Forever young: how AHL15 delays developmental phase transitions to prevent ageing in plants

Luden, T.

Chapter 1

Regulation of the life history of flowering plants

(Introduction)

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Summary

The life history strategy of plants can vary strongly between different plant species. Some annuals, such as the model plant *Arabidopsis thaliana* (Arabidopsis) germinate, grow, flower, set seed and die within a period of a few months, whereas woody perennials grow and flower every year and are among the longest lived organisms on this earth. The majority of our knowledge on longevity-associated traits in plants is based on research in Arabidopsis, which following germination undergoes four developmental phases from juvenile vegetative through adult vegetative and reproductive to the senescence phase. Here we will review the regulation of life history traits, with a focus on the juvenile-to-adult transition, called Vegetative Phase Change (VPC), the transition from vegetative to reproductive growth also called the floral transition, leaf senescence, and the maintenance of vegetative development during the reproductive stage in the context of life history strategy. We will also discuss how the protein AHL15 plays a role in each of these processes, and elaborate on the current knowledge of how AHL proteins function.

Keywords: *Arabidopsis thaliana,* vegetative phase change, phyllotaxis, flowering, floral transition, vernalization, inflorescence architecture, senescence, life history strategy, polycarpy, monocarpy, global proliferative arrest, AHL15, AHLs

Life cycle of flowering plants

The life cycle of the model plant *Arabidopsis thaliana* (Arabidopsis) and other seed plants begins with the germination of the seed. Seeds contain a dormant embryo, and germination is initiated when water is available (imbibition) and can be accelerated by other factors such as prior exposure to cold (stratification) (Iwasaki et al., 2022). Imbibed seeds will rapidly produce a radicle, an outgrowth of the embryonic root, followed by the expansion of the hypocotyl, which is a stem-like structure between the developing root and one or two embryonic leaves called cotyledons (Boyes et al., 2001) (Figure 1). Hypocotyl growth is responsive to light, and the hypocotyl will elongate when kept in dark or low-light conditions to ensure that the cotyledon(s) can emerge from the soil. The hypocotyl initially emerges with an apical hook in which the top of the hypocotyl is curved at an 180° angle, resulting in a downward position of the cotyledons and the stem cell zone called the shoot apical meristem (SAM), which helps to protect them when the shoot moves through the soil to reach the light. Upon exposure to light, the apical hook is resolved allowing the cotyledons to open, expand and produce chlorophyll to start photosynthesis (Abbas et al., 2013; Krahmer and Fankhauser, 2024). The primary root elongates by division and differentiation of cells in the root apical meristem, and lateral roots are produced by a specific stem cell zone in the root called the pericycle (Beeckman and De Smet, 2014). The aboveground tissues of the developing plant will be formed by the SAM as it produces new aerial organs. In Arabidopsis, the first pair of true leaves emerges simultaneously from the SAM after about one week, and subsequent leaves are formed one by one in a radial pattern. Leaves produced early on in the plant's life cycle are typically in a juvenile state, and over time the plant will gradually transition into an adult state, a process that is called vegetative phase change (VPC) (Poethig, 2013; Poethig and Fouracre, 2024). Before flowering, Arabidopsis plants grow as a rosette, meaning that leaves are produced from the SAM directly after one another without elongation of the stem in between. In addition to the SAM, axillary meristems (AMs) are located in leaf axils, which can either produce additional organs or remain dormant (Tian et al., 2014; Tian et al., 2019; Zhu and Wagner, 2020).

The floral transition is initiated during the plant's adult stage and its timing is strongly influenced by environmental conditions such as day length and temperature (Kinoshita and Richter, 2020). In Arabidopsis, flowering is promoted in long days and is repressed in short days, but this can be different in other species such as tomato whose floral transition is insensitive to day length (Lifschitz and Eshed, 2006), or chrysanthemum in which flowering is promoted

by short days (SharathKumar et al., 2021). In Arabidopsis, the flowering process starts with the formation of a primary inflorescence stem that emerges from the SAM in a process called bolting, and grows upwards to form flowers in a raceme: an indeterminate inflorescence from which new flowers emerge at the sides of the SAM, with the oldest flowers at the bottom and the youngest flowers at the top (Figure 1). Additional inflorescences can emerge from AMs in the rosette, which usually develop after the primary inflorescence has started elongating. The inflorescences also produce inflorescence AMs that can form secondary branches, resulting in a branched inflorescence architecture (Zhu and Wagner, 2020).

Arabidopsis is a self-pollinating plant, and upon fertilization, embryogenesis and seed formation occur in an elongated gynoecium, which develops into a silique that ripens as the rest of the plant undergoes senescence. The inflorescences continue to produce new flowers until the inflorescence meristem stops producing new stem cells and arrests its production of new flowers (Thomas, 2013; Balanzà et al., 2019). The life cycle of Arabidopsis is concluded when the whole plant has senesced and the siliques and seeds are ripened, resulting in the death of the plant and the release of seeds for a new generation (Figure 1).

Vegetative Phase Change

Plants generally undergo a juvenile and an adult growth phase during their vegetative state. The juvenile-to-adult transition during which plants shift from producing juvenile leaves to adult leaves and acquire the competence to flower is referred to as vegetative phase change (VPC). Juvenile Arabidopsis leaves are typically round in shape with smooth margins and lacking abaxial trichomes, whereas adult leaves are typically oblong or oval-shaped with serrated margins at the base and have trichomes on both sides of the leaf (Telfer et al., 1997; Rahimi et al., 2022a). This difference in phenotype between juvenile and adult leaves is called heteroblasty, and can be seen in many but not all plant species. Although heteroblasty is conserved, the specific traits associated with juvenile and adult leaves can differ widely between genotypes and species (Huijser and Schmid, 2011; Doody et al., 2022; this thesis).

VPC has generally been considered as a necessary transition in the plant to gain competence to flower (Huijser and Schmid, 2011; Hyun et al., 2017). However, recent findings have challenged the idea that VPC is a necessary step for the initiation of the flowering process. For example, growth in monochromatic blue light accelerates flowering time while the VPC is only

weakly affected (Spaninks et al., 2020). In addition, the timing of VPC also does not always correlate with the timing of the floral transition, indicating that these two processes are regulated independently (Hyun et al., 2017; Spaninks et al., 2020; Doody et al., 2022; this thesis).

The molecular pathway controlling VPC has been studied in detail in Arabidopsis, and is regulated primarily by the microRNAs *miR156* and *miR157*, and members of the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)

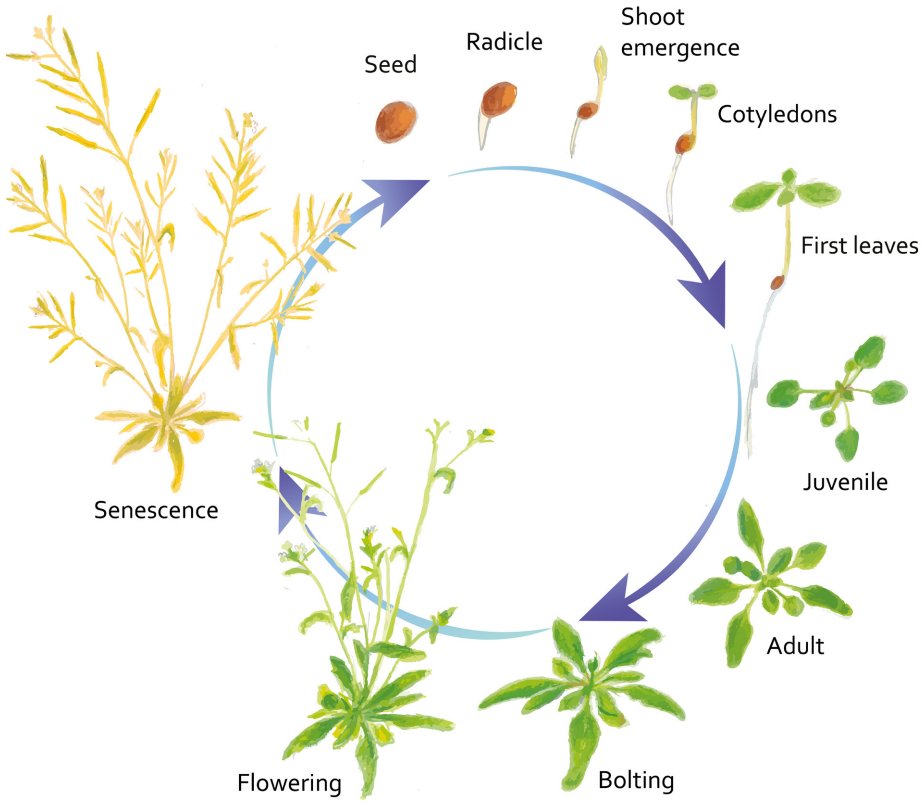

Figure 1: Life cycle of *Arabidopsis thaliana*. Germination of the seed starts with the emergence of the embryonic root (radicle), followed by the emergence of the shoot with an apical hook from which the two cotyledons will angle upwards and unfold once in the light. The first true leaves emerge symmetrically in a pair from the shoot apical meristem, and subsequent leaves are formed in a radial pattern. Juvenile leaves are rounded in shape with a long petiole, whereas adult leaves are more oblong with shorter petioles. The flowering process is induced in adult plants and starts with the formation of a bolt (the emerging primary inflorescence), and additional inflorescences are produced from axillary meristems in the rosette. Inflorescences form flowers in a raceme, an inflorescence type in which the oldest flowers are produced at the bottom and new flowers emerge from the top. After pollination, seeds are produced inside a silique. Upon flowering the senescence of the rosette is initiated, followed by senescence of the inflorescence tissues resulting in whole-plant death.

