The Nuts and Bolts of Stem Elongation

Unravelling the Role of ARABIDOPSIS THALIANA HOMEOBOX 1 in the Control of the Arabidopsis Rosette Habit

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Aan de basis van stengelelongatie

De rol van ARABIDOPSIS THALIANA HOMEOBOX 1 bij het behoud van de Arabidopsis rozetvorm

(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

General introduction

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It is predicted that the amount of arable land will reach a plateau between 2040-2050, while global populations are predicted to further increase (Alexandratos and Bruinsma, 2012). To cope with future demands for food, it is necessary to increase crop yield. Since the Green Revolution of the 1970s, impressive advances have been made in yield increase for staple crops, such as wheat, rice and maize. However, these successes have not yet been translated to the cultivation of rosette crops, such as lettuce, sugar beet, onions and cabbages. These plants are characterised by the formation of a rosette of leaves close to the ground, followed by the formation of an elongated stem (bolting) during reproductive growth. Premature bolting of crops reduces both quality and quantity of harvestable material. Surprisingly little is known about the molecular regulation of bolting, therefore an overview of current knowledge pertaining to bolting is presented here.

1.1 What is bolting?

1.1.1 The end of the rosette: a bolt move

Vegetative growth of rosette plants, such as *Arabidopsis thaliana* (Arabidopsis), is characterised by the formation of the rosette: a whorl of leaves that has little to no elongation between successive nodes. During reproductive growth, most rosette plants undergo a dramatic change in architecture, which results in the rapid formation of an elongated stem (bolting), and the formation of flowers (flowering). In monocarpic plants such as Arabidopsis flowering and seed set is followed by systemic senescence. In polycarpic species, senescence is limited to the flowering stem, allowing vegetative growth to resume from axillary meristems.

The rosette habit is widespread amongst flowering plants. It is observed in several contrasting environments, in both dicots and monocots, and in plants adopting annual, biennial and perennial life history strategies alike, and has also been lost and gained multiple times throughout Angiosperm evolution (Cohen, 2011; Givnish et al., 1999; Hao et al., 2017; Marks et al., 2011). The compactness of rosette plants provides several advantages compared to taller, less compact plants. In cold environments, biennial and perennial rosette plants overwinter as rosettes. The rosette reduces exposure to freezing temperatures as it can be covered and protected beneath snow (Larcher et al., 2010), and also may be protected by the warmer microclimate of the soil. Rosette habits are also very prevalent in arid or hot environments. It has been proposed that in these environments rosette formation facilitates water capture from fog (Martorell and Ezcurra, 2002). Besides protection from abiotic stresses, the compact rosette is also thought to prevent damage caused by large herbivores (de Bello et al., 2005; Fujita and Koda, 2015).

Little is known about the genetic processes that enable plants to form rosettes. Studies into Arabidopsis and *Brassica rapa* have determined that blue, red and far-red light perception through phytochrome and cryptochrome photoreceptors is needed for repression of internode elongation during rosette growth (Devlin, 1998; Devlin et al., 1996, 1997; Halliday and Whitelam, 2003; Mazzella et al., 2000). However, it is not known which downstream processes underlie this process.

Bolting dramatically changes the morphology of a rosette plant, with the bolting stem acting oppositely to the rosette stem: Internode elongation is repressed during rosette growth and promoted during bolting. Where the rosette is protective against damage, it follows that the stem is susceptible. However, the stem facilitates pollination and seed dispersal, essential for a successful outcome of the reproductive phase (Schaffer and Schaffer, 1979; Thomson et al., 2011). The formation of the stem drains resources from the leaves and roots, and rosette plants that switch to reproductive growth must therefore tightly regulate this fundamental developmental switch. Likely, the repression of the developmental programme of the rosette is a prerequisite for bolting. Therefore, understanding the regulation of bolting may also facilitate understanding of the rosette habit.

The architectural changes that occur as a result of bolting often have several undesirable effects on crops (Table 1.1). Therefore, if plants bolt before harvest, yield is affected. In leafy rosette crops, such as lettuce and cabbage, premature bolting reduces marketability by diminishing head compactness and crop quality as bolting leads to the formation of hardened stem tissue and bitter secondary metabolites in leaves (Guttormsen and Moe, 1985; Sessa et al., 2000). In tuberous rosette crops, such as sugar beet, bolting reduces yield of both the bolted plant and non-bolted, neighbouring plants. Bolting causes reallocation of nutrients from harvestable tissues to the stem, resulting in smaller roots containing less harvestable content, e.g. sugars. It has been reported that in sugar beet, the total sugar content of roots taken from bolted beets was 23% lower than in roots of non-bolted plants of the same age (Wood and Scott, 1975). Furthermore, bolted plants overshadow non-bolted plants, thereby inducing shade avoidance responses, which also reduces yield. An increase of 1% in bolting in a population of plants has been reported to reduce the total sugar yield of sugar beet and root chicory by 0.5-1% and 1.1-1.2%, respectively (Jaggard et al., 1983; Longden, 1989; Longden et al., 1975; Schittenhelm, 2001; Wood and Scott, 1975). Although rosette crops are generally not staple crops, they are both economically and nutritionally important. In 2017, five of the ten highest globally produced vegetable groups were rosette crops, and sugar beet was the 8th highest produced crop in the world (Food and Agriculture Organization of the United Nations, 2017)(Table 1.1). Therefore, small improvements in bolting resistance could have great impacts on yield.

Table 1.1: Rosette crops cultivated worldwide
Worldwide production of rosette crops (10^6 tonnes) in 2017 ranked in ascending order of production, in relation to vegetable crops or all crops (Food and Agriculture Organization of the United Nations, 2017). The effects of bolting on each crop type and most important environmental conditions controlling bolting are listed (i.e. photoperiod (phot), vernalization (vern) or ambient temperature (temp)) .

Rank (veg)	Rank (all crops)	Crop group	Production (10^6 tonnes)	Type	Effect of bolting	Factors controlling bolting
2	16	Onions, dry	97.9	tuberous	Bulb weight reduction [1]	phot, vern
4	24	Cabbages and other brassicas, including chinese cabbage, pak-choi, mustard cabbage (*Brassica rapa*), white, red, savoy cabbage, Brussels sprouts, collards, kale, kohlrabi (*Brassica oleracea*)	71.5	leafy & tuberous	Hardened stem tissue [2] loss of head compactness [2], lower fresh weight [3]	phot, vern
6	31	Carrots (*Daucus carota*) and turnips *(B. rapa var. rapifera*)	42.8	tuberous	Lignification, reduction of harvestable tissue	phot, vern
8	39	Garlic	28.2	tuberous	No or positive effect on bulb weight [4-5]	phot, vern, temp
9	40	Spinach	27.9	leafy	Loss of quality	phot
11	43	Lettuce (*Lactuca sativa*), chicory (*Cicorium intybus*) and endive (*Cichorium endivia*)	26.9	leafy	Bitter secondary metabolites [6], hardened stem tissue, loss of head compactness	temp
				tuberous	Reduction of root size [7-8], reduction of sugar yield [7] lignification of root [8]	temp, vern
12	44	Cauliflowers and broccoli (*Brassica oleracea* var. *botrytis*)	26	other	Marketability	phot, vern
19	81	Green onions (*Allium cepa*), shallots (*Allium ascalonicum*) and welsh onions (*Allium fistulosum*)	5.1	tuberous	Formation of hardened stem tissue [9]	phot, vern
21	100	Leeks (*Allium porrum*), chives *(Allium schoenoprasum*) and other alliaceous vegetables	2.2	leafy	Decrease in quality [10]	phot, vern
Non-vegetable rosette crops						
n/a	8	Sugar beet	301	tuberous	Reduction of harvestable tissue and sugar content [11-13]	phot, vern

References in table: 1: Kwon et al. (2016); 2: Guttormsen & Moe (1985); 3: Kitamoto et al. (2017); 4: Mathew et al. (2010); 5: Wu et al. (2016); 6: Sessa et al. (2000); 7: Schittenhelm et al. (2001); 8: Mathieu et al. (2018) 9: Yamasaki et al. (2000); 10: Kawagishi et al. (2009); 11: Wood & Scott (1975); 12: Jaggard et al. (1983); 13: Longden et al. (1989)).

1.1.2 Bolting is not flowering

Bolting and flowering both occur as a result of the reproductive phase change, a developmental phase transition that switches plants from the vegetative growth phase to the reproductive growth phase. Bolting is therefore often perceived as an integral part of the flowering response. Alternatively, the terms bolting and flowering are often, but wrongly, used interchangeably to refer to flowering only. For a number of reasons bolting and flowering should be treated as separate processes that are not under identical regulation. First, the stem and floral primordia are formed from different and distinct zones in the shoot apex (Section 1.3). Second, many negative effects of bolting, such as the loss of head compactness and formation of hardened tissue, are linked specifically to the initiation and formation of the stem, and not the formation of flowers. The build-up of bitter secondary metabolites in lettuce is also linked to initiation of the stem, and is not significantly affected by flowering (Sessa et al., 2000). Reduction of root yield is also linked to bolting and not flowering, as yield losses could be prevented in sugar beet by cutting down the stems of bolted plants. These plants were not flowering at time of cutting or at the time of harvest, thus the difference in yield could not be attributed to flowering (Wood and Scott, 1975). Third, bolting and flowering can be uncoupled. Bolting and flowering are not always initiated simultaneously or in the same order between species. In Arabidopsis, radish and *Brassica* species, floral buds are clearly visible upon initiation of bolting (Chen et al., 2018a; Guttormsen and Moe, 1985; Takahashi et al., 1994; Yoshida et al., 2010). However, in sugar beet, lettuce and chicory, no floral buds are visible before bolting, and there can be a long delay between the initiation of stem growth and the appearance of the first flower (Gianquinto, 1997; Hao et al., 2018; Mutasa-Göttgens et al., 2012). In these plants the cauline phase, referring to the part of the stem that carries cauline leaves rather than flowers, is extended. The cauline phase thus reflects the delay between initiation of bolting and flowering.

Another example of asynchronous induction of bolting and flowering is vegetative or floral reversion. After induction of bolting, plants sometimes revert to vegetative, non-elongation growth without proceeding to flowering. This is called vegetative reversion and has been observed in Arabidopsis, but also occurs regularly in vernalized sugar beet where it is the outcome of devernalization. In sugar beet, reversion causes bolted stems to stop elongating and form aerial rosettes, or to terminate growth and initiate new rosettes from rosette leaf axils. Sugar beet plants

that have started flowering will not revert to rosette growth (Reeves et al., 2007). Reversion from flowering to vegetative growth has been observed in several other species, including Arabidopsis, and several non-rosette plants, such as soybean, maize and tomato (Tooke et al., 2005).

In Arabidopsis, it has been proposed that under non-optimal growth conditions bolting is not controlled by the same regulatory processes as flowering (Pouteau and Albertini, 2011). Japanese bunching onion (*Allium fistulosum*) is a clear example of bolting and flowering regulation by different processes, as short days are needed for floral development, while long days are needed for bolting (Yamasaki et al., 2000).

In a similar vein, it is possible to induce bolting without inducing flowering. In sugar beet, radish, spinach, cabbage (*Brassica olearceae* var *capitata*), carrot and *Silene armeria* bolting can be induced under suboptimal conditions through application of the hormone gibberellin (GA). Flowering cannot be induced this way, and will only occur if plants are exposed to inductive environmental conditions (e.g. vernalization, photoperiod) (Hamano et al., 2002; Janick and Leopold, 1961; Mutasa-Göttgens et al., 2010; Suge and Rappaport, 1968; Talon et al., 1991). Similar findings have been reported for other *Brassica* crops, including kale and turnip (Wittwer and Bukovac, 1957). This uncoupling is even more pronounced in carrots where in certain conditions GA promoted bolting but repressed flowering (Nieuwhof, 1984).

The opposite, flowering without bolting, has also been observed. Both celery and celeriac require vernalization for bolting and flowering, but the vernalization requirement for flowering is shorter than the requirement for bolting (Booij and Meurs, 1995; Hanisova and Krekule, 1975). Celeriac plants vernalized for short periods will flower, but not bolt (Booij and Meurs, 1995). When grown in non-inductive conditions, some turnip varieties will form flower buds, but will not bolt (Takahashi et al., 1994). Similarly, blocking GA biosynthesis in radish and *S. armeria* only affects bolting and not flowering (Cleland and Zeevaart, 1970; Suge and Rappaport, 1968). In Arabidopsis, GA is essential for bolting, but not always for flowering: The GA biosynthesis mutant *ga1-3* does not bolt or flower in short-day conditions (SD), but flowers without bolting in long days (LD). Flowering can be restored in SD grown *ga1-3* plants through ectopic expression of floral pathway integrators, but bolting cannot (Blázquez et al., 1998; Moon et al., 2003). Several other mutants have been identified in Arabidopsis that flower but do not bolt, or exhibit severely impaired stem elongation. These include gain of function boundary gene mutants (Section

1.2), brassinosteroid signalling and biosynthesis mutants, and the *ARABIDOPSIS THALIANA HOMEOBOX1* (*ATH1*) overexpressor (Section 1.4)(Borghi et al., 2007; Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Kauschmann et al., 1996; Nakazawa et al., 2003; Norberg, 2005; Rutjens et al., 2009; Shuai et al., 2002).

1.2 The shoot apex and its control over rosette growth and bolting

The reproductive phase change is controlled by a number of internal and external factors, such as developmental age, temperature, photoperiod, light quantity and quality, and prolonged cold (Section 1.3). These signals are integrated at the shoot apex, which contains the shoot apical meristem (SAM) and the underlying rib meristem/rib zone (RM/RZ). The SAM contains the stem cells that give rise to all above-ground structures of the plant. To maintain a balance of organ initiation and stem cell maintenance, the SAM is subject to complex regulation, which has been subject of intense study (Gaillochet et al., 2015; Žádníková and Simon, 2014).

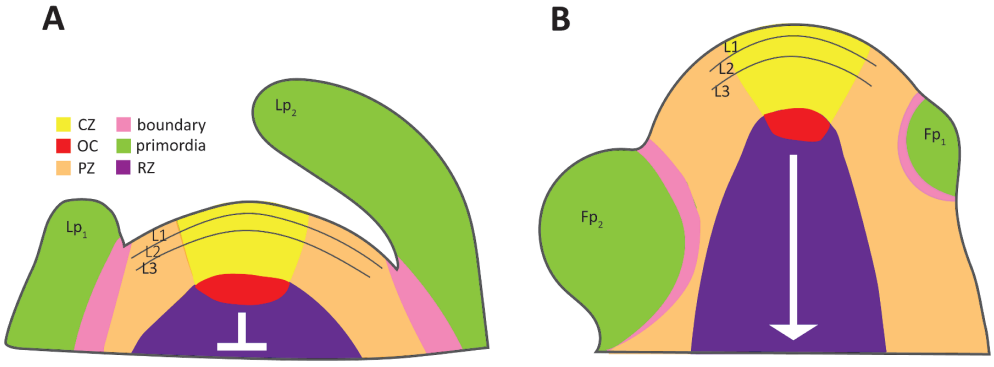

Fig. 1.1 Schematic overview of the Arabidopsis shoot apex during vegetative (A) and generative (B) growth The shoot apex can be divided according to cell lineage (L1/L2/L3) or by functional domain (central zone (CZ), organizing centre (OC), peripheral zone (PZ), rib zone (RZ), boundary, primordia). Leaf (LP) and floral primordia (FP) are marked. White bar (A) denotes mitotic inactivity of RZ during vegetative growth, white arrow (B) depicts direction of cell division during bolting.

1.2.1 Morphology of the shoot apex

The SAM can be subdivided into three clonal layers (L1, L2 and L3) or into three functional domains (central zone (CZ), peripheral zone (PZ) and boundary) (Fig. 1.1). Cells in the L1 and L2 divide anticlinally and give rise to the epidermis and ground tissue, respectively. Cells in the L3 divide more randomly and form the inner tissues, including the vasculature. The CZ contains the undifferentiated stem cells and the

organizing centre (OC). The OC consists of a group of mitotically inactive cells in the L3, basally to the stem cells. Stem cells divide slowly, displacing their daughter cells into the PZ where they proliferate and eventually differentiate into primordia (Reddy et al., 2004). The SAM is separated from primordia by a strip of non-dividing cells termed the meristem-organ boundary. Unlike cells of the PZ and CZ, where cell divisions are more random, cortical microtubuli of boundary cells are highly organised and their cell walls are more rigid, which constricts cell division angle and is correlated with a very low rate of cell division (Heisler et al., 2005, 2010). The boundary enforces the delicate balance between stem cell maintenance and differentiation. As such, disruption of the boundary results in organ fusion defects and/or complete loss of the meristem (Borghi et al., 2007; Ha et al., 2007; Hibara et al., 2006; Jun et al., 2010; McKim et al., 2008; Norberg, 2005; Shuai et al., 2002).

The RM and RZ are responsible for stem growth (bolting). Unlike the CZ and PZ, the RM and RZ have received much less attention in literature. The RM and RZ lie deep within the shoot apex, and therefore are very difficult to image, especially during vegetative growth. As a result, the few morphological studies of the shoot apex that include data on the RM/RZ have been conducted on shoot apices during reproductive growth. Another difficulty in studying the RM/RZ is that the definition of the RM and RZ is not always consistent, with both being referred to as a single tissue and as separate tissues.

Both the RM and RZ have been defined as an area containing clearly organized transverse cell files, which undergo increased cell division during bolting, giving rise to the stem (Metzger and Dusbabek, 1991; Peterson and Yeung, 1972; Sachs, 1991; Talon et al., 1991). The RM/RZ can be further divided into two sections: the RZ core and RZ periphery, which form the core and epidermal structures of the stem, respectively (Sachs, 1991). Cells of the core are supplied from the CZ, while cells of the periphery are supplied from the PZ (Bencivenga et al., 2016).

The most common consensus for the beginning of the RM is directly beneath the CZ (Bencivenga et al., 2016; Hempel and Feldman, 1994; Kwiatkowska, 2008; Medford, 1992; Sachs, 1991; Serrano-Mislata et al., 2017; Talon et al., 1991). Often, this area has also been referred to as the location of the RZ (Bencivenga et al., 2016; Boscá et al., 2011; Jacqmard et al., 2003; Mayer et al., 1998; Medford, 1992; Serrano-Mislata et al., 2017). No clear lower limit has been given to the RZ.

Even in literature where the RM and RZ are considered separate units, their behaviour and functions remain unclear. In some cases the RM has been defined as part of the L3, containing small, meristematic cells, while the RZ or subapical pith lies below the RM and consists of larger, vacuolated cells organized in ribs (Jacqmard et al., 2003). Similarly, sometimes the RM has been defined as the part of the L3 where the transcription factor *WUSCHEL* (*WUS*) is expressed, although this area also has been referred to as the RZ. (Yadav et al., 2009, 2014; Zhou et al., 2015). This L3-RM has been proposed to remain mitotically inactive during induction of bolting and flowering, while the RZ has been defined as an area that undergoes increased cell division during the induction of bolting (Jacqmard et al., 2003). A similar division has been proposed for the shoot RM and RZ of woody perennials, although here both the RM and RZ are proposed to be mitotically active during growth (Ruonala et al., 2008). Recent studies do not support the model of the Arabidopsis RM acting as a true meristem as it was shown that the RM/RZ acquires its cells from the overlying PZ/CZ rather than maintaining their own stem cell population (Bencivenga et al., 2016). As the consensus in Arabidopsis leans towards the RM and RZ being a single area, we will consider the RM and RZ a single entity referred to as the RZ.

The morphology of the shoot apex undergoes significant changes during the reproductive phase change. During vegetative growth (Fig. 1.1A), the shoot apex is flat, and the RZ is compact and mitotically inactive (Jacqmard et al., 2003). The reproductive phase change transforms the vegetative SAM into the reproductive inflorescence meristem (IM). This switch is associated with a strong increase in cell division in the CZ, leading to enlargement of the meristem, specifically in height. This leads to doming of the apex (Hempel and Feldman, 1994; Jacqmard et al., 2003; Kwiatkowska, 2008). The IM also ceases production of leaf primordia and switches to the formation of floral primordia (Fig. 1.1B). This results in the start of flowering. Initiation of bolting is governed by mitotic changes in the RZ, which strongly affect RZ morphology. Bolting induction increases mitotic activity in the RZ, whose cells divide periclinally to form longitudinal cell files. Initial stages of bolting require increased cell division in the RZ (Bencivenga et al., 2016; Metzger and Dusbabek, 1991; Sachs et al., 1959b, 1959a). Cells in the RZ periphery divide faster than cells in the RZ core. These two regions provide cells for the epidermal and core structures of the stem, respectively (Bencivenga et al., 2016; Sachs, 1991). This increase in

mitotic activity leads to the formation of elongated internodes between successive leaves, thus switching the plants from a rosette formation to stem formation. Cell elongation and differentiation of RZ cells does not occur in the apical parts of the RZ, but is thought to occur further down the developing stem (Bencivenga et al., 2016; Jacqmard et al., 2003; Sachs et al., 1959a).

