7. *SPL* genes differently expressed in the microarray experiment and their validation by next generation sequencing and qRT-PCR

(A) *SPL* genes differently expressed in the microarray samples. x-axis, different *SPL* genes; y-axis, relative expression levels. Error bars indicate the 4 biological replicates. J, JV, A and AV as in Fig.5. (B) Quantification of reads from 454 Roche sequencing for selected *SPLs*. x-axis, the four time points: J, JV, A, AV; y-axis, the number of reads divided by the total number of reads for the specific run and multiplied by 1,000,000. (C) qRT-PCR for selected *SPLs*. x-axis as in (B); y-axis, relative expression to the reference gene Aa *RAN3*. Error bars indicate technical replicates. (B and C) J and A as in Fig.5; JV, apices from plants grown for 2 weeks in LDs and then vernalized for 5 weeks ; AV, apices from plants grown for 8 weeks in LDs and then vernalized for 5 weeks.

4.5 Characterization of the SPL family of transcription factors in *A. alpina*

In Arabidopsis there are 17 genes encoding *SPLs*, although 2 of them (*SPL13* and *SPL17* also named *SPL13A*/*B*) are located in a 100% duplicated region of the *A. thaliana* genome. The *SPL* family in *A. alpina* was characterized to better understand the level of conservation between the two species. The family is defined by a conserved protein domain called the SBP-box. This domain is responsible for DNA binding and it is composed of 79 amino acid residues that are necessary and sufficient for the binding to a palindromic GTAC core motif (Birkenbihl et al., 2005; Cardon et al., 1999; Yamasaki et al., 2004). In all the Arabidopsis SPL proteins the SBP-box domain is highly conserved and encoded by the first and second exons. In order to identify the SPL family members in *A. alpina*, the predicted proteome was searched with the SBP-box domain of *A. thaliana* SPL proteins. In total 15 genes containing the SBP-box domain were identified, some of them with different splicing variants (not showed). The number of *A. alpina SPL* candidate genes was very similar to the number of known *SPL* genes in *A. thaliana*. To evaluate the evolutionary relationship between *A. thaliana* and *A. alpina*, to predict orthologs and potentially identify new family members, the deduced amino acid sequences of the 15 *A. alpina* SBP-box containing domain proteins and the amino acid sequences of the *A. thaliana* SPLs were aligned. In all the proteins the SBP-box is highly conserved (Fig.8). Two separate zinc binding domains and the nuclear localization signal characteristic of the domain were identified (Fig.8) (Birkenbihl et al., 2005; Yamasaki et al., 2004).

Phylogenetic analysis strikingly showed that almost all the *A. thaliana* genes are paired with an *A. alpina* one, suggesting high level of conservation during evolution (Fig.9A). In addition, these are good candidates to be the *A. alpina* orthologs of the *A. thaliana* genes. The putative Aa SPL5 was initially missing from the *A. alpina* SPL list. Aa *SPL5* cDNA sequence was manually searched by blasting the At *SPL5* cDNA sequence against the mRNA-seq reads. Several reads were found and assembled to determine the full length cDNA. Afterwards, the predicted amino acid sequence was obtained and added to the phylogenetic tree. For all the homologues, the conservation of synteny was analyzed using the local *A. alpina* genome browser (gbrowse) to compare flanking genes. Synteny was highly conserved (showed for Aa *SPL9*, Fig.10A). Finally, all the genes identified on the basis of the protein sequence showed a high degree of conservation also at the nucleotide levels.

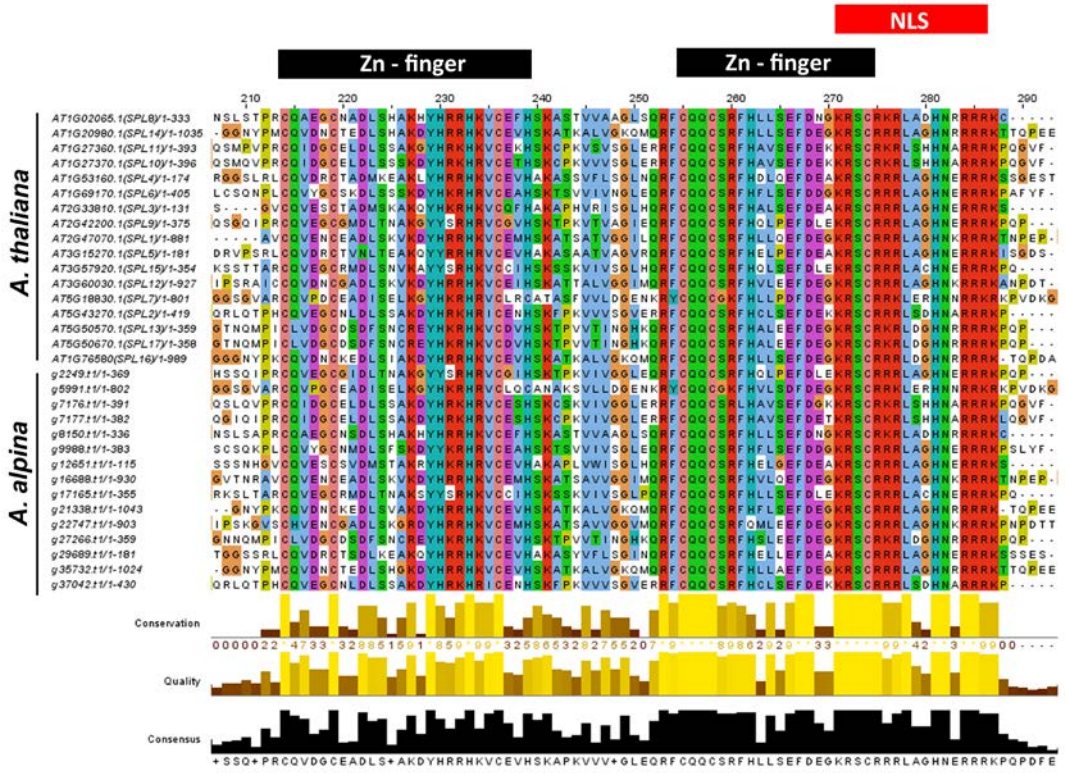

Figure 8. Conservation of the SBP-box in *A. thaliana* and *A. alpina* SPL proteins

Multiple sequence alignment was performed using the program ClustalW. The conserved Zinc finger domain (ZN-finger) and Nuclear Localization Signal (NLS) are indicated.

Members of the *SPL* family are known to be post-transcriptionally regulated by miR156 (Rhoades et al., 2002; Schwab et al., 2005). Thus, *A. alpina SPL* sequences were searched for the miR156 binding site. In *A. thaliana* miR156 binds with 100% pairing to *SPL2, SPL6, SPL9, SPL10, SPL11, SPL13, SPL15* and *SPL17*. The binding site is perfectly conserved in the *A. alpina* homologues of these genes (Fig.10A, asterisks). In these genes the miR156 binding site is included in the coding sequence. The other three *SPL* genes targeted by miR156 in Arabidopsis are *SPL3*, *SPL4* and *SPL5*. These are the shortest of the SPL genes, mainly composed by the SBP-box domain. In *A. thaliana* miR156 binds to the UTRs of *SPL3*, *SPL4* and *SPL5* with a few mismatches. The binding site is similarly conserved in the *A. alpina* homologues, with a few more mismatches (Fig.9B). Further experiments are needed to understand if the additional mismatches observed for Aa *SPL4* and Aa *SPL3* compromise the miR156 binding. miR156 binding sites are not found in the *A. alpina* homologues *SPL1, SPL7, SPL8, SPL12, SPL14* and *SPL16*, showing that the subfamily of miR156 regulated *SPLs* is also conserved in *A. alpina*.

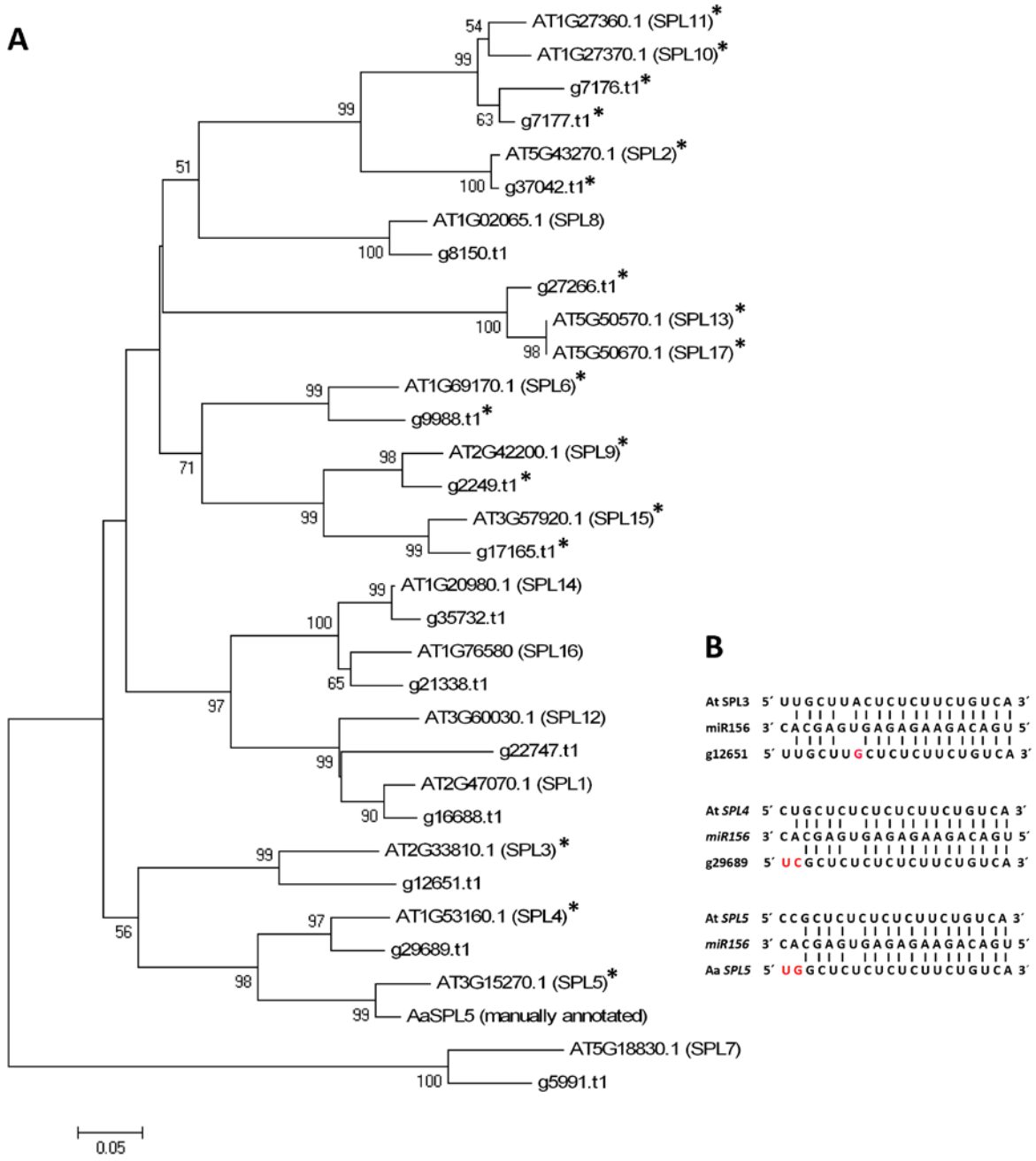

Figure 9. Phylogenetic tree for the SPL proteins of *A. thaliana* and *A. alpina*

(A) The tree was constructed from a complete alignment of 17 *A. thaliana* and 16 predicted *A. alpina* SPL amino acid sequences (starting with "g"). The full protein sequences were used for the phylogenetic reconstruction. Neighbour-joining algorithm was used with a bootstrapping analysis of 10,000 reiterations. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches when higher than 50% (MEGA4 software). Asterisks indicate the presence of the miR156 binding site. (B) Sequence differences in the miR156 binding site between *A. thaliana* and *A. alpina SPLs*. Alignment to miR156 is shown for *SPL3* (g12651), *SPL4* (g29689) and *SPL5* homologues. Indicated with red letters are the nucleotide changes.

4.6 Expression of Aa *SPL9* mRNA in *A. alpina* plants during development and in vernalization correlates with the timing of the juvenile to adult transition and flowering

The SPL family of transcription factors is highly conserved between *A. alpina* and *A. thaliana*. Five members of the *SPL* gene family were more highly expressed in the main apices of plants grown for 8 weeks in LDs than in those grown for 2 weeks (Fig.7A). Among them, Aa *SPL9* was chosen as a major candidate for a gene promoting the adult phase because it showed the highest expression difference between juvenile and adult apices already before vernalization. Aa *SPL9* sequence was identified in the genome of *A. alpina* and the cDNA in the transcriptomic data. These sequences showed that Aa *SPL9* is composed of 3 exons and 2 introns, like At *SPL9*. Moreover, Aa *SPL9* shares with At *SPL9* 86.1% identity at the amino acid level and 85.9% at the nucleotide level. Synteny of the region containing Aa *SPL9* is also conserved because the flanking genes are homologous, further supporting the idea that At *SPL9* and Aa *SPL9* are orthologs (Fig.10A). In the 3rd exon of Aa *SPL9* the miR156 binding site is 100% conserved, indicating that Aa *SPL9* might be under a similar molecular regulation.

Aa *SPL9* mRNA temporal expression pattern during development was followed by qRT-PCR. Aa *SPL9* mRNA levels increased at about 4-5 weeks after sowing and then stabilized (Fig.10B). Therefore, changes in Aa *SPL9* mRNA expression temporally correlate with the acquisition of reproductive competence (Wang et al., 2011b). Adult apices, but not juvenile, undergo the transition to flower when exposed to vernalization. In order to investigate the changes occurring during vernalization the levels of Aa *SPL9* mRNA were tested in the apices of juvenile and adult plants shifted to low temperatures for three months. The higher levels of Aa *SPL9* mRNA in adult apices stayed high during and after exposure to vernalization (Fig.10C, black bars). An additional peak of expression was transiently observed after 5 weeks in vernalization (Fig.10C, black bars). By contrast, the lower levels of Aa *SPL9* mRNA observed in juvenile apices prior to vernalization, did not change after the plants were shifted to vernalization (Fig.10C, white bars).

In *A. thaliana*, At *SPL9* is expressed in leaves and apices. Aa *SPL9* mRNA was tested in an *in situ* hybridization to evaluate its spatial expression pattern in the apex. Apices from juvenile and adult plants before vernalization showed similar expression patterns (Fig.10D and 10H). Aa *SPL9* mRNA was strongly and transiently upregulated only in the meristem of adult apices at 5 weeks in vernalization and then the levels decreased again at 12 weeks in

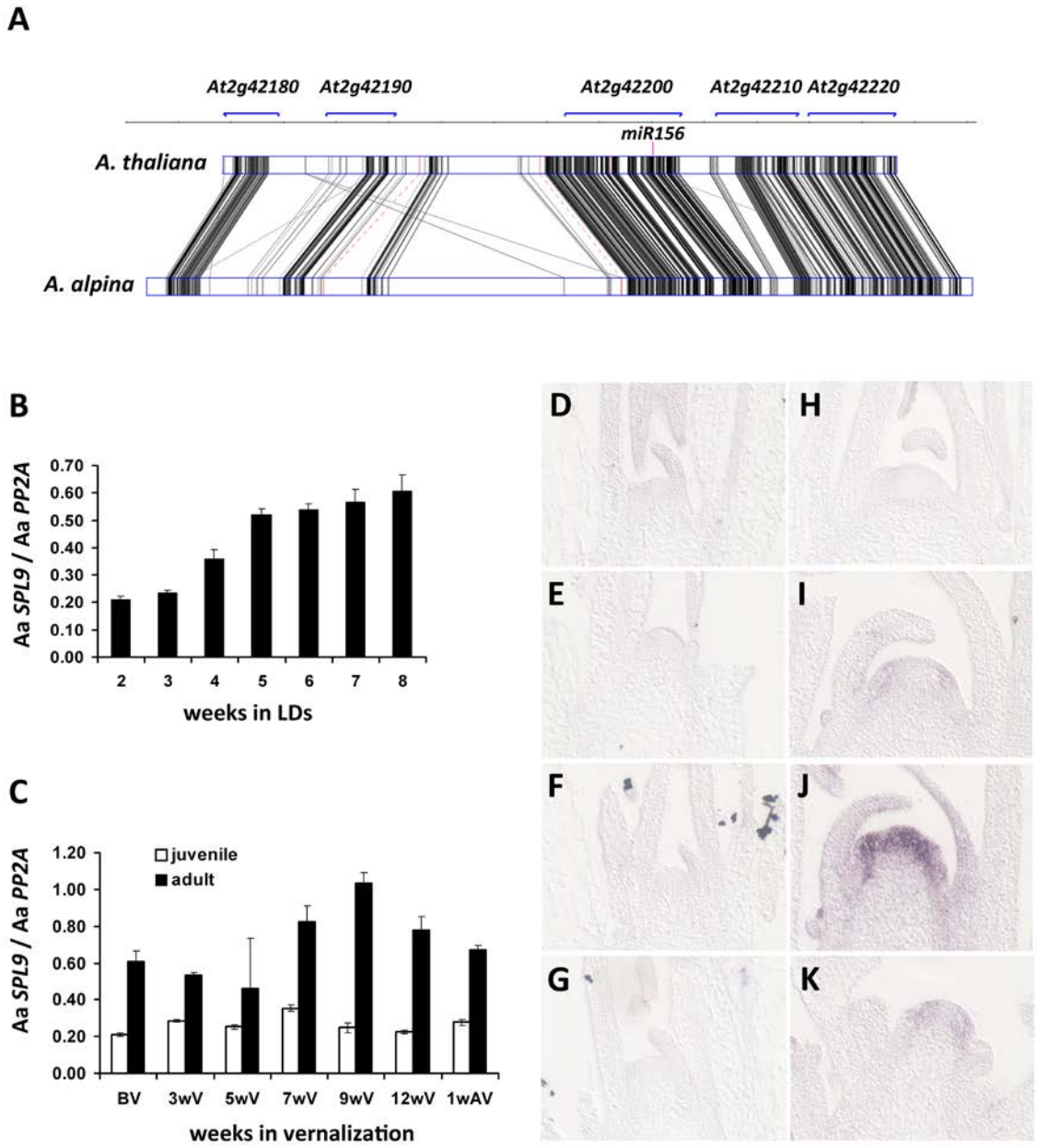

Figure 10. Aa *SPL9*: synteny conservation and expression analysis

(A) The alignment shows the synteny conservation between the genomic regions surrounding the *A. thaliana* (upper bar) and *A. alpina* (lower bar) *SPL9* loci. *At2g42200* is At *SPL9*. *At2g42180* and *At2g42190* are the proximal upstream genes in *A. thaliana*; *At2g42210* and *At2g42220* are the proximal downstream genes in *A. thaliana*. Blue lines below the gene names indicate the extent of the genes and the gene orientation. miR156 target site position is shown in pink. Lines between the two bars show conservation and rearrangement of the sequences. Black lines show conservation, the darker the colour the higher the conservation. Red lines show sequence inversion. (B) Aa *SPL9* qRT-PCR on the main apices of plants grown in LDs 20°C from 2 to 8 weeks. x-axis, weeks of growth in LDs; y-axis, relative expression to Aa *PP2A* mRNA. Error bars, technical replicates. (C) *SPL9* qRT-PCR on the main apices of plants transferred to vernalization. BV, before vernalization; 3wV to 12wV, number of weeks spent in vernalization before the harvesting; 1wAV, 1 week in LD after 12 weeks in vernalization. White histograms, juvenile apices (BV= 2 weeks), black histograms, adult apices (BV= 8 weeks). y-axis, relative expression to Aa *PP2A*. Error bars, technical replicates. (D to K) *SPL9* mRNA *in situ* hybridization of main apices. (D to G) juvenile apices, (H to K) adult apices. (D and H) before vernalization; (E and I) after 3 weeks in vernalization; (F and J) after 5 weeks in vernalization; (G and K) after 12 weeks in vernalization.

vernalization (Fig.10J and10K). Similar to qRT-PCR results, Aa *SPL9* mRNA pattern did not change in juvenile apices during vernalization (Fig.10E, 10F and 10G).