transcription factor (TF) family. Juvenile plants strongly express *miR156* and *miR157*, and their abundance decreases gradually over developmental time in a process that is controlled by the rate of cell division in the SAM (Cheng et al., 2021). The sequence of *miR156* and *miR157* is highly complementary to the mRNA sequence of many *SPL* genes, and causes post-transcriptional silencing of these genes via the RNAi pathway (Wang et al., 2009a). As miR156 abundance decreases over time, the post-transcriptional silencing of *SPLs* is reduced, resulting in a gradual increase of SPL protein levels (Wang et al., 2009a; Wu et al., 2009; Xu et al., 2016). SPLs regulate expression of several genes involved in leaf shape, trichome production, and flowering. For example, SPLs promote the expression of miR172, which targets the transcripts of a number of APETALA2 (AP2)-like genes that inhibit flowering and abaxial trichome formation, and thereby promote flowering and adult traits (Wu et al., 2009).

Perturbations in the miR156/SPL pathway can dramatically change the phenotype of the plant, resulting in a prolonged and ectopic juvenile state or an accelerated transition to the adult state depending on which part of the pathway is affected. For example, constitutive overexpression of *miR156* delays both the VPC and flowering time (Wu et al., 2009; Xu et al., 2016), and inflorescences of these plants produce rosette-like leaves from the inflorescence AMs that are called aerial rosettes (ARs) (Rahimi et al., 2022a). A similar phenotype can be seen in higher-order *spl* mutants, but this effect is less pronounced than in *MIR156* overexpressing plants. This can be explained by the fact that miR156 regulates as many as ten *SPL* genes, whereas only five *SPLs* were mutated in the highest-order *spl* knockout (Xu et al., 2016). On the other hand, plants overexpressing SPLs or those expressing miR156-insensitive *SPL* alleles (*rSPL)* show an accelerated VPC and flower early (Wang et al., 2009a; Wu et al., 2009; Xu et al., 2016).

Leaf development and rosette architecture

As the plant grows, new above-ground organs including leaves are produced from the pool of totipotent cells at the SAM. The pattern in which new leaves are formed is referred to as phyllotaxis, and the phyllotactic pattern varies between plant species. In Arabidopsis, phyllotaxis occurs in a radial pattern following Fibonacci's "golden angle", where each new leaf emerges at an angle of circa 137° relative to the one produced before (Kuhlemeier, 2017). Many other plant species show the same phyllotactic pattern: sunflower heads, pinecones, and pineapples all follow the same golden angle, although other phyllotactic patterns such as bilateral or fourfold symmetry or alternating

leaves are common as well.

Leaves develop from primordia in the periphery of the SAM, and the position of these primordia relative to one another determines the phyllotactic pattern. Primordia consist of groups of differentiating cells, and the central zone of the SAM contains totipotent cells (Kuhlemeier, 2017). Both the primordia and the cells in the SAM central zone continuously divide, resulting in an outward push of cells from the central zone to the periphery, and concomitant growth of the primordia. This results in in a pattern where the oldest, and largest, primordia are the most distant from the SAM, and new primordia arise closer to the SAM centre (Yin, 2021). In Arabidopsis and other plants with radial phyllotaxis, new primordia arise at the location that is most distant from previous primordia as a result of strong differences in local concentrations of the hormone auxin. Auxin promotes the division and differentiation of cells by activating specific target genes, and new primordia arise at local auxin maxima (Yin, 2021). This is demonstrated by the fact that primordium formation can be artificially induced by exogenous application of auxin (Reinhardt et al., 2000; Reinhardt et al., 2003).

The position of auxin maxima is determined by polar auxin transport which is regulated by auxin efflux carriers of the PIN-FORMED (PIN) family (Reinhardt et al., 2003). PIN proteins are predominantly located on the plasma membrane, where they are distributed asymmetrically (i.e. on one side of the cell). This asymmetric distribution establishes a directional auxin flow between cells (Okada et al., 1991). The position of PIN1, the most important PIN protein involved in phyllotaxis, is regulated by auxin in a positive feedback loop where a high local concentration of auxin stimulates efflux of auxin against the auxin gradient, resulting in local auxin hyperaccumulation (Reinhardt et al., 2003; Galvan-Ampudia et al., 2020). The high concentration of auxin then stimulates the formation of a primordium, whereas the vicinity is depleted of auxin, inhibiting the formation of other primordia. Together with the continuous division of cells in the SAM central zone, this results in a divergence angle of approximately 137° between subsequent leaves (Galvan-Ampudia et al., 2020). Disruptions in the transport of or response to auxin cause differences in phyllotaxis, and the absence of local auxin maxima in, for example, the *pin1* mutant or by inhibition of polar auxin transport abolishes the formation of primordia altogether (Reinhardt et al., 2000; Reinhardt et al., 2003).

In addition to the position and maintenance of local auxin maxima in the SAM, the size and number of cells in the SAM central zone also affects the phyllotactic pattern (Giulini et al., 2004; Yin, 2021). The SAM size is strongly

affected by the hormone cytokinin (CK), which promotes cell division in the SAM central zone (Leibfried et al., 2005). In parallel to the induction of primordium development by auxin, repression of CK signalling contributes to the phyllotactic pattern (Giulini et al., 2004; Besnard et al., 2014a; Besnard et al., 2014b). Knockout mutants of genes that negatively regulate the CK response show altered phyllotactic patterns as a result of the enhanced SAM size (Giulini et al., 2004), and the enhanced CK response results in the simultaneous initiation of multiple primordia (Giulini et al., 2004; Besnard et al., 2014a). This implies that CK promotes the outgrowth of newly arising primordia, and that repression of the CK response in developing primordia helps to time their outgrowth. The timing of primordium development is an important contributor to plant architecture, as this determines whether leaves are produced sequentially or in groups. Sequential initiation of primordia results in spiral or alternate phyllotaxis, whereas simultaneous initiation of primordia gives rise to opposing pairs or whorls of leaves. Finally, mechanical forces caused by cell expansion as well as stem torsion influence the phyllotactic pattern by affecting PIN polarity (Heisler et al., 2010; Yin, 2021).

The floral transition

Several factors can influence the floral transition, a process during which the plant undergoes several developmental changes that result in the production of an inflorescence and flowers (Figure 2A). In the absence of environmental stimuli, the developmental age of the plant will initiate the flowering process under influence of SPLTFs, most importantly SPL9 and SPL15 (Hyun et al., 2016; Xu et al., 2016). As the miR156 levels decrease with age and plants transition to the adult state, a threshold is reached in which the floral promoters overrule the floral inhibitors and flowering is initiated in a process called the age-dependent flowering pathway (Wang, 2014; Hyun et al., 2017).

Flowering cues are integrated by so-called floral integrators, such as SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and FLOWERING LOCUS T (FT). Together with SPL15, SOC1 promotes the expression of *miR172* and *FRUITFULL (FUL)* in the SAM (Hyun et al., 2016). FT is a mobile protein that is unable to directly bind DNA but promotes the expression of several flowering genes including *SOC1* when interacting with the DNA-binding TF FLOWERING LOCUS D (FD) in the SAM (Abe et al., 2005; Zhu et al., 2020). The genes targeted by SOC1 and the FT-FD complex promote differentiation of the SAM into an inflorescence meristem (IM) that produces bracts and flowers instead of leaves.