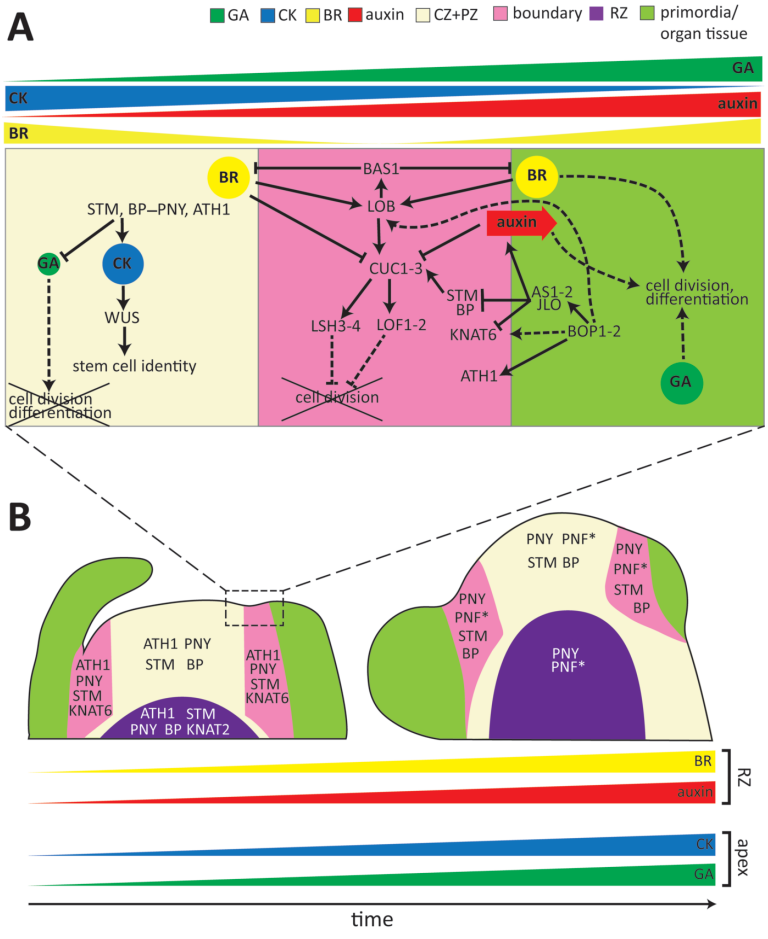

Fig. 1.2 Genetic networks controlling SAM maintenance and hormonal levels in Arabidopsis Schematic representation of hormonal levels in the central zone/peripheral zone (CZ+PZ), boundary and organ primordia and simplified overview of genetic networks controlling the balance between these three zones (A) and of reported TALE homeobox gene expression in the vegetative (left) and generative (right) shoot apex (B). Dashed arrows indicate indirect genetic interactions. TALE homeobox expression is based on reporter gene studies, except for PNF (*), whose expression domain is inferred from pny pnf mutant phenotypes. Coloured bars indicate changes in hormone levels in SAM domains (A) or between vegetative and generative shoot apices (B).

1.2.2 Molecular regulation of the shoot apex

Each zone of the shoot apex has distinct expression profiles, and in each zone hormonal levels are tightly controlled (Fig. 1.2A). The CZ and PZ are areas of low auxin and GA, and high CK and BR. Primordia have low levels of CK, but high levels of auxin, GA and BR. Auxin and BR are depleted from boundaries. A complex regulatory web of transcription factors, auxin, BR, GA and CK levels coordinates cell maintenance and cell differentiation in the shoot apex, allowing shoot growth.

The stem cell pool in the CZ is initiated by *WUS. WUS* is expressed in the OC, but can move to adjacent cell layers, and induces expression of the peptide *CLAVATA3* (*CLV3*). *CLV3* expression marks stem cells. The CLV3 peptide also diffuses to neighbouring cells, where it binds the CLV1 receptor, inducing a signalling cascade resulting in repression of *WUS* expression. Thus, cells situated further away from the OC lose their stem cell identity and differentiate (Daum et al., 2014; Lenhard and Laux, 2003; Schoof et al., 2000). In parallel to the WUS-CLV pathway, the transcription factor *SHOOT MERISTEMLESS* (*STM*) also maintains the stem cell population, together with its homologue *BREVIPEDICELLUS* (*BP*) (Byrne et al., 2002; Endrizzi et al., 1996). STM and BP are KNOTTED1-LIKE HOMEOBOX (KNOX) transcription factors, which can selectively form functional heterodimers with BEL1-LIKE HOMEODOMAIN (BLH) transcription factors. STM can interact with the BLH factors ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1), PENNYWISE (PNY; BELLRINGER; VAAMANA; REPLUMLESS; LARSON; BEL-LIKE HOMEODOMAIN 9 (BLH9)) and its homologue POUND-FOOLISH (PNF; BLH8) (Cole et al., 2006; Kanrar et al., 2006; Rutjens et al., 2009). This interaction was shown to be needed for STM function in stem cells (Rutjens et al., 2009). STM controls hormonal levels in the SAM by promoting cytokinin (CK) biosynthesis and repressing GA biosynthesis (Hay et al., 2002; Jasinski et al., 2005). In turn, CK promotes expression of *WUS* (Gordon et al., 2009). *STM* and *BP* are expressed throughout the SAM, excluding the primordia. Their expression is restricted from primordia by the organ-specific transcription factors ASYMMETRIC LEAVES1 (AS1), AS2 and JAGGED LATERAL ORGANS (JLO) (Lodha et al., 2013; Rast and Simon, 2012).

The hormone auxin is key for the development of lateral primordia. The CZ/PZ is associated with an auxin minimum, while lateral primordia are formed at sites of auxin maxima (Benková et al., 2003; Heisler et al., 2005; Reinhardt et al., 2003). This

process is tightly controlled through transport of auxin by the auxin efflux facilitator proteins PINFORMED1 (PIN1), PIN3, PIN4 and PIN7. PIN proteins are polarised on the plasma membrane towards cells with a higher auxin concentration, thereby transporting auxin to one location (Benková et al., 2003; Reinhardt et al., 2003). PIN1 polarity is regulated by the PINOID (PID) kinase (Friml et al., 2004). Floral primordia formation is disrupted or absent in *pin* and *pid* mutants, as well as in plants treated with auxin transport inhibitors (Benková et al., 2003).

The boundary is formed in a region with high mechanical strain, low brassinosteroids and adjacent to an area of high auxin (Bell et al., 2012; Gendron et al., 2012; Heisler et al., 2010). Laser ablation of the boundary affects these stresses and leads to rapid induction of *PIN1*, *PID* and *STM*. These factors are normally expressed strongly in the boundary, and could be considered pioneering factors during boundary development (Landrein et al., 2015).

To separate the stem cells from differentiating cells, the boundary must repress the stem cell and organ-specific developmental programmes as well as cell proliferation. This is achieved through the expression of a large number of transcription factors, collectively known as boundary genes. It should be noted that boundary genes are not exclusively expressed in boundaries—some boundary genes are also expressed in the meristem or primordia. In addition, some primordia-expressed genes, such as *AS1* and AS2 indirectly control boundary development from outside the boundary.

The transcription factors LATERAL ORGAN BOUNDARIES (LOB), CUP SHAPED COTELYDON 1 (CUC1), CUC2, CUC3 , LIGHT-DEPENDENT SHORT HYPOCOTYL 3 (LSH3, also known as ORGAN BOUNDARY 1), LSH4, LATERAL ORGAN FUSION1 (LOF1) and LOF2 are expressed exclusively in boundary tissue. LOB controls BR levels in the boundary by inducing expression of the BR catabolism enzyme *PHYB ACTIVATION TAGGED SUPPRESSOR* (*BAS1*). This causes depletion of BR in the boundary (Bell et al., 2012). Auxin levels are kept low in the boundary by JLO*,* while AS1 and AS2 stimulate *PIN1/3/4/7* expression, moving auxin away from the boundary and towards the primordium (Rast and Simon, 2012). BR and auxin repress the expression of *CUC1, CUC2* and *CUC3* (Gendron et al., 2012). On the other hand, expression of *STM* in boundaries stimulates *CUC* expression (Balkunde et al., 2017; Spinelli et al., 2011). Thus, *CUC1-3* expression is restricted to boundary tissue. In

the boundary, CUCs promote expression of *LSH3, LSH4*, *LOF1* and *LOF2* (Gendron et al., 2012; Lee et al., 2009; Takeda et al., 2011). Together, the CUCs, LSHs and LOFs repress cell proliferation. Stem cell identity is repressed in organ primordia by JLO, AS1 and AS2*,* which repress *STM* and *BP* expression, as do BLADE ON PETIOLE1 (BOP1) and BOP2. Interestingly, BOP1 and BOP2 (indirectly) promote the expression of the KNOX transcription factor *KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA 6* (*KNAT6*), BLH-transcription factor *ATH1* and boundary gene *LOB* (Ha et al., 2007; Jun et al., 2010; Khan et al., 2015; Rast and Simon, 2012). Finally, BRs promote *LOB* expression, creating a feedback loop on the system that reinforces boundary positioning (Bell et al., 2012).

Very little is known about genes that are expressed in the RZ, and even less is known about genes that regulate identity or activity of the RZ. The BLH-transcription factor *ATH1* is expressed in the shoot apices of vegetative plants, and has been linked to defects in stem elongation. Ectopic expression of *ATH1* represses bolting without affecting flowering, while *ath1* mutants exhibit defects in the subapical region of the SAM (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009). Unlike other non-bolting Arabidopsis mutants, such as auxin, BR or GA deficient mutants (Blázquez et al., 1998; Kauschmann et al., 1996; Timpte et al., 1992), ectopic expression of *ATH1* represses stem growth but not leaf growth (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009). Besides *ATH1*, *PNY* and *PNF* have also been linked to regulation of stem elongation. Stem elongation is reduced in *pny* mutants, and *pny* stems bear clusters of cauline leaves, flowers or siliques, interspersed with longer internodes. In *pny pnf* double mutants, no mitotic activity is detected in the RZ, and these plants do not bolt or flower at all (Bhatt et al., 2004; Byrne et al., 2003; Kanrar et al., 2006; Smith and Hake, 2003; Smith et al., 2004). No discernible phenotypes are observed in *pnf* single mutants. Loss of *ATH1* rescues stem height and clustering in *pny* and bolting and flowering in *pny pnf*, which marks *ATH1* as repressor of bolting and antagonist of *PNY* and *PNF,* while *PNY* and *PNF* promote bolting (Bao, 2009; Khan et al., 2012a, 2015)

The spatiotemporal expression domains of *ATH1, PNY* and *PNF* are in line with their perceived role in bolting (Fig. 1.3B). *ATH1* is expressed in the vegetative shoot

apex, including the RZ, and is downregulated during the transition to reproductive growth (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007). Similarly, *PNY* is expressed in the vegetative shoot apex, but is also expressed in the generative shoot apex (Andrés et al., 2014). The spatiotemporal expression pattern of *PNF* mRNA or PNF protein is unknown, but transcriptomic data suggest that it is very lowly expressed in shoot apices during vegetative growth but upregulated during the transition to reproductive growth (Rutjens, 2007). As *pny pnf* plants do not bolt and *pny PNF/pnf* hemizygotes form fused organs (Kanrar et al., 2006; Smith et al., 2004), it is likely that PNF acts only on the IM, including its meristem-organ boundaries, and the reproductive RZ.

BLH transcription factors form selective heterodimers with KNOX family transcription factors. Together, KNOX and BLH family members constitute the three-amino-acid-loop-extension (TALE) superfamily of homeodomain transcription factor proteins in plants (Bellaoui et al., 2001). BLH-KNOX heterodimerization is required for proper functioning of both proteins, affecting nuclear localization and DNA binding affinity (Bellaoui et al., 2001; Bürglin, 1997; Cole et al., 2006; Kim et al., 2013; Liu and Douglas, 2015; Rutjens et al., 2009; Smith and Hake, 2003; Viola and Gonzalez, 2009).

STM, ATH1, PNY and PNF can interact with the KNOX proteins BP, KNAT2 and KNAT6, which have also been linked to stem elongation. *BP* is expressed in the SAM and RZ and *bp* mutants form dwarfed stems (Lincoln et al., 1994; Venglat et al., 2002). This phenotype is further enhanced in *pny bp* double mutants (Smith and Hake, 2003). *KNAT2* and *KNAT6* are ectopically expressed in *pny* and *bp* plants, and loss of *KNAT6* was sufficient to rescue plant height of *pny* and *bp*. Furthermore, loss of *KNAT6* also rescued bolting and flowering in *pny pnf.* During vegetative growth *KNAT2* is normally expressed in the RZ and *KNAT6* is expressed in organ boundaries, but they are no longer expressed there during reproductive growth (Ragni et al., 2008).

Boundary genes also may play a role in the RZ. Both *LSH3* and *LSH4* are expressed in the vegetative but not the reproductive RZ (Takeda et al., 2011). Recently, ectopic expression of *LSH4* in the reproductive RZ of *pny* mutants has been linked to the dwarfed stems formed in *pny* plants. In *pny*, ectopic *LSH4* disrupts the mitotic angle of RZ cells and thus impairs stem elongation (Bencivenga et al., 2016). Additionally, increased or ectopic expression of *AS2, LOB, JLO*, *CUC3, BOP1* and *BOP2* results in repression of bolting and stem elongation, without affecting flowering (Borghi et

al., 2007; Nakazawa et al., 2003; Norberg, 2005; Shuai et al., 2002; Vroemen et al., 2003). However, these plants also exhibit several other growth defects, such as smaller or deformed leaves (Borghi et al., 2007; Norberg, 2005; Shuai et al., 2002). This raises an interesting question about the role of boundary genes in bolting, and the mechanisms through which the RZ is kept inactive during vegetative growth.

1.3 Regulation of the reproductive phase change

The reproductive phase change initiates both bolting and flowering and has been best studied in the context of flowering in Arabidopsis. This phase change is regulated by several internal and external factors that are sensed throughout the plant. To coordinate these inputs and translate them into the reproductive phase change, these signals are integrated at the shoot apex by the floral pathway integrators, mainly *SUPPRESSOR OF CONSTANS 1* (*SOC1*), *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*) and *FRUITFULL* (*FUL*). Floral regulatory signals can be divided into two categories (Fig. 1.3): floral enablers (developmental age, prolonged cold (vernalization), ambient temperature, autonomous) or floral promoters (photoperiod, light quality, GA). Floral promoting pathways directly stimulate expression of floral pathway integrators. Floral enablers act by repressing a floral repressive pathway, thus enabling plants to respond to subsequent floral promotive pathways. Much of what is currently known about the regulation of bolting in other plant species also pertains to these floral pathways, particularly the pathways under environmental control.

Environmental regulation of bolting has been studied in many crops (Table 1.1) but it was not always specified what the relative contribution of said environmental factor is to bolting and flowering, respectively. The little we know about bolting-specific regulation has been gained from studies on sugar beet and *Brassica* species. As outlined in section 1.1.2, there are a few examples of crops where one environmental factor has a stronger effect on bolting than on flowering, or *vice versa*. For example, photoperiod is a stronger requirement for bolting of celeriac than it is for flowering (Booij and Meurs, 1995). Ambient temperature is a potent inducer of bolting in chicory, while flowering has a stronger requirement for vernalization (Mathieu et al., 2018). However, there does not appear to be a universal environmental signal that strongly induces bolting or unlinks bolting from flowering in all rosette crops.

The three strongest environmental factors known to induce premature bolting in crops are photoperiod, high temperature and prolonged cold, yet little is known of the molecular mechanisms that control bolting in crops. As such, a brief overview of the multiple floral regulatory pathways of Arabidopsis will be given to help frame the context of bolting regulation of other rosette plants.

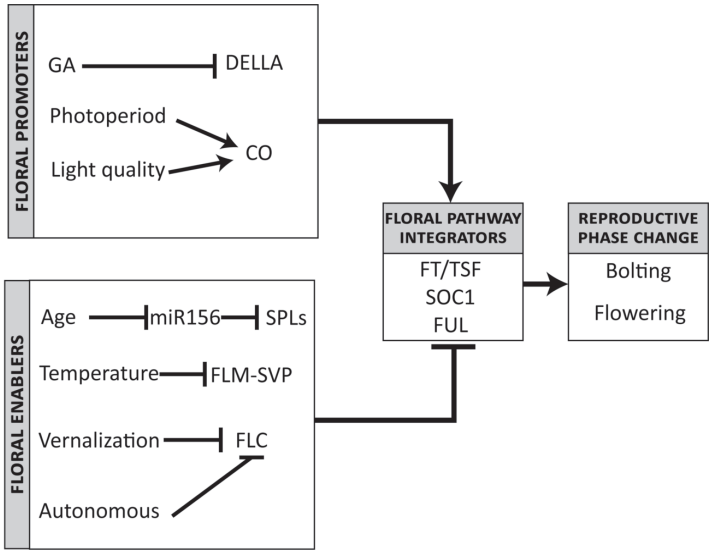

Fig. 1.3 Simplified presentation of genetic pathways controlling the reproductive phase change in Arabidopsis
Each pathway can be classified into floral enabling or floral promoting pathways. Pathways individually affect expression of floral pathway integrator genes, which in turn promote the reproductive phase change, consisting of the induction of bolting and flowering. Crosstalk between pathways is not depicted.

1.3.1 Floral enablers: Release of the brake

Ambient temperature pathway

Small changes in ambient temperature can have very strong effects on flowering time (Balasubramanian et al., 2006). In Arabidopsis, bolting and flowering is generally induced by increases within the ambient temperature range (12-27°C), which induces *SOC1* and *FT*. Pivotal factors in the ambient temperature pathway are the MADS-box transcription factors FLOWERING LOCUS M (FLM) and SHORT VEGETATIVE PHASE (SVP), which form a repressive complex at lower temperatures and repress *FT* and *SOC1* in leaves or the SAM. At high temperatures, this complex does not form due to differential alternative splicing of *FLM* and a decrease of SVP protein stability, thus alleviating repression of *FT* and *SOC1* (Lee et al., 2013; Posé

et al., 2013; Sureshkumar et al., 2016). Phytochromes, mainly *phyB* and *phyE,* also contribute to the ambient temperature response, by acting as repressors of *CO* and *FT* (Halliday and Whitelam, 2003; Halliday et al., 2003). It was recently shown that the ratio of active to non-active phyB protein is temperature-controlled, with plants accumulating lower amounts of active phyB when exposed to high night temperatures (Jung et al., 2016; Legris et al., 2016).

Sudden increases in ambient temperature are the primary cause of premature bolting in lettuce. Like in Arabidopsis, *LsSOC1* and *LsFT1* are upregulated by high temperature (Chen et al., 2018a, 2018b; Fukuda et al., 2011). Here, *SOC1* is upregulated in high temperature through binding of the heat shock factors LsHsfA1e and LsHsfA4c to the *LsSOC1* promoter, which stimulate *LsSOC1* expression. A higher expression level of *LsSOC1* during normal growth temperatures was also observed in early bolting, heat-sensitive lettuce cultivars, compared to more heat-resistant, late bolting cultivars (Chen et al., 2018a; Han et al., 2016). Similarly, bolting and flowering time was delayed in *LsSOC1-RNAi* plants, even under high temperatures (Chen et al., 2018b). High temperatures also strongly stimulate expression of the GA biosynthesis gene *LsGA3OX1* in the subapical part of the stem, which leads to a strong increase in GA1 levels that precede, and are required for, bolting (Fukuda et al., 2009; Liu et al., 2018b; Umetsu et al., 2011). It is currently not known if GA induces bolting in a SOC1-dependent manner, or whether this is an independent mechanism.

High ambient temperatures can also induce bolting in chicory, which normally requires vernalization prior to bolting. Growth of non-vernalized plants at 5°C above ambient temperature induced bolting in 42% of plants, thus overriding the vernalization requirement. Furthermore, not all plants that bolted progressed to the flowering stage, which suggests that while vernalization affects both flowering and bolting, temperature primarily promotes bolting (Mathieu et al., 2018).

Vernalization pathway

Prolonged exposure to cold temperatures results in vernalization. Arabidopsis accessions can be classified as summer annuals, which do not require vernalization, and winter annuals, which only flower after prolonged exposure to cold temperatures. Vernalization is a systemic signal and is sensed by the entire plant. Cells exposed to

vernalization and cells formed after exposure to cold retain an epigenetic memory of vernalization (Angel et al., 2011). Once other environmental conditions are favourable, reproductive growth commences. The vernalization pathway is centred on the cold-induced epigenetic silencing of the MADS-box transcription factor and floral repressor gene *FLOWERING LOCUS C* (*FLC*), whose product represses the expression of *FT* in leaves and *SOC1* in the shoot apex (Helliwell et al., 2006; Searle et al., 2006). Cold induces substantial changes in the chromatin state of the *FLC* locus and consists of two stages: Initial repression of *FLC* during cold and maintenance of *FLC* repression post-vernalization (Sheldon et al., 2002). Initial repression of *FLC* occurs rapidly after temperatures drop below a certain threshold, while stable repression of *FLC* requires longer cold exposure (Sheldon et al., 2002; Sung et al., 2006).

In most commercial *Brassicaceae* crops, such as (Chinese) cabbage, radish and turnip, bolting resistance is conferred solely through the vernalization pathway (Guo et al., 2004; Takahashi et al., 1994). The *B. rapa* genome, for example, encodes five orthologues to *FLC* (*BrFLC1-3, BrFLC3', BrFLC5*) and QTLs associated with bolting resistance have been independently mapped to variation at the loci of *BrFLC1*, *BrFLC2*, *BrFLC3* and *BrFLC2* (Kakizaki et al., 2011; Kitamoto et al., 2014).

The vernalization pathway in sugar beet differs from Arabidopsis and shows some distinction between regulation of bolting and flowering. Biennial sugar beet plants require both vernalization and long days for reproductive growth. Commercial sugar beet varieties are all vernalization-sensitive biennials, although variation exists in the vernalization requirements of different varieties. Current bolting sensitive cultivars require relatively short vernalization periods, while resistant cultivars require longer periods of cold or are more prone to devernalization, a reversion to a non-vernalized state caused by sudden increases in temperature or transfer to SD (Chiurugwi et al., 2013; Trap-Gentil et al., 2011). Interestingly, devernalization can cause plants to stop bolting, but bolting reversion is not observed in plants after they have started flowering (Reeves et al., 2007).