To conclude, expression analysis experiments showed that the increase of Aa *SPL9* mRNA observed by qRT-PCR before vernalization correlates with the timing of maturation in *A. alpina*, although the mRNA could not be detected by *in situ* hybridization at this time. Moreover, the levels of Aa *SPL9* mRNA in juvenile apices do not change during vernalization in agreement with the plants not being able to flower when exposed to the inductive stimuli. In adult apices, upon vernalization Aa *SPL9* mRNA increases again in the meristem shortly after the plant is induced to flower, suggesting a possible role for Aa *SPL9* in flowering.

4.7 Overexpression of Aa *rSPL9* in *A. alpina* causes early flowering

Aa *SPL9* mRNA levels change when plants acquire competence to flower. In *A. thaliana*, overexpression of At *SPL9* causes a very pleiotropic phenotype, inducing early flowering, shortening of the plastochron length and promotion of adult leaf characters (Wang et al., 2009a; Wang et al., 2008). Moreover, the *A. thaliana spl9 spl15* double mutant is less responsive to inductive photoperiod *(Schwarz et al., 2008)*. Flowering of *A. thaliana* is accelerated in response to vernalization from the seed stage whereas *A. alpina* needs to be older than 4 weeks in order to flower in response to cold treatment (Chandler and Dean, 1994; Koornneef et al., 1991; Wang et al., 2011b). To understand the role of Aa *SPL9* in the regulation of reproductive competence of *A. alpina*, Aa *SPL9* was overexpressed in *A. alpina*. Aa *SPL9* has a conserved miR156 target site indicating that this gene might be negatively regulated by the miRNA. Therefore, in order to obtain overexpression *in planta*, the miR156 target site was mutated at the nucleotide level, without changing the amino acid sequence and therefore preserving protein activity (Fig.11A) (Wang et al., 2008). miR56-resistant (Aa *rSPL9*) and wild-type (Aa *SPL9*) cDNA sequences driven by the 35S CaMV promoter were transformed into *A. alpina* plants carrying active or inactive alleles of *PEP1*. So far, one BASTA resistant line was recovered in the *pep1* background. The primary transformant *35S:Aa rSPL9 pep1* flowered in LDs with 10 leaves whereas *A. alpina pep1* flowers normally with about 23 leaves (Fig.11B) (Wang et al., 2009b). This plant showed additional vegetative phenotypes. All the leaves produced before flowering were small, serrated and lacked petioles, strongly resembling cauline leaves. Taken together, vegetative and reproductive phenotypes suggest that *Aa SPL9* promotes flowering in *A. alpina*. Nevertheless this result is still preliminary and more transgenic lines are being selected in order to confirm the phenotype. In

addition, obtaining *35S:Aa rSPL9* lines in the *PEP1* background would test the effect of the transgene on response to vernalization. Finally, comparison of *35S:Aa rSPL9* lines with those overexpressing the miR156 susceptible cDNA could provide an experimental indication of the effect of miR156 regulation of Aa *SPL9*.

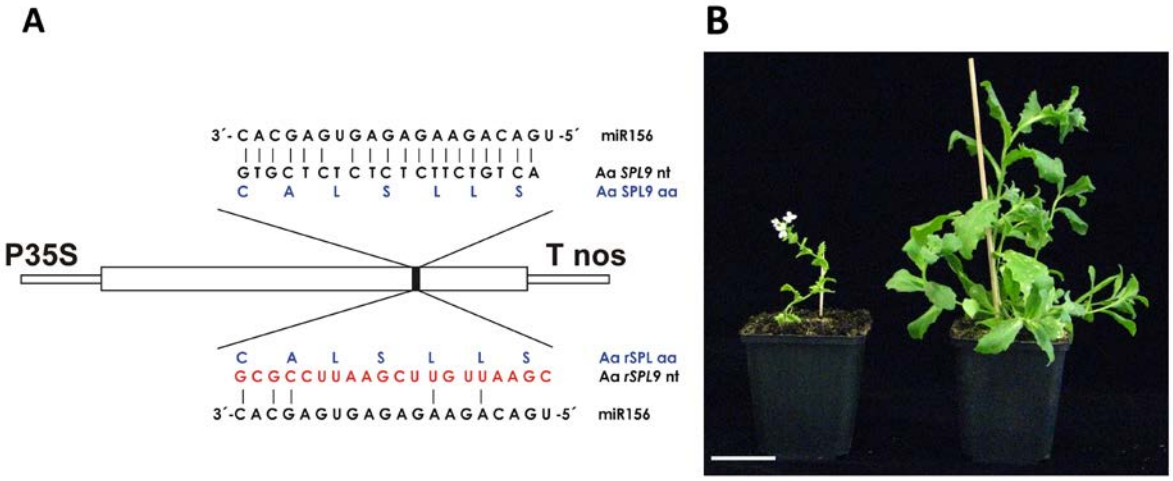

Figure 11. Overexpression of Aa *rSPL9* in *A. alpina*

(A) Constructs to overexpress Aa *rSPL9* into *A. alpina*. Aa *SPL* cDNAs with susceptible wild-type (upper sequence) or resistant mutant (lower red sequence) miR156 target sites were introduced into a vector driven by the 35S CaMV promoter and transformed into plants. (B) *35S:Aa rSPL9 pep1* (left) and *pep1* mutant (right). Scale bar = 5 cm.

4.8 Identification of *MIR156* genes in *A. alpina*

In *A. thaliana, SPL9* is post-transcriptionally regulated by miR156 (Rhoades et al., 2002; Schwab et al., 2005; Wang et al., 2008). Moreover, microarray analysis showed that additional miR156-targeted Aa *SPLs* were differently expressed between juvenile and adult apices (Fig.7A, Table II in the Appendix). Therefore, the involvement of miRNAs in the regulation of Aa *SPL9* expression and/or reproductive competence was tested. miRNAs are classes of small RNAs (20-22 nt long) that are generally encoded by several *MIR* genes found in different regions of the genome as well as in intronic areas. For the biogenesis of the mature miRNA, *MIR* genes are first transcribed by RNA Polymerase II enzyme into long single stranded precursors (pri-miRNAs). Pri-miRNAs can fold to form a hairpin and they are successively cleaved by a dicer-like enzyme (DCLs) to a pre-miRNAs. The pre-miRNA hairpin structures are important as they enable cleavage to a short double stranded miRNA

duplex. Finally, mature single stranded miRNAs are incorporated in the RNA-induced silencing complex (RISC) and guided to negatively regulate gene expression by inhibiting translation or cleavage (Voinnet, 2009).

miR156 is one of the most conserved and evolutionarily old miRNAs in plants (Axtell and Bowman, 2008; Poethig, 2009; Zhang et al., 2006). In *A. thaliana*, miR156 is encoded by 8 *MIR156* genes (*MIR156* a to h). Moreover, in *A. alpina* the miR156-target site in the *SPL* genes show high sequence similarity to *A. thaliana*, suggesting that the mature form of miR156 is most probably conserved as well. A computational approach was used to identify *A. alpina* miR156 precursors making use of the known *A. alpina* genome sequence and of the miR156 sequences publicly available in the miRBase database (http://mirbase.org/). Homologues of all eight *A. thaliana MIR156* genes were found in *A. alpina*, for which nucleotide sequences and synteny were conserved. Studies in *A. thaliana* showed that miRNA processing is strictly dependent on the secondary structure of the precursors (Mateos et al., 2010; Song et al., 2010; Werner et al., 2010). When folded to form a secondary structure, *A. alpina* pre-miRNAs showed hairpin loops some of them very similar in shape to the *A. thaliana* homologues (Fig.12). In *A. thaliana* experimental evidence for the processing of six pre-miR156 RNAs to a mature active miR156 have been reported (Wu and Poethig, 2006). This evidence, together with the high similarity observed between *A. alpina* and *A. thaliana* pre-miR156, suggests that the *A. alpina* homologues are likely to be similarly processed by the miRNA machinery. Moreover, in the stems of the pre-miR156 hairpins it is possible to detect the highly conserved double stranded miRNA duplexes and the mature miR156 (Fig.12 sequences highlighted in red).

These analyses show that the eight *MIR156* genes known in *A. thaliana* to encode for miR156 precursors are conserved in *A. alpina*. No duplications of these genes have been identified in the *A. alpina* genome. Nevertheless, when initially searching the genome using the mature miR156 sequences additional hits were identified that respond to the criteria used and described in the literature (Bonnet et al., 2004; Zhang et al., 2006). More experiments need to be performed to understand if these sequences encode additional pre-miR156s processed by the miRNA machinery to form mature miR156.

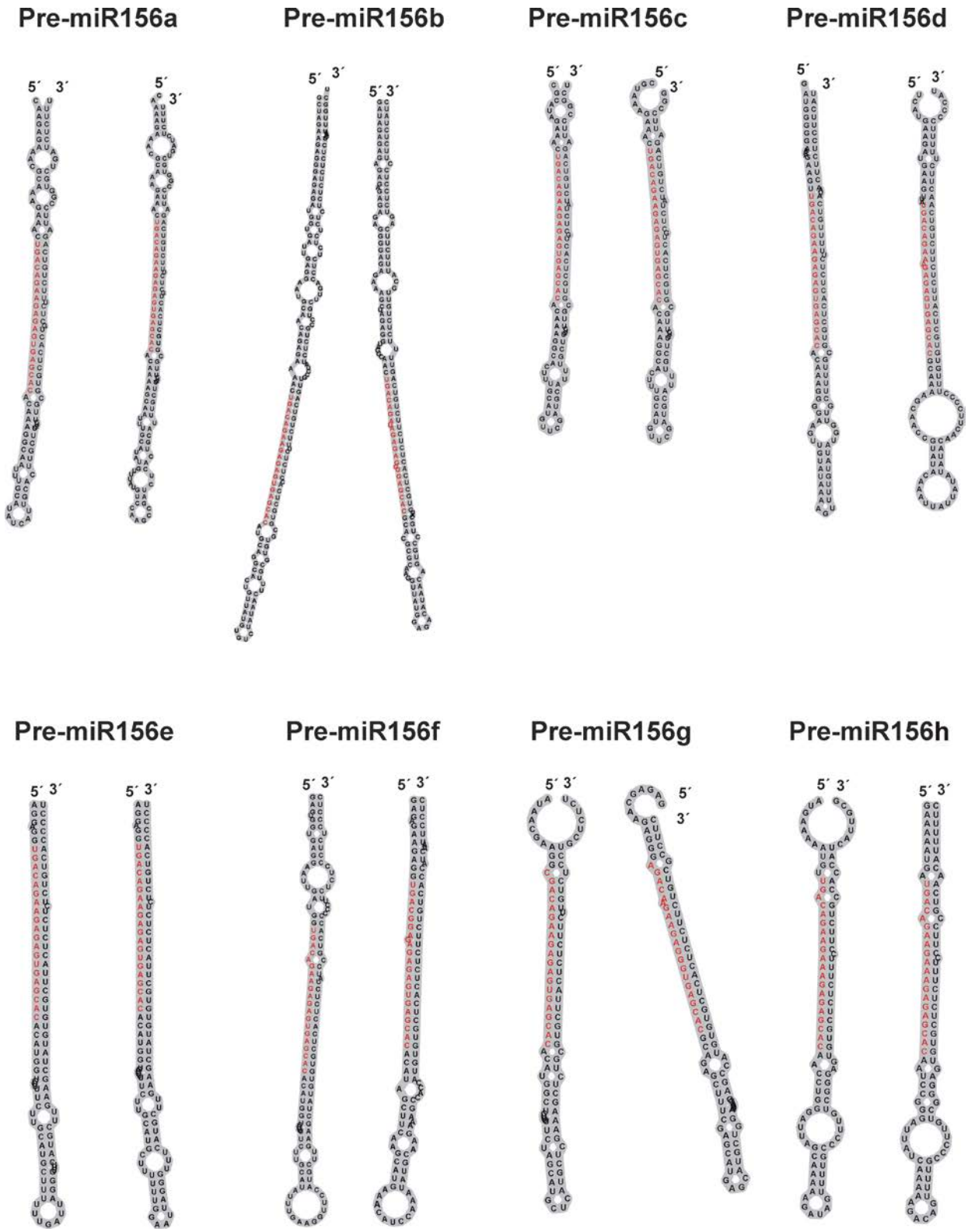

Figure 12. Secondary structure prediction of *A. thaliana* and *A. alpina* miR156 precursors (pre-miR156)

The software RNA fold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to predict the hairpin structure of *A. thaliana* and *A. alpina* pre-miR156s. For every pre-miR156 (a to h) the *A. thaliana* precursor is shown on the left side and the *A. alpina* on the right. Red letters highlight the mature miR156.

4.9 miR156 expression in apices of *A. alpina* inversely correlates with Aa *SPL9* expression pattern and with the acquisition of reproductive competence

In order to investigate the role of miR156 in Aa *SPL9* regulation and in the regulation of reproductive competence the same time courses used for the detection of Aa *SPL9* were used to quantify miR156 levels. A qRT-PCR technique specifically designed for small RNA detection was applied (see methods) (Yang et al., 2009). miR156 levels changed during development when plants were grown in LDs at 20°C for several weeks. miR156 was highly expressed at early stages of development and decreased when plants grew older (Fig.13A). miR156 expression dropped of about 12 folds after 3 weeks after sowing and reached trough levels at about 5 weeks in LDs. The temporal expression profile of miR156 during development inversely correlates with the expression profile of Aa *SPL9,* indicating that Aa *SPL9* might be a miR156 target (Fig.10B). In order to investigate the possible role of miR156 in regulating flowering, its expression levels were tested in a time course of juvenile and adult plants shifted to vernalization for 12 weeks. In main shoot apices of adult plants, miR156 expression was low before vernalization and did not change during the entire cold treatment (Fig.13B, black histograms). Interestingly, in juvenile apices the high miR156 levels observed before vernalization stayed high throughout three months of vernalization, which is sufficient to induce flowering of adult plants (Fig.13B, white histograms). miR156 expression levels observed by the end of vernalization correlate with the floral fate of the meristems. Apices of juvenile plants with high miR156 expression levels are still vegetative at the end of vernalization (Fig.13B) (Wang et al., 2011b). Apices from adult plants, with low miR156 expression and, by the end of vernalization, repressed *PEP1* expression, are flowering (Fig.13B)(Wang et al., 2011b; Wang et al., 2009b).

Once juvenile vegetative plants were returned to normal growing conditions after three months in vernalization, the decrease of miR156 expression was restored (Fig.13B V+1/2/3wAV). This decrease was comparable to what was observed in the growth of plants in LDs before vernalization, suggesting that vernalization prevents the downregulation of miR156 accumulation (Fig.13A). Several individuals from juvenile plants grown after vernalization in LDs for 1, 2 or 3 weeks were vernalized a second time and tested for flowering. Only plants grown for 3 weeks in LDs after the first cold treatment flowered when exposed to the second vernalization period (data not shown). This result shows that the flowering response again correlates with the miR156 levels reached before the second vernalization treatment.