Growth in long-day conditions can accelerate the induction of flowering in long-day plants due to the activation of *FT* expression in leaves. *FT* transcription is controlled by the diurnally regulated TF CONSTANS (CO), whose stability is day-length dependent. In Arabidopsis, CO levels are low in short days, and sufficiently high levels to initiate *FT* transcription are only reached in long-day conditions (>12h light) (An et al., 2004; Valverde et al., 2004; Maple et al., 2024). The FT protein is expressed in the leaf vasculature and from there it enters the phloem and moves to different locations where it regulates the expression of its target genes. In the SAM, it interacts with FD to promote the expression of flowering genes, but FT can also regulate branch outgrowth and sugar transport (Fichtner et al., 2021). The daylength-dependent expression of *FT* is responsible for the rapid flowering of Arabidopsis under long day, but the daytime-dependent (diurnal) regulation of CO stability and subsequent *FT* expression varies between species and differs in species that, for example, flower under short days.

In addition to developmental age and day length, environmental temperature also plays an important role in controlling flowering time. For example, most *Brassica* species require vernalization, a prolonged exposure to cold conditions, to flower. This cold requirement is conveyed by *FLOWERING LOCUS C (FLC),* a floral repressor that is expressed in the whole plant upon germination and targets the flowering-promoting genes *SOC1, FT,* and *SPL15* and represses their expression (Deng et al., 2011). *FLC* expression is gradually silenced upon cold exposure, and becomes permanently silenced in Arabidopsis after a threshold period of cold exposure is reached (Michaels and Amasino, 1999; Shindo et al., 2006; Duncan et al., 2015). *FLC* expression is promoted by the TF FRIGIDA (FRI) (Michaels and Amasino, 1999; Johanson et al., 2000), and cold conditions cause a perturbation in the FRI transcriptional complex (Hu et al., 2014), reducing *FLC* expression immediately. More importantly, vernalization induces a gradual silencing of the *FLC* gene during extended cold exposure by recruiting the Polycomb Repressive Complex2 (PRC2) that trimethylates the Lysine 29 of histone 3 (H3K29me3; a repressive epigenetic mark) (De Lucia et al., 2008; Angel et al., 2011). The FLC pathway helps to prevent precocious flowering during winter, and the mechanism with which *FLC* is silenced during vernalization is an ongoing subject of study and is a widely used model to study epigenetic silencing mechanisms.

Inflorescence architecture and flower production

In Arabidopsis, the inflorescence phyllotaxis is regulated similarly to the

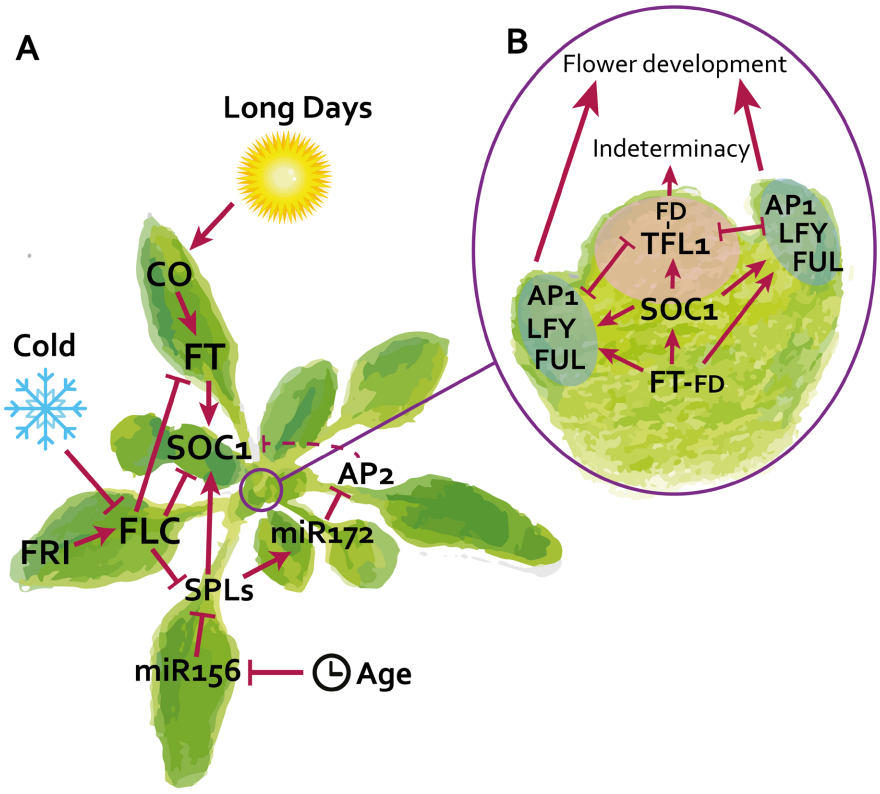

Figure 2: Induction of flowering in Arabidopsis. **A:** Long days promote the stability of the TF CONSTANS (CO) in leaves, which promotes transcription of *FLOWERING LOCUS T* (*FT*). The mobile FT signal travels to the SAM, where it binds to FD. This complex promotes flowering by inducing expression of *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*). SOC1 activates the expression of several other TFs, such as *APETALA1* (*AP1*), *LEAFY* (*LFY*), and *FRUITFULL* (*FUL*), that promote the transition from a vegetative to a reproductive meristem and the subsequent formation of flowers. Flowering is suppressed by FLOWERING LOCUS C (FLC), whose expression is promoted by FRIGIDA (FRI) and is suppressed by prolonged cold exposure (vernalization). In addition, flowering is repressed in young plants by high levels of miR156, which post-transcriptionally repress the *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) genes. Age reduces the expression of *miR156*, relieving the suppression of *SPL* genes and thereby increasing the expression of the flowering-promoting genes *SOC1* and *miR172*. miR172 targets the flowering-repressing gene *APETALA2* (*AP2*) and several of its family members.
B: Regulation of indeterminacy and inflorescence architecture in the SAM (area encircled in purple). FT binds FD in the SAM and promotes the expression of several flowering-promoting genes. TERMINAL FLOWER 1 (TFL1) competes with FT to bind FD. The TFL1-FD complex represses the expression of several flower-specifying genes (*AP1, LFY* and *FUL*) in the central zone of the IM to ensure its indeterminacy (area colored pink). On the other hand, in the peripheral zone of the IM the TFs AP1, LFY, and FUL initiate the flower developmental program by promoting the differentiation into a floral meristem that subsequently produces a flower (area colored blue).

rosette architecture: both tissue types are arranged in a spiral pattern. In order to produce flowers, primordia separate from the SAM and undergo a transition from the inflorescence identity of the IM to a floral identity, thereby becoming a floral meristem (FM). Each flower in the Arabidopsis inflorescence is produced by an individual FM that splits off from the IM, resulting in a raceme architecture in which the oldest flowers are produced at the bottom and new flowers are continuously produced near the SAM. The transition of IM- to FM identity is regulated by floral identity TFs, and in the SAM the balance between expression of TFs that convey either the IM or FM identity determines the architecture of the inflorescence (Prusinkiewicz et al., 2007; Azpeitia et al., 2021). IM-promoting TFs ensure indeterminacy of the SAM, whereas FMs are determinate and produce a single flower. To produce a raceme, TFs promoting the IM identity are generally expressed in the center of the SAM whereas TFs promoting floral identity are expressed in the primordia in the peripheral zone of the SAM, resulting in flowers that split off from the side and an indeterminate SAM in the center (Prusinkiewicz et al., 2007; Azpeitia et al., 2021).

Retaining at least some IM cells in the SAM is required for making an inflorescence that contains multiple flowers, because the complete transition of the IM to an FM would result in the production of a single flower. This can be seen in the *terminal flower 1* mutant, which does not express the *TERMINAL FLOWER 1 (TFL1)* gene that plays a role in preventing the transition of the IM to the FM fate. As its name suggests, the *tfl1* mutant produces only a single flower from each inflorescence (Alvarez et al., 1992; Bowman et al., 1993). TFL1 blocks the expression of floral identity genes in the center of the SAM and thereby ensures indeterminacy. This effect is achieved because TFL1 competes with FT to bind FD in the SAM. FT and TFL1 have opposite effects on FD target genes, resulting in a flowering-promoting effect by FT and a flowering-delaying effect by TFL1 (Zhu et al., 2020). *TFL1* expression is controlled in part by SOC1 and other flowering-inducing genes that restrict its expression to the center of the SAM, where TFL1 represses the transition to FM identity (Ratcliffe et al., 1999; Liu et al., 2013; Azpeitia et al., 2021).