Regulation of the reproductive phase change in sugar beet centres on an antagonistic pair of FT homologues, BvFT1 and BvFT2. *BvFT1* is a repressor of reproductive growth and it represses *BvFT2*, which promotes reproductive growth (Pin et al., 2010). Vernalization leads to downregulation of *BvFT1* and upregulation of *BvFT2,* and the opposite occurs during devernalization (Pin et al., 2010). *BvFT2* promotes

both bolting and flowering but, in the context of vernalization, appears to have a stronger effect on flowering. In *BvFT2-RNAi* and *BvFT1-BvFT2-RNAi* lines, *BvFT2* is not induced post-vernalization, while *BvFT1* is still downregulated. These plants never flower but can bolt, although bolting is often extremely delayed or abortive (Pin et al., 2010, 2012; Vogt et al., 2014). This suggests that there is a *BvFT2*-independent route regulating bolting after vernalization, but it is currently not known which factors act in this pathway.

It is possible that vernalization-induced DNA and RNA methylation regulates bolting independently of *BvFT1* and *BvFT2.* Vernalization has a strong effect on global DNA and RNA methylation in the shoot apex of sugar beet. The *BvFT1* and *BvFT2* genes are not differentially methylated as a result of vernalization, which suggests that the methylation pathway is distinct from the BvFT1/BvFT2 pathway (Hébrard et al., 2013; Trap-Gentil et al., 2011). Bolting resistant cultivars have a higher level of DNA and RNA methylation both before and during vernalization, and these levels do not decrease upon vernalization, unlike in sensitive cultivars (Hébrard et al., 2015; Trap-Gentil et al., 2011). The sugar beet orthologue of *FLC, FLOWERING LOCUS 1* (*BvFL1*), is affected by vernalization-induced methylation (Reeves et al., 2007; Trap-Gentil et al., 2011). Variation in sequence and DNA methylation of *BvFL1* has been linked to differences in bolting time of several wild and cultivated beets (Frerichmann et al., 2013; Trap-Gentil et al., 2011). However, unlike in Arabidopsis, *BvFL1* only plays a minor role in the vernalization response, as it only contributes significantly to the response during the first three weeks of vernalization, and does not affect the vernalization pathway in the leaves (Hébrard et al., 2013, 2015; Reeves et al., 2007; Trap-Gentil et al., 2011). Moreover, *BvFL1* does not appear to regulate *BvBTC1* and *BvFT1*, only *BvFT2* (Vogt et al., 2014). Probably, *BvFL1* has a general effect on the reproductive phase change and does not affect bolting specifically.

Age and autonomous pathways

The age and autonomous pathways enable the reproductive phase change independently of environmental signals. The age pathway is responsible for transitioning plants from the juvenile growth phase to the adult vegetative growth phase. During the juvenile growth phase, most plants are unable to respond to floral inductive signals as several floral pathway integrators and floral meristem identity genes are repressed. Juvenile plants express high levels of the microRNA156

(miR156), which repress a subset of *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) transcription factors (Wang et al., 2009). The levels of *miR156* decline over time, leading to an increase in expression of *SPLs*, which indirectly repress the *APETALA2-like* (*AP2-like*) family of floral repressors (Aukerman and Sakai, 2003; Jung et al., 2011; Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009). The autonomous pathway consists of seven proteins involved in RNA processing and epigenetic regulation: FCA, FPA, FLOWERING LATE KH MOTIF (FLK), FY, FLOWERING LOCUS D (FLD), FVE, and LUMINIDEPENDENS (LD). These proteins enable the reproductive phase change by down-regulation of the floral repressor *FLC* (Liu et al., 2007, 2010; Luo et al., 2015; Simpson et al., 2003; Yu et al., 2011). This repression occurs independently of vernalization.

1.3.2 Floral promoters: A foot on the accelerator

Photoperiod

Photoperiod can be a potent inducer of the reproductive phase change, but its effect is species-specific. Arabidopsis is a facultative long day (LD) plant, meaning that plants are able to flower in short day (SD) conditions, but flowering is induced much faster in LDs. Other species could be obligate SD or LD plants, meaning that they will only transition to reproductive growth when grown in SD or LD conditions, respectively. Lastly, there are daylength neutral plants, where flowering is not regulated by photoperiod. Central to photoperiodic regulation is the zinc-finger transcription factor *CONSTANS*, which is expressed in leaves and promotes expression of *FT* in LD conditions (Suárez-López et al., 2001; Valverde et al., 2004). FT protein is subsequently transported from the leaves to the shoot apex, where it induces the reproductive phase change (Corbesier et al., 2007; Jaeger and Wigge, 2007).

Sugar beet is an obligate long day plant that occurs as annual or biennial (vernalization requiring) varieties. Activation of the photoperiod route in annual sugar beet plants is sufficient for plants to bolt and flower, while biennial sugar beets require both LDs and vernalization. The photoperiod pathway, like the vernalization pathway of sugar beet, affects expression of *BvFT1* and *BvFT2*. Photoperiodic regulation of these factors requires the diurnally expressed pseudo response regulator *BOLTING TIME CONTROL 1* (*BvBTC1*) and B-box transcription factor *BvBBX19*, located on the B and B2 loci, respectively. BvBTC1 promotes expression of *BvFT2*, while BvBTC1 and BvBBX19

are thought to interact and repress *BvFT1*, acting as an analogue to CO (Dally et al., 2014; Pin et al., 2012). During domestication of sugar beet, plants were selected that adopted a biennial growth strategy and thus only bolt after vernalization. These plants consistently carry recessive *Bvbtc1* alleles or non-functional *Bvbbx19* alleles, which no longer regulate *BvFT1* (Dally et al., 2014; Frerichmann et al., 2013; Höft et al., 2018; Pin et al., 2012).

Photoperiodic requirement for bolting and for flowering differs in sugar beet. Biennial sugar beet plants are able to bolt in SD if vernalized and treated with GA, but will not flower (Mutasa-Göttgens et al., 2010). In a similar vein, annual sugar beet plants transformed with a *BvBTC1-RNAi* construct do not bolt in LD unless vernalized, although bolting is extremely delayed and abortive in these plants. Like SD-grown biennials, they never flower (Pin et al., 2012).

A similar effect is observed in spinach, which is related to sugar beet. Photoperiod is the main factor linked to premature bolting of spinach and transfer of SD-grown spinach to LDs rapidly induces bolting and flowering. This induction is coupled to a rise in GA levels, and GA treatment of SD-grown plants induces bolting but not flowering (Janick and Leopold, 1961), like in sugar beet (Mutasa-Göttgens et al., 2012). Also, like sugar beet, the spinach genome contains two FT orthologues, *SoFT1* and *SoFT2*, which share significant homology with *BvFT1* and *BvFT2*. Both genes are lowly expressed in SD and *SoFT2* is induced in LD conditions. *SoFT1* is not completely downregulated in LD and therefore it is not known whether *SoFT1* has a functional role in bolting of spinach (Abe et al., 2014).

In celeriac, which requires vernalization and long days for induction of bolting and flowering, the threshold for induction by photoperiod differs for bolting and flowering. Transient exposure to inductive photoperiods is sufficient to induce flowering and maintain it even after plants are transferred to non-inductive SD photoperiods, while bolting requires continued growth in long days (Booij and Meurs, 1995).

Light quality

Light quality-mediated flowering depends on the ratio of red to far-red (R:FR) light. As plants absorb red light, but reflect far-red light, a low R:FR ratio signifies shading by neighbouring plants. Low R:FR ratios induce the shade avoidance response, which,

amongst other effects, promotes early bolting and flowering (Wang and Wang, 2015). The R:FR ratio is measured by phytochrome receptors phyA, phyB, phyD and phyE, which are activated by red light and inactivated by FR light. Active phyB, phyD and phyE repress the reproductive phase change, primarily by repressing FT and CO (Cerdán and Chory, 2003; Devlin et al., 1996; Halliday et al., 2003; Wollenberg et al., 2008).

Gibberellins

The third promoter of the reproductive phase change is the phytohormone GA. In Arabidopsis, GA is essential for bolting and flowering in SD conditions, but only essential for bolting in LDs (Blázquez et al., 1998; Regnault et al., 2014; Wilson et al., 1992).

GA regulates gene expression through the SCF-mediated degradation of DELLA family proteins.DELLA proteins act as repressors of the GA response by binding to transcription factors and modulating their activity. They can promote transcription factor degradation, or interfere with DNA binding or transcriptional activity, but can also act as transcriptional cofactors themselves (Feng et al., 2008; Fukazawa et al., 2014; Hyun et al., 2016; Li et al., 2016a, 2016b; Yamaguchi et al., 2014). In the presence of GA, DELLA proteins are ubiquitinated and degraded (Dill et al., 2004; Griffiths et al., 2006; Murase et al., 2008). Arabidopsis has five *DELLA* genes, *GIBBERELLIN INSENSITIVE* (*GAI*), *REPRESSOR OF GAI* (*RGA*), *RGA-LIKE1* (*RGL1*), *RGL2* and *RGL3,* each with distinctive functions and expression patterns. Flowering and stem elongation is regulated by RGA and GAI (Dill and Sun, 2001; King et al., 2001).

In SDs, increased GA at the shoot apex promotes the reproductive phase change, but GA activity in the leaves does not appear to be important for this process (Yu et al., 2012). In vegetative shoot apices GA levels are low, allowing RGA and GAI to repress expression of *SOC1*, *LFY* and multiple *SPLs*, including *SPL3* and *SPL9* (Eriksson et al., 2006; Hyun et al., 2016). Additionally, RGA and GAI can physically interact with SPL15, inhibiting its transcriptional activity (Hyun et al., 2016). Over time, GA levels in the shoot apex gradually increase, although GA biosynthesis in the shoot apex does not change (Eriksson et al., 2006). It is thought that GA precursors, but not bioactive forms of GA, are biosynthesized in the leaves, and transported to the shoot apex where they are converted to bioactive GA_1 and GA_4 (Regnault et al., 2015). As

a result of this rise in GA, RGA and GAI are degraded, releasing SPL15 protein and enabling expression of *SOC1, LFY* and *SPL3/9.* SPL15 and SOC1 subsequently interact and induce the reproductive phase change (Hyun et al., 2016).

The GA-biosynthesis mutant *ga1* does not bolt or flower in SD. It is possible to restore flowering in *ga1-3* mutants in these conditions through ectopic expression of *SOC1, LFY, CO* and *FT,* but bolting is never restored in these plants (Blázquez et al., 1998; Eriksson et al., 2006; Moon et al., 2003). In line with this, LD-grown *ga1* mutants flower without bolting (Wilson et al., 1992). This suggests that GA has a dual function, whereby it promotes the reproductive phase change but is separately also required for induction of bolting.

In contrast to its role in SDs, the role of GA in LDs is not limited to the shoot apex, and it appears to have a more systemic role in promoting the reproductive phase change. In LD, GA induces expression of *SPLs* and *LFY* in both the leaves and the shoot apex (Yu et al., 2012). Moreover, in plants switched from SD to LD, GA biosynthesis is rapidly increased throughout the entire rosette, including the leaves (Xu et al., 1997).

As mentioned previously, GA application can induce bolting in many crops grown in non-inductive conditions, without inducing flowering (Section 1.1.2). In addition to this, high levels of GA coinciding with bolting have been observed in spinach, lettuce and *S. arneria* (Fukuda et al., 2009; Liu et al., 2018b; Talon et al., 1991; Zeevaart, 1971), which will be discussed in-depth in Section 1.4.

1.3.3 Floral meristem identity genes

Activation of the floral pathway integrators is required for initiation of the reproductive phase (Fig. 1.3). These integrators promote expression of floral meristem identity genes in the SAM (Abe et al., 2005; Hyun et al., 2016; Liu et al., 2008; Wigge et al., 2005; Yamaguchi et al., 2005). The main floral meristem identity genes are *LEAFY* (*LFY*), *APETALA 1* (*AP1*) and *CAULIFLOWER* (*CAL*) and their expression is required for the formation of floral meristems, thus flowering (Liljegren et al., 1999).

Interference with floral signalling pathways generally seems to affect the timing of both bolting and flowering (Cerdán and Chory, 2003; Devlin, 1998; Lee et al., 2013; Macknight et al., 1997; Moon et al., 2005; Posé et al., 2013; Simpson et al., 2003; Suárez-López et al., 2001; Wahl et al., 2013). GA is an exception to this, as repression

of the GA pathway in LD only affects bolting and not flowering (Blázquez et al., 1998; Wilson et al., 1992). Disruption or ectopic expression of floral pathway integrators also affects the timing of both bolting and flowering time (Ferrándiz et al., 2000; Liu et al., 2008; Moon et al., 2005; Wahl et al., 2013; Yamaguchi et al., 2005). On the other hand, loss of the floral meristem identity genes *LFY* and *AP1* affects only flowering and not bolting (Huala and Sussex, 1992; Ruiz-García et al., 1997). Floral meristem identity genes therefore should be viewed as flowering-specific factors, while upstream genes should be considered regulators of the reproductive phase change in general.

1.4 Internal regulation of bolting

The reproductive phase change is associated with significant changes in internal signals, e.g. phytohormone levels. Gibberellin is generally classified as the bolting hormone, but significant changes in auxin, brassinosteroids (BR), cytokinins (CK) and jasmonic acid (JA) have also been observed in bolting or bolting-sensitive plants, and there is some evidence to suggest that also ethylene has a role in regulating the timing of bolting and subsequent internode elongation (Achard et al., 2007; Frugis et al., 2001; Hao et al., 2018; Ogawara et al., 2003; Yoshida et al., 2010).

1.4.1 The timing of bolting

Prior to bolting, increases in GA, auxin and CK have been observed in shoot apices of rosette plants (Fig. 1.2B). Induction of the reproductive phase is correlated with an increase in endogenous GA levels in Arabidopsis, and this has also been observed in spinach, lettuce and *S. armeria* (Eriksson et al., 2006; Fukuda et al., 2009; Liu et al., 2018b; Talon et al., 1991; Xu et al., 1997; Zeevaart, 1971). In *S. armeria*, photoperiodic induction of bolting leads to a 9-fold increase of bioactive GA_1 in an area of 0-0.5 mm from the shoot apex tip, which includes the SAM and RZ (Talon et al., 1991). Strong increases in GA_1 have been also been observed further away from the apex tip during bolting induction of *S. armeria* and also in lettuce (Fukuda et al., 2009; Talon et al., 1991). Bolting induction strongly upregulates *LsGA3OX1* expression in lettuce, and bolting is no longer induced by high temperature in *Lsga3ox1* loss of function mutants (Fukuda et al., 2009; Umetsu et al., 2011). During bolting induction, GA_3 and GA_4 levels also gradually increase in lettuce leaves, and

LsGA2OX2 GA catabolism genes are downregulated in both the leaves and the stem (Liu et al., 2018b). In these conditions, GA levels peaked one day before bolting was visible. This GA peak also preceded a rapid increase of auxin levels in the developing stem, while auxin levels in the leaves remained unchanged. A single application of GA to the whole shoot was also sufficient to induce bolting and increased auxin levels in both stems and leaves (Hao et al., 2018; Liu et al., 2018b). This suggests that mobile GA may induce bolting via the induction of auxin. In Arabidopsis, GA promotes auxin transport by regulating the PINFORMED (PIN) family of auxin efflux carriers (Löfke et al., 2013; Salanenka et al., 2018; Willige et al., 2011). Interestingly, in both *pin6* mutants and *35S::PIN6* overexpressors, bolting and flowering time is increased (Ditengou et al., 2018), which suggests that auxin homeostasis plays an important role in regulating the timing of bolting.

CK might also play a role in the timing of bolting. In Arabidopsis, induction of bolting and flowering was associated with a strong increase in isopentenyl (IP)-type CK, initially in the leaves and later in the SAM (Corbesier et al., 2003). Increased CK-levels in leaves have also been linked to early bolting in lettuce, and bolting induction also increased protein abundance of CK signalling proteins in lettuce leaves (Frugis et al., 2001; Hao et al., 2018). The timing of bolting in Arabidopsis is also affected by CK, as LD-grown plants treated with IP-type CKs bolted early, while CK receptor mutants bolted late. However, CK treated plants also flowered early, and CK receptor mutants flowered late and exhibited several floral defects (He and Loh, 2002; Nishimura et al., 2004). Thus, it is possible that CK affects the reproductive phase change as a whole and does not specifically regulate bolting. Unlike the abovementioned hormones, the phytohormone jasmonic acid (JA) has been linked to repression of bolting. In sugar beet, radish and qing gen cai (*B. rapa* var. *chinensis*) rosette crops, JA has also been linked to bolting repression. Application of JA or JA-analogues could repress inflorescence internode elongation in these crops, even after vernalization (Koda et al., 2001; Takada et al., 2013; Yoshida et al., 2010). A sharp decline in levels of the fatty acid and JA precursor α-7Z,10Z,13Z-hexadecatrienoic acid (16:3) was observed prior to bolting in vernalized radish plants. In shoot tissue of early bolting cultivars, this decline occurred earlier than in late bolting plants. These plants also contained higher starting levels of 16:3 (Yoshida et al., 2010). In sugar beet, a decline in JA levels occurred in non-vernalized plants, suggesting that JA levels can also be regulated independently of vernalization (Koda

et al., 2001).In Arabidopsis, JA-treatment delays bolting and flowering (Khan et al., 2015). However, the role of JA in repressing bolting might not be universal, as higher levels of JA were correlated with bolting induction or early bolting in lettuce and garlic (Hao et al., 2018; Wu et al., 2016).

JA signalling requires SCF-mediated degradation of negative regulators of JA responses: the JASMONATE ZIM-DOMAIN (JAZ)-family proteins (Thines et al., 2007). In Arabidopsis, JA-treatment delays bolting and flowering and reduces internode elongation (Khan et al., 2015). The *jazD* mutant, which lacks seven of the ten *JAZ* genes, is late bolting and flowering in both LD and SD conditions. It is thought that JA represses bolting and flowering by indirectly repressing *FT*, possibly through mutual antagonism between JAZ and DELLA proteins (Guo et al., 2018; Hou et al., 2010; Yang et al., 2012; Zhai et al., 2015).

Lastly, ethylene affects the timing of bolting, but its effect is ambiguous. Arabidopsis plants treated with the ethylene precursor ACC bolt and flower late in both SD and LD. Ethylene also reduces biosynthesis of GA, which would suggest that it is a negative regulator of bolting and flowering time (Achard et al., 2007). However, inactivation of negative regulators of the ethylene signalling pathway also results in late bolting (Ogawara et al., 2003).

1.4.2 The effects of hormones on the RZ and initial stages of bolting

As described in Section 1.2.1, the RZ drives internode elongation during bolting. Upon bolting induction, the RZ of rosette plants undergoes increased cell division, coupled with a switch from random divisions to periclinal divisions (Bencivenga et al., 2016; Jacqmard et al., 2003; Metzger and Dusbabek, 1991; Peterson and Yeung, 1972). Analysis of the shoot apices of the rosette plants *Hyoscyamus niger* and *Samolus parviflorus* revealed that exogenous GA application induced bolting by promoting periclinal cell divisions in the RZ within 24 hours of application (Sachs and Long, 1957; Sachs et al., 1959a). CK treatment of SD-grown *Sinapis alba* also caused significant increases in mitosis in the RZ and CZ, which had previously also been observed during initial stages of bolting induction of plants transferred to LD conditions. However, despite this increase in RZ activity, CK application alone was not sufficient to induce bolting or flowering (Bernier et al., 1977). In Arabidopsis, CK does not localize to the RZ during induction but can instead be found throughout

the rest of the shoot apex (Corbesier et al., 2003). In contrast, studies of auxin reporter lines in Arabidopsis showed that auxin is present in the RZ of bolting plants, while absent from the RZ of vegetative plants (Brunoud et al., 2012; Shi et al., 2018) (Fig. 1.3B). Suppression of BR activity from the RZ of vegetative plants may also be important in repressing activity of the RZ during rosette growth. The *PHYB ACTIVATION TAGGED SUPPRESSOR 1* (*BAS1*) gene, which encodes a BR catabolic enzyme, is strongly expressed in the RZ during vegetative growth but not during reproductive growth (Fig. 1.2B) (Sandhu et al., 2012).

In vegetative shoot apices the GA catabolism enzymes *GIBBERELLIN-2-OXIDASE1* (*GA2OX1*) and *GA2OX4* are also expressed in the RZ (Jasinski et al., 2005; Li et al., 2019). GA application promotes organization of the RZ into cell files, but does not induce cell elongation in the RZ itself (Sachs and Long, 1957; Sachs et al., 1959b; Talon et al., 1991), which matches recent findings that show that cell division and not elongation is the major contributor to initial stages of bolting in Arabidopsis (Bencivenga et al., 2016). Nevertheless, later stages of bolting do require cell elongation, which occurs further away from the apex tip (Bencivenga et al., 2016). As no clear lower limit has been given to the RZ, it is difficult to determine whether this process occurs in the RZ itself, or in the inflorescence stem.

1.4.3 The effects of hormones on stem elongation

Activity of GA, auxin and BR is necessary in the stem. BR biosynthesis mutants, such as *dwarf1* (*dwf1*) and *dwf4* form shorter stems, while the BR receptor mutant *brassinosteroid insensitive 1* (*bri1*) does not bolt at all, like GA biosynthesis null mutants. The gain of function AUX/IAA mutant *auxin resistant 2* (*axr2*) is also severely impaired in stem elongation. All these mutants are still able to flower, with the exception of SD-grown GA-deficient plants. However, it should be noted that growth of other organs, e.g. leaves, is generally also impaired in all these mutants (Azpiroz et al., 1998; Blázquez et al., 1998; Kauschmann et al., 1996; Timpte et al., 1992; Wilson et al., 1992).