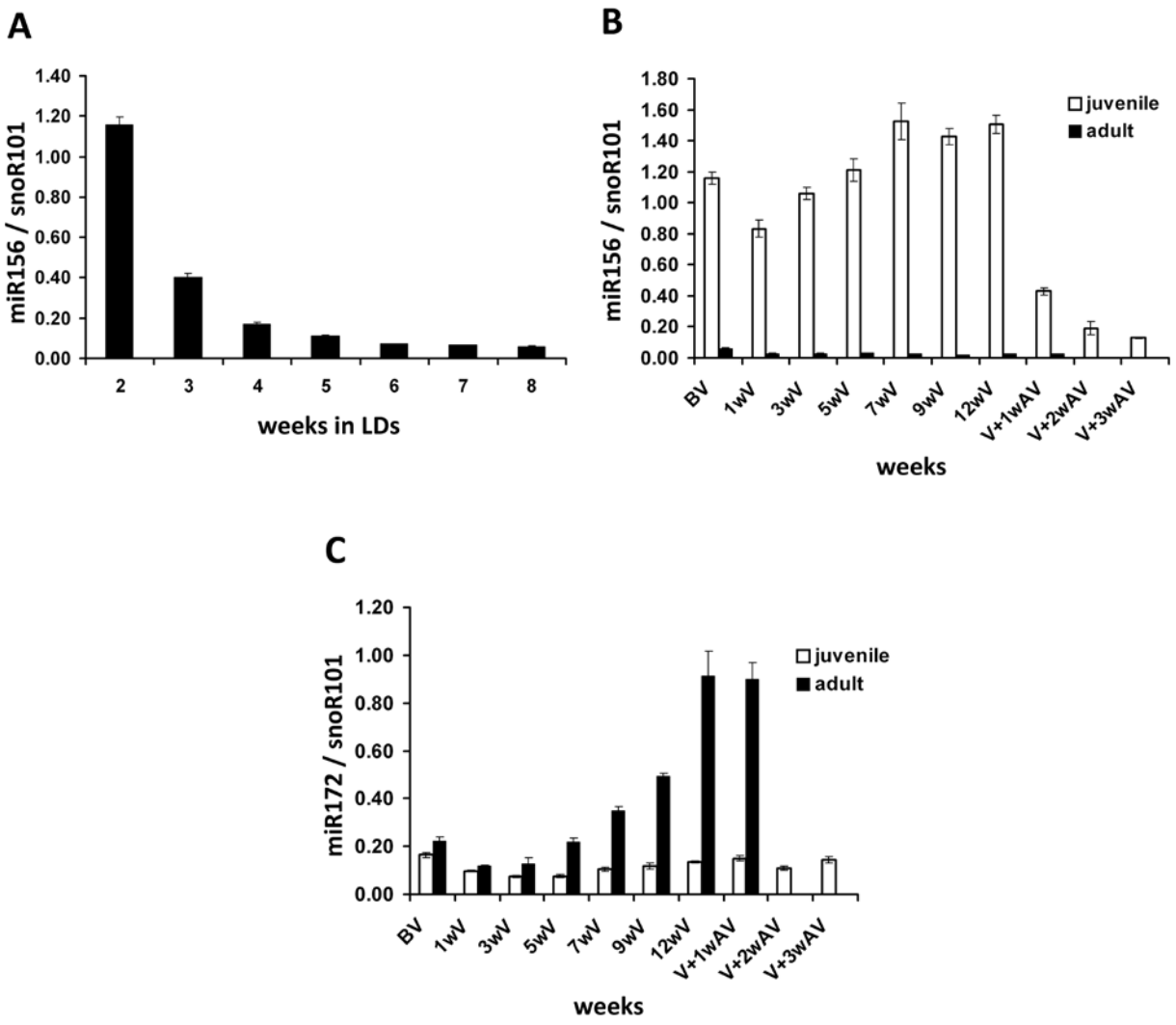

Figure 13. Expression of miR156 and miR172 during development and in vernalization

miR156 expression in apices (A and B). (A) Expression during development in LDs and 20°C conditions. x-axis, weekly harvest. y-axis, relative expression (B) Expression in main apices of plants shifted to vernalization. BV, before vernalization; 3wV to 12wV, number of weeks spent in vernalization before the harvesting; V+1wAV/2wAV/3wAV, 1/2/3 weeks in LD after 12 weeks in vernalization. White histograms, juvenile apices (BV= 2 weeks), black histograms, adult apices (BV= 8 weeks). (C) miR172 expression in apices. The rest as in B. Expression levels normalized to snoR101. Error bars, technical replicates.

4.10 miR172 expression in apices of *A. alpina* correlates with floral development

During development of Arabidopsis, maize and rice plants the decrease of miR156 levels is complementary to an increase of miR172 levels, so that the latter increase as the plant age (Chuck et al., 2007; Lauter et al., 2005; Tanaka et al., 2011; Wu et al., 2009). In addition, in *A. thaliana* At SPL9 regulates the expression of miR172 by directly binding to the promoter of *MIR172b* locus (Wu et al., 2009). In order to understand if the same regulatory module is

conserved in *A. alpina* the expression levels of miR172 were analysed by qRT-PCR before and during vernalization in the same time course used for miR156 and Aa *SPL9* expression. Surprisingly, miR172 levels were similar between juvenile and adult plants and remained at similar levels even when plants were grown for several months in LDs (Fig.13C BV). During vernalization, miR172 expression was upregulated only in adult plants induced to flower (Fig.13C). These data suggest that in *A. alpina* miR172 plays a role only upon induction of flower development but not in the regulation of the age pathway as it was proposed for *A. thaliana*. In addition, the data also suggest that during development Aa *SPL9* expression is not sufficient to activate expression of miR172.

4.11 Levels of miR156 correlate with age-related leaf traits

In *A. thaliana* and other species miR156 is expressed in several tissues including leaves. Its role has been largely characterized in those organs where miR156 is necessary for the expression of juvenile leaf characters (Chuck et al., 2007; Schwab et al., 2005; Wang et al., 2011a). Age-related leaf characters vary according to the species and in *A. alpina* the heteroblastic changes occurring during development have not been characterized. For example, in *A. thaliana* the presence of trichomes on the abaxial side of the leaf blade is the most frequently used trait to distinguish between juvenile and adult leaves (Chien and Sussex, 1996; Telfer et al., 1997). In *A. alpina,* abaxial leaf trichomes are present on all leaves. However, leaf shape and size visibly change during development also in *A. alpina*. Leaves produced at the beginning of development are smaller and round (Fig.14A, node 1 and 2). Leaves produced later in time are more elongated and exhibit increasing serration (Fig.14A).

The level of miR156 in leaves collected at different nodes in plants growing in LDs for 5 weeks was characterized to assess whether this correlates with leaf morphology. miR156 levels were high in lower leaves, produced early in development, and decreases in leaves produced later at higher nodes (Fig.14B). As observed for other species, high expression of miR156 correlates with juvenile leaf traits and, in *A. alpina,* with reduced serration and size. The expression of miR156 was also measured in leaves of plants growing in vernalization. Although the rate of leaf initiation was much lower than for plants grown at 20°C, differences in leaf shape could still be observed among leaves on the same plant (Fig.14C). Moreover, leaves growing in vernalization showed changes in leaf traits which also correlated with changes in miR156 expression (Fig.14C). This observation supports the idea that juvenile

plants undergo the progression to adult in vernalization, but that the process progresses much more slowly. Whether changes in leaf shape are directly linked to changes in meristem

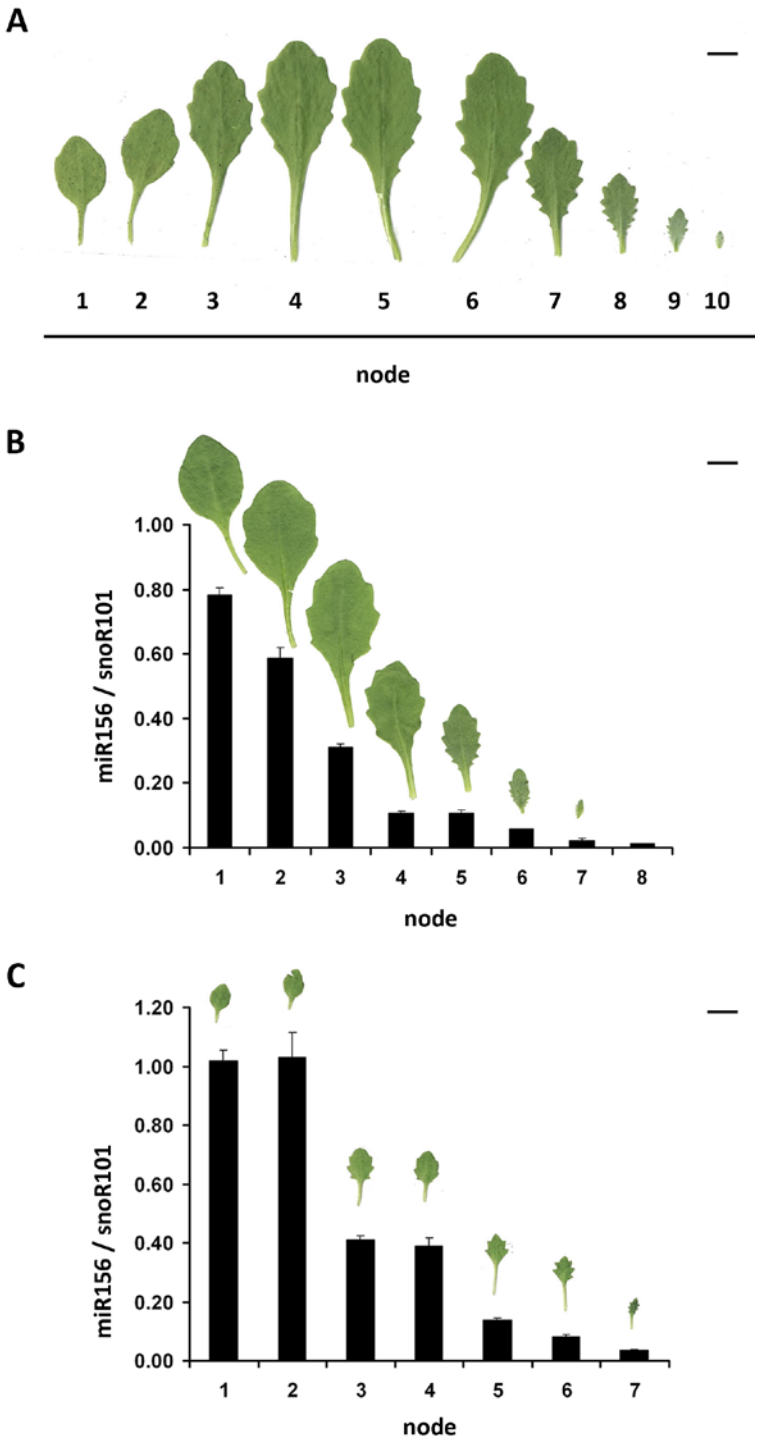

Figure 14. Heteroblasty in *A. alpina* and miR156 expression in leaves

(A) Appearance of *A. alpina* leaves produced at different nodes in a plant grown in LDs for 5 weeks. Leaf at node 1 is the first true leaf after cotyledons. Leaf at node 10 is the last produced at the time of harvesting. At the time of harvesting, most of the leaves were not yet fully expanded. (B and C) miR156 expression in leaves at different nodes. Above the histogram is shown the shape of the collected leaves (B) Plants grown for 4 weeks in LDs, 20°C. x-axis, node number according to the temporal appearance; y-axis, relative

expression to snoR101 (C) Plants grown for 2 weeks in LDs, 20°C then transferred in vernalization for 23 weeks. x-axis and y-axis as in (B). Error bars, technical replicates. Scale bars = 1 cm.

competence is not clear, but leaf traits could be used as morphological markers to track phenotypically the levels of expression of miR156 in transgenic plants (Poethig, 2009). In the case of *A. alpina*, leaf serration is the most evident trait whose changes correlate with miR156 levels.

4.12 *A. alpina* plants overexpressing *MIR156* strongly repress flowering and show prolonged production of leaves with juvenile traits

A *35S:MIR156b* construct was introduced into *A. alpina* using *A. tumefaciens* to investigate the biological function of miR156 in *A. alpina*. The *35S:MIR156b* construct, which was already used to overexpress miR156 in *A. thaliana,* was transformed in *A. alpina* Pajares plants carrying active or inactive alleles of *PEP1 (Schwab et al., 2005)*. Six independent transgenic lines were recovered in the *PEP1* background. All the lines showed very pleiotropic phenotypes including prolonged production of leaves with juvenile features (Fig.15A and 15C). Transgenic plants produced even at very late nodes, only small and round leaves that showed very mild serration of the blade (Fig.15A, 15B and 15C). These leaves strongly resemble the juvenile leaves produced during early development of wild-type plants (Fig.14A, nodes 1, 2 and 3). Similar to what has been reported in *A. thaliana,* maize, rice and tomato these lines showed also additional phenotypes such as increase in branching and leaf number that gave the plant a characteristic bushy phenotype (Chuck et al., 2007; Schwab et al., 2005; Xie et al., 2006; Zhang et al., 2011).

The active allele of *PEP1* represses flowering in warm temperatures, therefore in order to test the ability of these transgenic lines to flower, wild-type control plants and transgenic lines were grown for 10 weeks in LDs and then vernalized for 3 months. As expected, vernalization induced flowering of all of the control plants. Interestingly, none of the six lines carrying the transgene flowered after vernalization, indicating that miR156 represses flowering of *A. alpina* (not shown).

In *A. thaliana,* plants overexpressing *MIR156b* are extremely late flowering when grown in SDs but flowering is only mildly delayed when they are grown in LDs (Schwab et al., 2005). Moreover, photoperiod induces expression of members of the *SPL* gene family, promoting flowering (Lal et al., 2011; Wang et al., 2009a). Based on these observations *A.*

alpina transgenic lines were vernalized for 20 weeks in LDs instead of the standard SD conditions. Comparable vegetative propagated branches obtained from the same mother T1

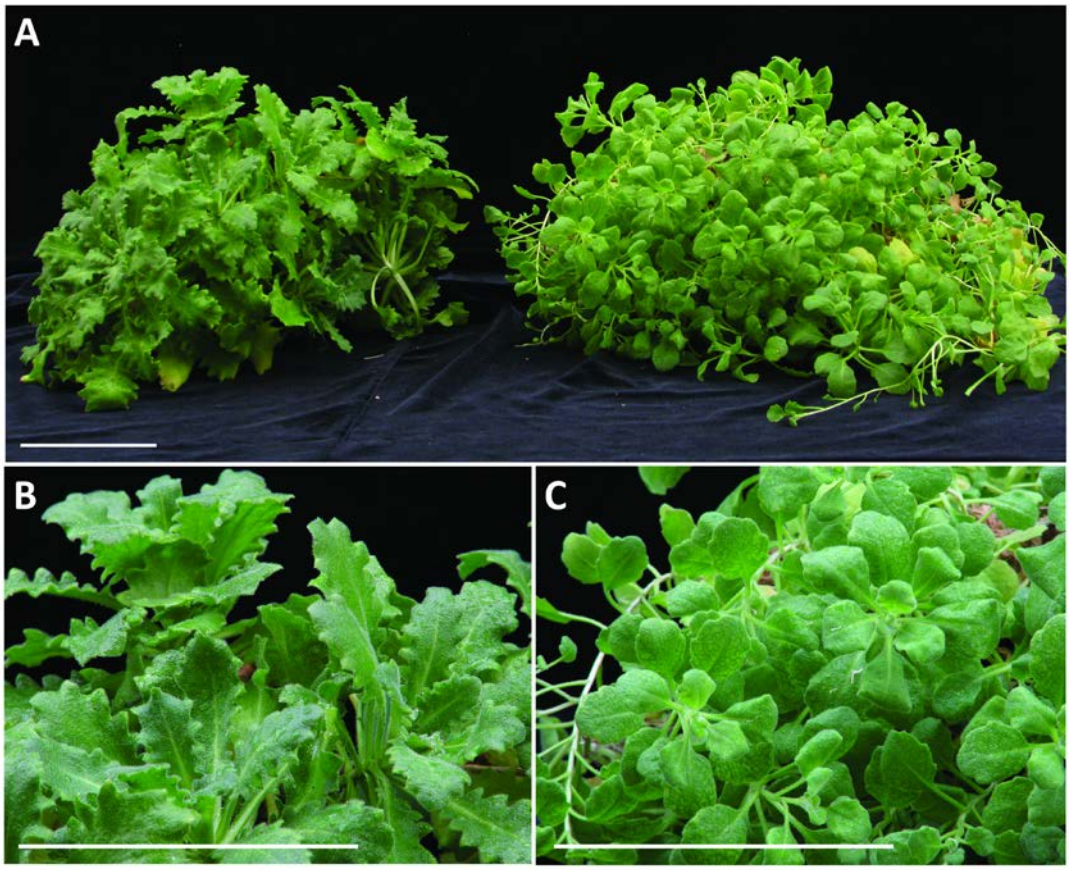

Figure 15. Vegetative phenotype of *35S:MIR156b PEP1*

(A to C) Phenotype of *35S:MIR156b A. alpina PEP1* plants. (A) Comparison of *A. alpina PEP1* (left) and *35S:MIR156b PEP1* grown for 6 months in LDs. (B) Close up of the *A. alpina PEP1* leaves. (C) Close up of the *35S:MIR156b PEP1* leaves. Scale bars =10 cm

plants were vernalized in SDs and LDs and then compared for flowering. Clones vernalized in LDs grew much more than those in SDs but, when returned to normal temperature again, none of them flowered (not shown).

The plant hormone gibberellin promotes flowering in *A. thaliana* in the absence of inductive stimuli (Moon et al., 2003b). Moreover, it also plays a role in the promotion of adult leaf traits in *A. thaliana* and maize indicating that it might regulate shoot maturation (Chien and Sussex, 1996; Evans and Poethig, 1995; Telfer et al., 1997). In *A. alpina*, application of exogenous GAs did not induced flowering in juvenile plants (Wang, 2007). This indicates that low GA levels are not responsible for the juvenile incompetence to flower in response to

floral stimuli. However, we treated the transgenic lines with exogenous GA to test whether this would induce flowering through other flowering pathways. Four *35S:MIR156b PEP1* T1 lines (#14, #12, #9 and #6) and a control wild-type plant were vegetatively propagated in four clones each. The clones were grown for 7 weeks (with the exception of the clones from line #6, grown in LDs for 11 weeks) and then vernalized for 16 weeks. During the last two months in vernalization 2 clones for each T1 original plant (and wild-type) were treated with 100 μM $GA_{3,}$ while the other 2 clones (and wild-type) were mock treated as controls. When returned to warm temperatures, all the wild-type clones flowered while none of the clones from the transgenic lines underwent the reproductive transition regardless of the treatment received during vernalization (data not shown). Taken together these results show that neither long photoperiod, nor application of bioactive GA can induce flowering in *PEP1* plants overexpressing *MIR156b*, indicating that miR156 is a strong repressor of flowering in *A. alpina*.

35S:MIR156b construct was introduced to *A. alpina pep1* background, which does not need vernalization to flower (Wang et al., 2009b). Several transgenic lines were recovered, all of them displaying strong vegetative phenotypes as observed in the *PEP1* background (Fig.16A and 16B). *A. alpina pep1* plants flower without vernalization after 2-3 months in LDs and with about 23 leaves (Wang et al., 2009b) (Fig.16A, left side). The transgenic lines did not flower after 3 months (Fig.16A, right side). However, when grown in LDs for several months (6-7 months), some side shoots eventually flowered and set seeds normally but the majority still grew vegetatively (Fig.16C and 16D). *A. alpina pep1* flowers without vernalization making characterization of its juvenile phase difficult. However, when miR56 and miR172 levels were tested in *A. alpina pep1* they showed the same temporal pattern as in *PEP1* plants, suggesting that the maturation programme operates in *pep1* plants (Fig16E and 16F; Fig.13A and 13C).