FM identity is conveyed by TFs such as FUL, LEAFY (LFY), and APETALA1 (AP1) (Bowman et al., 1993; Liu et al., 2013; Azpeitia et al., 2021). Their expression is initially promoted by SOC1, the FT-FD complex, SPL3, and other flowering-promoting TFs (Xu et al., 2016; Zhu et al., 2020; Azpeitia et al., 2021). *LFY* is the first to be expressed, and acts as a pioneer TF to promote expression of *AP1* and other target genes (Jin et al., 2021). AP1 promotes the expression of flower-specific genes and blocks the expression of *TFL1*, which

enforces the FM identity in domains in which AP1 is expressed (Ratcliffe et al., 1999; Prusinkiewicz et al., 2007; Azpeitia et al., 2021). Knockout of FM-specific genes usually results in defects in the flower architecture: *lfy* mutants produce indeterminate branches with many bracts instead of flowers, and when flowers are eventually made they are subtended by many additional bracts (Schultz and Haughn, 1991; Weigel and Nilsson, 1995). *ap1* loss-of-function plants produce flowers with defective petals or no petals at all (Alejandra Mandel et al., 1992), and double mutants of *ap1* and its close homolog *cauliflower (cal)* produce an enlarged, cauliflower-like IM with many FMs that do not develop into flowers (Bowman et al., 1993). The proper control of IM and FM boundaries is required to ensure functional inflorescence and flower architectures, and changes in their expression patterns explain the variation in inflorescence architectures that have evolved (Harder and Prusinkiewicz, 2013). In addition, human selection for mutations in IM- and FM identity genes have resulted in the various *Brassicaceae*- and other vegetables that are cultivated worldwide: cauliflower, for example, is a *Brassica oleraceae* variety with mutations in its *ap1* and *cal* orthologs (Azpeitia et al., 2021). Thus, knowledge of the TFs shaping the inflorescence architecture is of fundamental as well as agricultural interest.

Leaf senescence

Soon after bolting and the formation of the first flowers, Arabidopsis plants will gradually start to relocate their nutrients from the rosette leaves to the developing flowers and seeds through a process called senescence. Senescence is a developmental process during which the chlorophyll (Chl) and other components of a plant organ (primarily leaves) are systematically degraded and transported for reuse to other, newly developing parts of the plant, and can contribute to a plant's health and reproductive success (Lim et al., 2007a; Schippers et al., 2015). Proper regulation and a timely onset of senescence is important for the plant's ability to cope with a sudden change in nutrient availability and for securing sufficient nutrients for their offspring (Schippers et al., 2015). For example, it was shown that seed protein content correlates with early leaf senescence in wheat and barley, indicating that senescence positively impacts the seed quality (Uauy et al., 2006; Jukanti and Fischer, 2008). On the other hand, delayed leaf senescence can increase the photosynthetic capacity of those leaves and thereby increase the final seed yield, even if the quality and protein content of the individual seeds is somewhat reduced (Guo et al., 2021). Delayed senescence can also be a valuable trait for commercially cultivated food- and ornamental crops, as the yield and shelf life of leafy crops

and the shelf life of cut flowers is increased when senescence is delayed (Zhou and Yang, 2023). Senescence is therefore an agriculturally important trait and improved control of plant senescence can help to increase the fitness of plants in nutrient-limited conditions, improve nutritional qualities, and improve the marketability of crops and reduce food waste.

The most commonly studied and most visible type of senescence is leaf senescence, which can be induced via several processes: sequential, induced, reproductive, and post-harvest senescence. Sequential or age-dependent senescence is the process during which older leaves are degraded and their nutrients are relocated to newly developing leaves. This process is induced primarily by developmental age and is unaffected by reproduction or other developmental processes (Zentgraf et al., 2004; Schippers et al., 2015). The timing of sequential senescence has been proposed to be regulated by the metabolic rate and the accumulation of oxidative stress over a leaf's life time, and mutants with reduced photosynthetic efficiency show a delayed senescence phenotype (Lim et al., 2007a). Senescence can also be induced by several external factors, such as treatment with plant hormones, dark incubation, salt- or drought stress, environmental temperature, or pathogen infection (Guo and Gan, 2005; Bresson et al., 2018). Reproductive senescence is induced upon flower- and seed production. It ensures that the plant's resources are relocated from the leaves to the newly developing seeds, providing the maximum amount of resources to the next generation, and can be delayed in some species by removal of the reproductive structures (Schippers et al., 2015). Finally, post-harvest senescence is the process in which senescence is induced when a plant is cut from its roots or when leaves are removed from the plant. In all cases, the expression of a large set of senescence-associated genes is induced, which eventually results in the degradation of Chl and other macronutrients.

Leaf senescence is regulated by several plant hormones, most notably ethylene. Ethylene promotes fruit ripening, etiolation (stem elongation in dark conditions), and plays a central role in promoting leaf senescence (e.g. Guo et al., 2021). Other hormones that can induce senescence are strigolactones, abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA), whereas CK and auxin have been shown to delay the onset of leaf senescence (Guo and Gan, 2005; Ueda and Kusaba, 2015). Interestingly, ethylene-induced senescence can only occur when a leaf has reached a certain age, as ethylene application could only induce senescence in Arabidopsis leaves that were at least 25 days old (Jing et al., 2005). In addition to hormone treatment, senescence

can be externally induced by placing plants or detached leaves in the dark. This adaptive trait helps a plant relocate its nutrients away from leaves that do not catch sufficient sunlight for photosynthesis to new leaves (Schippers et al., 2015). Ethylene treatment and dark incubation are two commonly used methods to induce leaf senescence in an experimental setup to investigate

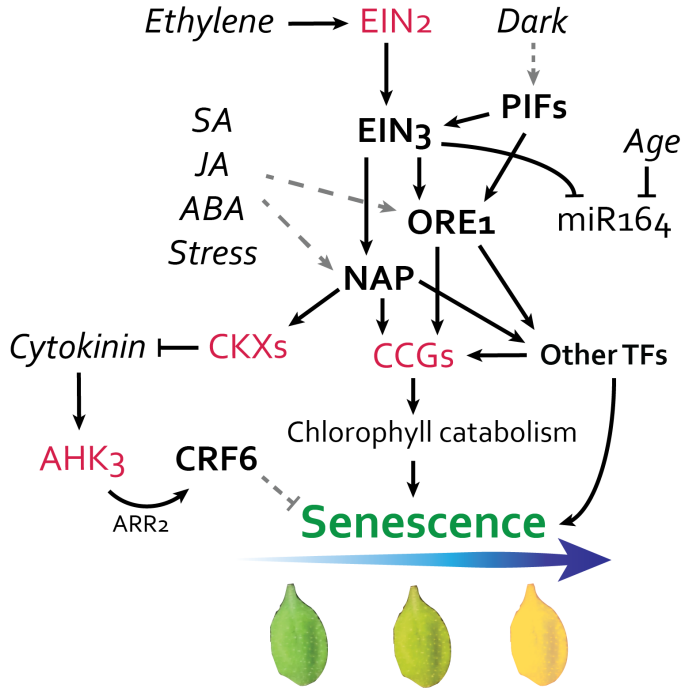

Figure 3: A simplified overview of regulation of leaf senescence in *Arabidopsis thaliana*. Senescence can be induced by ethylene, which is perceived by receptors such as ETHYLENE RESISTANT1 (ETR1) and via ETHYLENE INSENSITIVE2 (EIN2) activates the TF ETHYLENE INSENSITIVE3 (EIN3). EIN3 promotes the transcription of several TF-encoding genes, of which the most important are *ORESARA1* (*ORE1*) and *NAC-LIKE ACTIVATED BY AP3/PI* (*NAP*). NAP and ORE1 then promote the expression of several Chlorophyll Catabolic Genes (*CCGs*) either directly or indirectly via other TFs. *ORE1* mRNA is targeted by miR164, whose transcription is repressed by EIN3 and decreases with age, thereby regulating the age-dependent senescence pathway. Senescence can be induced by several other hormones (e.g. JA, SA, ABA) or external stressors that promote the expression of the senescence-inducing TFs *NAP* and *ORE1* in an ethylene-independent manner. Dark treatment activates PHYTOCHROME INTERACTING FACTOR (PIF) 4 and PIF5, which promote senescence by inducing the transcription of *EIN3* and *ORE1*. Finally, cytokinin represses senescence via its receptor ARABIDOPSIS HISTIDINE KINASE 3 (AHK3), which phosphorylates ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2). Phosphorylated ARR2 promotes transcription of the TF *CYTOKININ RESPONSE FACTOR 6* (*CRF6*), which inhibits senescence by an unknown mechanism. During the senescence process, NAP also induces expression of the *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) proteins, which degrade cytokinin and thereby relieve CK-mediated suppression of senescence. TFs are in indicated in green, other types of protein are indicated in lack, and hormones and other factors affecting senescence are italicized.

senescence under controlled conditions (Bresson et al., 2018).