Auxin, GA and BR all stimulate both cell division and cell elongation (Gonzalez et al., 2010; Sachs and Long, 1957; Sachs et al., 1959a; Timpte et al., 1992). GA most likely controls stem height through a combination of both processes. The *rosette* (*ros*) mutant of rapid cycling *B. rapa* is GA deficient and forms a much shorter inflorescence

stem, with each stem internode containing both fewer and shorter cells (Rood et al., 1990; Zanewich et al., 1990). In seedlings, GA promotes reorganization of cortical microtubuli, which facilitates longitudinal elongation of hypocotyl cells (Locascio et al., 2013; Vineyard et al., 2013). Possibly, GA has a similar effect on the cells of inflorescence stems.

Ectopic expression of the GIBBERELLIC ACID STIMULATED IN ARABIDOPSIS THALIANA 5 (GASA5) peptide represses GA signalling by increasing *GAI* expression. Stems of these plants contained smaller cells, and stems elongated at a significantly slower rate (Zhang et al., 2009). Homologues of GASA genes have also been identified in lettuce and sugar beet. In sugar beet, a homologue of *GASA2* was robustly upregulated in plants that were induced to bolt in SD using a combination of vernalization and GA (Mutasa-Göttgens et al., 2012). Sugar beet grown this way does not flower, which suggests that *GASA2* may exclusively regulate bolting in sugar beet. In lettuce, differential expression of *GASA* family genes was also observed during bolting induction, and between bolting sensitive and bolting resistant cultivars (Han et al., 2016; Liu et al., 2018b).

Possibly, BR promotes stem elongation primarily through cell elongation, as cell height and not cell number was affected in the dwarfed stems of *dwf4* mutants (Azpiroz et al., 1998). Analysis of *axr2* stems suggests that auxin has a similar effect in the epidermis and pith of the stem, but affects both cell division and elongation in the stem cortex (Timpte et al., 1992). Auxin promotes expression of the GA biosynthesis genes *GA20OX1* and *GA20OX2,* which are expressed in the stem and required for stem elongation (Frigerio et al., 2006; Rieu et al., 2008). It is likely that a delicate balance of auxin levels must be maintained in the stem during bolting. Disruption of PIN1 and PIN6-mediated basipetal auxin transport, observed in the stems of *pin1* mutants and *35S::PIN6* overexpressors, leads to auxin accumulation in the stem to growth inhibiting levels, thereby reducing the rate of stem elongation (Cazzonelli et al., 2013; Ditengou et al., 2018). Conversely, *pin6* mutants have a higher stem elongation rate than wild-type plants (Ditengou et al., 2018), again linking the importance of auxin homeostasis to bolting. Auxin transport is tightly interlinked with GA as GA promotes transport of auxin through inflorescence stems through PIN placement. As a result, *ga1-3* and *gai* mutants are also impaired in auxin transport through the stem and accumulate less PIN1 protein (Löfke et al., 2013; Salanenka et al., 2018; Willige et al., 2011).

Ethylene can modulate GA-mediated stem elongation through *ETHYLENE RESPONSE FACTOR 11 (ERF11*). ERF11 interacts with RGA and GAI, an interaction that is thought to be mutually antagonistic. Increased ethylene thereby can increase sensitivity to GA. Furthermore, ectopic expression of *ERF11* represses ethylene and stimulates GA biosynthesis, which leads to an increase in cell elongation and thereby in internode length. Furthermore, stem elongation in the weak *ga1-2* biosynthesis mutant can be rescued by the gain of function *erf11-1D* allele.

Crosstalk between GA and JA also affects internode elongation, albeit in an antagonistic manner. This antagonism is clearly observed in plants that lack *CALCIUM DEPENDENT PROTEIN KINASE 28* (*CPK28*). CPK28 promotes GA biosynthesis while repressing JA biosynthesis, and *cpk28* mutants form severely shorter stems than wild-type plants. Stem elongation was rescued by either GA application or inactivation of JA biosynthesis, which shows that the ratio between GA and JA controls elongation of the stem (Matschi et al., 2013, 2015).

1.5 Perspectives

Bolting is an agriculturally relevant, fundamental developmental process in rosette plants. Yet, the process of bolting is poorly understood, with most of the knowledge on bolting linked directly to known floral regulatory mechanisms. The maintenance of the rosette habit during vegetative growth is an equally important process, as the rosette habit defines vegetative growth of rosette plants and is crucial for the production of several rosette crop-derived vegetables. Yet, the rosette has received even less attention than bolting. Molecular control of bolting is still largely unknown, as are the molecular mechanisms that prevent internode elongation during rosette growth. Although GA is crucial for bolting in Arabidopsis, elevated GA biosynthesis or constitutive GA signalling does not disrupt rosette formation (Coles et al., 1999; King et al., 2001), which suggests that there are factors present during rosette growth that block sensitivity to elongation signals such as GA.

To understand the mechanisms behind the regulation of bolting and the formation of the rosette, the following questions need to be answered.

1. How is the rosette habit maintained during vegetative growth?
2. What molecular changes occur at the shoot apex that underlie the switch from rosette growth to stem growth?
3. What is the contribution of shoot apex-expressed TALE homeobox transcription factors to the maintenance of the rosette and the regulation of bolting?

Thesis outline

In this thesis we investigate the role of the BLH family transcription factor ATH1 in the regulation of the rosette habit and bolting of *Arabidopsis thaliana*.

In **Chapter 2** we combine confocal imaging, genetics, expression analyses, and pharmacological experiments to show that *ATH1* is a crucial factor for maintaining rosette habit and repressing bolting in Arabidopsis in a separate but converging pathway to GA. Elongation of the RZ is correlated with loss of ATH1 from the SAM and RZ. This elongation already occurs during vegetative growth of *ath1-3* mutants, resulting in elongation of rosette internodes (heterochronic bolting). Heterochronic bolting requires GA and is enhanced by GA, leading to loss of rosette habit in *ath1-3* mutants. ATH1 promotes expression of the boundary genes *LOB, BOP1, BOP2* and *LSH4*. We show that *LOB* expression is almost entirely dependent of *ATH1*, and an important component of ATH1-mediated repression of internode elongation. It is discussed that convergence of ATH1- and GA-mediated signals most likely occurs downstream of LOB.

In **Chapter 3**, we use pharmacological experiments to investigate the hormonal induction of heterochronic bolting further. Our data suggest that ATH1 blocks sensitivity of rosette internodes to auxin and BR, partially through *LOB*. We also show that loss of *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) or *PIF7*, major regulators of hypocotyl elongation, (partially) suppress heterochronic bolting at high temperature or in low R:FR conditions, respectively. We also show that combined

application of auxin and BR is sufficient to induce heterochronic bolting in wild type plants, and reduces expression of *ATH1*.

In **Chapter 4** we perform an EMS mutagenesis screen on *ath1-3* to identify genetic components that enhance or suppress heterochronic bolting. We classified these mutants as *enhancer of ath1-3 rosette internodes* (*eri*) or *suppressor of ath1-3 rosette internodes* (*sri*) mutants. We obtained four *ath1-3 eri* and nine *ath1-3 sri* mutants, identifying putative causal mutations in three *ath1-3 sri* mutants. We show that BR is essential for GA or auxin-induced heterochronic bolting. Additionally, identification of *ath1-3 sri* mutants carrying mutations in *BIG* or *WUS* suggest that *ATH1* maintains rosette habit through multiple routes.

In **Chapter 5** we investigate the role of ATH1-interacting class I KNOX transcription factors (KNAT2, KNAT6, BP and STM) and putative ATH1-PNY antagonism in ATH1-mediated repression of internode elongation. We show that *KNAT2* and *KNAT6* are required for ATH1-mediated repression of internode elongation, whereas STM also contributes to suppression of heterochronic bolting. Strikingly, we show that PNY has a dual role in the regulation of internode elongation, acting synergistically with ATH1 during rosette growth and antagonistically to ATH1 during stem growth.

This thesis is concluded in **Chapter 6**, where a summary of the most important findings from the presented work is given, and is discussed in a broader context.

CHAPTER 2

ARABIDOPSIS THALIANA HOMEOBOX 1 and gibberellins form two independent but converging pathways controlling rosette habit and bolting in *Arabidopsis thaliana*

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Abstract

Rosette plants, such as lettuce, sugar beet and the model plant Arabidopsis thaliana, undergo a dramatic change in growth habit during their life cycles. Vegetative growth is characterized by the formation of a compact whorl of leaves (the rosette) whereas reproductive growth is characterized by rapid elongation of internodes resulting in the formation of an elongated stem (bolting). Bolting is both a fundamental developmental process in rosette plants and an agriculturally undesirable trait, as premature bolting significantly affects crop yield. The hormone gibberellin (GA) is essential for bolting in Arabidopsis but little is known about factors that promote the rosette phase. Here, we show that the transcription factor ATH1 is key in maintaining rosette growth and repressing bolting in Arabidopsis, independently of GA. The rib zone (RZ) of vegetative *ath1-3* mutants morphologically resembled the RZ of bolting plants, leading to strong rosette internode elongation during vegetative growth (heterochronic bolting). GA-treatment of *ath1-3* during vegetative growth enhanced heterochronic bolting and disrupted the rosette habit, while chemical inhibition of GA restored rosette growth. ATH1-mediated repression of internode elongation required the boundary gene *LOB*, which together with the boundary genes *BOP1*, *BOP2* and *LSH4* is targeted by ATH1. Neither *ATH1* or its boundary gene targets were regulated by GA, which suggests that ATH1 represses internode elongation independently of GA. Thus, *ATH1* and GA form two independent pathways that converge to control internode elongation and thereby specify rosette growth and bolting in Arabidopsis.

Introduction

From deserts to mountain ranges, dicots to monocots, tundras to the tropics, rosette plants are found far and wide. Rosette plants, including the model plant species *Arabidopsis thaliana*, are typified by vegetative growth as a rosette (a tightly packed whorl of leaves close to the ground), followed by the formation of a stem (bolting) during reproductive growth. These two contrasting growth forms have contrasting functions: The rosette is thought to protect plants from (a)biotic stresses, while the stem facilitates pollination and seed dispersal (Fujita and Koda, 2015; Larcher et al., 2010; Schaffer and Schaffer, 1979; Soons et al., 2004). In rosette crops such as Brasscicaceae crops, lettuce and sugar beet, prolonging rosette growth and preventing bolting is of agricultural importance, as bolting negatively affects crop quality and yield (Guttormsen and Moe, 1985; Longden et al., 1975; Sessa et al., 2000). Environmental factors such as temperature, daylength and vernalization can induce bolting (Fukuda et al., 2009; Guttormsen and Moe, 1985; Mathieu et al., 2014), yet very little is known about the molecular regulation of the rosette habit.

Growth of the rosette is coordinated at the shoot apex, which contains the shoot apical meristem (SAM) and the rib zone/rib meristem (RZ/RM). The SAM is responsible for the formation of shoot tissue and consists of a central population of stem cells (central zone; CZ), flanked laterally by the dividing cells of the peripheral zone (PZ) and basally by the rib meristem (RM) and rib zone (RZ) (Bencivenga et al., 2016; Reddy et al., 2004). The CZ and PZ are separated by a strip of nondividing cells termed the boundary (Reddy et al., 2004; Žádníková and Simon, 2014). During vegetative growth the SAM is flat, the RM and RZ are compact and mitotically inactive, and cells from the PZ differentiate and form leaf primordia. During the transition to reproductive growth, the meristem transforms into a domed inflorescence meristem (IM), where the PZ forms floral primordia, and the cells in the RM and RZ proliferate to form the stem (Bencivenga et al., 2016; Jacqmard et al., 2003; Kwiatkowska, 2008; Peterson and Yeung, 1972; Sachs et al., 1959a; Serrano-Mislata et al., 2017). While the roles of the PZ and CZ have been well studied, the RM and RZ have received less attention due to two complications: First, definition of the RM and RZ is not consistent, thus these terms have been used both synonymously and separately. Second, as the RM and RZ lie deep within the shoot apex, they have been inaccessible for thorough microscopic examination during vegetative growth.

During reproductive growth, the RM/RZ is organised into transverse cell files (ribs) and undergoes increased cell division during reproductive growth, giving rise to the stem (Bencivenga et al., 2016; Peterson and Yeung, 1972; Sachs, 1991; Serrano-Mislata et al., 2017). Recently, application of novel imaging techniques has shed light on the cellular origins and nature of the RM/RZ in reproductive meristems. Here, it was shown that this region can be divided into a fast dividing peripheral RZ, and a slower dividing central RZ, whose cells originate from the PZ and CZ, respectively (Bencivenga et al., 2016). As the RM/RZ region appears to lack a true meristematic identity, we will refer to this region solely as the RZ.

The plant hormone gibberellin (GA) can induce cell division in the RZ of some rosette plants, which correlates with the onset of stem growth (Mutasa-Göttgens et al., 2010; Peterson and Yeung, 1972; Sachs et al., 1959a). In short day (SD)-grown Arabidopsis, GA is essential for both bolting and flowering and rising GA levels in the shoot apex have been linked to the onset of reproductive growth (Eriksson et al., 2006; Wilson et al., 1992). In long days (LD) GA is essential for bolting, but not flowering (Blázquez et al., 1998). GA elicits its downstream response through the degradation of DELLA family proteins, which act as master repressors of GA signalling. In Arabidopsis, this family consists of five members, of which *GIBBERELLIC ACID INSENSITIVE* (*GAI*) and *REPRESSOR OF GAI* (*RGA*) repress stem elongation (Dill and Sun, 2001; King et al., 2001; Serrano-Mislata et al., 2017). Although GA is required for elongation of the inflorescence stem, it does not appear to be crucial for rosette formation. Arabidopsis *della* mutants, mutants with increased GA levels and GA-treated plants bolt earlier but never lose their rosette habit—there still is a clear distinction between compact rosette internodes and elongated stem internodes (Coles et al., 1999; Galvão et al., 2015; Park et al., 2013; Rieu et al., 2008). These plants undergo a clear bolting transition despite elevated GA signalling. This suggests that during vegetative growth, other regulatory components overrule GA-mediated internode elongation and thereby maintain rosette growth.

The BEL1-LIKE HOMEODOMAIN (BLH) homeodomain transcription factor ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) is a negative regulator of internode elongation. It is expressed in the vegetative SAM, floral primordia and basal organ boundaries of the shoot. *ATH1* expression in the SAM is downregulated during the

reproductive phase change (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007). Plants ectopically expressing *ATH1* no longer bolt, or they produce much shorter stems, which contain cells that are significantly smaller than those in wild-type stems. In contrast, *ATH1* overexpressors flower normally (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007; Rutjens et al., 2009). In a similar vein, the subapical region of *ath1* loss of function mutants is more elongated compared to wild-type plants (Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009). This raises the question whether ATH1 acts as a regulator of the rosette habit, repressing bolting during vegetative growth.

Here, we show that ATH1 is a key regulator of *Arabidopsis thaliana* rosette growth and instils a block on internode elongation independently of GA, thereby preventing bolting. Bolting induction coincides with downregulation of ATH1 in the CZ and RZ, and elongation of the RZ in wild-type plants. On the other hand, *ath1* loss-of-function mutants already show an elongated RZ during vegetative growth, which is reflected by visible elongation of rosette internodes (heterochronic bolting). While exogenous GA application is not sufficient to induce heterochronic bolting in wild-type plants or to restore wild-type bolting in plants constitutively expressing *ATH1*, elongation of rosette internodes in *ath1* requires active GA signalling and exogenous application of bioactive GA results in complete loss of rosette habit. This suggests that ATH1 and GA act in two converging, but independent pathways to regulate rosette habit and bolting.

Expression of the boundary genes *BLADE ON PETIOLE 1* (*BOP1*) and *LIGHT SENSITIVE HYPOCOTYL 4* (*LSH4*) is induced in *35S::ATH1* overexpressors and reduced in *ath1-3* seedlings, while expression of the boundary gene *LATERAL ORGAN BOUNDARIES* (*LOB*) is severely diminished in *ath1-3*. This marks these genes as targets of *ATH1*. Moreover, loss of *LOB* leads to heterochronic bolting in response to exogenous GA-treatment, although this phenotype is weaker than in *ath1-3* mutants. As boundary genes repress tissue growth (Norberg, 2005; Shuai et al., 2002; Takeda et al., 2011), ATH1 likely maintains rosette growth in part through positive regulation of *LOB*.

RESULTS

Loss of *ATH1* from the shoot apical meristem results in the elongation of the rib zone

Vegetative growth of Arabidopsis is characterised by the formation of a compact rosette, where successive leaves are formed without producing elongated internodes. This morphology was not observed in *ath1-3* loss of function mutants, as *ath1-3* rosette internodes were significantly elongated (Fig. 2.1F). This partial loss of rosette habit might reflect changes in (activity of) the vegetative RZ of *ath1-3* mutants. Previous studies showed that *ath1-3* mutants form a more elongated subapical region, which includes the RZ (Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009). Activation of the RZ is necessary for stem elongation during reproductive growth, i.e. bolting (Bencivenga et al., 2016; Jacqmard et al., 2003; Sachs et al., 1959a), but this region of the shoot apex has thus far been poorly studied, especially during vegetative growth.

To compare the RZ of vegetative *ath1-3* mutant plants with that of both vegetative and bolting wild-type plants, Col-8 and *ath1-3* plants were grown in conditions that were non-inductive to bolting and then transferred to bolting-inductive conditions. Under these conditions, bolting is induced uniformly at population level with first macroscopic signs of bolting, scored as a 0.5 cm extension of the stem, visible seven days after transfer. Plants kept in non-inductive conditions served as control. Shoot apices were imaged 0, 1 and 4 days after shifting. Wildtype plants (Col-8) retained in non-inductive conditions exhibited a weakly curved apex, from which blade-shaped leaf primordia (Sarojam et al., 2010) initiated at the flanks (Fig. 2.1A). Based on morphological landmarks defined previously (Bencivenga et al., 2016), the CZ and RZ were defined as areas at respectively 0-45 µm and 45-90 µm from the apex tip, and 15 µm from the main axis of the apex (Fig. 2.1E). The RZ of the control plants was compact and RZ cells showed a random organisation (Fig. 2.1A). These morphological characteristics are typical of vegetative development and are reflected in the formation of a compact rosette (Jacqmard et al., 2003; Kwiatkowska, 2008). After one day in inductive conditions, there were no apparent changes in morphology, while after four days, both flowering and bolting had been initiated. The meristem was domed, forming rounded floral primordia, and cells of the RZ were elongated and organised into transverse cell files (Fig. 2.1A), all typical for the reproductive Arabidopsis IM (Bencivenga et al., 2016; Kwiatkowska, 2008). Strikingly,

elongated, transverse cell files were already present in the RZ of *ath1-3* mutants kept in non-inductive conditions. Moreover, transfer to inductive conditions did not cause observable changes in morphology of the *ath1-3* RZ, although floral transition was observed 4 days after induction, as in Col-8 (Fig. 2.1B). Comparison of cell heights of the CZ and RZ showed that bolting induction increased cell height of Col-8 RZ cells, but not those of *ath1-3*. Until day 4 of induction, when Col-8 elongation caught up to *ath1-3,* RZ cells were shorter in Col-8 than in *ath1-3*, , (Fig. 2.1D), indicating that the *ath1-3* RZ is already active during vegetative development. Unlike the RZ, the *ath1-3* CZ was still responsive to inductive conditions as CZ cells in both Col-8 and *ath1-3* increased in height after transfer (Fig. 2.1D).The increased elongation of the RZ in vegetative *ath1-3* is reflected in the morphology of the *ath1-3* rosette, as *ath1-3* mutants form elongated rosette internodes (Fig. 2.1F). The formation of elongated

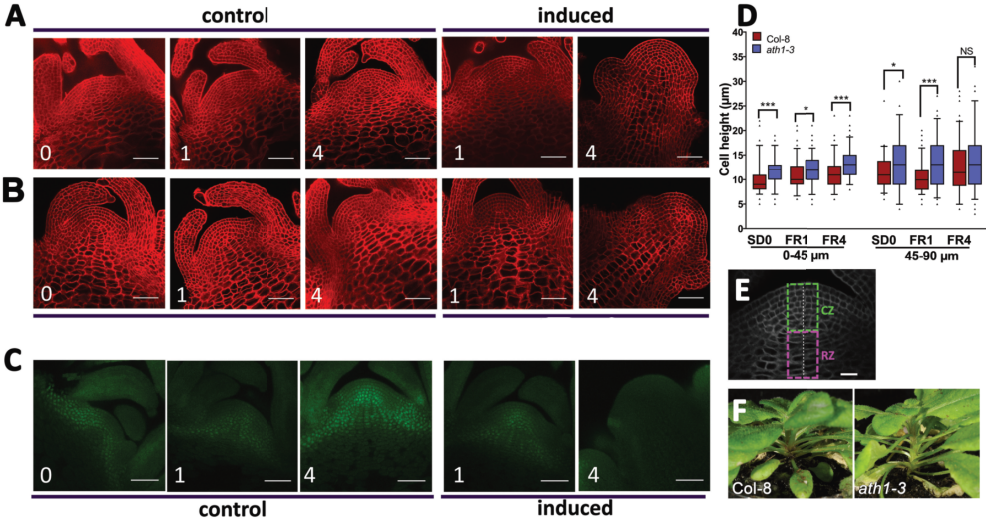

Fig. 2.1: ATH1 function correlates with an inactive rib zone
A-B: Representative confocal images of Col-8 (A) and *ath1-3* (B) shoot apices stained with mPS-PI. Plants were grown for 5 weeks in SD, then induced to bolt or kept in control conditions. Samples were taken 0, 1 and 4 days after transfer. Scale bar denotes 50 µm.
C: Representative confocal images showing spatiotemporal expression of 5-week old SD-grown ATH1:ATH1-GFP plants induced to bolt or retained in control conditions. Samples were taken of four apices at 0, 1, 4 and 7 days after transfer to FR. Images represent a maximum projection of 5 z-slices. Scale bar denotes 50 µm.
D: Sizes of CZ and RZ for apices imaged in A. Average areas were determined for medial sections from 4 separate apices per genotype, time point and condition (asterisks denote $p<0.05$ (*), $p<0.0001$ (***) or $p>0.05$ (NS)).
E: Schematic overview of regions of shoot apex analysed for CZ (0-45 µm from top of apex) and RZ (45-90 µm from top of apex) cell size. Cells within 15 µm of the axis were analysed. Scale bar represents 15 µm.
F: Rosettes of 60-day old Col-8 and *ath1-3* grown in non-inductive SD conditions.

rosette internodes in the *ath1-3* mutant is akin to bolting during vegetative growth, therefore we propose that this phenotype is termed “heterochronic bolting”.