Taken together, the results obtained by overexpressing *35S:MIR156b* in *A. alpina*, indicate a conserved role for miR156 in the regulation of heteroblasty through evolution. Moreover miR156 strongly represses flowering in *A. alpina* in both *PEP1* and *pep1* genetic backgrounds. Interestingly, differently from *A. thaliana*, growth in LDs does not seem to accelerate flowering in the *A. alpina* transgenic lines, indicating that photoperiod might have a more important role in floral induction in *A. thaliana* than it has in *A. alpina*. None of the conditions tested so far were successful to strongly accelerate flowering in *35S:MIR156b* plants making it difficult to test reproductive competence in these plants. To overcome this problem, *A. alpina* plants have also been transformed with the miR156 target mimicry

construct that may induce early flower by blocking miR156 activity (Franco-Zorrilla et al., 2007).

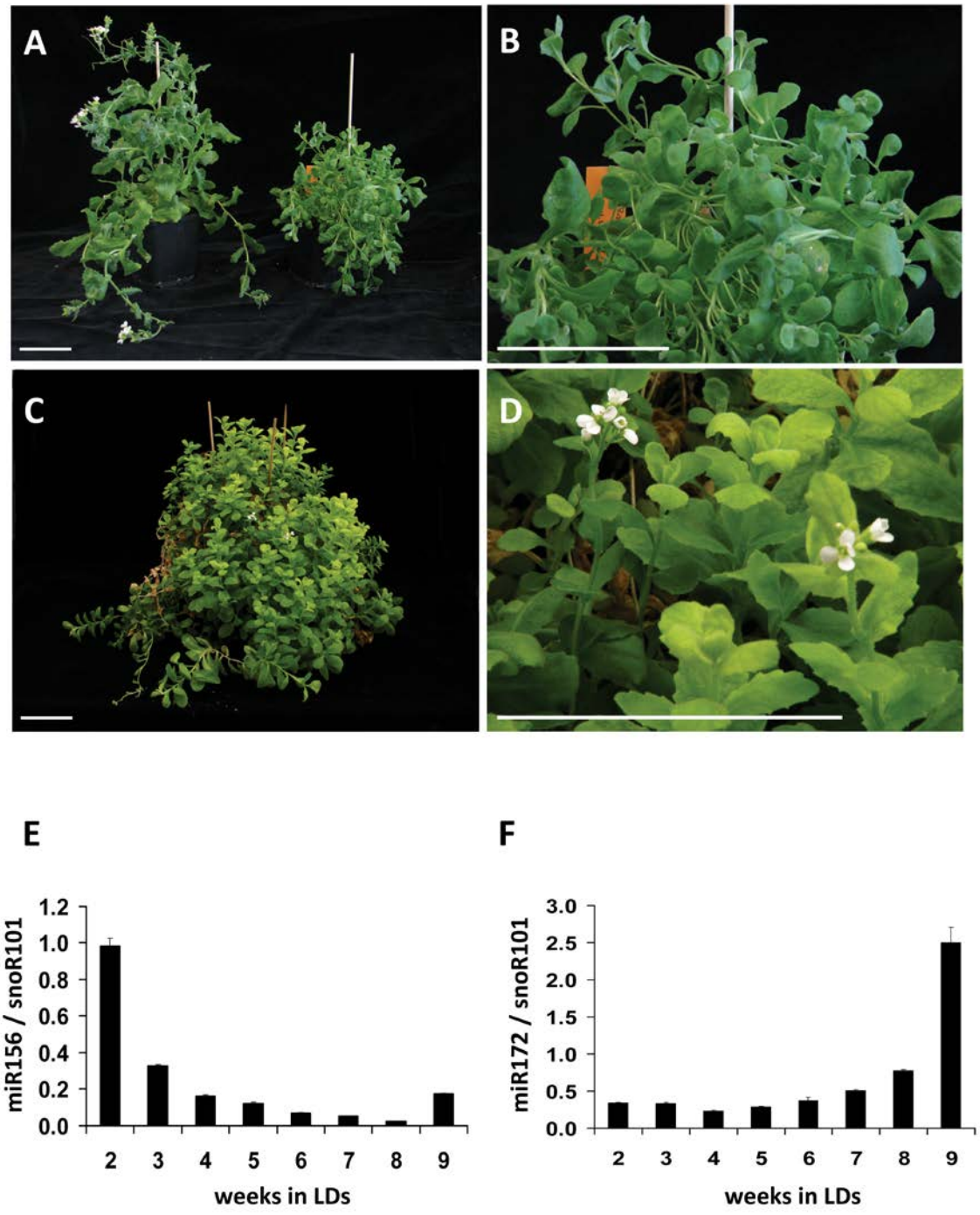

Figure 16. Vegetative phenotype of *35S:MIR156b pep1* plants and miRNA expressions in *A. alpina pep1*

(A to D) Phenotype of *35S:MIR156b* in *A. alpina pep1*. (A) Comparison between *A. alpina pep1* (left) and *35S:MIR156b pep1* grown for 3 months in LDs. *A. alpina pep1* has flowered. Siliques are already formed. *35S:MIR156b pep1* is still growing vegetatively. (B) Close up of the *35S:MIR156b pep1* leaves. (C) *35S:MIR156b pep1* grown for 6 months in LDs. A few side shoots are forming flowers. (D) Close up of the flowers in the *35S:MIR156b pep1* plants. Scale bars = 7 cm. (E and F) qRT-PCR expression data for miR156 and miR172 in *A. alpina pep1*. x-axis, weeks in LDs; y-axis, relative expression to snoR101. Error

bars indicate technical replicates. (E) Expression of miR156 during development in LDs. (F) Expression of miR172 during development in LDs. At 9 weeks in LDs *A. alpina pep1* starts to flower based on production of visible floral buds.

These transgenic lines are expected to contribute to the understanding of miR156 role in *A. alpina* development and floral induction.

4.13 miR156 levels in main shoot apices of *A. thaliana* young plants do not change in vernalization

During the three months necessary to saturate the vernalization requirement of *A. alpina* Pajares, miR156 levels did not change (Fig.13B). Especially in juvenile plants exposed to vernalization, miR156 levels stayed high preventing juvenile plants from flowering after vernalization. However, although plants grow more slowly in vernalization, by the end of the three months juvenile plants did produce a few new leaves suggesting that their development does progress in the cold (data not shown). Moreover, once plants were returned to normal growth conditions a decrease of miR156 occurred immediately (Fig.13B, AV). Taken together, these results demonstrate that growth temperature has a role in the control of miR156 decrease during development.

Studies in *A. thaliana* and other species have not focused on the relationship between temperature and miR156 regulation during development. Therefore, in order to understand whether the observations made in *A. alpina* can be extended to *A. thaliana,* a similar vernalization experiment was performed using *A. thaliana* acc. Columbia. *A. thaliana* plants were grown at 20°C in SDs to avoid the effect of photoperiodic induction and to be more comparable with published experiments (Wang et al., 2009a; Wu et al., 2009). Two weeks after sowing half of the seedlings were transferred to vernalization, while the others were kept at 20°C. Weekly harvest was performed until week 9, when *A. thaliana* plants growing at 20°C bolted. In the apices of plants growing at 20°C, miR156 levels gradually decreased as previously reported (Fig.17A, black histograms) ((Wang et al., 2009a; Wu et al., 2009; Wu and Poethig, 2006). However, when young seedlings, with high levels of miR156, were shifted to grow at 4°C, the levels of miR156 did not change (Fig.17A, white histograms). This experiment showed that miR156 levels do not change when plants are grown at 4 °C for 7 weeks, and this effect is common to *A. alpina* and *A. thaliana*.

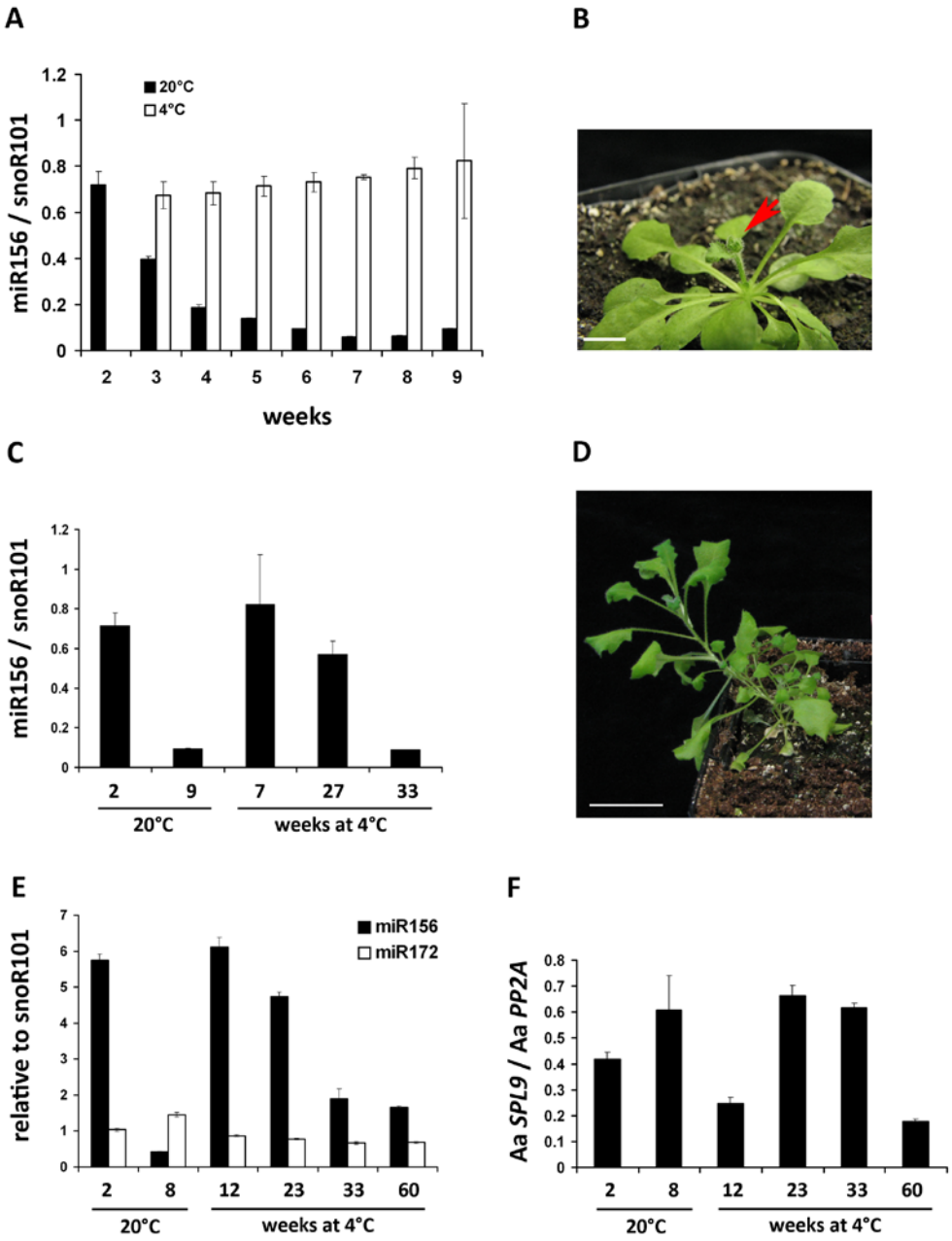

Figure 17. Growth of *A. thaliana* and *A. alpina* plants at 4 °C

(A to C) Experiments conducted in *A. thaliana* acc. Columbia. (A) miR156 levels in apices of *A. thaliana* plants grown in SD 20°C until flowering (black histograms) or shifted to grow in vernalization after 2 weeks at 20°C (white histograms). x-axis, weeks of growth. (B) *A. thaliana* plant bolting after 33 weeks in vernalization. The red arrow points to the inflorescence and floral buds. Scale bar = 1 cm (C) miR156 levels in apices of *A. thaliana* plants shifted to vernalization after two weeks at 20°C and grown at 4°C until bolting. On the x-axis, 2 and 9 are the time points of plants growing at 20°C. 7, 27 and 33 are the time points in vernalization after growth for 2 weeks at 20°C. Plants growing at 20°C for 9 weeks or at 4°C for 33 weeks were bolting. (D to F) Experiments conducted in *A. alpina*. (D) *A. alpina* plant grown in vernalization for 60 weeks. This plant is still growing vegetatively. Scale bar = 2 cm. (E) miR156 (black histograms) and miR172 (white histograms) levels in apices of *A. alpina* plants shifted to vernalization after 2 weeks at 20°C and grown at 4°C for up to 60 weeks. On the x-axis, 2 and 8 are the weeks at 20°C, 12, 23, 33 and 60 are the weeks in vernalization after growth for 2 weeks at 20°C. (F) Aa *SPL9* mRNA expression. x-axis as in (E). In all the qRT-PCR experiments, miRNA levels were normalized to snoR101. Aa *SPL9* mRNA was normalized to Aa *PP2A* mRNA. Error bars indicate technical replicates.

4.14 The decrease of miR156 levels in *A. thaliana* and *A. alpina* occurs slowly when plants are grown at 4°C for over 20 weeks

By the end of the vernalization treatment, 12 weeks for *A. alpina* or 7 weeks for *A. thaliana*, miR156 levels in main shoot apices of young seedlings were as high as they were before vernalization (Fig.13B and 17A). To understand if the decrease of miR156 is blocked during vernalization or if it is only occurring at a lower rate at low temperatures, *A. alpina* and *A. thaliana* plants were grown in vernalization for longer and the main shoot apices tested for miR156 expression. *A. thaliana* plants bolted in vernalization after 33 weeks at 4°C (Fig.17B). miR156 levels were also reduced in these plants after 27 weeks and eventually dropped to similar levels as in plants grown for 9 weeks in LD (Fig.17C).

Unlike *A. thaliana, A. alpina* plants were grown at 4°C for more than 15 months (60 weeks) but never flowered (Fig.17D). Nevertheless, *A. alpina* juvenile plants slowly developed in vernalization producing by the end of the 60 weeks more than 20 leaves (Fig.17D). Similarly to *A. thaliana,* miR156 levels started to fall after 23 weeks in vernalization and stabilized after 33 weeks (Fig17.E, black histograms). During the 60 weeks of growth in vernalization miR156 expression never reached the low levels observed in adult shoot apices. This could explain why vegetative growth was maintained when vernalized juvenile plants were returned to normal growing conditions after 33 or 60 weeks at 4°C (not shown). Moreover, miR172 levels did not change during vernalization of these plants, consistent with the fact that plants failed to initiate

flowering (Fig.17E, white histograms). This result again shows that in *A. alpina* the increase in expression of miR172 does not directly follow the decrease in miR156 expression, in contrast to what was described in *A. thaliana (Wu et al., 2009)*.

Aa *SPL9* transcription in main shoot apices of plants vernalized for 60 weeks was also tested. As previously observed, the low expression of Aa *SPL9* mRNA in juvenile apices did not increase during the first 3 months of cold treatment (Fig.10C and 17F). A first increase in Aa *SPL9* transcription was observed after 23 weeks in vernalization and maintained after 33 weeks. In these samples Aa *SPL9* mRNA levels were as high as in adult plants before vernalization but when some individuals were moved to normal growth conditions flowering did not occur (not shown). Surprisingly, in the last vernalized sample, Aa *SPL9* mRNA levels were even lower than in juvenile plants before vernalization (Fig.17F). In this experiment Aa *SPL9* mRNA expression pattern did not mirror miR156 accumulation and fluctuated during vernalization. However, although the levels of Aa *SPL9* mRNA changed during vernalization, we never observed the second peak reached in apices of adult plants during cold treatment and

possibly related to flowering (Fig.10C and 10J). This could explain the repression of flowering and it needs further investigation.

To summarize, the decrease of miR156 levels in apices is not blocked during vernalization but it occurs at a very low rate and it is only observed after greatly extended vernalization periods. The unknown mechanism regulating miR156 decrease seems to be conserved between *A. alpina* and *A. thaliana*. *A. thaliana* plants are fast growing and bolting could be observed after eight months of growth at 4°C. miR156 levels in the floral buds sample were low, allowing flowering to proceed even in SDs. For *A. alpina*, 60 weeks of growth at 4°C were not enough to permit the shift to reproductive development. Interestingly, in *A. alpina* miR156 levels seemed to stabilize between the last two time points, spanning a period of 6 months. Therefore, although the decrease seems to be conserved between the two species, we cannot exclude the possibility that in *A. alpina* miR156 will never reach adult trough levels in juvenile plants exposed to 4°C and that these plants would never flower in vernalization. The transcription of Aa *SPL9* during the year in vernalization is difficult to interpret but could suggest the importance of the second increase in mRNA observed during vernalization in adult apices. This second peak of Aa *SPL9* mRNA expression must be independent of miR156 regulation, since it occurs in adult plants at the time miR156 levels are already low. Moreover, we know from studies in *A. thaliana* that other factors additional to miR156 regulate *SPL* gene transcription.

4.15 Temperature affects the rate of miR156 decrease

To further investigate the role of temperature in the rate of miR156 decrease we tested miR156 levels during the development of plants grown in LDs at the intermediate temperature of 15°C. miR156 levels were high at the beginning of development and fell with time (Fig.18A). Decrease in miR156 accumulation seems to be slower at 15°C compared to 20°C. However, the results of these two independent experiments cannot be directly compared. At 20°C miR156 expression reached trough levels at 5-6 weeks, whereas at 15°C constant low levels were observed after 6-7 weeks. Moreover, Aa *SPL9* transcript levels in main shoot apices of plants grown at 15°C mirrored miR156 accumulation as it does at 20°C (Fig.18B). At 20°C, Aa *SPL9* mRNA started to increase 4 weeks after sowing and reached a plateau of expression after 5 weeks (Fig.10B). When plants were grown at 15°C the transcriptional increase seemed to occur more gradually, starting 5-6 weeks after sowing (Fig.18B).