Induction of senescence triggers the expression of a set of genes that are involved in the catabolism of Chl and other macromolecules. Their expression can be induced via several regulatory pathways depending on the method of senescence induction. Chl degradation occurs in several steps, and involves the activity of the genes *NON-YELLOW COLORING1 (NYC1), NON-YELLOW COLORING-LIKE (NOL)* to convert Chl-*b* to Chl-*a*, which is then further degraded to pheophytin by genes in the *NON-YELLOWING (NYE)* class. The PHEOPHYTINASE (PPH) enzyme is responsible for hydrolyzing pheophytin into pheophorbide *a* and phytol, and pheophorbide *a* is subsequently catabolized by PHEOPHORBIDE A OXYGENASE (PAO), resulting in a loss of green color (reviewed by Hörtensteiner and Kräutler, 2011). Knockout of any of these catabolic genes results in a stay-green or delayed senescence phenotype, and loss-of-function of TFs that promote their expression directly or indirectly has a similar effect. On the other hand, genes that negatively regulate senescence have a stay-green phenotype when overexpressed, and may also show accelerated senescence when knocked out.

Several central regulators of senescence have been identified, and cross-talk between different senescence-inducing pathways is common (Figure 3). One central regulator of senescence is the TF ETHYLENE INSENSITIVE 3 (EIN3), which is activated by EIN2, a key regulator of ethylene perception that itself is activated by ethylene receptors such as ETHYLENE RESISTANT 1 (ETR1) (Alonso et al., 1999). EIN3 promotes transcription of the NAC-family TFs *ORESARA1 (ORE1)* and *NAC-LIKE ACTIVATED BY AP3/PI (NAP)*, which promote transcription of several Chl catabolic genes and of other TFs involved in senescence (Qiu et al., 2015). In addition, *ORE1* transcripts are targeted by miR164, whose expression is decreased over plant age and thereby conveys an age-dependent regulation of senescence (Kim et al., 2009). Expression of *miR164* is repressed by EIN3, resulting in a release of post-transcriptional silencing of *ORE1* in addition to the direct activation of *ORE1* transcription by EIN3 (Li et al., 2013). *EIN3* activation and *ORE1* transcription can also be induced by PHYTOCHROME INTERACTING FACTORS (PIF) 4 and 5, which are induced by dark treatment (Song et al., 2014). *ORE1* transcription is also induced indirectly by salt stress and ABA, and the transcription of several ORE1 targets including Chl catabolic genes can be induced by other hormones like JA and SA and by salt stress (Balazadeh et al., 2010; Woo et al., 2019). Because of the complex interaction between several TFs that regulate senescence, there is a large overlap between different senescence pathways, which makes it difficult

to identify at what level a stay-green mutant is affected.

Unlike the senescence-promoting hormones ethylene, JA, SA, and ABA, CKs repress the progression of leaf senescence. Although much remains to be understood about the role of CK in repressing senescence, it has been shown that it depends specifically on the CK receptor ARABIDOPSIS HISTIDINE KINASE 3 (AHK3) which causes phosphorylation of the ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2) upon CK perception (Kim et al., 2006). Phosphorylated ARR2 then promotes the expression of cytokinin-responsive genes and indirectly represses expression of Chl catabolic genes (Kim et al., 2006). AHK3 also induces the expression of CYTOKININ RESPONSE FACTOR 6 (CRF6), which is partially responsible for the repressive effect of CK on senescence (Zwack et al., 2013). In contrast, the expression of the CK-degrading *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) genes is upregulated during senescence by NAP and possibly also by other factors, resulting in decreased CK levels and subsequent release of senescence inhibition (Hu et al., 2021; this thesis). Application of exogenous CK delays senescence, and transgenic plants expressing a plant- or *Agrobacterium*-derived *ISOPENTENYL TRANSERASE* (*IPT*) CK biosynthesis gene under a senescence-specific promoter are significantly longer-lived than wild-type plants (Jordi et al., 2000; McCabe et al., 2001; Guo et al., 2021). Progression of senescence thus not only requires the activation of a regulatory pathway that induces the expression of Chl catabolic genes, but also requires the active down-regulation of CK levels in order to lift the repressive effects of CK on senescence.

Life history strategies of higher plants

The life cycle of Arabidopsis is that of a typical monocarpic plant: reproductive senescence ensures that all resources are reallocated from the senescent tissue to the ripening seeds, followed by the senescence and death of the aboveground meristems in a process that is called general proliferative arrest (GPA) (Balanzà et al., 2018; Wang et al., 2023). In monocarpic plants, reproductive senescence and GPA result in the death of the whole plant upon seed maturation. In contrast, polycarpic plants are able to maintain vegetative growth independently of flowering, which allows for a longer life span in which several, often seasonal flowering cycles can be attained (Thomas, 2013; Kiefer et al., 2019; Figure 4). In addition to the ability to flower repeatedly, the formation of a cambium ring in the stem producing wood (secondary growth) and the production of a more complex root network are traits typically associated with polycarpic species (Glover et al., 2010).

The terms "annual" and "perennial" are often used interchangeably with the terms "monocarpic" and "polycarpic", but these terms are misleading because the life span of a plant is not necessarily reflective of its life history. For example, some monocarpic plants can live for decades before initiating the flowering program, but are unable to return to their vegetative stage upon flowering. This process is also known as a "death bloom" and can be observed in several species, although it is not always clear whether these species are truly monocarpic or whether they can regenerate from below-ground rhizomes (Simcha, 2017). On the other hand, the capability to maintain vegetative growth during and after flowering in polycarpic plants does not always imply a long

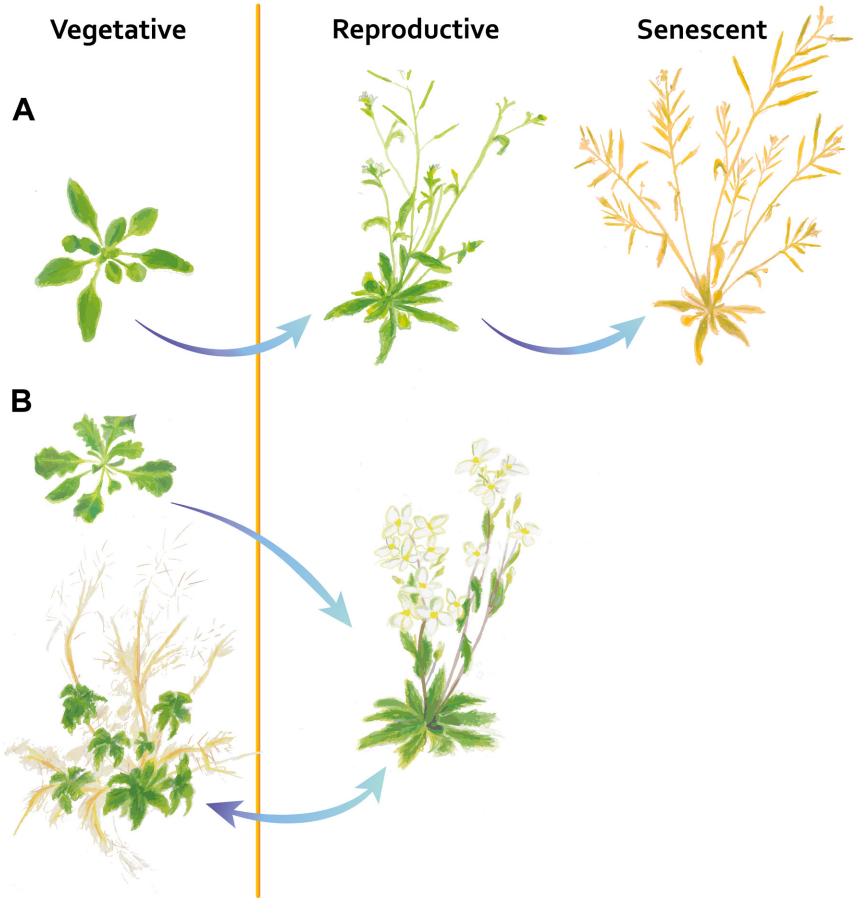

Figure 4: Schematic representation of the monocarpic and polycarpic life history strategy. **A:** Monocarpic plants like *Arabidopsis thaliana* transition from a vegetative to a reproductive phase in which flowers and seeds are produced, followed by a general proliferative arrest of the meristems, whole-plant senescence and death. **B:** Polycarpic plants like *Arabis alpina* transition between the vegetative and reproductive phase and back. During flowering, some meristems retain their vegetative identify and stay alive while reproductive structures arrest, senesce and die.

life span, because their survival might be affected by other factors than their capacity to maintain vegetative growth. For example, species such as the ornamental plant Petunia (*Petunia hybrida)* are commercially referred to as "annual" but are actually (sub)tropical polycarps that cannot survive the cold winter temperatures of the (Northern) countries in which they are cultivated (Vandenbussche et al., 2016). In addition to temperature, limited nutrient availability and exposure to pathogens or herbivores can be factors that limit the life span of polycarpic species. Finally, many species including those of the *Brassicaceae* family of which Arabidopsis is a member are often referred to as "winter annuals" or "biannuals": these species require vernalization (i.e. winter cold) to flower, but die soon after seed set. These are in fact monocarpic species that germinate in spring, but will survive over the winter because the repression of flowering has not yet been relieved (Schiessl et al., 2019). The terms "monocarpic" and "polycarpic" are therefore preferred over other terms, because they refer to the plant's developmental traits rather than its expected life span.