ATH1 is expressed in the shoot apices of vegetative plants and downregulated in the IM (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007). To determine the spatiotemporal localization of the ATH1 protein during bolting induction, we imaged *ath1* shoot apices complemented with an a ATH1:ATH1-GFP reporter construct (Fig. 2.1C). In non-inductive conditions, ATH1-GFP was detected throughout the SAM, predominantly at the RZ and lateral boundaries of the meristem. ATH1-GFP was still present one day after induction, but was no longer expressed in the meristem after 4 days of induction. This loss of expression coincides with the initiation of elongation of the RZ in Col-8 plants, which is the start of bolting (Fig. 2.1A). At this time, ATH1-GFP reappeared in incipient floral primordia from stage 1 onwards, which is in line with *ATH1* mRNA expression (Khan et al., 2015). As loss of ATH1 expression from the meristem coincided with activation of the RZ (Fig. 2.1A, C) and the start of bolting, and *ath1-3* already exhibits RZ activation and internode elongation during rosette growth (Fig. 2.1B), it can be concluded that ATH1 prevents RZ elongation during the rosette phase and absence of ATH1 allows elongation of the RZ needed for bolting.

Rosette habit cannot be disrupted by GA in the presence of *ATH1*

While vegetative *ath1-3* mutants exhibit elongation of rosette internodes, rosette habit is only partially lost (Fig. 2.1F). This indicates that loss of *ATH1* alone is not sufficient to induce strong elongation of rosette internodes. The phytohormone GA plays an important role in internode elongation in many plant species and is a major regulator of bolting in Arabidopsis and other rosette plants. We therefore treated the shoot apices of both LD- and SD-grown *ath1-3* plants with GA4+7. This GA application greatly enhanced elongation of rosette internodes in *ath1-3* in LD conditions (Fig. 2.2C-D, 2.S1B), and lead to complete loss of rosette habit in SD-grown plants (Fig. 2.2E). In LD, we saw a linear relationship between concentration of applied GA and elongation of rosette internodes. A similar treatment of Col-8 plants had no such effect, and also did not induce heterochronic bolting in SDs (Fig. 2.2C-D). This suggests that ATH1 represses internode elongation by reducing sensitivity to GA or inhibits the downstream GA response. Earlier work shows that GA application cannot rescue stem elongation in non-bolting 35S::ATH1 overexpressors (Gómez-Mena and

Sablowski, 2008) (Fig. 2.S2), which suggests that GA has no effect on elongation in the presence of ATH1. This is reflected in our findings in wild-type rosettes.

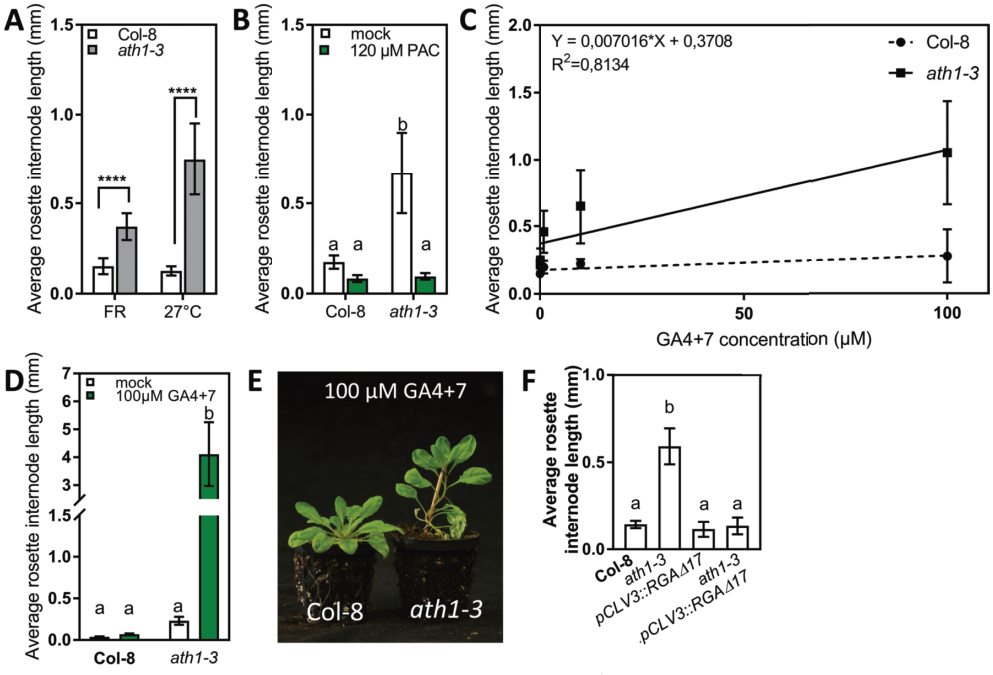

Fig. 2.2: ATH1 represses heterochronic bolting induced by GA4+7
A: Average rosette internode elongation of WT and *ath1-3* grown in FR or 27°C conditions. Asterisks indicate p<1·10-4 in unpaired 2-tailed Student's t-tests, n=14.
B: Average length of rosette internodes of mock or 120 µM paclobutrazol (PAC)-treated Col-8 and *ath1-3* grown in FR conditions (n=14, 14, 13, 13).
C: Average length of Col-8 and *ath1-3* rosette internodes grown in LD and treated with 0-100 µM GA4+7 (n= 10). Solid line represents linear relationship between GA concentration and elongation calculated for *ath1-3*; equation and R^2 are displayed. Dotted line represents predicted linear relationship between GA and elongation for Col-8 (not statistically significant, see Supplemental Table 2.S1).
D: Average length of Col-8 and *ath1-3* rosette internodes grown in SD and treated with a mock solution or 100 µM GA4+7 (n=9, 6, 10, 10).
E: Representative 51-day old GA4+7 plants from D.
F: Average length of Col-8, *ath1-3*, *pCLV3::RGAΔ17* (homozygous and heterozygous plants), *ath1-3 pCLV3::RGAΔ17* (homozygous) plants grown in FR conditions (n=10, 9, 13, 16).
Error bars represent standard deviation from the mean, letters above graphs denote statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test.
For reporting of statistical tests, see Supplemental Table 2.S1.

We next tested if endogenous GA is required for heterochronic bolting in *ath1*-3. We grew Col-8 and *ath1-3* in LD conditions under a low red : far red (R:FR) ratio or

at 27°C. Both of these conditions are known to promote bolting and flowering in Arabidopsis (Galvão et al., 2015; Wollenberg et al., 2008), and lead to increased GA production (Hisamatsu et al., 2005). In both conditions, *ath1-3* rosette internodes were significantly more elongated, while those of Col-8 were compact under all conditions (Fig. 2.2A, Fig. 2.2C, 2.S1B). Treatment of plants with the GA biosynthesis inhibitor paclobutrazol (PAC), completely suppressed heterochronic bolting of *ath1-3* mutants both when grown in FR-enriched conditions or at 27°C (Fig. 2.2B, 2.S1A). In addition, when crossed with *pCLV3::rgaΔ17* plants, which express a GA-insensitive form of the DELLA repressor protein RGA in the shoot apex, heterochronic bolting was no longer observed in FR-grown *ath1-3* mutants (Fig. 2.2F).

RGA has been previously identified as a binding target of ATH1 (R. Sablowski, personal communication) and *ATH1* as a binding target of RGA (Serrano-Mislata et al., 2017). This puts RGA as a potential point of convergence between the ATH1 and GA pathways. To determine whether *RGA* is a transcriptional target of ATH1, we measured expression of DELLA genes in *ath1-3* mutants. qRT-PCR analysis revealed that *DELLA* gene expression is not altered in *ath1-3*. *ATH1* expression is also not misregulated in mutants lacking all five DELLA proteins (*della*) (Fig. 2.S3). This suggests that ATH1 and DELLA act largely independently. Nevertheless, heterochronic bolting is not observed in *ath1-3* mutants in absence of GA, and GA alone cannot rescue 35S::ATH1 or induce heterochronic bolting in WT plants. Therefore, taken together these findings show that the combination of loss of ATH1 and activation of GA signalling is required for releasing repression of internode elongation during rosette growth.

ATH1 represses internode elongation through positive regulation of *LOB*

To gain more understanding into how ATH1 represses internode elongation during rosette growth, we next focused on ATH1 targets. Organ boundary genes have previously been implicated as negative regulators of growth, and loss of *ATH1* results in organ boundary defects in flowers and between stem and cauline leaves. Like *ATH1*, ectopic or constitutive overexpression of some organ boundary genes represses bolting, but not flowering (Bao, 2009; Gómez-Mena and Sablowski, 2008; Lin et al., 2003; Norberg, 2005; Shuai et al., 2002; Takeda et al., 2011). Furthermore, the organ boundary genes *LOB*, *BOP1*, *BOP2* and *LSH4* were recently identified as putative direct targets of ATH1, based on ChIP-seq data (R. Sablowski, personal

communication). To determine whether ATH1 controls expression of these genes, plants expressing a fusion protein of ATH1 with the rat glucocorticoid receptor hormone-binding domain (HBD) under the control of the CaMV 35S promoter (*35S::ATH1-HBD*) were grown in liquid culture. Nuclear translocation of ATH1 was induced by addition of dexamethasone (DEX). As positive control, expression of the KNOTTED1-LIKE HOMEOBOX transcription factor genes *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*) and *KNAT6* was measured. Both genes were previously observed to be under direct and positive control of ATH1 (M. Proveniers, unpublished data). As seen in Fig. 2.S4 nuclear expression of ATH1 resulted in increased expression of *KNAT2, KNAT6, BOP1, BOP2,* and *LSH4*. Except for *BOP2*, similar results were observed in the presence of both DEX and the translation inhibitor cycloheximide (CHX), suggesting that *BOP1,* and *LSH4*, like KNAT2 and KNAT6, are under direct control of *ATH1*. This is supported by significant reduction of expression of *BOP1* and *LSH4* in an *ath1* mutant background (Fig. 3.3). As *LOB* expression seems to be controlled by DEX treatment itself and *BOP2* expression by CHX, definite conclusions about ATH1 as upstream regulator of these genes cannot be drawn (Supplemental Fig. 2.S4). However, as *LOB* expression, unlike *BOP2*, was almost completely diminished in *ath1-3* plants (Fig. 2.3A), it can be concluded that *LOB* is an ATH1 target. Our previous data suggest that, to regulate rosette habit, ATH1 and GA act in two converging, but independent pathways. To determine if both pathways converge on co-regulation of any of these organ boundary genes, expression of *LOB*, *BOP1, BOP2* or *LSH4* was measured in mock or GA4+7-treated Col-8 and *ath1-3* seedlings, and in the global *della* mutant. As GA had no effect on boundary gene expression in both Col-8 and *ath1-3* (Fig. 2.3A) and boundary gene expression was also unchanged in *della* mutants (Fig. 2.S4), this most likely is not the case.

Next, the role of these organ boundary genes in ATH1-mediated internode elongation was analysed. Given the strong effect of an *ath1* mutation on *LOB* expression, we focussed on LOB and tested *lob* loss-of-function mutants for heterochronic bolting. While no rosette internode elongation was observed in mock-treated *lob-3* mutants, heterochronic bolting could be induced in *lob-3 by* GA4+7 treatment (Fig. 2.3B-C), although these plants were less elongated than GA4+7-treated *ath1-3*, Moreover, introduction of the *lob-3* mutation enhanced the heterochronic bolting phenotype of *ath1-3* mutants (Fig. 2.3B-C). Taken together, these data suggest that ATH1

represses internode elongation partly through the induction of *LOB*. As elongation of rosette internodes of *ath1-3 lob-3* double mutants displayed similar sensitivity to GA4+7-treatment as in *ath1-3,* in line with aforementioned data, this most likely occurs independently of GA signalling.

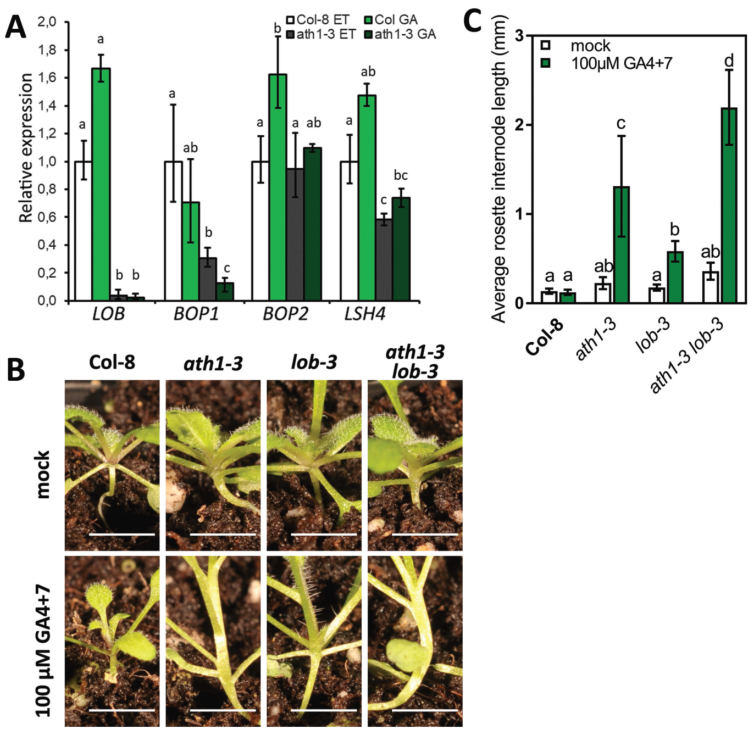

Fig. 2.3: ATH1 represses internode elongation through LOB
A: Relative expression of *LOB*, *BOP1*, *BOP2* and *LSH4* in 7-day old SD-grown seedlings determined by qRT-PCR. Error bars represent standard deviation from the mean. Expression was determined in mock (0.1% ethanol) or 100 µM GA4+7-treated Col-8 and *ath1-3* seedlings.
B: Rosettes of representative 18-day old, LD-grown plants used in C. Scale bar denotes 5 mm
C: Average rosette internode elongation of LD-grown Col-8, *ath1-3*, *lob-3* and *ath1-3 lob-3* which were treated with a mock solution or 100µM GA4+7 (n=10).
Error bars denote standard deviation of the mean. Letters above graphs represent homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test (see Supplemental Table 2.S1).

Discussion

ATH1, a key regulator of the rosette phase?

The rosette habit characterises the vegetative growth phase of rosette plants like Arabidopsis. However, very little is known about how the rosette habit is maintained. Here, we show that the transcription factor ATH1 is a key factor for promoting the

rosette phase and repressing bolting. *ATH1* is expressed in the SAM shortly after germination, coinciding with leaf initiation (Proveniers et al., 2007) until the end of the vegetative phase (Fig. 2.1C). This period encompasses the entire rosette phase of Arabidopsis. In plants switched from non-inductive to inductive conditions, downregulation of ATH1 coincides with expansion of the RZ and the onset of bolting. This expansion of the RZ already occurred in the vegetative RZ of *ath1-3* mutants (Fig. 2.1), which form elongated rosette internodes (heterochronic bolting). In extreme cases, loss of *ath1-3* results in a complete loss of rosette habit (Fig. 2.2). On the other hand, ectopic expression of ATH1 sustains rosette growth into the reproductive growth phase, thus repressing bolting (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009). Both GA-deficient mutants and mutants that ectopically express boundary genes, also exhibit impaired growth of other tissues, such as leaves, flowers and siliques (Kauschmann et al., 1996; Norberg, 2005; Shuai et al., 2002; Wilson et al., 1992). In *ATH1* overexpressors, elongation of these tissues is not affected, even though ATH1 controls boundary gene expression and represses GA sensitivity. This highlights the rosette-specific, local action of *ATH1*, positioning ATH1 as a specific regulator of rosette habit rather than a general regulator of growth.

ATH1 likely targets several genes to repress internode elongation during rosette growth. One of the routes through which *ATH1* represses internode elongation is likely through induction of multiple boundary genes. Ectopic expression of boundary genes represses bolting (Norberg, 2005; Shuai et al., 2002; Vroemen et al., 2003). *ATH1* targets the boundary genes *LOB, LSH4, BOP1* and *BOP2* (R. Sablowski, personal communication), and loss of *ATH1* reduces expression of *LSH4*, *BOP1* and, most strongly, *LOB* (Fig. 2.3A, Fig. 2.S4A). Rosette internodes of *lob-3* mutants elongate in response to GA, but less than those of *ath1-3* (Fig. 2.3C). Therefore, *ATH1* likely does not act through *LOB* alone, but possibly also through *BOP1* and *LSH4*. Ectopic expression of *BOP1* induces the hormone jasmonic acid (JA), which represses stem elongation (Khan et al., 2015). However, the effects of *BOP1* or *LOB* on RZ activity are unknown. It was recently shown that the boundary gene *LSH4*, which is normally expressed in the RZ and boundary of vegetative plants, is repressed in the RZ during the reproductive phase. This allows correct orientation of RZ cell divisions, which are needed for proper stem elongation (Bencivenga et al., 2016; Takeda et al., 2011). Possibly, *LSH4* expression in the RZ relies on ATH1, linking *LSH4* downregulation to

ATH1 downregulation. To understand how boundary gene function affects rosette habit, it would be informative to study changes in RZ cell divisions during vegetative growth of *lsh4, lob* and *bop1* mutants, and compare LSH4, LOB and BOP1 localization between wild-type and *ath1* shoot apices during vegetative growth and bolting.

ATH1 expression is light regulated (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007; Quaedvlieg et al., 1995). Interestingly, heterochronic bolting and disruption of the rosette habit has previously been observed in plants lacking multiple photoreceptor mutants, i.e. the red light receptors phytochrome A (phyA), phyB and phyE and blue light receptor CRYPTOCHROME 1 (CRY1) (Devlin, 1998; Halliday and Whitelam, 2003; Mazzella et al., 2000). Similar to *ath1-3,* this phenotype is enhanced by FR light and high ambient temperature, which raises the question whether phyA, phyB, phyE and CRY1 (indirectly) promote *ATH1,* and whether the heterochronic bolting phenotypes of these mutants are caused by loss of *ATH1* expression. Other findings linking *ATH1* to photoreceptors is that *bop1* and *bop2* mutations enhance heterochronic bolting of *phyB* (Zhang et al., 2017a). *BOP1* and *BOP2* have been identified as both targets (R. Sablowski, personal communication) and upstream regulators (Khan et al., 2015) of *ATH1*. Therefore, both ATH1 and one of its boundary gene targets might be absent in *bop1/2 phyB* mutants, preventing repression of internode elongation during vegetative growth. To understand the relationship between photoreceptors and *ATH1* in the control of rosette habit, it is necessary to determine whether induction of *ATH1* during vegetative growth requires photoreceptor activity. Analysis of heterochronic bolting of *ath1* in photoreceptor mutant backgrounds will also help understand whether photoreceptors and *ATH1* operate in parallel pathways, or whether photoreceptors require *ATH1* in controlling the rosette habit.

Combined but independent function of ATH1 and DELLA staves off bolting

During vegetative growth of Arabidopsis, two independent blocks on internode elongation are in place: ATH1 and DELLA. During vegetative growth, *ATH1* levels are high (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007) and GA levels are low (Eriksson et al., 2006), meaning that DELLAs repress GA signalling. We show that ATH1 overrides GA action, as in the presence of *ATH1*, rosette habit is not disrupted by GA application or conditions that increase endogenous GA, i.e. FR light or high ambient temperature (Fig. 2.2). However, loss of *ATH1* alone is not enough

to enable internode elongation during rosette growth—this also requires GA (Fig. 2.2B, F). Once both of these blocks are removed, complete loss of the rosette habit, *i.e.* removing morphological distinction between rosette internodes and stem internodes, is possible (Fig. 2.2DE).

This regulation is mirrored during reproductive growth. Plants that are switched from non-inductive to inductive conditions exhibit both a rapid decline of ATH1 in the SAM (Fig. 2.1C) and a rapid increase of GA biosynthesis (Xu et al., 1997), leading to degradation of DELLA in the shoot apex.

Yet, loss of one repressive signal is again not sufficient to overrule the other. Bolting cannot be rescued in *ATH1* overexpressors through exogenous GA application (Gómez-Mena and Sablowski, 2008). Downregulation of ATH1 alone is insufficient to induce internode elongation as GA biosynthesis and signalling mutants also do not bolt (Blázquez et al., 1998; Serrano-Mislata et al., 2017).

Thus, independent repressive action of ATH1 and DELLA signalling can maintain rosette growth, and the combined loss of both of these repressors is necessary for ceasing rosette growth and initiating bolting. It is possible that the function of these two independent blocks on internode elongation is to control the timing of bolting, ensuring that rosette growth is maintained until both environmental and internal conditions for bolting are favourable.