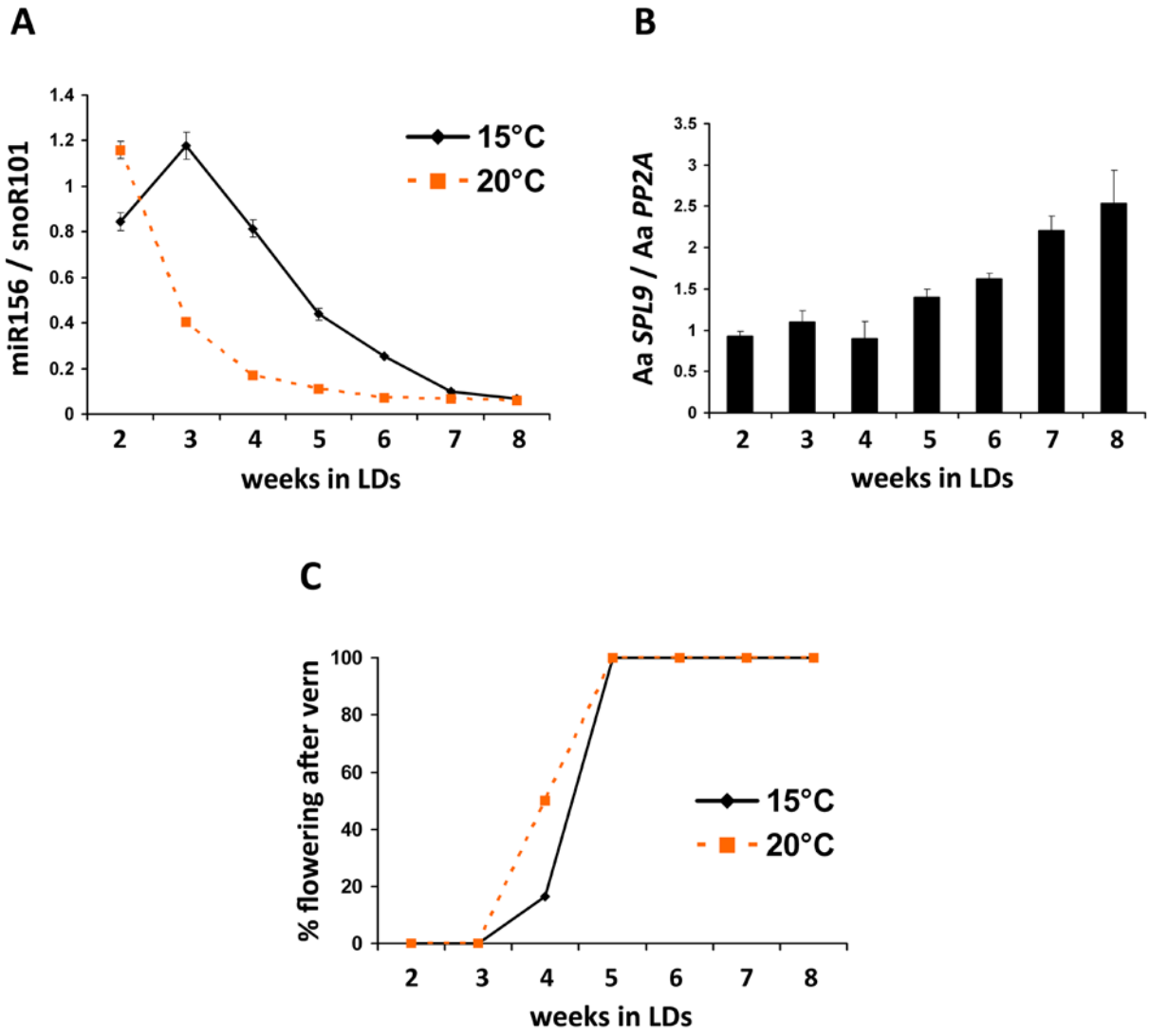

Figure 18. miR156 and Aa *SPL9* mRNA levels and flowering behaviour of *A. alpina* plants growing at 15°C

(A) miR156 levels in apices of *A. alpina* plants grown in LDs 15°C (black line). The dashed orange line represents miR156 levels at 20°C as control (Fig.13A). Experiments at 15°C and at 20°C were not performed in parallel. x-axis, weeks of growth at 15°C before vernalization. y-axis, miR156 levels relative to snoR101. (B) Aa *SPL9* mRNA expression in plants grown in LDs 15°C. x-axis as in (A). y-axis, mRNA levels relative to Aa *PP2A* mRNA. (C) Percentage of flowering plants after 16 weeks in vernalization. x-axis, weeks of growth at 15°C (black line) or 20°C (orange dashed line, Wang et al., 2011) before vernalization. y-axis, percentage of flowering plants after vernalization.

In order to test the effect of different rates of miR156 decrease on the acquisition of reproductive competence, we grew plants at 15°C and transferred 12 individuals each week to vernalization for 3 months, after which they were returned to 20°C and tested for flowering.

All the plants grown for at least 5 weeks at 15°C flowered after vernalization whereas plants shifted after only 2 or 3 weeks did not respond by undergoing floral induction (Fig.18C, black line). 16% of the individuals that experienced vernalization after 4 weeks at 15°C flowered after induction, whereas 45% flowered when plants were grown at 20°C for the same amount of time.

These data show that the fall of miR156 levels takes place more gradually and is delayed when plants experience lower temperature. Consistently with Aa *SPL9* post-transcriptional regulation by miR156, Aa *SPL9* mRNA also increases later in development when plants are grown at 15°C. However, temporal expression differences were not clearly reflected in a delayed acquisition of meristem competence to respond to floral induction. All plants grown at 20°C or 15°C for 5 weeks similarly flowered after vernalization. 30% more plants flowered when grown for 4 weeks at 20°C compared to 15°C before vernalization. Whether this difference is significant and indicates a faster maturation occurring at higher temperatures need to be further investigated.

4.16 miR156 is expressed in axillary shoots apices and its levels decrease during development

All the experiments reported until now referred to the acquisition of competence in the main shoot apex but physiological changes during development occur in parallel in the main shoot and in the axillary shoot branches. Moreover, in *A. alpina* axillary shoots play an important role to sustain polycarpic flowering (Wang et al., 2011b; Wang et al., 2009b). Acquisition of reproductive competence correlates with miR156 levels in the main shoot apex. Moreover, overexpression of *MIR156* delays flowering in the main shoot apex but also in the axillary shoots. miR156 is additionally expressed in the leaves where its levels correlate with the age-related shape of the leaves. In *A. alpina*, leaves produced on the axillary shoot branches show similar changes in leaf serration during development as observed in the main shoot, suggesting that changes in miR156 levels might confer these differences (Fig.19A). Whether miR156 is expressed also in the axillary shoot apices and its abundance changes during maturation was tested. The firsts axillary shoots grew out at the axes of the first two true leaves after 5 weeks in LDs. Their appearance was considered the first time point in a time course following shoot growth until week 10. miR156 is expressed in the axillary apices and its expression changes during development similarly to what observed in the main shoot apex (Fig.19B). This preliminary result, together with the previously reported characterization of

flower behaviour in axillary shoots, suggests that miR156 levels might allow or prevent flowering after vernalization in axillary shoots (Wang et al., 2011b).

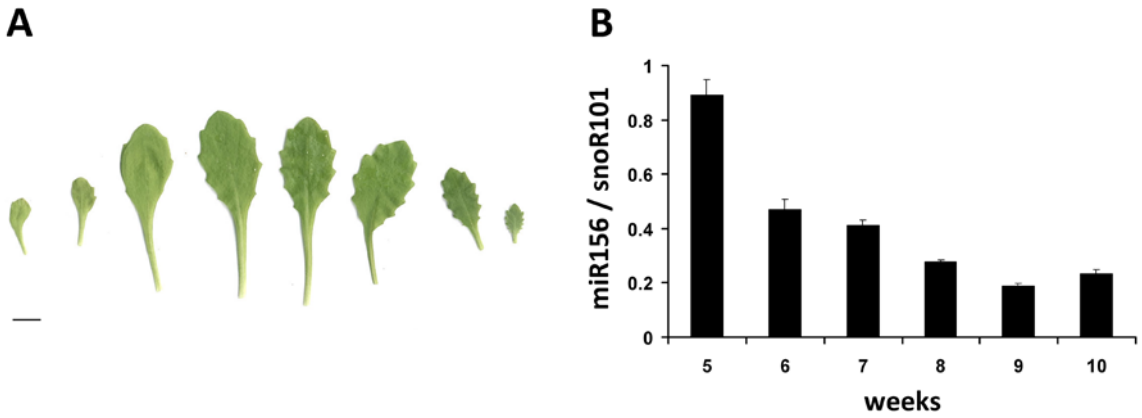

Figure 19. miR156 expression is axillary shoot apices

(A and B) Axillary shoots produced during the first year of growth. (A) Heteroblastic leaf changes in axillary shoots. Scale bar = 1 cm. (B) miR156 levels in apices of the 1st and 2nd axillary shoots produced during development. x-axis, weeks in LDs. Axillary shoots at node 1 and 2 start to be visible at week 5 in LDs. Their development was followed until week 10. y-axis, miRNA levels normalized to snoR101.

4.17 Physiological characterization of reproductive competence acquisition in vegetative axillary shoots during the second year of growth

In our experiments, plants were normally grown for 2 or 8 weeks in LDs and then vernalized for 12 weeks (Fig.20A, Year I). When adult plants (8 weeks old) were returned to LDs the main axis flowered after two weeks in LDs and new vegetative shoots (6-10) appeared at nodes below the inflorescence (Fig.20C, after vernalization). The axillary shoots produced below the main inflorescence maintained vegetative growth after flowering (Wang et al., 2009b). To test if these shoots were not flowering because they also had a juvenile phase, flowering plants were grown in LDs for different weeks and then vernalized again (Fig.20A, Yellow box, Year II). In plants grown in LDs for more than 4 weeks most of the axillary shoots flowered after vernalization (Fig.20B and 20C, > 4 wLDs + 12wV). In plants grown in LDs for less than 4 weeks a smaller percentage of axillary shoots flowered after vernalization whereas the majority remained vegetative (Fig.20B and 20C, 2wLDs +12wV). The number of reproductive and vegetative shoots in control plants vernalized for additional 12 weeks without experiencing any LDs was also counted. Plants grown for 24 consecutive weeks in vernalization produced a similar number of vegetative shoots as the plants whose flowering

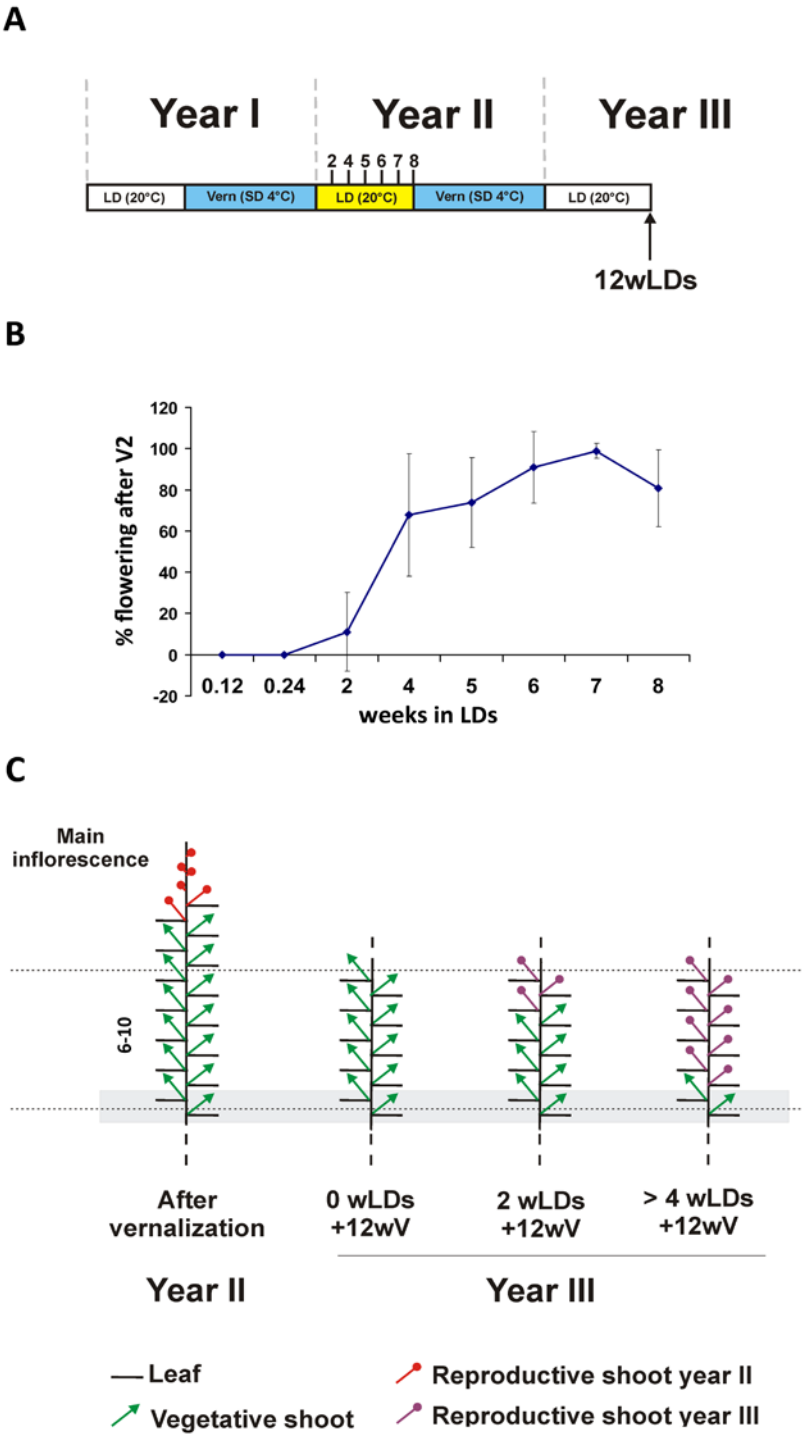

Figure 20. Acquisition of reproductive competence in the axillary shoots growing below the main inflorescence

(A) Experimental design. Plants were grown for 8 weeks in LDs (20°C) and then vernalized for 3 months (Year I). In Year II plants were grown for different lengths of time in LDs (20°C) before being exposed to a second vernalization. When returned to LDs (Year III) the axillary shoots were scored for flowering after 12 weeks. Blue boxes, vernalization periods. White boxes, growth in LDs. Yellow box, growth in LDs for different lengths of time (number of weeks indicated above). (B) Percentage of flowering shoots scored in Year III following the second vernalization in relationship to the number of weeks of growth in LDs (20°C) in Year II. x-axis, weeks in LDs (20°C) before the second vernalization. 0.12, axillary shoots were scored

was scored after the first 12 week vernalization (6-10). None of these axillary shoots flowered after vernalization, indicating that axillary shoots need to grow in LDs before being able to respond to vernalization (Fig.20B, 0.12 and 0.24. Fig.20C, 0 wLDs + 12wV). Moreover, the length of the exposure to LDs determines the flowering response similarly to what we previously observed for the SAM. These data suggest that axillary shoots have independent juvenile phases.

The axillary shoots growing closer to the inflorescence responded more to vernalization and flowered (Fig.20C, Year III). This was quite surprising since these shoots were produced in higher nodes and therefore grown in the axes of younger leaves compared to the shoots at lower nodes. However, scanning electro microscope (SEM) studies had previously demonstrated that axillary shoots closer to the SAM develop earlier than the ones in lower nodes (Wang et al., 2009b). Moreover, not all the axillary shoots flowered after vernalization. At lowest nodes the axillary shoots were less developed and did not flower regardless of the time spent in LDs before vernalization (Fig.20C, grey box). This could be explained by the very compacted internodes occurring in this region and by failure of axillary shoot outgrowth. It indicates that many factors can influence the flowering fate of axillary shoots.

for flowering after the first vernalization; 0.24, plants grew in vernalization for 24 consecutive weeks without any LD in Year II. y-axis, percentage of flowering shoots after the second vernalization. (C) Schematic representation of vegetative and reproductive shoots after the first vernalization (Year II) and after the second vernalization (Year III). Dotted lines delimitate the axillary shoots of interest. Black small lines, leaves produced at every node. Green arrows, vegetative shoots. Red lines, reproductive shoots flowering in Year II (main inflorescence). Purple lines, reproductive axillary shoots flowering in Year III. Grey box highlights the shoots that never flower.

5. CONCLUSION AND DISCUSSION OF FUTURE WORK

5.1 Conclusions

The aim of the study was to investigate the molecular mechanisms regulating meristem competence to flower in *A. alpina*. I made use of the close relationship between *A. alpina* and *A. thaliana* and firstly showed that it is possible to distinguish differentially expressed *A. alpina* genes on a genome wide scale using an Arabidopsis microarray (4.2 and 4.3). Combining interspecific microarray hybridizations with candidate gene approaches identified several members of the miR156-regulated *SPL* gene family as being differently transcribed in reproductively competent and incompetent shoot apices (4.4). The high degree of conservation between the members of the *SPL* families in the two species included the sequence of the miR156 target site, leading to the hypothesis that the homologues of these genes in *A. alpina* were probably under similar post-transcriptional regulation to that described in *A. thaliana* (4.5). I showed that the progressive reduction in miR156 levels during development temporally correlates with the acquisition of reproductive competence and that the rate of the decrease seems to be temperature dependent (4.9, 4.14 and 4.15). During vernalization miR156 levels change very slowly, so that high miR156 levels before vernalization are maintained through the vernalization period and correlate with repression of flowering in apices of juvenile plants, whereas low levels before vernalization in adult plants allow the shift to the reproductive transition during vernalization. A similar decrease in miR156 levels was observed in the first axillary shoots growing in LDs, suggesting that miR156 might play similar functions in main and axillary shoots (4.16). The role of miR156 as a floral repressor was confirmed by the late flowering phenotype of transgenic plants overexpressing *MIR156* in *A. alpina* (4.12). Interestingly, in contrast with what was previously described for *A. thaliana*, LDs could not accelerate the strong flowering delay observed in these transgenic lines.

Among the Aa *SPL* genes regulated by miR156 to exert its pleiotropic functions, the transcriptional pattern of Aa *SPL9* during development in LDs was inversely related to miR156 accumulation (4.6). A transgenic line overexpressing Aa *SPL9* cDNA with a mutated miR156-target site showed accelerated transition to reproductive development (4.7).

In several species miR156 has been linked to miR172 in regulating the vegetative phase transition. They show opposite temporal expression patterns during development. In *A. thaliana* this has been proposed to be due to At SPL9/10 proteins binding to the promoter of *MIR172b* activating its transcription. In contrast, miR156 and miR172 levels do not inversely correlate in *A. alpina* apices suggesting that miR172 might not be involved in the acquisition of reproductive competence in this species (4.10).

Finally, to investigate the role of juvenility in the regulation of *A. alpina* polycarpic flowering, a physiological characterization of the acquisition of reproductive competence of axillary shoots growing in the second year was carried out (4.17).