Monocarps and polycarps are both common in nature, and species vary widely in their lifespan and the overall biomass that can be achieved. The polycarpic life history strategy underlies the largest and longest-living organisms in the world, such as the giant redwood trees (*Sequoiadendron giganteum*) and clonally growing poplar groves (*Populus tremuloides*) that can reach ages of over 1000 years (Thomas, 2013). On the other hand, the monocarpic life history strategy is inherent to, or has been selected for in many economically important crops such as the *Brassicaceae* (cabbage and mustard family), pulses, and grains such as maize, rice, and wheat (Thomas, 2013). A likely cause for this is that monocarps relocate the maximum amount of resources to their fruits or seeds, which improves the yield of monocarpic plants compared to polycarpic plants in the same season (Jukanti and Fischer, 2008). In addition, many polycarpic species require a longer time period to achieve the competence to flower and thereby are slower to form fruits than monocarpic species (Kiefer et al., 2019). It is also possible that polycarpic species have not performed better than monocarps in cultivation systems where crops are completely harvested at the end of each season, resulting in inadvertent selection for monocarpy. Finally, some polycarpic crops are cultivated as monocarps and are harvested yearly, despite being able to renew their vegetative cycle. However, despite the apparent preference for monocarpy, polycarpic traits can also be beneficial to agricultural systems, and recent efforts have been made to reintroduce polycarpic traits to monocarpic grain species

(Glover et al., 2010; Zhang et al., 2023). The increased complexity and depth of a polycarp's root system compared to that of a monocarp, for example, can result in a more efficient uptake of nutrients and water in the soil, reducing the demand for fertilizer and irrigation (Glover et al., 2010). In addition, the annual plowing and replanting of a field is a major contributor to soil erosion and salinification, and cultivating crops over multiple years can reduce this issue. Finally, established plants are often more resilient to competition from weeds and pathogen attack than seedlings, reducing the losses due to disease and the need for herbicide and pesticide application (Glover et al., 2010).

Regulation of monocarpic and polycarpic traits on a molecular level

The maintenance of vegetative development in polycarpic species relies on repression of floral identity in some of the AMs of the plant during the flowering process. In monocarpic plants all meristems transition into an inflorescence- or floral fate or become dormant upon flowering, whereas polycarpic plants can prevent this transition in some of their AMs and can reactivate dormant buds (Ponraj and Theres, 2020; Vayssières et al., 2020). Although this process is not entirely understood, a few players have been identified in this process by comparing Arabidopsis with *Arabis alpina,* a polycarpic relative in the *Brassicaceae* family. In this section, we will discuss which genes have been shown to play a role in the maintenance of vegetative identity and other traits associated with life history in plants.

The aforementioned floral repressor FLC is one of the best-studied genes involved in maintenance of vegetative identity. In addition to a global repression of the flowering program, it was shown that FLC can repress flowering genes locally in AMs of flowering *Arabis alpina* (Lazaro et al., 2018). In vernalization-sensitive Arabidopsis, *FLC* expression can be permanently silenced by prolonged cold exposure, resulting in flowering followed by reproductive senescence and GPA (Michaels and Amasino, 1999). In contrast, vernalization causes only a temporary silencing of the *FLC* homolog *PERPETUAL FLOWERING 1 (PEP1)* in *A. alpina*, and *PEP1* expression is restored upon return to ambient temperatures. This causes a transition to the flowering state in some meristems during vernalization while in others PEP1 represses this transition and thereby helps retain the vegetative identity (Wang et al., 2009b; Hyun et al., 2019; Vayssières et al., 2020). In addition, *A. alpina pep1* knockout mutants do not require vernalization and lose most of their polycarpic traits (Wang et al., 2009b). It was shown that in contrast to *A. alpina, FLC* expression

is not reinitiated after flowering in Arabidopsis or in the monocarpic *Arabis montbretiana* (Kiefer et al., 2017). Recently, this differential regulation of *FLC* expression between mono- and polycarpic species was confirmed by crossing sets of mono- and polycarpic species from two *Brassicaceae* genera (Zhai et al., 2024). Moreover, transgenic Arabidopsis plants expressing polycarpic *FLC* homologs under their native polycarpic promoter gained a polycarpic phenotype, indicating that *FLC* expression after flowering is sufficient to induce a polycarpic life history strategy (Zhai et al., 2024).

Although differences in the cis-regulatory elements in the *FLC* loci of mono- and polycarpic species largely explain life history, it is not yet fully known which other genes are involved and which genes act upstream of *FLC* to reset its expression. Some additional regulators have been identified in *A. alpina:* for example, reactivation of *PEP1* expression in AMs after vernalization is regulated in part by the *A. alpina* AP2 homolog PEP2 (Lazaro et al., 2019). In addition, the *A. alpina* homolog of *TFL1* is expressed more highly in the SAM of young plants and in AMs, and AaTFL1 blocks the transition to flowering in these tissues in parallel to PEP1 (Wang et al., 2011). Interestingly, TFL1 homologues appear to be more important for the life history strategy of *Rosaceae* species than FLC, indicating that polycarpic life history can be achieved in multiple ways (Kurokura et al., 2013). Overall, it is clear that the maintenance of vegetative AMs contributes to the polycarpic life history strategy by ensuring the plant's capability to produce new organs during and after flowering.

Wood formation is another trait associated with polycarpy, and recent findings have indicated that the regulation of flowering and wood formation are at least partially linked. The floral integrator *SOC1* and the floral identity gene *FUL* have been shown to act as important moderators of wood formation and other polycarpic traits (Melzer et al., 2008). In addition to a significantly delayed flowering time and polycarpic traits, the *soc1 ful* double mutant showed a strongly enhanced secondary growth in the inflorescence stems, indicating that these two genes act together to repress wood formation. In addition, the AMs on the inflorescences of *soc1* single- and *soc1 ful* double mutants produced many rosette-like leaves in ARs, indicating that these axillary meristems have a (partially) vegetative identity similar to what occurs in polycarpic plants (Melzer et al., 2008; Dorca-Fornell et al., 2011). The development of the *soc1 ful* double mutant strongly resembles that of polycarpic species, because the overall longevity and biomass of these plants was also increased to more than 14 months (Melzer et al., 2008). The phenotype of this double mutant strongly implies that SOC1 and FUL play an important role in repressing longevity and

are required for the monocarpic life history strategy of Arabidopsis.

An important component of the monocarpic life history strategy is the ability to stop flower development before nutrient availability is too low to support the production of new fruits (Thomas, 2013; Wang et al., 2023). This can be achieved by proper timing of GPA. GPA timing is regulated by several processes, in which the TFs FUL and AP2 play important roles. In addition to its roles in promoting flower production and repressing wood formation, FUL is also an important regulator of GPA (Balanzà et al., 2018; Balanzà et al., 2019). In Arabidopsis, the single knockout of *ful* delays GPA by 3-4 weeks resulting in a longer life span and higher seed yield (Balanzà et al., 2018). *FUL* is expressed in the IM and restricts its proliferative capacity by indirect repression of *WUSCHEL (WUS),* a gene involved in maintenance of the stem cell pool in meristems. By restricting *WUS* expression, the proliferation of the meristem is also restricted, which limits the number of flowers and fruits that can be produced from a single inflorescence meristem (Balanzà et al., 2018). The floral inhibitor AP2, on the other hand, promotes stem cell proliferation by promoting *WUS* expression directly and indirectly, and its induction prolongs the proliferation of IMs (Martínez-Fernández et al., 2020). Merelo et al. (2022) showed that IM arrest is initiated around three weeks after bolting in Arabidopsis as a result of decreased meristem size and decreased cell division. This is caused by a decrease in the levels of CKs that promote cell division, and a subsequent decrease in *WUS* expression (Merelo et al., 2022). At five weeks after bolting, the IM stops producing new flowers in WT plants, but *ful* mutants proceed to produce flowers at a low rate, due to a higher CK concentration in the IM at this time point. AP2, on the other hand, promotes CK biosynthesis and downregulates genes associated with bud dormancy (Martínez-Fernández et al., 2020). Earlier research has indicated that FUL represses *AP2* expression and promotes the expression of the CK-degrading *CKX3* and *CKX5* genes, thereby reducing CK levels in the IM (Balanzà et al., 2014; Bemer et al., 2017). Taken together, IM longevity is stimulated by AP2-induced CK production and cell division, and limited by the repressive action of FUL on these processes.