Where do ATH1 and gibberellin signalling converge?

ATH1 and DELLA both repress internode elongation, but act in independent pathways. Therefore, the question remains whether or where these two pathways converge. GA does not regulate *ATH1* or its targets *LOB, BOP1* and *LSH4* (Fig. 2.3, 2.S3, 2.S4). Loss of *LOB* makes plants susceptible to GA-induced induction of heterochronic bolting (Fig. 2.3). Therefore, if ATH1 and DELLA signalling converges to repress internode elongation, this occurs further downstream of *LOB*. *LOB* promotes catabolism of brassinosteroid (BR) hormone in organ boundaries through *PHYB ACTIVATION TAGGED SUPPRESSOR 1* (*BAS1*), and low BR levels are required for proper boundary formation (Bell et al., 2012; Gendron et al., 2012). It is possible that low BR might also be necessary for rosette growth. BR stimulates cell division and cell elongation, and the BR signalling mutant *brassinosteroid insensitive1* (*bri1*) exhibits non-bolting phenotypes paired with an overall decrease in growth (Kauschmann et al., 1996).

Furthermore, *BAS1* is expressed in the RZ during vegetative but not generative growth (Sandhu et al., 2012). BRs are also repressed by DELLAs: RGA physically interacts with the BR-induced transcription factors *BRASSINAZOLE RESISTANT1* (*BZR1*) and *BRI1-EMS SUPRESSOR1* (*BES1*), preventing BZR1 and BES1 binding to DNA (Bai et al., 2012a; Li et al., 2012b). Thus, localized repression of BR levels and signalling, caused by converging function of ATH1 and DELLA, might enable rosette formation.

GA also regulates growth of other tissues, such as the hypocotyl, where DELLA is part of a larger regulatory network that is tightly interlinked with multiple hormones (Bai et al., 2012b; Oh et al., 2014; Ragni et al., 2011). Therefore, ATH1 and DELLA signalling might converge through other hormones, such as auxin or jasmonic acid (JA). Auxin promotes internode elongation and reporter expression suggests that auxin is low in the RZ during vegetative growth and high during reproductive growth (Brunoud et al., 2012; Shi et al., 2018; Timpte et al., 1992). Moreover, low BR and low auxin characterise meristem-organ boundaries (Žádníková and Simon, 2014), and as ATH1 targets boundary genes, it is possible that ATH1 function (indirectly) affects auxin. The boundary gene and ATH1 target *BOP1* promotes JA (Khan et al., 2015), which represses stem elongation (Koda et al., 2001; Takada et al., 2013; Yoshida et al., 2010). GA affects auxin transport, and antagonizes JA signalling (Hou et al., 2010; Löfke et al., 2013; Willige et al., 2011). Therefore, investigating hormonal crosstalk in the vegetative RZ may help elucidate the convergent action of ATH1 and DELLA on internode elongation.

Understanding bolting is important for the cultivation of rosette crops

Since the Green Revolution of the 1970s, huge advances have been made in yield increase for staple crops, such as wheat, rice and maize. However, these successes have not yet been translated for the cultivation of rosette crops, such as lettuce, sugar beet, onions and cabbages where premature bolting is a major cause of yield loss.

In leafy crops bolting leads to formation of hardened tissues in the leaves and stem, loss of head compactness, and can lead to build up of bitter-tasting compounds in crops like lettuce (Guttormsen and Moe, 1985; Sessa et al., 2000). In sugar beet, sugar yields have been reported to be up to 29% lower in bolted plants than non-bolted plants of the same age (Longden et al., 1975) as bolting in tuberous crops

causes reallocation of resources from roots and tubers into stem tissue. Although rosette crops are not staple foods, they are most important: In 2017, five of the ten highest globally produced vegetable types were rosette crops. Sugar beet, though not a vegetable, was the 8th highest produced crop in the world in 2017 (Food and Agriculture Organization of the United Nations, 2017). Therefore, small improvements in rosette growth and bolting resistance could have great impacts on yield.

Currently, very little is known about the molecular regulation of bolting in crops. As a result, current practice to reduce premature bolting involves restricting the growth season and using vernalization requiring varieties, practices which fail to utilize the full potential of the growing season of these crops. In Arabidopsis, ATH1 represses bolting independently of GA and without affecting flowering, which makes it a promising factor to be studied in the context of breeding more bolting-resilient crops. Further investigation into the interplay of ATH1, boundary genes and hormonal regulation of internode elongation therefore will open up novel insights into both fundamental and agricultural questions.

Materials & Methods

Plant material and growth conditions

The *Arabidopsis thaliana* Col-8 and Ler accessions were used as wild types in this study. The *ath1-3* (Proveniers et al., 2007), *lob-3* (Bell et al., 2012), *35S::HA*-ATH1 (Proveniers et al., 2007), 35S*::ATH1-HBD* (*35Spro:ATH1-HBD*) (Rutjens et al., 2009)*, ET22* (Shuai et al., 2002), *pCLV3::RGAΔ17* (Galvão et al., 2012) and *della* (Feng et al., 2008) mutants have been described previously.

All lines were in the Col-8 background, except *35S::HA-ATH1* , *lob* ET22*,* and *della* (Ler background). The *ath1-3 pCLV3::RGAΔ17* double mutant was obtained by crossing *ath1-3* and *pCLV3::RGAΔ17,* followed by genotyping (for primer sequences, see Supplemental Table 2.S2). *ATH1:ATH1-GFP* and *ath1-3 lob-3* were kind gifts from Robert Sablowski.

Seeds were stratified for 2 days in darkness at 4°C and planted on soil or on plates containing sterile Murashige & Skoog medium (pH=6.0, 0.8% plant agar; Duchefa). Plants were grown in short days (SD; 8 hours light/16 hours dark) or long days (LD; 16 hours light/8 hours dark) under white fluorescent lights (Sylvania, Luxline Plus

Cool White) or in far-red enriched conditions (FR; long days, under red, blue and far red LED light; R:FR ratio 1.34). All plants were grown at 120 µmol/m^2/s light at 70% relative humidity, and at 22°C or 27°C.

For bolting induction, plants were grown for 5 weeks in noninductive, SD conditions. Half of the population was transferred to LD FR conditions to induce bolting (induced), while the other half was kept in SD (control). Apices were sampled 0, 1 and 4 days after transfer and processed further for imaging.

Phenotypic analyses

Average elongation of rosette internodes was determined by measuring the height of the total rosette using a digital calliper, and dividing the height by the number of rosette leaves. The first leaf to form a secondary shoot was defined as the first cauline leaf. Continuous GA treatments of the shoot apex were performed by growing plants for 1 week on MS plates containing 100 µM GA4+7 (GA; Duchefa) or 0.1% ethanol (mock), followed by transfer to soil where treatment was continued 3 times a week by applying 1 µl of GA or mock solution supplemented with 0.01% Silwet-L77 (Momentive) onto the shoot apex. Treatment was performed until opening of the first flower.

For paclobutrazol treatments, seeds were germinated on top of nylon membrane (Sefar Nitex 03-100/44) on MS agar plates, and then transferred to MS agar plates containing 120 µl paclobutrazol (PAC; Sigma Aldrich) or 0.1% DMSO (mock). Plants were transplanted to soil on day 7; treatment was continued by spraying plants three times a week with mock or PAC solution supplemented with 0.01% Silwet L-77. Differences in rosette internode elongation were tested in IBM SPSS 24 using 1-way ANOVA followed by Tukey's post-hoc test (α=0.05).

Confocal microscopy

Dissection and staining of apices with mPS-PI was performed as described previously (Bencivenga et al., 2016; Truernit et al., 2006). Meristems were imaged at a resolution of 0.25 x 0.25 x 0.5 µm using a Leica SP5 confocal microscope with a 20x/0.75 long working distance objective. Excitation was at 561 nm and emission filters were set to 571-700 nm. Apices for GFP imaging were cleared using the ClearSee method (Kurihara et al., 2015) and imaged using a Leica SP5 confocal microscope (20x/0.75

long distance working distance objective, resolution 0.25 x 0.25 x 0.5 µm, excitation at 488 nm, detection at 502-521 nm).

Analysis of cell heights

Images were processed in ImageJ using custom scripts described previously (Bencivenga et al., 2016). To examine changes in cell elongation medial sections of the images were segmented in 2D, and the heights of cells within 15 µm of the main axis, and 0-45 µm or 45-90 µm from the apex summit (defined as CZ and RZ, respectively; Fig. 2.1E) were measured in R (www.r-project.com). Differences in cell heights were compared using Mann-Whitney U statistical tests (α=0.05).

Analysis of gene expression

For analysis of gene expression in plate-grown seedlings, Col-8, *ath1-3,* Ler and *della* were grown in SD conditions on MS plates containing 0.1% ethanol or 100 µM GA4+7 (Col-8 and *ath1*-3 only). Seedlings were sampled on day 7. For analysis of induction of ATH1 targets, Col-8 and the dexamethasone (DEX)-inducible overexpressor *35S::ATH1-HBD* were grown in bottles containing 100 ml half-strength MS medium (pH=5.7) in LD, shaking at 225 RPM. On day 7, plants were incubated for 4 hours with a control solution (0.1% ethanol or 0.1% DMSO), 10 µM DEX or a combination of 10 µM DEX and 35 µM cycloheximide (DEX+CHX) and harvested. All samples were snap-frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. All experiments were repeated three times to obtain three biological replicates. RNA was extracted from seedlings as described previously (Oñate-Sánchez and Vicente-Carbajosa, 2008). RNA was treated with DNAseI (ThermoScientific), followed by cDNA synthesis from 1 µg RNA using RevertAid reverse transcriptase and Ribolock RNAse inhibitor (ThermoScientific) according to manufacturer's instructions. qRT-PCR was performed for *ATH1, GAI, RGL2, RGL3, KNAT2, KNAT6, LOB, BOP1*, *BOP2* and *LSH4* (For primer sequences see Supplemental Table 2.S2) using the Viaa7 real-time PCR system (Applied Biosystems). The *At5g15400* gene was used as endogenous control. Relative expression was calculated using the ΔCT method. Differences between mutant and wild-type ΔCT values were statistically analysed using Student's t-test in IBM SPSS Statistics 24 (α=0.05).

Supplemental figures and tables

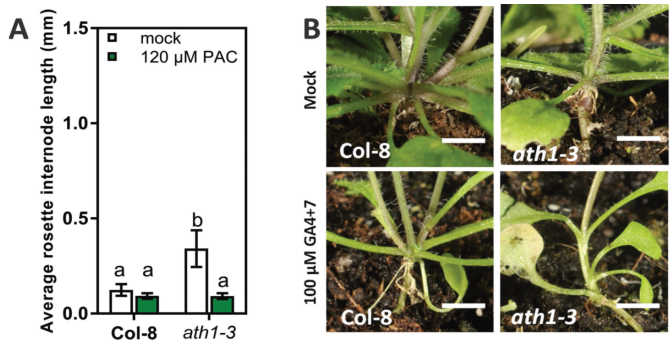

Fig. 2.S1: The effects of paclobutrazol and GA4+7 on heterochronic bolting of *ath1-3*

A: Average length of rosette internodes of Col-8 and *ath1-3* grown at 27°C in LD and treated with 0.1% DMSO (mock) or 120 µM paclobutrazol (PAC; n=20, 19, 21, 21).Error bars denote standard deviation of the mean. Letters above graphs represent homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test (see Supplemental Table 2.S1).

B: Representative phenotypes of 41-day old Col-8 and *ath1-3* plants grown in LD at 22°C and treated with a mock solution or 100 µM GA4+7.

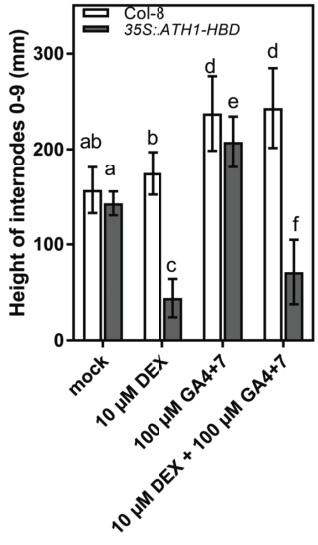

Fig. 2.S2: Plant height of *35S::ATH1-HBD* overexpressors is not restored by GA4+7 application

Height of stem internodes 1-10 in LD-grown Col-8 and the DEX-inducible *35S::ATH1-HBD line*, treated with a mock (0.1% ethanol) solution, 10 µM DEX, 100 µM GA4+7, or a combination of DEX and GA (n=31, 30, 35, 35, 30, 31, 30, 37). Letters denote statistically homogeneous groups defined with 1-way ANOVA followed by Tukey's post-hoc test (see Supplemental Table 2.S1).

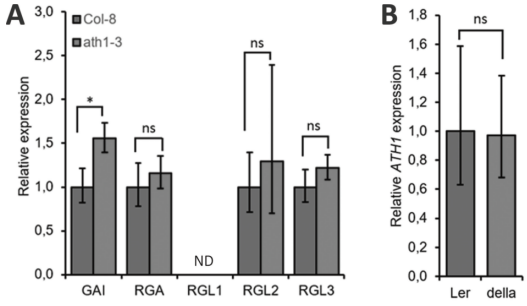

Fig. 2.S3: Relative expression of *ATH1* or *DELLA* genes in 7-day old SD-grown seedlings determined by qRT-PCR

A: Expression of *GAI, RGA, RGL1, RGL2* and *RGL3* in Col-8 and *ath1-3* seedlings. Expression of *RGL1* could not be detected in seedlings (ND).

B: Expression of ATH1 in 7-day old SD-grown Ler and global *della* mutant seedlings.

Error bars represent standard deviation from the mean. Differences in expression were compared using 2-tailed independent t-tests, asterisk denotes p<0.5, ns denotes p>0.5 (see Supplemental Table 2.S1).

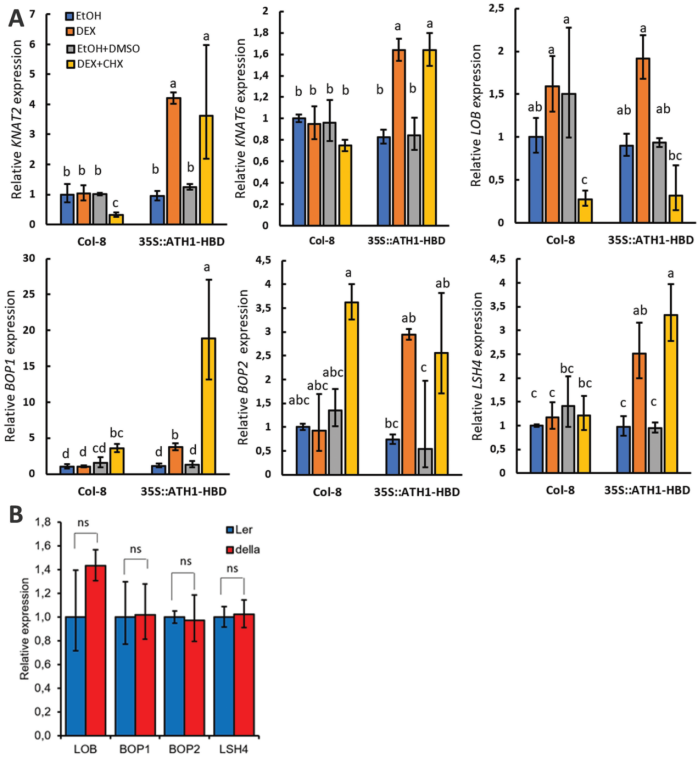

Fig. 2.S4: Expression of putative ATH1 targets in *35S::ATH1-HBD* or *della* seedlings

A: Expression of *KNAT2*, *KNAT6*, *LOB*, *BOP1* and *BOP2* in Col-8 and the DEX-inducible *35S::ATH1-HBD* determined by qRT-PCR. Seedlings were grown in liquid culture for 7 days and treated with 0.1% ethanol (EtOH), 10 µM DEX (DEX), a combination of 0.1% ethanol and 0.1% DMSO (EtOH+DMSO) or a combination of DEX and 35 µM cycloheximide (DEX+CHX). Differences in expression were compared using 1-way ANOVA, letters denote homogeneous groups determined with Tukey's post-hoc test (α=0.05) (Continued on next page.)

(Continued from previous page)
B: Expression of *LOB*, *BOP1*, *BOP2* and *LSH4* in 7-day old SD-grown Ler and *della* seedlings determined by qRT-PCR.
Error bars represent standard deviation from the mean. Differences in expression were compared using 2-tailed independent t-tests (α=0.05).

Table 2.S1: Outcome of statistical tests

Figure	Test	Outcome
2.2A	Independent 2-tailed Student's t-test; FR	t(26)=9.477; p=6.4E-10
	Independent 2-tailed Student's t-test; 27°C	t(12)=11.12; p=8.2E-8
2.2B	1-way ANOVA	F(3; 42)=68.723; p=3.0E-16
2.2C	Linear regression F-test for *ath1-3*	Y=0.007016*X + 0.3708; R^2=0.8134; F(1; 3) = 13.08, p=0.036;
	Linear regression F-test for Col-8	Y=0.001089*X + 0.1771; R^2=0.74; F(1; 3)=8.94; p=0.058
2.2D	1-way ANOVA	F(3; 31)=38.4; p=1.5E-10
2.2F	1-way ANOVA	F(3; 44)=153.5; p=2.5E-23
2.3A	1-way ANOVA (*LOB* dCT)	F (3; 8)=45.8; p=4.2E-9
	1-way ANOVA (*BOP1* dCT*)*	F(3; 6)=5.11; p=3.6E-4
	1-way ANOVA (*BOP2* dCT)	F(3; 7) = 7.059; p=0.016
	1-way ANOVA (*LSH4* dCT)	F (3; 8) = 14.0; p=0.002
2.S1A	1-way ANOVA	F (3; 77) = 108.03; p=1.6E-27
2.S2	1-way ANOVA	F(7; 251)=211.7; p=1.9E-101
2.S3A	Independent 2-tailed Student's t-test (GAI dCT)	t(4) = 3.475; p=0.038
	Independent 2-tailed Student's t-test (RGA dCT)	t(4) = 0.863; p=0.437
	Independent 2-tailed Student's t-test (RGL2 dCT)	t(4) = 0.637; p=0.559
	Independent 2-tailed Student's t-test (RGL3 dCT)	t(4) =1.59; p=0.187
2.S3B	Independent 2-tailed Student's t-test (ATH1 dCT)	t(4)=-0.089; p=0.934
2.S4A	1-way ANOVA (*KNAT2* dCT)	F(11; 21)=16.64; p=5.7E-8
	1-way ANOVA (*KNAT6* dCT)	F(7; 12)=13.29; p=8.5E-5
	1-way ANOVA (*LSH4* dCT)	F(7; 13)=11.34; p=1.2E-4
	1-way ANOVA (*BOP1* dCT*)*	F(11; 21)=20.07; p=1.0E-8
	1-way ANOVA (*BOP2* dCT)	F(7; 13)=5.28; p=0.005
	1-way ANOVA (*LSH4* dCT)	F(7; 13)=10.76; p=1.6E-4
2.S4B	Independent 2-tailed Student's t-test (LOB dCT)	t(4) = 1.443; p=0.282
	Independent 2-tailed Student's t-test (BOP1 dCT)	t(4) = 2.186; p=0.282
	Independent 2-tailed Student's t-test (BOP2 dCT)	t(4) = -0.234; p=0,826
	Independent 2-tailed Student's t-test (LSH4 dCT)	t(3) = 0,096; p=0.930

Table 2.S2: List of primer sequences used in this study

Primer name	Sequence	Application	Reference
LOB qPCR-F	TGCGTCGGAGCCATCTCTTATC	qRT-PCR	
LOB qPCR-R	AGTCAGCATTAGCTGCGTCGAG	qRT-PCR	
BOP1 qPCR-F	AGCTTGGAGCAGCTGATGTGAAC	qRT-PCR	
BOP1 qPCR-R	ACCATTTCAGCCGCAATGTGAAG	qRT-PCR	
BOP2 qPCR-F	GGAAGGTATGAGTCGGCATC	qRT-PCR	Andrés et al., 2015
BOP2 qPCR-R	TGCATGCCCCTCTTCTTAAT	qRT-PCR	
LSH4 qPCR-F	ACCAATTCGGCAAGACTAAGGTTC	qRT-PCR	
LSH4 qPCR-R	AGCAGCTCTAAGACGGCCAATG	qRT-PCR	
At5g15400 qPCR-F	GGGCACTCAAGTATCTTGTTAGC	qRT-PCR	
At5g15400 qPCR-R	TGCTGCCCAACATCAGGTT	qRT-PCR	
GAI-qF	ACTCGTTGGAAGGTGTACCG	qRT-PCR	
GAI-qR	AACTCGGTCAGGTCCATCAC	qRT-PCR	
RGA-qF	CTCGCGACGGATACTGTTCA	qRT-PCR	
RGA-qR	TAGAACTCGCCGGAAGAGGA	qRT-PCR	
RGL1-qF	GGTTCACGGAATCGCTACAT	qRT-PCR	
RGL1-qR	ATGCCTCTCTACCCGGTCTT	qRT-PCR	
RGL2-qF	AATTCCGGGTCTTTCTGCGT	qRT-PCR	
RGL2-qR	TGAGAGTCAACGAGCACCAC	qRT-PCR	
RGL3-qF	TGGTCTAACGACGGAGAGGT	qRT-PCR	
RGL3-qR	CCACCACTGTTACGAGACCC	qRT-PCR	
RGL3-2-qF	AGCTGTTAGCGACGGTTAAGGC	qRT-PCR	
RGL3-2-qR	ACGTCACCGTTATGGTTCGCTTC	qRT-PCR	
113353-F	TTTGTAGTTCAAGAGAAAAGCTTGA	Genotyping *ath1-3* (113353-F/R, 113353-F/LBb1)	Proveniers et al., 2007
113353-R	GGCGGGTTTTCGGATCTACATT		
LBb1	GCGTGGACCGCTTGCTGCAAC		
RGAd17-FWD	ATCATCACCAATTCCAAGGTC	Detecting RGAΔ17 deletion in pCLV3::RGAΔ17	
RGAd17-REV	GGAAGAGGAGGAGGATTAAGC		

CHAPTER 3

ARABIDOPSIS THALIANA HOMEOBOX 1 maintains rosette habit by mediating sensitivity to auxin, brassinosteroids and gibberellins

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Abstract

To elongate or not to elongate along the shoot axis is a fundamental question for rosette plants. Seedling establishment requires elongation of the hypocotyl, rosette growth requires inhibition of internode elongation, and reproductive growth requires rapid elongation of stem internodes (bolting). There is an overlap in hormonal (e.g. gibberellin, auxin, brassinosteroids) and environmental inputs (e.g. light, temperature) that control hypocotyl elongation and bolting. However, it is not known why these signals do not induce internode elongation during rosette growth. Here, we show that the TALE homeobox transcription factor *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) promotes the rosette habit by mediating sensitivity to gibberellin, brassinosteroids and auxin, partially through its direct target *LATERAL ORGAN BOUNDARIES* (*LOB*). Exogenous application of BR, auxin or GA induced heterochronic bolting in *ath1-3* and *lob-3* mutants. Combined application of auxin and BR was sufficient to induce heterochronic bolting in plants carrying a wild-type *ATH1* allele, but not in plants expressing *ATH1* from the Cauliflower Mosaic Virus 35S (35S) promoter. Heterochronic bolting of *ath1-3* was enhanced by *bzr1-1D,* and repressed by chemical inhibition of GA or BR biosynthesis. However, loss of *DELLA* in auxin-BR treated plants did not enhance heterochronic bolting, suggesting that *ATH1* controls elongation at multiple regulatory points. Repression of heterochronic bolting could also be achieved through loss of *PIF*7 in FR conditions, or could be partially repressed by loss of *PIF4* at 27°C. Taken together, these results suggest that *ATH1* instates a robust lock on internode elongation during rosette growth.