5.2 The use of heterologous microarray hybridization

When the project was initiated the genome sequence of *A. alpina* had not been determined and Next Generation Sequencing (NGS) technologies were still under development. Therefore, an Arabidopsis microarray was chosen to perform transcriptional profiling in *A. alpina*, exploiting the expected sequence similarities of the genomes of *A. alpina* and the model species *A. thaliana*. This approach resulted in the identification of thousands of genes that were differently expressed between samples. Expression differences for some genes could later be reproduced by other techniques confirming that it is possible to use *A. thaliana* microarray to correctly detect *A. alpina* transcriptional differences. Studies in other Brassicaceae species also used *A. thaliana* microarrays to understand many biological processes but in most studies commercially available Arabidopsis arrays were used (van de Mortel and Aarts, 2006). In this study, a customized array that combines features from oligo set and long oligo arrays was used, aiming to further improve detection. However, despite the early enthusiasm for this type of approach, the establishment of NGS techniques quickly substituted the use of microarrays to study gene expression in non model species, because this does not rely on hybridizations that are dependent on sequence similarities.

Currently, a draft of the full genome of *A. alpina* is available, and manual annotation of a wide range of genes suggests that this contains most of the gene space. Transcriptomic studies can now be efficiently performed using NGS and the expression differences observed can be directly compared to the *A. alpina* genome. In addition to the reliable detection of transcriptional differences, sequencing could allow the discovery of new or highly diverged genes in *A. alpina* compared to *A. thaliana* that might be related to its perennial life strategy.

Furthermore, the availability of *A. alpina* sequences facilitates the validation of the transcriptomic discoveries and hypotheses.

In the work presented in this thesis, microarray results were mainly used to investigate the function of the regulatory module involving miR156 and *SPL* homologues in the regulation of reproductive competence in *A. alpina*. Moreover, many more genes showed differential expression in these comparisons suggesting further biologically important information can still be extracted from these data. For example, genes exhibiting a transcriptional pattern similar to the one reported for Aa *FUL*, Aa *LFY* or Aa *TFL1*, whose expression patterns change in adult apices during the early phases of floral transition, could represent downstream targets of reproductive competence genes. In contrast, genes whose expression patterns change during vernalization in both juvenile and adult apices could be good candidates to investigate the transcriptional changes triggered by chilling temperatures. Furthermore, mainly genes promoting the adult phase have been investigated. However, a similar number of genes were more highly expressed in juvenile apices. Among them, genes known in *A. thaliana* to repress flowering such as the *AP2-like* genes *SMZ* and *SNZ*, were identified and could represent candidates for repressors of the adult phase (Mathieu et al., 2009; Schmid et al., 2003).

5.3 miR156 expression in main shoot apices and its proposed role in *A. alpina* flowering

miR156 is highly expressed in *A. alpina* apices early in development and decreases in expression over time. These temporal changes are common to all species in which miR156 has been studied although in some cases only expression levels for the whole seedlings were reported (Chuck et al., 2007; Wang et al., 2009a; Wu and Poethig, 2006). In *A. alpina*, miR156 expression reaches trough levels after 5 weeks of growth in LDs (20°C). The temporal pattern of miR156 expression precisely correlates with the acquisition of reproductive competence in *A. alpina* since only plants that have been grown for more than 4-5 weeks in LDs (20°C) will flower upon vernalization (Wang et al., 2011b). In adult competent plants, trough miR156 expression levels reached before vernalization do not change during the 3 months of cold treatment. Low miR156 expression during vernalization could allow adult apices to undergo the floral transition.

Juvenile plants that have been grown for less than 4-5 weeks in LDs and then transferred to vernalization for 3 months do not flower (Wang et al., 2011b). Interestingly, in

these plants miR156 levels in apices do not decrease during the whole vernalization treatment, although these plants grow and produce new leaves. The high levels of miR156 during vernalization correlate with the maintenance of vegetative growth in juvenile plants. Furthermore, overexpressing *MIR156b* in *A. alpina* strongly delayed flowering confirming the role of miR156 as a floral repressor. A strong floral delay was observed in both *A. alpina* plants carrying an active or inactive allele of *PEP1* indicating that vernalization and age act additively to promote flowering and that vernalization cannot overcome the repression by miR156. Similar flowering delays were also observed in other species although the effect of miR156 was less pronounced. For example, miR156 overexpression in maize, rice and tomatoes delayed flowering by only a few weeks (Chuck et al., 2007; Xie et al., 2006; Zhang et al., 2011). Plants overexpressing *MIR156b* in *pep1* mutant background produced the first flowers 4 months later than *pep1* plants would do. This is very similar to what was observed for *A. thaliana MIR156* overexpressing plants grown in SDs where flowering occurs after several months principally in axillary shoots (Schwab et al., 2005). Interestingly, *35S:MIR156 A. thaliana* plants are not insensitive to inductive photoperiod and exposure to LDs can drastically overcome the reproductive delay caused by miR156 so that transgenic lines flower only slightly later compared to wild-type plants (Schwab et al., 2005; Wang et al., 2009a). In contrast, LDs did not visibly accelerate flowering of *35S:MIR156b A. alpina* plants. This could indicate that photoperiod plays a less important role in *A. alpina* than in *A. thaliana* or that the age pathway has a much stronger control on flower induction. The way photoperiod overcomes miR156 repression in *A. thaliana* has not been directly tested. In *A. thaliana*, LDs do not affect miR156 levels directly but the transcripts of several *SPL* genes are upregulated when plants are shifted from SDs to LDs (Schmid et al., 2003; Wang et al., 2009a). However, the strong effect of LDs observed on *35S:MIR156* lines is presumably through a parallel (photoperiod) pathway that activates floral integrator genes downstream of the *SPLs (Adrian et al., 2009)*. In fact, although the expression levels of *SPL* mRNAs have not been tested in *MIR156* overexpressing plants shifted to LDs, even natural levels of miR156 can suppress the effects of *SPL* overexpression (Wu and Poethig, 2006). The contribution of the photoperiodic pathway to flowering of *A. alpina* is largely unknown. The *A. alpina* otholog of the At FT gene has been identified and causes precocious flowering when overexpressed in *A. thaliana* (Adrian et al., 2010; Wang, 2007). Similarly to At *FT*, Aa *FT1* mRNA is expressed in the leaves under LDs and its expression falls in SD (vernalization). Nevertheless, an increase is observed in apices of adult plants upon vernalization induction but the levels of expression are lower compared to the expression in leaves of plants grown in LDs. To gain some insights

into the role of FT in *A. alpina*, transcription of *FT* in *A. thaliana* and *A. alpina 35S:MIR156* plants shifted to LDs will be compared in order to understand if in *A. alpina* mRNA accumulation of Aa *FT* is for example prevented. Further comparisons between the two plant systems will shed light on the differences and similarities between the two species in the photoperiodic and age regulation of flowering.

Although photoperiod can overcome the effect of miR156 overexpression in *A. thaliana*, the *35S:MIR156* lines were still less responsive to LD treatment than wild-type *A. thaliana* plants, indicating that low miR156 levels allow flowering by conferring reproductive competence (Schwarz et al., 2008). However, wild-type *A. thaliana* seedlings were previously shown to gain the ability to respond to photoperiodic induction accelerating flowering after only 4 days of growth in SD whereas miR156 levels still take some weeks to reach trough levels (Mozley and Thomas, 1995; Wang et al., 2009a). Acquisition of reproductive competence during development was shown in the accession Landsberg *erecta* (Ler) whereas all the more recent experiments involving miR156 were conducted in Columbia (Col), suggesting that the discrepancy observed could be related to the different genotypes used in the studies. Therefore, characterization of miR156 level decrease in Ler and /or physiological studies to undestand the timing of reproductive competence acquisition in Col must be performed to understand if different genotypes have different miR156 regulation or if, as supported by *MIR156* overexpressing lines, flowering in *A. alpina* can simply proceed even with high levels of miR156.

A. alpina could represent a better system than *A. thaliana* to test miR156 regulation of reproductive competence. However, the flowering delay observed in *35S:MIR156b* is so extreme as to hamper feasible comparisons to wild-type plants on the acquisition of reproductive competence. Therefore, *A. alpina* plants were additionally transformed with the *35S:MIM156* target mimicry construct (Franco-Zorrilla et al., 2007). In these lines miR156 activity should be prevented, favouring acceleration of flowering and facilitating comparison of meristem competence to respond to inductive vernalization. This loss of function approach will reveal the significance of miR156 when expressed at wild-type levels in *A. alpina*.

The current model for flowering in *A. alpina* Pajares involves the downregulation of miR156 and upregulation of *SPLs* during development as well as the downregulation of the floral repressor *PEP1* during vernalization (Wang et al., 2009b). At the molecular level, vernalization causes in both juvenile and adult plants upregulation of Aa *SOC1* mRNA whereas the transcriptional level of some other flowering genes specifically change in adult apices upon floral induction (Wang et al., 2011b). For example, mRNA of the floral repressor

Aa *TFL1* is downregulated during vernalization only in adult apices while transcription of the floral identity gene Aa *LFY* is upregulated only in adult apices. Microarray expression data also showed that Aa *FD* and Aa *FUL* mRNA levels were upregulated upon floral induction specifically in adult apices. Moreover, in *A. thaliana* the miR156-regulated SPLs promote transcription of floral integrator and floral meristem identity genes. For example, At SPL9 promotes the transcription of At *FUL*, At *SOC1* and At *AGL42* whereas At SPL5 regulates At *LFY*, At *AP1* and At *FUL* mRNAs (Wang et al., 2009a; Yamaguchi et al., 2009). Such regulation by SPLs could also explain flowering in adult *A. alpina* plants. Low apical levels of miR156 by the end of vernalization and higher expression of *SPL* genes may induce flowering by promoting the expression of genes such as Aa *FUL* and Aa *LFY* in *A. alpina*.

Aa *TFL1* was also shown to regulate the age response to vernalization in *A. alpina (Wang et al., 2011b). A. alpina* lines in which the levels of Aa *TFL1* have been reduced using dsRNAi exhibit reduced length of the juvenile phase. miR156 expression pattern in LDs is not affected in *35S:Aa TFL1 dsRNAi* lines, decreasing at about 4-5 weeks after sowing as in wild-type plants (Bergonzi, unpublished). One possibility is that in adult plants SPLs activate floral meristem identity genes such as Aa *AP1* and Aa *LFY*, and that Aa AP1 and Aa LFY transcription factors in turn repress Aa *TFL1*. Such negative regulation of *TFL1* has been described in *A. thaliana* (Bradley et al., 1997; Ratcliffe et al., 1999). Further experiments to understand the regulatory network linking miR156, Aa *SPLs* and Aa *TFL1* for the control of maturation in *A. alpina* are being performed.

5.4 Conservation of the role of miR156 in vegetative phase change

A. alpina 35S:MIR156b transgenic lines are very late flowering but also show additional vegetative phenotypes. Leaves produced at different nodes were all small and round, even after several months of growth. They resemble the juvenile leaves produced at the first 2-3 nodes in wild-type plants. Indeed when miR156 expression was measured in *A. alpina* wild-type leaves I observed that, as reported recently for several woody trees, juvenile leaves had higher levels of miR156 than adult leaves (Wang et al., 2011a). Moreover, plants overexpressing miR156 produced more axillary shoots and, together with prolonged vegetative growth, resulted in a very bushy phenotype. This has been observed in *A. thaliana* but it is also very evident in monocots, where it represents a phenotype with important implications for plant yield and seed production (Chuck et al., 2007; Singleton, 1951; Xie et al., 2006). Recently, the branching phenotype caused by miR156 overexpression in rice was

shown to be mediated by Os *SPL14 (Miura et al., 2010; Xie et al., 2006)*. Curiously, when in the 50s Singleton described the phenotype of the maize Corn Grass mutant, only recently shown to be caused by miR156 overexpression, he associated it to a perennial type of growth that could have represented the grass-like ancestor of maize (Chuck et al., 2007; Singleton, 1951).

Overexpression of At *MIR156b* in *A. alpina* indirectly also showed that the *A. alpina* microRNA machinery can recognize and process the *A. thaliana* miR156 precursor. Several studies reported that the shapes of the hairpin loops, more than the sequences, are important for the processing of miRNAs precursors (Mateos et al., 2010; Song et al., 2010; Werner et al., 2010). *A. thaliana* and *A. alpina* pre-miR156s present similar secondary structures. Moreover, the phenotype of the transgenic *A. alpina* plants suggests that the *A. thaliana* hairpin has been processed and that the miR156 produced is most probably repressing the Aa *SPLs*.

5.5 Regulation of Aa *SPL9* mRNA and its role in *A. alpina* flowering

In *A. thaliana* miR156 negatively regulates several members of the *SPL* gene family (Gandikota et al., 2007; Rhoades et al., 2002; Schwab et al., 2005; Wang et al., 2008; Wu and Poethig, 2006). Based on the conservation of the SBP-box motif in evolution, we identified the *SPL* genes in *A. alpina*. Phylogenetic analysis showed that the two species have a similar number of homologues genes and sequence conservation revealed that also the subgroup of miR156-regulated *SPLs* is conserved. The only exceptions could be Aa *SPL3* and Aa *SPL4* where an additional nucleotide miss match might influence miR156 binding in *A. alpina*. Analysis of expression levels of different SPLs in *MIR156* overexpressing lines will provide further evidence of Aa *SPL* regulation.

miR156 plays several roles in plant development and detailed studies in *A. thaliana* showed that SPL proteins share redundant functions but can also specifically regulate the expression of flowering genes and leaf traits. Aa *SPL9* showed the highest expression difference between adult and juvenile apices already before vernalization and thus it represented a good candidate to study competence to flower. Aa *SPL9* ranked as the 59th gene of the 2901 genes that showed statistically significant higher expression in adult apices (adj. p-value < 0.05). In *A. thaliana*, At *SPL9* was reported to be one of the earliest genes expressed already during vegetative development and *spl9 spl15* double mutant showed a reduced response to photoperiodic induction similar to that of *35S:MIR156* plants (Schmid et al., 2005; Schwarz et al., 2008). Quantitative PCR confirmed that Aa *SPL9* mRNA changes

are complementary during development to miR156 decrease and correlate with the acquisition of mersitem competence. These differences in expression were not detected by *in situ* hybridization but this could be due to the lower sensitivity of this method. qRT-PCR and *in situ* hybridization consistently showed that in juvenile plants exposed to vernalization Aa *SPL9* mRNA level does not change whereas in adult apices it further transiently increases upon induction. This second peak in mRNA abundance was also observed when *A. thaliana* plants were shifted to LDs, suggesting a conserved role in flowering that is independent of miR156 regulation (Wang et al., 2009a; Wang et al., 2008). In *A. thaliana* At *SPL9* was mainly expressed in leaf primordia and leaf anlagen before vernalization, a pattern that has been related to the role of At *SPL9* in plastochron length control (Wang et al., 2008). Upon photoperiodic induction, At *SPL9* mRNA increases in the flanks of the meristem, in the provascular strands below the shoot apical meristem and transiently in floral anlagen and early floral primordia (Wang et al., 2009a; Wang et al., 2008). In *A. alpina*, Aa *SPL9* mRNA expression upon induction resembles the one of At *SPL9* and after 3 weeks in vernalization mRNA starts to be visible in the meristem flanks. After 5 weeks in vernalization Aa *SPL9* mRNA seems to be strongly expressed also in the central apical zone, which was not previously observed in Arabidopsis. Aa *SPL9* mRNA level in the inflorescence meristem is lower again, in agreement with qRT-PCR data and with *A. thaliana* studies.

Overexpressing Aa *SPL9* cDNA with a mutated miR156 target site that prevents binding and repression by miR156 accelerated flowering in *A. alpina pep1*. Interestingly, all the produced leaves resembled cauline leaves suggesting a strong acceleration from vegetative to reproductive development. In *A. thaliana*, plants overexpressing At *rSPL9* under the 35S CaMV promoter were not recovered (Wang et al., 2008). However, the miR156-resistant form was successfully misexpressed under different promoters and never showed such a strong phenotype in which only cauline leaves are produced. After flowering from the main shoot, *A. alpina 35S:MIR156b pep1* plants produced axillary shoots indicating that the transgenic plant maintained perennial growth similar to *pep1* plants.

Although miR156 levels decrease similarly in *pep1* and wild-type plants, suggesting that the maturation process is taking place in both genotypes, the fact that *pep1* flowers without vernalization makes it a more difficult system to study acquisition of reproductive competence. However, flowering can be further accelerated in *pep1* plants by exposure to prolonged cold treatment (Albani, unpublished). Therefore, in order to compare *35S:Aa rSPL9 pep1* and *pep1* in their response to inductive vernalization, a characterization of the juvenile phase of the *pep1* mutant is currently being performed by growing plants in LDs and

shifting them to vernalization at different times after germination to accelerate flowering. In addition, the *35S:Aa rSPL9 pep1* line will be crossed to *A. alpina PEP1* and more lines overexpressing miR156 are currently under selection in the wild-type *PEP1* background where they will be more clearly tested for reproductive competence to flower. The 35S CaMV constitutive promoter has been shown in some cases to cause abnormal genes expression (Hsu et al., 2011). Thus, lines expressing *rSPL9* and *SPL9* under the Aa *SPL9* promoter will also be generated.