Proliferative arrest of IMs is also accelerated by the fruits it has produced. An arrested IM can be reinitiated by pruning the siliques on that inflorescence (Hensel et al., 1994; Balanzà et al., 2019; Ware et al., 2020; Merelo et al., 2022), and IM proliferation is not arrested in male-sterile plants that are unable to produce seeds (Hensel et al., 1994). This indicates that seeds or fruits produce a mobile signal that represses IM proliferation, and it was shown that auxin acts as such a mobile signal (Ware *et al.*, 2020). Auxin represses the IM proliferation

when its levels are high, and Ware et al. (2020) showed that fertilized siliques export auxin into the polar auxin transport system that transports auxin from the SAM to the rest of the plant. High auxin export from the fruits interferes with auxin export from the SAM, resulting in its accumulation at the SAM and subsequent inflorescence arrest, similar to how auxin prevents the formation of new leaf primordia during the vegetative stage. This effect is achieved only by fruits in close proximity to the apical IM, as removal of proximal fruits could delay IM arrest whereas removal of distal fruits had no effect on the SAM. In addition, local silique- or flower removal does not affect the development of other inflorescences on the same plant, showing that the auxin-induced SAM arrest is a local process (Ware et al., 2020). Together these data indicate that reproductive senescence in monocarpic plants is not necessarily linked to IM arrest, which explains why polycarpic plants can produce inflorescences that senesce while other tissues are unaffected.

AHL15 as a regulator of vegetative development

Recently, the AT-HOOK MOTIF NUCLEAR LOCALIZED (AHL) family protein AHL15 was shown to negatively regulate various longevity phenotypes (Karami et al., 2020; Rahimi et al., 2022a; Rahimi et al., 2022b). The *AHL* gene family is conserved in all land plants and contains 29 members in Arabidopsis (Zhao et al., 2014). Knockout mutants of *ahl15* and other single *ahl* mutants show few or mild developmental- or other phenotypic defects, indicating that these genes act redundantly (Zhao et al., 2013; Karami et al., 2020). Knowledge on AHL function is therefore based initially on the comparison of mutants that overexpress *AHL* genes to wild-type plants.

AHL15 and several closely related *AHLs* have been shown to delay several developmental processes when overexpressed. For example, overexpression of *AHL22, AHL27* or *AHL29* inhibits hypocotyl elongation (Street et al., 2008; Xiao et al., 2009; Zhou et al., 2013; Favero et al., 2016), *AHL15* overexpression delays VPC (Rahimi et al., 2022a), and several *AHL* genes delay flowering when overexpressed (Lim et al., 2007b; Street et al., 2008; Yun et al., 2012; Karami et al., 2020; Tayengwa et al., 2020). Leaf senescence can be delayed by overexpression of *AHL27* (Lim et al., 2007b) and *AHL15* (this thesis). Finally, *AHL15* overexpressing plants show a delayed proliferative arrest and whole-plant senescence, indicating that this gene may be involved in regulating life history strategy (Karami et al., 2020).

AHL15 and developmental phase transitions

AHL15 is expressed abundantly in the early embryo, the seed, and juvenile plants, and as the plant matures its expression is gradually limited to the AMs in the rosette and on the inflorescence (Zhao et al., 2013; Karami et al., 2020). This expression indicates that AHL15's primary function is in embryonic and juvenile development. The phenotypes caused by *AHL15* overexpression also suggest that AHL15 represses ageing and the transition to an adult state: Overexpression of *AHL15* in immature zygotic embryos results in the formation of somatic embryos (Karami et al., 2021), and *AHL15* overexpression was shown to delay the VPC by indirectly repressing several *SPL* genes independent of *miR156* (Rahimi et al., 2022a). Expression of *AHL15* in the IM results in leafy inflorescences with ARs (Karami et al., 2020; Rahimi et al., 2022a), and induction of a fusion protein of AHL15 and the glucocorticoid receptor (AHL15-GR) in inflorescences by dexamethasone treatment causes a similar phenotype (Karami et al., 2020). It was shown that *AHL15* expression in the AMs is repressed by SOC1 and FUL and that the AR phenotype of the *soc1 ful* double mutant is alleviated when crossed with *ahl15*, indicating that AHL15 is involved in repressing the reproductive state of inflorescence AMs (Karami et al., 2020). Finally, the inflorescence stems of *soc1 ful* and *AHL15* overexpressing plants show increased radial growth and enhanced xylem production, whereas *ahl15* loss-of-function results in a relative reduction of this trait in both wild-type and *soc1 ful* mutant background, again indicating that AHL15 acts antagonistically to SOC1 and FUL (Rahimi et al., 2022b). Taken together, these phenotypes show that AHL15 acts as a positive regulator of vegetative growth in several developmental stages, but its precise targets and mode of action remain unknown.

Molecular mechanism of AHL proteins

Like all AHL proteins, AHL15 contains two characteristic domains: the AT-hook domain that can bind the minor groove of AT-rich DNA, and the Plant-Prokaryote Conserved (PPC) domain that facilitates protein-protein interactions (Zhao et al., 2013; Zhao et al., 2014). Within the *AHL* gene family, two types of AT-hook domains (type I and II) and two types of PPC domains (type A and B) have evolved (Zhao et al., 2014). The AHL family can be divided into two subclades based on the type and number of AT-hook and PPC domains: clade A AHL proteins contain one type-I AT-hook as well as a type-A PPC domain, whereas clade B AHLs contain a type-B PPC domain and one type-II AT-hook that is sometimes

accompanied by a type-I AT-hook (Zhao et al., 2014). Clade-A AHLs have been shown to aggregate into complexes of dimers and trimers with themselves and with other AHL proteins (Zhao et al., 2013). Generally, members of clade A have been studied in more detail than clade B AHLs, although in recent years increasing attention has been dedicated to clade B AHLs.

The discovery of mutant alleles with structural modifications in the AT-hook and PPC domains has helped to understand the function of AHL proteins. Mutations in either of the two characteristic AHL domains can give rise to dominant-negative alleles. These are mutations that alter the protein structure resulting in stronger phenotypic changes than loss-of-function mutations where the gene is no longer expressed (Zhao et al., 2013; Karami et al., 2020). Mutation or removal of the AT-hook domain causes a loss of DNA binding ability, as was seen when a spontaneous mutation in the activation-tagged *AHL29* gene resulted in dominant-negative phenotypes in Arabidopsis (Street et al., 2008). This dominant-negative *ahl29* mutant lacked a functional AT-hook domain and produced longer hypocotyls than both wild type and *ahl29* loss-of-function mutants. A similar mutant lacking the core of the PPC domain also showed a dominant-negative phenotype, indicating that both domains are crucial for AHLs to function properly (Zhao et al., 2013).

Interaction with both AHL and non-AHL proteins is facilitated by the PPC domain. Yeast-2-Hybrid (Y2H) experiments have shown that AHLs can dimerize via their PPC domain, and it is thought that AHLs bind DNA as multimers (Zhao et al., 2013; Seo and Lee, 2021). Within the PPC domain, a conserved GRFEIL motif called the G motif is crucial for the interaction with non-AHL proteins and for transcriptional activation by AHLs, and deletion of this motif also causes dominant-negative phenotypes (Zhao et al., 2013; Karami et al., 2020). One such dominant-negative mutant that lacks the G motif is the *ahl15ΔG* mutant described by Karami et al. (2020), where introgression of an allele with a mutated G-motif into a heterozygous background (*ahl15-/+*) causes dominant-negative phenotypes similar to the removal of the AT-hook domain in *ahl29* (Figure 5). A similar effect was obtained by fusion of the large reporter protein GUS to AHL15, indicating that the fused GUS protein interfered with the PPC domain. This loss of the G-motif had such a strong impact on AHL15 functioning that plants carrying this allele were only viable if at least a single copy of the wild-type *AHL15* allele was present, showing that dominant-negative mutations can have severe phenotypic effects and highlighting the importance of AHLs during embryogenesis.

Although both types of dominant-negative alleles cause strong phenotypic

defects, they appear to cause these defects in different ways. Mutation or removal of the AT-hook domain prevents the resulting protein from binding DNA, but not from interacting with other proteins, and may act in a dominant-negative manner by reducing the DNA-binding ability of the AHL complex it is incorporated into (Zhao et al., 2013). On the other hand, deletion of the G motif might reduce transcriptional activation by AHLs by inhibiting interaction of the AHL with non-AHL proteins such as TFs or chromatin remodellers (Zhao et al., 2013). This impaired interaction likely reduces access to the DNA for these interactors, causing changes in transcriptional regulation. Thus, both types of mutations prevent the proper functioning of the AHL complex, but the nature of this disruption differs between the two types of mutant alleles.