Introduction

Plants are subjected to a continuously changing, often unpredictable, environment. Optimal growth in these conditions requires control of developmental processes by environmental and internal signals. Plants integrate these varying environmental cues and modify developmental programmes accordingly. This is done, in part, through regulation by a complex web of hormones, which have both unique and overlapping functions. Rosette plants, such as *Arabidopsis thaliana*, switch between elongated and non-elongated growth habits, dependent on their developmental phase. Upon germination, initial shoot growth of Arabidopsis relies on elongation of the hypocotyl, a process that is affected by hormones and environment alike. After germination and photomorphogenesis, the shoot apical meristem (SAM) is activated and produces true leaves. Unlike the hypocotyl, the internodes between successive leaves do not elongate. Instead, a compact whorl of leaves is formed, termed the rosette. Rosette growth persists for the remainder of the vegetative growth phase, but ends at the start of the reproductive growth phase, during which new internodes rapidly elongate, leading to the formation of an elongated stem (bolting). Bolting is initiated by environmental factors such as photoperiod and temperature (Fukuda et al., 2009; Jacqmard et al., 2003). Activity of hormones, especially gibberellin (GA), auxin and brassinosteroids (BR), is also necessary for bolting—both for bolting initiation and subsequent elongation (Blázquez et al., 1998; Eriksson et al., 2006; Fukuda et al., 2009; Hou et al., 2008; Kauschmann et al., 1996; Liu et al., 2018b; Timpte et al., 1992). These hormones and environmental signals also promote hypocotyl elongation during seedling establishment (Chapman et al., 2012; Hornitschek et al., 2012; Nemhauser et al., 2004; Oh et al., 2014; Stavang et al., 2009), but do not cause elongation of rosette internodes. This raises the question as to how internode growth is blocked in rosettes. Understanding how rosettes maintain their non-elongated growth habit, requires and an understanding of the networks that are at play during hypocotyl and stem elongation.

GA, auxin and BR are key hormones that promote elongation both in the hypocotyl and the stem. Both GA and auxin signalling primarily involves the degradation of negative regulator proteins, belonging to the DELLA and AUX/IAA families, respectively. The DELLA proteins GIBBERELLIN INSENSITIVE (GAI) and REPRESSOR OF GA1-3 (RGA) and AUX/IAA factor AUXIN RESISTANT 2 (AXR2; IAA7 repress stem

elongation (Dill and Sun, 2001; Peng et al., 1997; Timpte et al., 1992). DELLA and AUX/IAA proteins primarily repress downstream signalling by interacting with and sequestering transcription factors, for example resulting in changing a transcription factor from an activator to a repressor of transcription. DELLA proteins can interact with several different classes of transcription factors (Feng et al., 2008; Hyun et al., 2016; Li et al., 2016b; de Lucas et al., 2008; Park et al., 2013), while AUX/IAA proteins interact with members of the AUXIN RESPONSE FACTOR (ARF) family (Wang and Estelle, 2014). GA and auxin cause SCF-mediated ubiquitination and subsequent degradation of DELLA and AUX/IAA, respectively (Ariizumi et al., 2011; Dill et al., 2004; Griffiths et al., 2006; Murase et al., 2008; Wang and Estelle, 2014).

Brassinosteroid signalling differs significantly from that of auxin and GA. The BR response is orchestrated by the transcription factors *BRASSINAZOLE RESISTANT 1* (*BZR1*) and *BRI1 EMS SUPPRESSOR1* (*BES1*, also known as *BZR2*), which regulate BR-responsive target genes. In absence of BR, the BRASSINOSTEROID INSENSITIVE-2 (BIN2) kinase phosphorylates BZR1 and BES1, which are transported out of the nucleus and degraded (He et al., 2002; Ryu et al., 2010). BR activates BRI1, inducing a cascade of kinase signalling that eventually leads to the degradation of BIN2 (He et al., 2002; Ryu et al., 2007, 2010; Wang et al., 2008). This allows dephosphorylation and nuclear accumulation of BES1 and BZR1 and induction of the BR response (Wang et al., 2002; Yin et al., 2002).

Given the overlap in processes that are regulated by auxin, BR and GA, it is not surprising that these hormones have significant effect on each other's function. This crosstalk is observed at the levels of hormonal biosynthesis and transport, signalling and in target gene regulation.

Auxin and BR promote GA biosynthesis (Chapman et al., 2012; Frigerio et al., 2006; Stewart Lilley et al., 2013; Unterholzner et al., 2015), and auxin promotes BR biosynthesis (Azpiroz et al., 1998; Chung et al., 2011). The role of GA on auxin and BR levels is more complex. GA has both a positive and negative effect on BR biosynthesis, and promotes auxin transport (Löfke et al., 2013; Salanenka et al., 2018; Stewart Lilley et al., 2013; Willige et al., 2011).

At the signalling level, RGA and GAI can interact with and inhibit BZR1 and BES1 (Bai et al., 2012a; Gallego-Bartolomé et al., 2012; Li et al., 2012b). Additionally, RGA

also can sequester ARF6 (Oh et al., 2014). Conversely, it has also been observed that BR can promote RGA stability (Stewart Lilley et al., 2013). BZR1 and ARF6 can also interact *in vitro* and promote each other's function: auxin enhances BZR1 binding to DNA, while BR enhances ARF6 binding to DNA. ARF and BZR1/BES1 DNA binding motifs have been found in close proximity to each other, therefore it is likely that ARF6 and BZR1 act in a larger complex (Nemhauser et al., 2004; Oh et al., 2014). A likely member of this complex is the transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4), which can interact with both ARF6 and BZR1 (Oh et al., 2012, 2014). Like ARF6, BZR1 and BES1, PIF4 is also negatively regulated by DELLA proteins and BIN2 (Bernardo-García et al., 2014; Li et al., 2016a; de Lucas et al., 2008). Several studies have shown that there is a significant overlap between genes regulated by BR, auxin and GA, and by targets bound by ARF6, BRZ1 and PIF4 (Feng et al., 2008; de Lucas et al., 2008; Nemhauser et al., 2004; Oh et al., 2014; Vert et al., 2008).

The PIF family of transcription factors (PIF1, PIF3-7) redundantly control hypocotyl and petiole elongation, and act as major signalling hubs, integrating not only auxin, BR, and GA signalling, but also light, temperature and (a)biotic stress signals (Paik et al., 2017). The interactions between PIF4, DELLA, ARF6 and BZR1 are deemed so critical for hypocotyl elongation that it has been proposed that these four components form a central signalling hub for the control of cell elongation, the BAP-D module (Oh et al., 2014).

In Chapter 2, we identified the three-amino-acid-loop-extension (TALE) homeobox transcription factor *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) as a key factor for maintaining rosette growth. *ATH1* is expressed in the SAM throughout rosette growth and is downregulated prior to bolting (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007). Ectopic expression of *ATH1* represses bolting (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009), while loss of *ATH1* during vegetative growth enables elongation of rosette internodes (heterochronic bolting; Chapter 2). *ATH1* is thought to act in an independent pathway to GA to regulate internode elongation, as heterochronic bolting is strongly enhanced by GA (Chapter 2), but *ATH1* overexpressors cannot be rescued with exogenous GA-application (Gómez-Mena and Sablowski, 2008). *ATH1* targets the boundary gene *LATERAL ORGAN BOUNDARIES* (*LOB*) (Chapter 2). *LOB* represses cell division and

elongation in meristem-organ boundaries by repressing BR function through PHYB ACTIVATION TAGGED SUPPRESSOR 1 (*BAS1*), which promotes catabolism of brassinolide and its direct precursor castasterone (Bell et al., 2012; Turk et al., 2005). Loss of *LOB* enhances heterochronic bolting of *ath1* mutants. Heterochronic bolting is also enhanced by far-red (FR) light and high ambient temperature, which are conditions that also increase GA, BR and auxin production or signalling (Hisamatsu et al., 2005; Ibañez et al., 2018; Kozuka et al., 2010; Stavang et al., 2009; Tao et al., 2008). This suggests that repression of rosette internode elongation may rely on inhibition of multiple hormonal pathways.

Here, we show that *ATH1* mediates sensitivity to auxin, GA and BR and PIFs, in a partially *LOB*-dependent manner, thereby repressing internode elongation during rosette growth. Chemical inhibition of BR or GA repressed heterochronic bolting in *ath1-3*, showing that endogenous GA and BR are sufficient to induce heterochronic bolting of *ath1-3* in FR or at 27°C. The combination of exogenous auxin and BR application was sufficient to induce heterochronic bolting in wild-type plants, thereby overriding the action of endogenous *ATH1*. However, loss of *DELLA* did not enhance this phenotype, suggesting that GA may also act independently to auxin and BR. As *ath1-3* mutants were consistently more elongated than any other genotype, it is highly likely that ATH1 represses multiple routes of elongation to maintain rosette habit.

Results

Heterochronic bolting requires brassinosteroids, independently of GA

In Chapter 2, we showed that *ATH1* promotes the rosette habit by repressing internode elongation. ATH1 is a positive regulator of the transcription factor *LOB*, which is required for *ATH1*-mediated repression of internode elongation and represses BR function. To determine whether brassinosteroids are required for heterochronic bolting, we measured heterochronic bolting in Col-8, *ath1*-3, and *lob-3* plants grown in LD conditions and treated them with 1 µM epi-brassinolide (BL). We observed a small, but statistically significant effect of BL on *ath1-3* and *lob-3* rosette internodes, although *lob-3* plants were less elongated than *ath1*-3. Rosette internodes of Col-8 plants did not elongate (Fig. 3.1A). As LOB promotes BR catabolism through BAS1, and is a direct target of ATH1, these findings suggest that ATH1 regulates BR levels through LOB. However, as *lob-3* mutants appear less

sensitive to applied BL than *ath1-3* mutants, it is possible that *ATH1* also has a *LOB*--independent effect on BR levels. We next tested the effect of BL on *ath1-3* plants grown in FR conditions or at 27°C, as these conditions already induce heterochronic bolting in *ath1-3* (Fig. 3.1B-E). BL treatment again did not induce heterochronic bolting in Col-8, and only had a small effect on *ath1-3* grown at 27°C. As both high temperature and FR light promote BR function or signalling, perhaps BR levels are increased to saturating levels and exogenous BL application therefore does not induce an added downstream response on rosette internodes. To determine if BR function is necessary for heterochronic bolting, we also tested the effect of 1 µM of the brassinosteroid biosynthesis inhibitor brassinazole (BRZ) at 27°C and in FR conditions. BRZ treatment of *ath1-3* completely repressed heterochronic bolting, therefore we concluded that heterochronic bolting indeed requires BR function (Fig. 3.1B-E). As the effect of exogenous BL treatment was weak and Col-8 plants did not respond to BL, it is likely that BR levels themselves are not strongly limiting, but BR signalling might be.

If BR signalling is limiting for induction of heterochronic bolting, we expected that constitutive activation of BR signalling would enable heterochronic bolting. To test whether elevated BR signalling enhances heterochronic bolting of *ath1-3,* we crossed *ath1-3* with the gain of function *bzr1-1D* mutant and grew Col-8, *ath1-3, bzr1-1D* and *ath1-3 bzr1-1D* plants in LD and FR conditions. The bzr1-1D protein can no longer be phosphorylated by BIN2 and therefore BR signalling is active in *bzr1-1D* even in the absence of BR (Wang et al., 2002). Thus, if *ATH1* represses heterochronic bolting by repressing BR function, it was expected that both *bzr1-1D* and *ath1-3 bzr1-1D* mutants exhibited heterochronic bolting in standard LD conditions. In both standard LD conditions and FR conditions, *ath1-3 bzr1-1D* double mutants were significantly more elongated than *ath1-3* single mutants (Fig. 3.2), resulting in a loss of rosette habit (Fig. 3.2A). Boundary defects of the *ath1-3* mutant, such as stem-cauline leaf fusions and reduced floral organ abscission (Bao, 2009; Gómez-Mena and Sablowski, 2008) were also strongly enhanced in the *ath1-3 bzr1-1D* double mutant (Fig. 3.S1).

Contrary to these results, *bzr1-1D* single mutants only elongated in FR conditions (Fig. 3.2C). While this shows that *BZR1* function promotes heterochronic bolting, the lack of heterochronic bolting of *bzr1-1D* in standard LD conditions suggests

that an additional elongation signal, present in FR conditions, is required to induce heterochronic bolting.

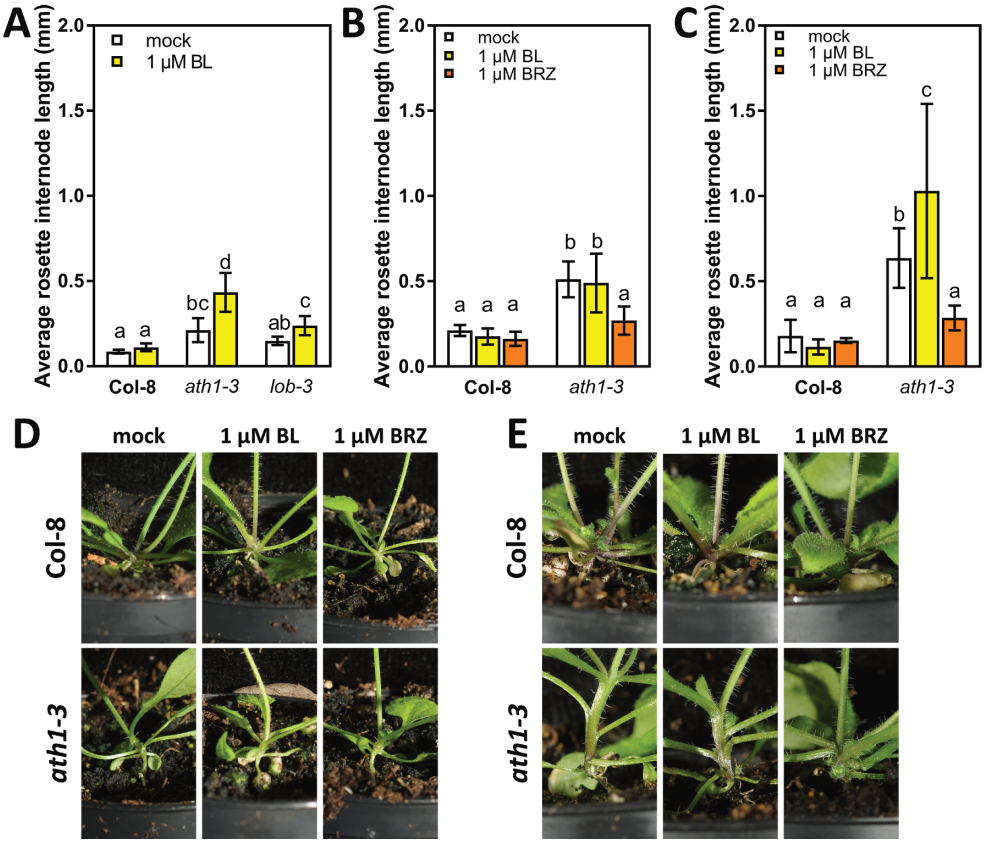


Fig. 3.1: Brassinosteroids are required, but not limiting for heterochronic bolting in *ath1-3*
A: Average rosette internode elongation of Col-8, *ath1-3* and *lob-3* grown in standard LD conditions and sprayed with 0.1% DMS (mock) O or 1 µM BL (n=10).
B-C: Average rosette internode elongation of Col-8 and *ath1-3* mutants grown in LD at in FR conditions (B; n=8, 10, 10, 9, 8, 10) or at 27°C (C; n=8, 5, 10, 10, 5, 10) and treated with 0.1% DMSO (mock), 1 µM BL or 1 µM BRZ.
D: Representative phenotypes of 26-day old plants from B.
E: Representative phenotypes of 32-day old plants from C.
Error bars represent standard deviation of the mean. Letters denote statistically homogeneous subsets as defined by 1-way ANOVA followed by Tukey's post hoc test (α=0.05). For outcome of tests, see Supplemental Table 3.S1.

Besides BIN2, BZR1 is also negatively regulated by the DELLA proteins RGA and GAI, which interact with BZR1 and prevent BZR1 binding to target DNA. Both high ambient temperature and FR light can induce GA biosynthesis, and thus can release BZR1 from this repression (Gallego-Bartolomé et al., 2012; Galvão et al., 2015;

Hisamatsu et al., 2005; Li et al., 2012b; Stavang et al., 2009). Furthermore, GA is required for heterochronic bolting in *ath1-3* mutants (Chapter 2). To test if GA is required for bzr1-1D-induced heterochronic bolting, we treated FR-grown Col-8, *ath1-3*, *bzr1-1D* and *ath1-3 bzr1-1D* with the GA biosynthesis inhibitor paclobutrazol (BZR). PAC repressed heterochronic bolting in both *ath1-3* and *bzr1-1D* single and double mutants, suggesting that GA is needed for BR-induced heterochronic bolting (Fig. 3.2C-D).

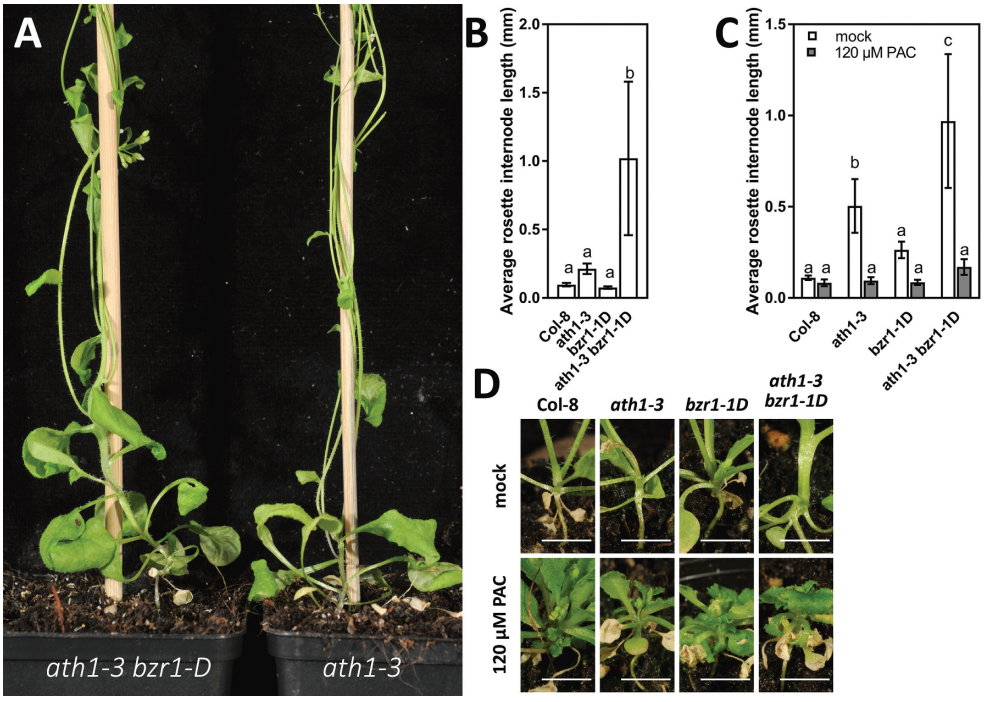


Fig. 3.2 Enhanced BR signalling promotes heterochronic bolting
A: Heterochronic bolting phenotype of 47-day old LD-grown *ath1-3* and *ath1-3 bzr1-1D* mutants.
B: Average internode elongation of LD-grown Col-8, *ath1-3, bzr1-1D* and *ath1-3 bzr1-1D* (n=10, 10, 9, 10).
C: Average internode elongation in FR-grown Col-8, *ath1-3, bzr1-1D* and *ath1-3 bzr1-1D* treated with 0.1% DMSO (mock) or 120 µM PAC (n=10, 10, 10, 10, 9, 9, 9, 10).
D: Photos of representative plants from C. Plants are 34 days old, scale bar denotes 5 mm.
Lowercase letters denote statistically homogeneous subsets as defined by 1-way ANOVA followed by Tukey's post hoc test (α=0.05). For outcome of tests, see Supplemental Table 3.S1.