5.6 miR172 and miR156 expression patterns are not complementary in development of *A. alpina*

In Arabidopsis and maize, miR156 and miR172 work together to promote vegetative phase change and flowering (Chuck et al., 2007; Lauter et al., 2005; Wu et al., 2009). Their effects on plant development are opposite. miR156 is expressed early during vegetative development and regulates juvenile leaf traits as well as preventing acquisition of competence to flower. The decrease of miR156 levels during development correlates with an opposite increase in miR172 in several species (Aukerman and Sakai, 2003; Chuck et al., 2007; Jung et al., 2007; Tanaka et al., 2011; Wu et al., 2009). Moreover, in *A. thaliana* the complementary pattern is mediated by At SPL9/SPL10 proteins that bind to the promoter of *MIR172b* to induce an increase in miR172 levels (Wu et al., 2009). Similarly, the maize *Corngrass1* mutant, which overexpresses a tandem miR156, shows lower miR172 levels (Chuck et al., 2007). In *A. thaliana*, transgenic lines overexpressing *MIR172* exhibit pleiotropic phenotypes including acceleration of flowering, appearance of adult leaf traits and defects in floral organ identity (Aukerman and Sakai, 2003; Chen, 2004; Wu et al., 2009; Yant et al., 2010). miR172 is considered to play a role in the acquisition of reproductive competence based on its expression pattern (Fornara and Coupland, 2009). However, plants with constantly higher or lower expression levels of miR172 were not tested for competence to respond to inductive photoperiod to flower. In *A. alpina,* the inverse correlation between the levels of the two miRNAs is not observed. While at the beginning of development miR156 expression decreases in a few weeks by several folds, miR172 expression does not dramatically change. Moreover, miR172 levels did also not significantly change during 2 or even 4 months of growth in LDs. However, miR172 levels did show pronounced upregulation at the end of vernalization in adult apices, at the time floral mersitems are established. The lack of correlation between miR172 levels and acquisition of reproductive competence in *A. alpina*

was further supported by the levels in juvenile plants grown for more than a year in vernalization. miR156 decreased after several weeks at 4°C but miR172 abundance did not change for the whole 60 weeks of treatment, in agreement with the lack of floral buds in these plants. Moreover, in *A. alpina pep1* plants, while miR156 decreases similarly as in wild-type plants, miR172 does not change during the first 6-7 weeks but is strongly upregulated at week 8 and 9 in LDs, when flower buds are formed. These results indicate that miR172 is not involved in the acquisition of reproductive competence in *A. alpina*. Since the regulatory module miR156-SPLs-miR172 is considered to be evolutionarily conserved between monocots and dicots, the differences observed in *A. alpina* could be due to species-specific changes in the regulation of *MIR172* genes. For example, differently from *A. thaliana*, Aa SPL9 might not bind to the promoter of *MIR172* during development in LDs. Moreover, we can not exclude that the possibility offered by the *A. alpina* system to clearly separate between acquisition of reproductive competence during development and floral induction when plants are shifted to vernalization might also have facilitated the detection of the temporal lapse between miR156 decrease and miR172 increase. Therefore, it is possible that also in other species miR172 exclusively correlates with flowering but that it is difficult to observe it due to the rapid progression from one phase to another and due to the impossibility to temporally separate vegetative development from floral induction.

The strong upregulation of miR172 at the end of vernalization suggests a role for miR172 in the regulation of flower development. In *A. thaliana*, miR172 spatially restricts the mRNA of the floral organ identity gene At *AP2* to whorls 1 and 2 and this might be conserved in *A. alpina* (Chen, 2004). In *A. thaliana*, miR172 also regulates the reproductive phase transition downregulating *AP2* and other *AP2-like* genes (Aukerman and Sakai, 2003; Chen, 2004; Kim et al., 2006; Schmid et al., 2003; Schwab et al., 2005; Yant et al., 2010). Interestingly, although miR172 expression levels do not significantly change during development, the mRNA of several *AP2-like* genes changed in juvenile and adult apices before vernalization, based on 454 sequences and microarray hybridization data. Especially *SMZ* and its paralog *SNZ* were exclusively expressed in juvenile apices whereas no reads or hybridization signals were detected in adult apices. However, in *A. thaliana* miR172 can regulate *AP2-like* genes by both mRNA degradation and translational inhibition, making it difficult to draw any conclusion with expression differences (Aukerman and Sakai, 2003; Chen, 2004). miR172 in *A. thaliana* is also regulated by the photoperiod, autonomous and ambient temperature pathways *(Jung et al., 2007; Lee et al., 2010)*. Whether these regulatory patterns are conserved in *A. alpina* is still unknown.

5.7 The role of temperature in the developmental regulation of the decrease in miR156

Acquisition of reproductive competence requires the decrease of miR156 during development before exposure to floral inductive stimuli. How the decrease is achieved is still largely unknown. The observation that miR156 levels did not change during vernalization in juvenile apices led to the formulation of two hypotheses. The first hypothesis was that vernalization could play an active role blocking the decrease of miR156, and the second was that temperature more than vernalization could control the rate of the decrease. Moreover, miR156 levels did not decrease when *A. thaliana* plants were vernalized, indicating that this unknown mechanism was a more general process and not specific to *A. alpina*. A decrease in miR156 expression was observed when *A. alpina* and *A. thaliana* were grown for a longer time at 4°C, favouring the hypothesis that low temperature influences the rate of miR156 decrease. Moreover, the speed of miR156 decrease observed in plants grown at 15°C showed a slower rate than at 20°C but faster than at 4°C, further supporting this idea. Mechanistically, whether temperature affects the levels of miR156 by acting on precursor transcription or on miR156 biogenesis needs to be tested.

miR156 has been reported to be induced by ambient temperature in *A. thaliana* and more recently in orchids (An et al., 2011; Lee et al., 2010). In contrast, the data in *A. alpina* and *A. thaliana* showed that miR156 expression remains almost constant when plants are shifted to 4°C and decreases slower at 15°C. However, the observations that in *A. thaliana* miR156 is more highly expressed at 16°C than at 23°C was reported for only one time point after seedlings were grown for 10 days at the two different temperatures, without really testing if a shift to a different temperature could change miR156 expression (Lee et al., 2010). Therefore it is possible that such a difference was observed as a consequence of the different rate of miR156 decrease during development and not because of active induction of transcription at 16°C.

Temperature influences several aspects of plant development including growth. *A. alpina* plants grow slower at lower temperature. This could suggest a correlation between miR156 decrease and number of nodes produced by the plant. Similarly, a recent study on vegetative phase change showed that a signal produced by Arabidopsis leaf primordia mediates the decline of miR156 in the shoot (Yang et al., 2011). However, *A. alpina* plants grown for 60 weeks in vernalization produced more than 20 leaves and still showed higher miR156 levels than plants grown for 5 weeks in LDs and that have produced about 10 leaves.

Thus, these data indicate that levels of miR156 in the apex do not correlate with the number of leaves produced under different growth temperatures.

From a biological point of view, it would be tempting to speculate that temperature, by regulating the rate of miR156 developmental decrease, could influence the timing of competence acquisition. Although the time of the year in which *A. alpina* seeds germinate in nature is still unknown, the temperatures experienced in autumn before the inductive winter could control flowering the following spring by influencing the rate of miR156 reduction and therefore assuring that smaller plants do not undergo reproduction in spring. However, although miR156 levels decreased slower at 15°C, plants grown for 5 weeks at 15°C before exposure to vernalization still flowered indicating that acquisition of reproductive competence was not delayed in these plants. In order to further investigate the role of temperature, the same experiment is now being performed at 11°C. Characterization of miR156 decrease and acquisition of reproductive competence at different temperatures will provide further insights into the role of temperature in the regulation of this process.

5.8 Maturation of axillary shoots and role of juvenility in polycarpy

A. alpina is a polycarpic perennial. Following vernalization the main apex and established axillary shoots in older nodes will flower (Wang et al., 2011b; Wang et al., 2009b). Other axillary shoots will not undergo reproduction and will remain vegetative until the following year. In *A. alpina* and *Malus domestica* vegetative growth after flowering is mainly maintained by axillary shoots proximal to the terminal inflorescence (Foster et al., 2003; Wang et al., 2009b). These shoots are formed at the axes of the leaves produced during vernalization and they grow out when returned to normal conditions contemporary to the appearance of the floral buds. The flowering response of these axillary shoots is highly dependent on the time spent to grow in normal conditions before the following vernalization. This result suggests that each meristem undergoes maturation independently in *A. alpina*.

It was previously shown that Aa TFL1 represses flowering similarly in young main apices and in small axillary shoots (Wang et al., 2011b). Moreover, miR156 levels decrease during axillary shoot development as observed in the main shoot. Aa TFL1 and miR156 data together suggest that in *A. alpina* reproductive competence in main and axillary shoots is regulated through similar mechanisms. Moreover, overexpression of miR156 delays flowering also in axillary shoots. However, these plants show a very pleiotropic phenotype involving several aspects of shoot development. For example, in *MIR156* overexpressing

plants apical dominance is compromised so that when plants undergo the floral transition it is normally observed first in the axillary shoots (Schwab et al., 2005). In addition, miR156 controls branching so that *35S:MIR156* lines result in a highly branched vegetative phenotype suggesting that miR156 repression of flowering is not dependent on shoot length or shoot outgrowth. Stem thickness of the main shoot and of side branches of the transgenic lines appears reduced suggesting that, as previously reported for *A. thaliana*, miR156 levels might influence meristem size and that this might be related to reproductive competence (Schwarz et al., 2008).

How, in the same individual, vegetative and reproductive growth are maintained contemporaneously is largely unknown. In poplar this is largely due to two FT paralogs showing differentiated expression during the seasons and opposite functions (Hsu et al., 2011). In *A. alpina*, the different timing of shoot growth and maturation, combined with the unstable repression of *PEP1* in the whole plant, contributes to the polycarpic life strategy that sustain flowering for several consecutive years (Wang et al., 2011b; Wang et al., 2009b). These two studies are successful example of the use of comparative biology to understand diversity in life history traits. It illustrates how in some cases species-specific traits can evolve through alteration of timing or patterns of expression of regulatory transcription factors (Prud'homme et al., 2007).

5.9 Concluding remarks

The use of related species to understand the regulation of molecular processes can reveal similarities and differences between the two species. If similarities are found, using a related species to the established model species might offer a new perspective and a better system to investigate evolutionarily conserved processes. For example, the study of miR156 and *SPL9* functions in *A. alpina* added new evidence to their roles in the regulation of meristem reproductive maturation. Moreover, while studying the miR156 response to vernalization in *A. alpina*, an unknown role for temperature in the developmental regulation of miR156 was revealed. If differences are found between the two species, comparison can provide insights into both systems. For example, the different responses to long photoperiod observed in *A. alpina* and *A. thaliana* plants overexpressing miR156 provided the first hints that photoperiod and age play different roles in flowering in the two species. Finally, these findings obtained by comparing juvenility in annual and perennial plants can contribute to a more general understanding of the mechanisms underlying evolution of life history strategies.

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APPENDIX

Table I. 454 Roche Sequencing

Number of reads obtained and median read length.

Sample name	Number of sequences	Length (median in bp)
2wLDs	1.027.496	367
8wLDs	1.116.119	174
2wLDs+5wVern	589.851	357
8wLDs+5wVern	312.501	365

Table II. *SPL* gene expression in the microarray hybridization

	MEAN (J)	ST DEV (J)	MEAN (JV)	ST DEV (JV)	MEAN (A)	ST DEV (A)	MEAN (AV)	ST DEV (AV)
***SPL1*#**	914.87	56.21	1193.26	177.30	929.46	79.44	852.04	168.78
***SPL7*#**	446.74	10.84	430.57	16.37	397.76	25.45	421.69	19.23
***SPL8*#**	175.68	9.28	176.34	13.45	177.24	11.04	172.41	5.58
***SPL12*#**	244.08	9.57	269.67	29.17	259.04	6.32	256.86	9.76
***SPL14*#**	1654.84	179.54	1584.07	139.04	1793.22	69.97	1829.16	341.52
***SPL16*#**	435.46	30.98	509.91	66.22	413.56	51.68	431.18	31.00
SPL2	418.01	16.95	393.69	43.45	753.15	42.40	766.00	98.94
SPL9	919.81	123.57	826.42	103.55	2300.38	365.98	2158.45	202.14
SPL6	224.78	13.89	240.71	22.06	327.79	9.69	356.23	21.36
SPL13	693.13	66.86	730.35	150.91	1447.82	121.80	1542.83	158.21
SPL4	233.92	15.32	245.14	9.05	263.34	4.59	401.16	61.63
SPL5	132.54	18.50	147.74	38.15	239.57	28.56	1211.71	270.32
SPL15	261.98	6.64	252.01	7.66	285.35	10.25	435.71	105.39
SPL3	205.94	6.25	203.10	8.92	402.16	71.37	208.78	14.69
SPL10	270.99	3.47	270.48	12.98	277.39	5.01	273.84	7.38
SPL11	211.53	16.11	240.54	11.53	347.79	7.34	365.51	12.36
SPL16	435.46	30.98	509.91	66.22	413.56	51.68	431.18	31.00

SPLs not targeted by miR156

(Mean and standard deviation are based on 4 biological samples/time point)

Table III. Primer list

ID	Gene	Sequence(5'-3')	Purpose	Sense
B187	Aa*SPL9*	(attB1)ATGGAGATGGGTTCCAACTCTG	Cloning in pDONR221	F
B188	Aa*SPL9*	(attB2)TCAGAGAGACCAGTTGGTATGG	Cloning in pDONR221	R
B194	Aa*SPL9*	GACTCAAACTGGCCTTAAGCTTGTTAAGCAATCCGGATCAGCCACAAG	Change miR156 target site	F
B195	Aa*SPL9*	GATCCGGATTGCTTAACAAGCTTAAGGCGCAGTTTGAGTCGCCCATTCCC	Change miR156 target site	R
B183	Aa*SPL9*	(T3)GCCACAAGACAACAACAAC	*in situ* hybridization	F
B184	Aa*SPL9*	(T7)CTTTGATGCAAAGAGAAGTAG	*in situ* hybridization	R
B217	Aa*PP2A*	AGTATCGCTTCTCGCTCCAG	qRT-PCR	F
B218	Aa*PP2A*	AACCGGTTGGTCGACTATTG	qRT-PCR	R
B156	Aa*SPL9*	CAGATAAACGGTGGAACGAC	qRT-PCR	F
B157	Aa*SPL9*	CTTTGATGCAAAGAGAAGTAG	qRT-PCR	R
B163	miR156	CGCGAGCTCAGAATTAATACGACTCACTATACGGCGGTGCTCAC	Key primer universal	R
F408	miR172	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACATGCAG	Key primer universal	R
F409	miR172	GGCGGAGAATCTTGATGATG	qRT-PCR	R
B148	AasnoR101	TTTTACAGGTAAGTTCTTGTTG	qRT-PCR	F
B149	AasnoR101	AGCATCAGCAAACCAATAGTT	qRT-PCR	R
B164	miR156	TGACAGAAGAGAGTG	qRT-PCR	F
B165	miR156	CGCGAGCTCAGAATTAATACGA	qRT-PCR	R
B166	miR172	AGAATCTTGATGATGC	qRT-PCR	F
B146	AtsnoR101	CTTCACAGGTAAGTTCGCTTG	qRT-PCR	F
B147	AtsnoR101	AGCATCAGCAGACCAGTAGTT	qRT-PCR	R
B185	Aa*SPL13*	AGAGCTCGAGAACAGCATCG	qRT-PCR	F
B186	Aa*SPL13*	CTGCACTCGCATTCTCAAaC	qRT-PCR	R
B160	Aa*SPL5*	CCAGCTGGCTCTCTCTCTTC	qRT-PCR	F
B161	Aa*SPL5*	TGCGGATAGACATCCATTATAGG	qRT-PCR	R
W113	Aa*RAN3*	CACAGGAAAAACCACATTCGT	qRT-PCR	F
W114	Aa*RAN3*	CCATCCCTAAGACCACCAAAT	qRT-PCR	R

Predicted cDNA sequences of miR156-regulated Aa *SPL* genes

G37042 (Aa *SPL2*)

ATTTTGCTGGTCAGTGTCTGATGGAGTGTAATGCGAAGCCATCGTTGCAGTGGGAATGGGATAATTTAATATCAT
TTGGTACTTCATCAGCTGAAATTCCTAGAAAGCTACGACCAATGGAGTGGGAAATTGATGGGTTTGATTGCACTT
CCCTCTATTCGTCCAGCTTTGCAGCAGTAGCTAATGGTGGTGGTTCAGGTTCTGACCTAGCTCAAGCATTCTCGA
AAAGCTCAAAGTCAACTTCCATCAGCTCTTCGTCAACTGAAGTGAGAACACACAATTTTACATCCGAAGCTGGTG
AAAGTCTTCCTGGAGAACTGGGCAGCAGTGAAGAGTTTGCAAAGGGAATCGATGCTTCGCCGAGTCTTGAACTTT
CCTTTGGCTCTGGTGATCCGGTTCTTGGTTTGAAGCTTGGTAAGCGAACATATTTTGAAGACTTTTGGGAAGTGG
AGAATGCAAAAGGTTCGGCACTTCCAGTGAGCCTCGCATCATCTGTTTCTCCGGTAAAGAGATCCAAAACCATTC
CGCAGAGGTTACAAACTCCTCACTGCCAAGTTGAAGGCTGTAATCTCGATCTCTCATCAGCTAAAGACTATCATC
GGAAACACAGGATTTGTGAAAACCATTCAAAGTTTCCGAAAGTCGTTGTGAGTGGCGTAGAACGTCGGTTCTGCC
AACAGTGCAGCAGGTTCCACTGTCTCTCTGAGTTTGATGAGAAGAAACGTAGCTGTCGCCGGCGTCTCTCTGATC
ACAATGCAAGACGTCGCAAGCCAAATCCTGGGAGGACATTTGATGGGAAGCAACATATGGATTTTGGATGGAACA
GATTTGCACTTGTCCATCCAAAAAGTGAGGAAAAGTTTCTATGGCCAAGTTCTAATCCCACACCATCAAGAGGGT
TGATTCTGCAACCTGCAAAGACCGAGACTTCCAATAAGCTGTTCACTAAGAACTGTGGATTTGGATTGTTGGACC
CCAAAACCaAAACCTCGAGAACTGAGTTATTCAGTAAAGAAAAGGTCATAATCTCTTCACACATGGGTACTTCTC
AAGATCTTGATGGTGCTCTCTCTCTTCTGTCAAATTCAACATCATGGGCTTCCTCTGACCCACCAAGACGTTTCT
CCCTTGACCACCATCCCACAAGCAACCTCCAACCACCCGTTGCTGACCGGTCTGTGTCTCAACTCAATTCAGTGT
CCGGCTATTGGCAGCCAGACGCACCAGCAGTGGGCCAAACAGCTCTGCATAGAAACGGTGTAGGCCAGTTTAATG
AGAACTACTTCAGCTTTAACCAGTTTTATAACTTATGAAAGTGTATTATGCCCTTGAATCCTGTAAGGATCtTTT
TTATGCAAGGATCGAGCAGGCGTAGTGTTAAATTTAAGATAGGATCTGTGAAACTTTCAGACACCAAGTCCTTTC
TTCTTTATTGCCCATTTATTTTCATGGGTTAGAGAACTGTGACAATTGTCTTGTGATGTTATAAATTCTAATTTC
TAAA

G12651 (Aa *SPL3*)