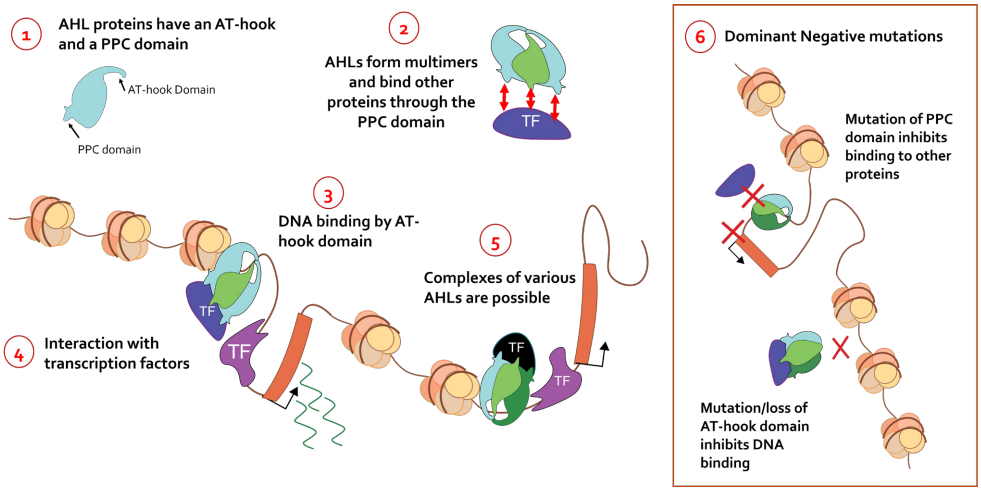

Figure 5: Overview of AT-HOOK MOTIF NUCLEAR LOCALISED (AHL) protein function. AHL proteins have two functional domains: the DNA-binding AT-hook domain and the PPC protein-interaction domain (**1**). AHLs can form multimers comprised of different AHL types (indicated in green and blue) and interact with other classes of proteins, such as TFs, via the PPC domain (**2**). AHL complexes bind the minor groove of AT-rich DNA via their AT-hook domains (**3**).
AHLs can form (heterotrimeric) complexes with other AHL proteins, and the interaction with other proteins might depend on the composition of the AHL complex (different types of TFs are colored dark blue, purple, or black). AHL-TF complexes can either promote transcription (**4;** arrow indicates transcription start site and wavy lines represent RNA transcripts) or repress transcription (**5**), depending on the composition of the complex. Finally, mutating either the AT-hook or PPC domain can result in dominant-negative effects (**6**). Without an AT-hook domain, AHLs lose the ability to bind DNA, whereas disruption of the PPC domain by mutation or by fusion with a large protein like GUS interferes with their ability to interact with other proteins. Both situations are likely to reduce the ability of the complex to regulate transcription, resulting in an altered transcription of AHL-bound genes.

Several studies have investigated the proteins with which AHLs can interact. Y2H and co-immunoprecipitation followed by mass spectrometry (coIP-MS) experiments using AHLs as bait have consistently identified a range of other AHL proteins as interactors, but other types of proteins have also been found. Among these, TFs of the TCP (TEOSINTE BRANCHED 1, CYCLOIDEA, PROLIFERATING CELL FACTOR) family have been identified in Y2H (Zhao et al., 2013; Yin et al., 2018), but most interactors are non-TF nuclear proteins. Interestingly, multiple studies have shown that AHLs can interact with proteins that form part of chromatin remodelling complexes. For example, Lee and Seo (2017) showed that AHL29 interacts with Actin-related Protein 4 (ARP4), a protein in the SWI2/SNF2-RELATED 1 (SWR1) complex that facilitates exchange of HISTONE 2A (H2A) for its variant H2A.Z, and that AHL29 recruits this complex to the *YUCCA9* (*YUC9*) locus, thereby repressing *YUC9* expression (Lee and Seo, 2017). Xu et al. (2013) showed that AHL16 is involved in silencing transposable elements (TEs) by interacting with MULTICOPY SUPPRESSOR OF IRA1 4 (MSI4) and MSI5, both of which are subunits of a complex that mediates HISTONE 3 (H3) deacetylation (Xu et al., 2013). AHL22 interacts with multiple HISTONE DEACETYLASE (HDA) proteins (Yun et al., 2012), and it was recently shown that it also interacts with the transcriptional repressors FAR1-RELATED SEQUENCE (FRS) 6 and 12 at matrix attachment regions (MARs) that anchor the DNA to the nuclear matrix (Xu et al., 2024). It was shown that the presence of a MAR in the *ETTIN/ARF3* locus was required for its silencing by AHL21, and that AHL29 promotes H3K9me2 deposition at this locus (Ng et al., 2009). H3K9me2 is a repressive chromatin modification that is involved in, among others, heterochromatin silencing (Xu and Jiang, 2020). Multiple chromatin immunoprecipitation (ChIP) experiments have shown that *AHL* overexpression can lead to increased levels of the repressive mark H3K9me2 and depletion of the permissive mark H3Ac, implying that AHLs are involved primarily in depositing modifications that are considered as silencing marks (Ng et al., 2009; Yun et al., 2012; Xu et al., 2013; Xiong et al., 2020). In addition to the evidence showing that AHLs are directly involved in silencing TEs, *FT, YUC9,* and *ETTIN,* RNA-seq data also show that the majority of differentially expressed genes in *AHL* overexpressing lines are downregulated (Favero et al., 2020; Karami et al., 2022, this thesis), further supporting the notion that AHLs play a role in supressing gene expression. Considering that various AHL interactors are subunits of chromatin remodelling complexes, it is most likely that AHLs affect gene expression by recruiting or facilitating the activity of chromatin remodelling complexes that remove the active mark H3Ac and promote the

deposition of repressive marks, especially H3K9me2. However, the precise role that AHL proteins play in this process remains to be investigated.

Thesis outline

This thesis focuses on the effects of AHL15 on various aspects of plant longevity. In **chapter 2**, we investigated the natural variation in life history strategy in the monocarpic plant Arabidopsis using AR formation as longevity phenotype, and show that profound differences in life history strategy are tightly linked to the expression of the floral repressor *FLC*. We also show that the expression of the floral integrator *SOC1* is negatively correlated with the AR phenotype. Expression of *AHL20*, a closely related homolog of *AHL15,* also correlated with the AR phenotype, indicating that this gene may play a role similar to AHL15 in polycarpy. Silencing of *FLC* by vernalization removed the AR phenotype, indicating that *FLC* acts upstream of all other genes tested in this chapter.

The longevity of leaves can be determined by measuring their Chl content. In order to measure leaf Chl content in a rapid and non-destructive manner, we developed an ImageJ plugin to quantify Chl based on digital images. This plugin, which is described in **chapter 3**, provides a non-invasive method to measure leaf Chl content and can be applied in a high-throughput, low-tech and low-cost experimental setup, e.g. for senescence measurements but also for other applications in which Chl content is a relevant parameter.

In **chapter 4**, we show that the longevity in Arabidopsis leaves can be significantly extended by overexpressing *AHL15.* We show that the expression of several senescence marker genes and the genes encoding the cytokinin-degrading CKX2, CKX3 and CKX5 enzymes is upregulated in dark-incubated detached leaves, but that their expression is delayed or inhibited upon activation of AHL15 in these leaves. Together, these data show that the delayed senescence phenotype seen in *AHL15* overexpressing plants relies on a reduced degradation of the phytohormone cytokinin.

In **chapter 5**, we dive into the molecular mechanism of AHL15 and describe its DNA binding sites and its effect on DNA structure and transcription in order to better understand how it can induce such dramatic changes in plant development. We show that AHL15 has profound effects on transcription and development when activated in seedlings, but that these changes do not rely on changes in chromatin accessibility. Our results indicate that AHL15 binding is enhanced near their transcription start- and end sites of genes that are differentially expressed upon AHL15 induction, and that AHL15 binding near the transcription start site is strongest near upregulated genes, whereas AHL15

binding to the transcription end site is strongest for downregulated genes. In addition, we compared the genome-wide binding sites of AHL15 to those of various epigenetic marks, and show that they do not coincide at the same location and that AHL15 is found predominantly at the borders of regions marked by epigenetic modifications. Finally, we show that AHL15 binds similar target regions as the AT-hook containing HMGA-family protein GH1-HMGA2/HON5, which suggests that AHL15 could have a similar function as this protein in regulating gene loop formation.

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