AGAGAGAGTTGAATTTTAGAGATGAGTATGAGAAGAAGCAAAGCAGAAGGGAAGAGGAGTTTAAGAGAAATGAGT
GAGGAAGAAGAAGATGATTATGATACTCCTGAGGAAGAAGAAGCTTTGGAGAAGAAGCAGAAAGGCAAAGGTACG
AGTAGTAGTAGTAGTAATCATGGAGTTTGTCAGGTGGAGAGTTGTTCTGTGGACATGAGCACAGCCAAACGCTAC
CACAAACGACACAGAGTCTGCGAGGTTCATGCCAAAGCTCCTCTTGTTTGGATCTCCGGTCTTCACCAACGTTTC
TGCCAGCAATGCAGCAGGTTTCACGAGCTCGGTGAGTTTGATGAAGCCAAGCGGAGTTGCAGGAGACGCTTAGCT
GGACACAACGAGAGAAGGCGGAAAAGCACGAATGAAGAATAAAGACGGTGAAAGATCTAAAATCTTGATATGAAG
GTTAATGAAATAGTCTTTGCTTGCTCTCTTCTGTCAGTCTCTTTAGCTCCTTGTAATCCTCTGTGTGTGTATCTC
TGTTTCTCTATATTGCCTGCAATCATACCTATCTGCTAAACCTAAGACATAATTAAATGCTTTTGAGACTAAGTA
AATGCTTGTTGGAATA

G29689 (Aa *SPL4*)

AATATCACCAAGTTTCATTGCTTTAACCTACTAGAGTTTCTTTCCTTCAAGAACCATCTTTTGAAGACTCTTTAC
TCTACCCAAAATGGAGGGTAAAAGAACAACACAAGGACAAGGCTACATGAAAAACAAGGCTAGTGTGTCTTACCT
TGTGGAAGAAGAAATGGAGAATGATATTGATGGAGAAGAGGAAGAGAAGAGGAACCGAGTGAATAAAGGGTATAG
TGGCAACACCGGTGGCTCATCGCGGTTGTGTCAAGTAGATAGATGCACATCTGATCTAAAAGAAGCTAAACAGTA
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TTGTCAACAATGCAGCAGGTTTCATGAGCTCTTGGAGTTTGATGAAGCTAAAAGAAGTTGTAGGAGGCGTCTCGC
TGGACATAATGAGAGGAGAAGGAAGAGCTCTAGTGAAAGTTTTGGAGAAGGATCGAGTAGTGGTCGAAGAGGAAT
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GGAGACTAATCGAAAGCCACGGTCACTATGAAGTTTCAGAGCCACGAATGAGAGATGTTTTGCTACGGATTTCAC
ATCTTTTTATGACATTTGATCGAGTTGAGGACTTCTTGGTTTGACATAACCTAGGAGTAGATTACAAGATATGAA
TAAAATCGATTGGGAGTA

G9988 (Aa *SPL6*)

TTTTCAATATTAGTGATTGATGGATTCTTGGAGTTACGGAAGAAGCGTTTTCTTGGCTAATGAATTGGAGAATGA
TGATCATGTCTTGATGTCCAATGGATTTAGTGATGTTTCCATTGCCAAAGTAGCTTCTTCTTCTTCTTGTGTTGT
TCCTTCGAGTTTGTTTCCTGTGGAGGAGGAAGAGAATGAATTTTCAAAGTTCTCTAGTGTGGCAACTGAAGAATT
GGCTAGGATAGATTTTAAATTAAGCAGCTTTTTGGATTATGGGAACGATGATACTTCCACTAGAGCTTTTGCTCT
TCGTGGTAAAAAGTCTCGAGCTTTAAACTCGTGCTCGCAGAAACCATTGTGTCAAGTGTATGGTTGCAATATGGA

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TGTTCTGCACAAGGACAGAGCAAGGCTTCTTGGTTTGATGATTTATTGGATTGGTGAGATGGTTAGATTAGGGGA
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GTTCTGAACAAATTTGCTTCAGATTTCAATGTTCTGCTTCTTTCTCAGGGTTTCAGATTGTATATGTAAATGTAA
TGAATATTCGCTGAAAAA

G2249 (Aa *SPL9*)

CACTCTCGTCTCTTTTTTtACCTTCTCTTCTGTCTCTCTCTCTCCTTTCTCTGAACCCAAAACAAGTCAAAATCA
GGGAAGCCATAATCTTCTTTGCTTTCTTCTCCTTTAGTCCTTTCTCTTAAACCGAAACAGTTAGGTTTTACAGAG
AAAGACTGATGAGTTTCTGTCTGAGTTAGAGGAAACCAAAATGGAGATGGGTTCCAACTCTGGCCCTAGACATGG
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CCAAAAGATCTACTTTGAGGATGGTGGTGGATCCGGTTCTTCTTCCTCCGGTGGGTCAAACAGAAGGGTACGTGG
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GTTATCTGATCAACATCATCAAAGTAGGAGACAGTACATGGAAAGTGAGAACACAAGGGCTTATGACTCTTCTTC
TAACCATACCAACTGGTCTCTCTGAGTCTTCTTTAGACTACTTCTCTTTGCATCAAAGAATCTTGTTACAATGAA
AAATGATTCTGCAATATCTTATCTTTTTGCTCCTTTTCTTCTACTAAATAAACCAAACAATTATTG

G7176 (Aa *SPL10*)

GATTTGTTGCTCTGGCTTTTGGTAGTAGGTCTATGAGATCAACCCTCATCTTGAATGGACTGCAACATGTTATCT
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ACTGAGTGGGAAGTTGAGAAAGGTGAAGGAATTGAATCTATAGTTCCATGTTTCGCCGCCCTCGAGAGAGTCAGT
AGTGTCTCTCCCACCAGTTTCTGGCACACTGCTGTATCGAAAAGCTCACAGTCCACCTCTATAAACTCATCATCT
CCCAAACTCAAACATtGCAATCTTGCATCAGAAAGTTCCCCTGGAGATTCTTGCAGCAACATAGATTTTGTCCAA
GTGAAGGCATCCCCAGCTTTAGACCTATCTGTTGCGTCAGCTGAATCAGATCTTTCTTTAAAACTAGGAAAGCCG
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GTTGTCGCTCGGAAGAAATCTAAATCGTGTGGTCAGAGCTTGCAAGTCCCACGTTGCCAAATTGATGGCTGTGAA
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TCCAACTTTCATCTTCCTGACAAAGGTATTGGAGAATGCTCACGGGGCATCCAAGAATCTCAAGATTTCTACAGT
GCTCTCTCTCTTCTGTCAACCTCTTCGGAATCACGTGGGACCAAACACAATCCCCTGATCGAAGCACAGCCGATA
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GTTTTCAAATTAAATAAAAAGGTGTACCGGTTACTCGGTTATCCTCGGTTTGTAGATACTTTCCAAACCTATCAG
ATCAACTTCTTCCTGGATCTTGTTGTATGTTATCTTCGTTACTGTCTCAGACATGTCTACTGTTTCATAAACAGA
TATACATTGTTTTCAATAAATGTTTGCATCTTGT

G7177 (Aa *SPL11*)

TTCTATGAAATCAACCCACTTCTTCAATGGACTGCAACATGACATCTCCGTTGCAGTGGGACTGGGATCATTTGA
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CTTCCCTCGAGAGAGCCCGTAGTGGTTCTGCGAATACTTTCCGGCACTCTGCTGCACCGAAAAGCGCACAGTCCA
CCTCTATCAACTCATCGTCTCCTAAAATCAAACATTGCAAGCTTGCATCAGAAAATTCTCCTGGAGATTCTTGCA
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TCGATCTCTCATCTGCCAAGGATTATCATCGTAAGCACAGAGTCTGCGAAAGCCATTCAAAGTGCCCTAAAGTTA
TTGTGGGTGGCCTGGAACGTCGATTCTGCCAACAGTGTAGCAGGTTCCATGCTGTCTCTGAATTCGATGAAACGA
AACGAAGCTGCCGAAAACGTCTTTCTCATCATAATGCGAGGCGTCGCAAGCTGCAAGGAGTATTTCCATTGAATC
CAGAAAGGGTGTACAATCGAAGACAGCAAACAAATATGCTGTGGAATGGGTTCTCCCTTAACACGAGATCCGAAG
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TCTCGACCGGGAAGTCCAACCTTCAACTTCAGGGCAAAGGTGTGGGAGAATACTCAGGGGTCCTCCATGAATCTC
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CGCAGCCACTTTCATCACTCTTCTTTTCATATGATGTTGTACCAAAATAGATGAGTAAGTAATGTCTAGTTTGTA
AAATCAGGTTAGCTCCGATCTGTGGATACTTGTCCAAGCCTATCAAAACATACATCATTCTTGATCGATCATGTT
GATGCTACTTTCGTATCTTTATCCTTCTTTTTCACACTGTTAACAAAACACTAAAAAGTGAAGATCTCTCTCTCT
TTGTTAATAGTAAATCAAGTAGAACGAATTTA

g27266 (Aa *SPL13/17*)

TGAGTGAAGGCGGAAAACAACAACAACAACATAAAAACACAATCTTTGAATGGACTGGAACTTCAAACTCAGCTC
GGGGTATTTGTCGGAATTTGAACAAGAATCTGTGCCTGATTTAACCCCAATCGATGGCTCGATCTCGTTCGGTGG
GTCATCACCAAAAGGGGACTTCTCATTTGATCTGAAACTTGGAAGAAACATTGGAAGCTCCTCCTCCTCTGCTTT
TGGTAATACAGAGCAAGTGATAAGTCTTAGTAAGTGGAAAGAGAGATCTATGTCAAAACCTGATGTTTCAAGAGC
CTCTTCATCGTCCAAGAGGACAAGAGGCAATGGTCTCGGGAACAACCAGATGCCGATTTGCTTGGTTGATGGATG
TGATTCTGATTTTAGTAACTGTAGAGAGTATCATAAGAGGCATAAGGTCTGTGAGGTTCATTCCAAAACTCCTGT
CGTTACAATCAATGGCCATAAGCAGAGGTTTTGCCAACAATGCAGCAGGTTTCATTCCTTGGAAGAGTTTGATGA
AGGGAAGAGAAGTTGTAGGAAACGTCTCGATGGACACAATCGTAGAAGACGGAAGCCTCAGCCTGATCATATTTC
CCGTCCTGCAAACTTCTTCACCGGTTTCCAAGGTACTAAATTGCTGGAGTTCTCTGGTGGTTCACATGTGTTTCC
AACTACATCTGTGGCGAACCCAAGCTGGGGAAATGGTCTTGTAAGCGTCGCTATGGCTAATGGTTCGAGTTACGG
GCAGAACCAGGGCTTTTCTGGTTCTTCTCCTGCAAAGACAGGCATAATGTTCCCAATCTCTTCTTCTCCAAACAG
CAGTAGAATCGCAGGAAAACAGTTCCCTTTCTTGCAAGAAGAAGAGAGCTCGAGAACAGCATCGTTGTGTGAGAG
AATGACGAGTTGCATCCATGACTCCGATTGTGCTCTCTCTCTTCTGTCATCCTCCTCATCAGTCCCTCATTTGCT
TCAACCGCCACTTTCTTTGTCCCAAGAAGCAGTAGAGACAGTTTTTTATGGCTCCGGATTGTTTGAGAATGCGAG
TGCAGTCTCTGATGGATCGGTTATATCCGGTAATGAGGCTGTGGCTCTTCCACAGACGTTCCCGTTTCACTGGGA
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TTTTTCATTGCTCTTGTTAAATGCTGAATTGTAGGTGGTCATGGACAATCGATTACTTGATGTGTAGCCCCCAAc
TAGCACCAATTAAGAGCTAAGATCAGTAGATAACTCTTTATGTACACACATTTCATTTCCAGTACTAAAAAAAGT
CTTAATCG

G17165 (Aa *SPL15*)

ATTTTATTTCCTTTCTTCTTCTTGGTCCTTTCTATTTCAAGGCTACAGAGAGTGAGATATGAGCAAAAGCATATC
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GAGTCAGGTGGTTCATCTTCAACAGAGTCATCTTCACTAAGTGGTGGACTAAGGTTTGGTCAAAAGATCTACTTT
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AGGTGCCAAGTGGAAGGTTGTAGAATGGATCTAACCAATGCAAAAAGTTACTACTCAAGACACAAAGTTTGTTGC
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CTGCTTTCTGAGTTTGATTTAGAGAAAAGAAGTTGTCGTAGACGACTCGCTTGCCATAACGAACGACGAAGAAAG
CCACAAGCTACAACTAGTCTTTTGAGTTCTCGTTACGCTAGAACTGCTCCATCTCTTTACGGGAATGCAAATTCT
GCGATGATTCGTAGCGTTTTGGGAGATCCTACAGCTTGGGCAACCGCAAGATCAGCGATGCGATGGTCTGGACCG
GAAAGGGAAAGCCATCAAGTTATGAATGTTTTCTCATCACATGGAAGCTCAAGCTTTACTACAACATGTCCAGAG
ATGATGATGAACAACAATGGCACAGACTCAAGCTGTGCTCTCTCTCTTCT
GTCAAACACAAACCCAAATCAGCAGCTACAGCAACATCAACTTCAGACACCAACCAATGTATGGAGACCATCGTC
AGGTTTCAATCCAGTGATTGCCGATAGGGTTACAATGGCGCAGCCACCACCCGTTTCAATCCATAATCAGTACCT
GAACAACCAAACTTGGGAGTTTACGACCGGTGAAAAGAGCAATTTGCAGTATATGTCGCCTGTTTTGGGACCGAG
TCAAATCTCCCAGCCAGCTGATTTCCAGATTAGTAATGGCTCGACAATGGGTGGATTCGAGCTGTCTCATCATCA

ACAGGTTCTGAGGCAATACATGGAACCTGAGAACACAAGAGCTTATGACTCTTCACCTCAACATTTCAATTGGTC
TCTTTGATGAGTGTTTTCTCTTTCACCCTTTAAAGACCTTTTTTGGATTAGCTTTTTCATCTGCACCAAGAGCTT
TACCTGTGGAAGGTTGCTACTAAGACTTGATAGTCTCAAGATCTTATCAATTATCTGTTCTATGCTACTTAAAAC
CGGGGAATGATACCGGATAATGGCTTTCGATTTGGATATTATTCTAGTGGCTTTGGCTTTTGATCTTTAAGCCCT
TTGATATTATGCTGTTGATGTTTTGTTTA

Aa *SPL5* CDS (manually annotated)

ATGGAGGGTCAGCGAACACAACGTAGGGGTTACTTGAAGGAAAAGGCTACAGTCTCCAGCCTTGTTGAAGAAGAA
ATGGAGAATGACATGGATGAAGAAGAGGAGGATGGAGGAGAAGAAGACAAAAGGAGGAGAATGATGGAGAGAGTT
AGAGGTCCTATCACCGACCGTGTTCCATCACGACTGTGCCAGGTCGATAGGTGCGCTGCTAATCTGACTGAGGCC
AAGCAGTATCACCGCAGACACAGAGTCTGTGAAGTTCATGCAAAGGCATCTGCTGCAACAGTTGCAGGCGTCAGG
CAACGGTTTTGTCAACAATGTAGCAGGTTTCATGAGCTACCAGAGTTTGATGAAACTAAAAGAAGCTGCCGGAGG
CGCTTAGCTGGACACAATGAGAGGAGGAGGAAGAGCTCTGGTGAAAGTTTTGGAGAAGGGTCAGGCCGGAGAGGG
TTTAGTGGTCAACTGATCCAGACTCAAGAAAGAAACAGGGTAGACATGAAACTTCCTATGACCAACTCATCGTTA
AAACGACCACAGATCAGATAA

List of abbreviations

General abbreviations

°C	degree Celsius
µg	microgramm
3', 5'	3-prime, 5-prime
A	Adult plants
A. alpina	*Arabis alpina*
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
Aa	*Arabis alpina*
At	*Arabidopsis thaliana*
AV	Adult vernalized plant
AV	after vernalization
bp	basepair
b-Zip	Basic Leucine Zipper Domain
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CDS	coding sequence
ChIP	Chromatin immune precipitation
Col	Columbia
Cy3	Cyanine 3 dye
Cy3	Cyanine 3 fluorescent dye
Cy5	Cyanine 5 dye
Cy5	Cyanine 5 fluorescent dye
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
dsRNAi	double strand-RNA-interference
GA	gibberellic acid
h	hour
H2A.Z	Z variant of histone protein H2A
H3K27me3	tri-methylatedlysin 27 at histone 3
J	Juvenile
JV	Juvenile + vernalization,

kb	kilobase
LD	long day
Ler	Landsberg *erecta*
M	Mol
mer	oligomer
miR	micro-RNA
mRNA	messenger RNA
Mya	million year ago
ncRNA	non coding RNA
nt	nucleotide
PCR	polymerase chain reaction
Pop	poplar, Populus
Pp	Physcomitrella patens
PRC2	polycomb repressive complex 2
RMA	Robust multichip Average
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	retro transcribed-PCR
SAM	shoot apical meristem
SBP-box	SQUAMOSA PROMOTER BINDING PROTEIN - box
SD	short day
Seq	sequencing
UTR	untranslated region
UV	ultraviolet light
V	vernalization
w	week
ZT	zeitgeber time

Amino acids

Alanine	A
Cysteine	C
Aspartic acid	D

Glutamic acid	E
Phenylalanine	F
Glycine	G
Histidine	H
Isoleucine	I
Lysine	K
Leucine	L
Methionine	M
Asparagine	N
Proline	P
Glutamine	Q
Arginine	R
Serine	S
Threonine	T
Valine	V
Tryptophane	W

Abbreviations of gene and protein names and ncRNA

AGL24	AGAMOUS LIKE 24
AGL42	AGAMOUS LIKE 42
AP1	APETALA 1
AP2	APETALA 2
Cg	Corn grass
CO	CONSTANS
COLDAIR	cold assisted intronic non coding RNA
COOLAIR	cold induced long antisense intragenic RNA
FCA	-
FD	FLOWERING LOCUS D
FLC	FLOWERING LOCUS C
FLM	FLOWERING LOCUS M
FRI	FRIGIDA
FT	FLOWERING LOCUS T
FUL	FRUITFUL

FVE	-
LFY	LEAFY
miR156	microRNA 156
miR172	microRNA 172
PNF	POUND-FOOLISH
PNY	PENNYWISE
SMZ	SCHLAFMUTZE
SNZ	SCHNARCHZAPFEN
SOC1	SUPPRESSOR OF OVEREXPRESSOR OF CONSTANS 1
SPL (1-17)	SQUAMOSA PROMOTER BINDING LIKE PROTEIN (1-17)
SVP	SHORT VEGETATIVE PHASE
TOE1/2/3	TARGET OF EAT 1/2/3

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Erklärung

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Köln, den 5. September 2011

Sara Bergonzi

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Lebenslauf

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