It is possible that ATH1 and GA antagonistically regulate heterochronic bolting through regulation of BR. This would mean that in our FR conditions, enough GA is present to release bzr1-1D protein from DELLA repression, while GA levels are limiting in LD. If this is the case, GA-treatment of *bzr1-1D* or BL treatment of the

global *della* mutant, where neither BZR1 or BES1 can be sequestered by DELLA, would be sufficient to induce heterochronic bolting in standard LD conditions. GA induced heterochronic bolting in *ath1-3*, but did not induce heterochronic bolting in *bzr1-1D* and neither did BL treatment of *della* (Fig. 3.3). This means that GA is needed, but not sufficient, for heterochronic bolting. As the *bzr1-1D* and *della* backgrounds all still contain wild-type *ATH1* alleles, we concluded that apart from BR and GA, ATH1 blocks additional elongation signals to repress internode elongation during rosette growth.

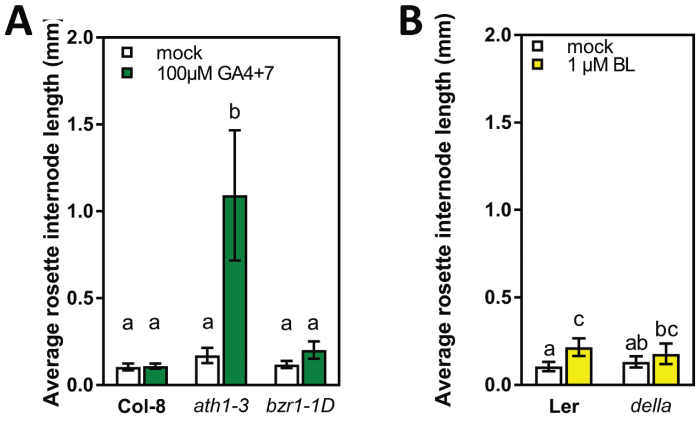


Fig. 3.3: Increased GA and BL signalling or application does not induce heterochronic bolting
A: Average internode elongation of Col-8, *ath1-3*, *bzr1-1D* grown in LD conditions, treated with 100 µM GA4+7 or 0.1% DMSO (mock; n=10).
B: Average internode elongation of Ler and *della* grown in LD at 22°C (B; n=10), treated with 1 µM BL or 0.1% DMSO (mock).
Error bars represent standard deviation from the mean, lowercase letters denote statistically homogenous subsets as defined by Tukey's post-hoc test (α=0.05). For outcome of tests, see Supplemental Table 3.S1.

Auxin and brassinosteroids combined induce heterochronic bolting

Auxin plays a major role in in elongation during shade avoidance and high temperature responses, acting synergistically with BR and GA (Chung et al., 2011; Frigerio et al., 2006; Oh et al., 2014; Stavang et al., 2009; Tao et al., 2008). Therefore, auxin might also promote heterochronic bolting in concert with BR or GA. To this end, we tested the effect of application of 0-5 µM of the synthetic auxin picloram to Col-8 and *ath1-3* shoot apices, in LD conditions. Picloram did not induce heterochronic bolting in Col-8 plants. In *ath1-3* mutants, however, clear elongation of rosette internodes was observed in plants treated with 1 µM or 5 µM of picloram (Fig. 3.4A) and there was a linear relationship between picloram concentration and average rosette internode

length (R^2=0.948; F(1; 3)=55.18; p=0.0050). Picloram at 5 µM had a repressive effect on general growth in plants, which were smaller and bolted later. At 10 µM of picloram, plants died (data not shown). As 5 µM of picloram showed the greatest elongation response, we performed subsequent experiments with 5 µM picloram unless stated otherwise.

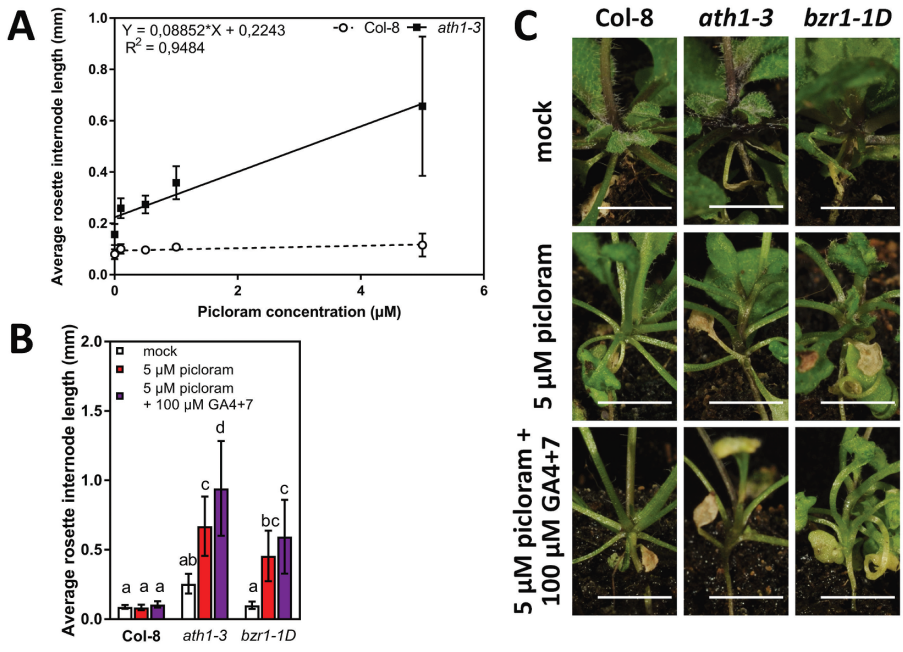


Fig. 3.4: Auxin and brassinosteroids promote heterochronic bolting in Arabidopsis
A: The effect of 0-5 µM of picloram on average internode elongation in Col-8 and *ath1-3* grown in LD conditions (n=10).
B: Average internode elongation of LD-grown Col-8, *ath1-3* and *bzr1-1D* treated with 0.1% DMSO (mock), 5 µM picloram or a combination of 5 µM picloram and 100 µM GA4+7 (n=10).
C: Representative phenotypes of plants depicted in B. Plants are 28 days old. Scale bar denotes 5 mm. For outcome of statistical tests, see Supplemental Table 3.S1. Letters denote statistically homogeneous subsets as defined by 1-way ANOVA followed by Tukey's post hoc test (α=0.05). Error bars represent standard deviation from the mean.

To determine if auxin is required for brassinosteroid-induced heterochronic bolting we tested the effect of picloram, and combined application of picloram and GA4+7 on *bzr1-1D*. Picloram alone was sufficient to induce heterochronic bolting in both *ath1-3* and *bzr1-1D*. There was only a small added effect of simultaneously treating *bzr1-1D* with picloram and GA, although there was a stronger effect on *ath1-3* plants treated with this combination (Fig. 3.4B). Therefore, auxin and brassinosteroids are likely the main hormones involved in heterochronic bolting, with GA acting in

parallel. Neither picloram, or picloram and GA4+7 induced heterochronic bolting in Col-8 (Fig. 3.4B-C).

To determine whether increased BR and auxin induce heterochronic bolting, Col-8, *ath1-3, bzr1-1D* were grown in LD conditions and treated with picloram or a combination of picloram and BL. The same hormones were also tested on *lob-3* to investigate a possible involvement of *LOB*. The combination of picloram and BL severely impaired growth of all plants, arresting growth in several cases (data not shown), so the experiment was repeated using 1 µM picloram instead of 5 µM. Like *ath1-3* and *bzr1-1D*, *lob-3* mutants also elongated when treated with picloram. However, rosette internodes of picloram-treated *lob-3* plants were less elongated than those of *ath1-3*, which again suggests that ATH1 acts through additional targets besides *LOB*. Col-8, *ath1-3* and *bzr1-1D* treated with both picloram and BL showed elongated internodes (Fig. 3.5). Additionally, *ath1-3, lob-3* and *bzr1-1D* plants that were treated with both picloram and BL did not elongate significantly more than plants treated with picloram alone (Fig. 3.5A). Probably, BR signalling is already at an optimal level to induce elongation, not only in *bzr1-1D* but also in *ath1-3* and *lob-3*. As such, picloram application alone suffices to induce internode elongation in these mutants.

As combined picloram and BL treatment can induce heterochronic bolting in Col-8 plants, which express a wild-type *ATH1* allele, we tested if either or both of these hormones could rescue stem elongation in the DEX-inducible *35S::ATH1-HBD* overexpressor. DEX-treated *35S::ATH1-HBD* plants are able to bolt, although stem elongation is reduced compared to wild-type plants (Bao, 2009). Picloram, BL or combined picloram and BL treatment of DEX-treated *35S::ATH1-HBD* plants had no effect on the elongation of the first 10 stem internodes and also did not induce heterochronic bolting (Fig. 3.6A-B). Probably, picloram and BL treatments reduce *ATH1* expression in Col-8, which does not occur when *ATH1* is expressed from a 35S promoter. The effect of picloram and BL on *ATH1* expression was tested on plate-grown PATH1::GUS reporter lines. There was a strong reduction of GUS expression in seedlings grown on combined picloram and BL, which suggests that combined picloram and BL induces heterochronic bolting in wild-type plants by repressing *ATH1* expression (Fig. 3.6C).

Combined picloram + BL treatment reduces *ATH1* expression but picloram + BL treated *ath1-3* mutants are nevertheless more elongated than Col-8 plants (Fig.

3.5A). Therefore, while auxin and BR are needed for heterochronic bolting, ATH1 probably blocks sensitivity to other elongation signals as well. Possibly, GA signalling explains this additional elongation, thus the combined effect of picloram and BL was tested in *della* mutants. However, loss of *della* function did not cause any additional elongation in picloram + BL treated plants, compared to Ler (Fig. 3.5B, D). As *della* mutants contain a functional copy of *ATH1, ATH1* likely directly controls GA-induced targets rather than affecting GA signalling.

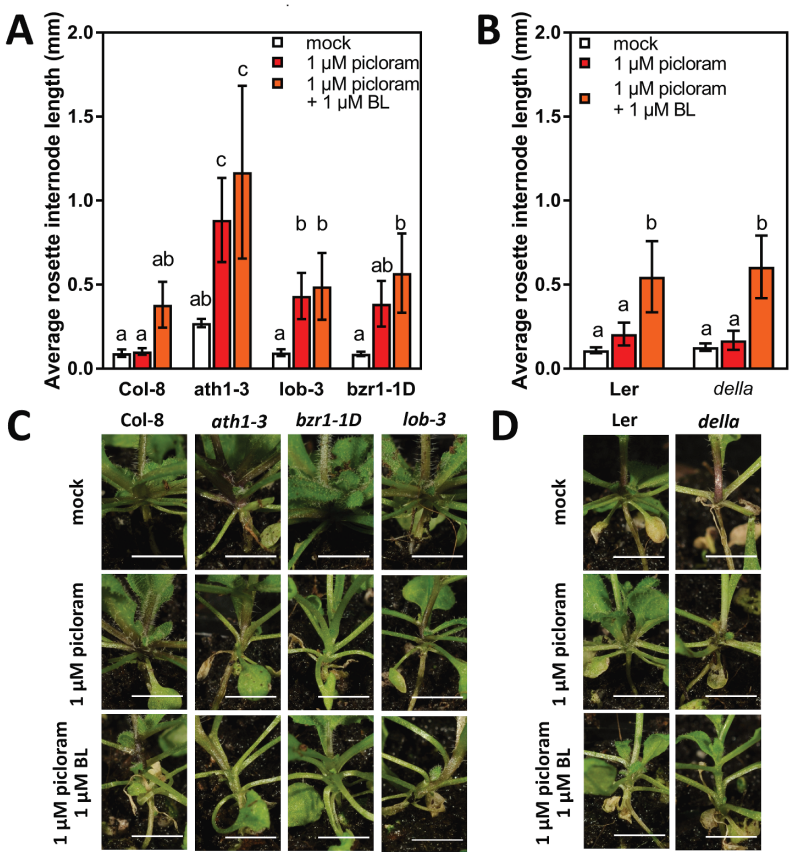


Fig. 3.5: Auxin and brassinosteroids promote heterochronic bolting in Arabidopsis
A-B: Average internode elongation of LD-grown Col-8, *ath1-3*, *lob-3* and *bzr1-1D* (A; n=10) or Ler and *della* (B; n=10). Plants were treated with 0.1% DMSO (mock), 1 µM picloram or a combination of 1 µM picloram and 1 µM BL.
C-D: Representative phenotypes of plants measured in A (C) and B (D). Plants are 32 days old. Scale bar denotes 5 mm.
Error bars represent standard deviation from the mean, lowercase letters denote statistically homogenous subsets as defined by Tukey's post-hoc test (α=0.05). For outcome of tests, see Supplemental Table 3.S1.

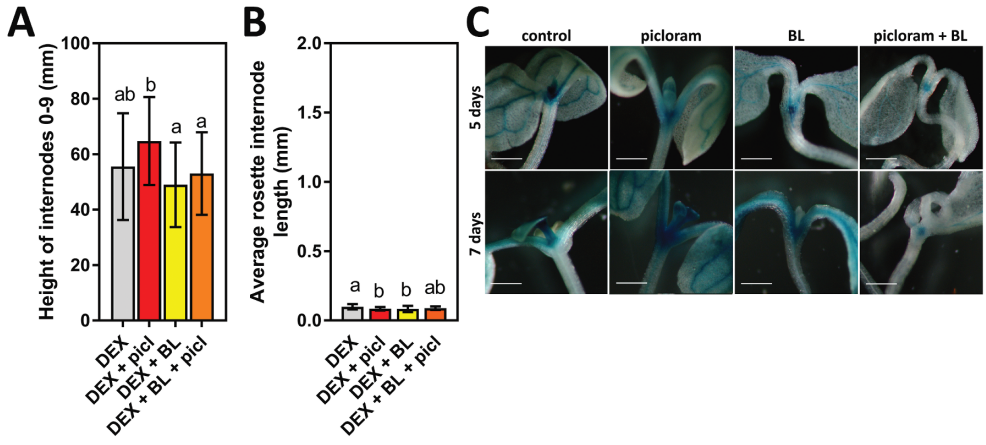


Fig. 3.6: Combined picloram and BL inhibits endogenous *ATH1* expression
A-B: Height of first 10 internodes (A) or average rosette internode elongation (B) of 35S::ATH1-HBD plants treated with 10 µM DEX supplemented with 0.1% DMSO (n=35), 1 µM picloram (picl; n=38), 1 µM BL (n=31) or a combination of 1 µM picloram and 1 µM BL (n=37). Letters denote statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test (α=0.05), see Supplemental Table 3.S1. Error bars represent standard deviation from the mean.
C: *PATH1::GUS* expression in 5 or 7-day old seedlings grown on 0.1% DMSO, 1 µM picloram, 1 µM BL or a combination of 1 µM picloram and 1 µM BL. Scale bar represents 0.5 mm.

PIF4 and *PIF7* are required for heterochronic bolting in *ath1-3* at 27°C or in FR conditions

PIF family transcription factors act downstream of DELLA and are master regulators of hypocotyl elongation, integrating signals such as high temperature, FR light, GA, auxin and BR to promote elongation (Feng et al., 2008; Li et al., 2016a; de Lucas et al., 2008; Oh et al., 2014). *PIF4* is an essential factor for elongation responses at high ambient temperatures (Koini et al., 2009), and was identified as a ChIP target of ATH1 (R. Sablowski, personal communication). In low R:FR conditions, *PIF4* is redundant to *PIF7,* which is essential for elongation in FR (Leivar et al., 2008) but not at high temperature. Thus, we hypothesized that heterochronic bolting of *ath1-3* requires *PIF* function. As such, we generated *ath1-3 pif4-2* and *ath1-3 pif7-1* double mutants and grew them at 27°C or in FR conditions. In FR conditions, *pif7-1* completely suppressed heterochronic bolting of *ath1-3* (Fig. 3.7A, F). *PIF7* function appeared to be specific to elongation in FR conditions, as *ath1-3 pif7-1* mutants grown at 27°C, or treated with 100 µM GA4+7 or 5 µM picloram elongated similarly to *ath1-3* single mutants (Fig. 3.7B-C). On the other hand, heterochronic bolting of *ath1-3 pif4-2* was partially suppressed 27°C, and slightly reduced in FR conditions (Fig. 3.6D-E, G).

These findings reflect the roles of PIF4 and PIF7 in hypocotyl and petiole elongation, but suggest that other *PIFs* may act redundantly with *PIF4* at 27°C.

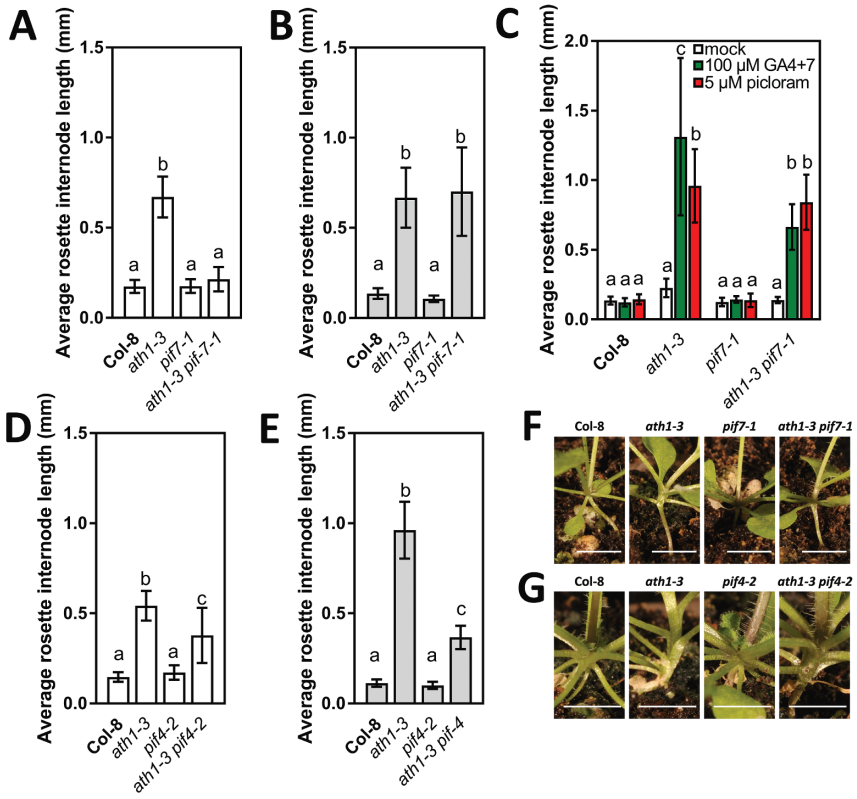


Fig. 3.7: Heterochronic bolting requires activity of multiple PIFs
A-C: Average rosette internode elongation of Col-8, *ath1-3*, *pif7-1* and *ath1-3 pif7-1* plants grown in FR conditions (A; n=10), at 27°C (B; n=10) or in LD conditions and treated with 0.1% DMSO (mock), 100 µM GA4+7 or 5 µM picloram (C; n=10, 10, 9, 10, 10, 10, 9, 10, 10, 9, 10, 10)
D-E: Average rosette internode elongation of Col-8, *ath1-3*, *pif4-2* and *ath1-3 pif4-2* plants grown in FR conditions (D; n=10) or at 27°C (E; n=10).
F: Photos of representative 22-day old plants of A.
G: Photos of representative 30-day old plants of E.
Scale bars denotes 5 mm.
Error bars represent standard deviation from the mean, lowercase letters denote statistically homogenous subsets as defined by Tukey's post-hoc test (α=0.05), see Supplemental Table 3.S1.

If ectopic PIF4 or PIF7 is indeed responsible for elongation of rosette internodes, overexpression of *PIFs* may be sufficient to induce heterochronic bolting at 27°C or in FR. To this end, we tested the *35S::PIF4-HA* overexpressor in these conditions. Heterochronic bolting was not observed in *35S::PIF4-HA* plants grown at 27°C, nor in *35S::PIF4-HA* plants grown in FR conditions (Fig. 3.8A-B). Since PIF protein is post-

translationally regulated, it is possible that overexpression of *PIF4* is not sufficient to generate high levels of active PIF protein. As PIF4 proteins can be bound and sequestered by DELLA proteins, we tested the effect of 100 µM GA4+7 on *35S::PIF4-HA* overexpressors in FR conditions. Although total rosette internode elongation remained unchanged in GA-treated *35S::PIF4-HA*, GA greatly reduced the total number of rosette leaves formed prior to bolting (8.2±1.3 vs 2.5±0.70). As a result, average rosette internode elongation was significantly increased in GA-treated *35S::PIF4-HA*, although not clearly visible (Fig. 3.8B). To confirm whether GA did induce heterochronic bolting, *35S::PIF4-HA* was grown in short-day (SD) conditions, where plants form more rosette internodes. In SDs, elongation of rosette internodes was observed in GA-treated *35S::PIF4-HA*, but unlike *ath1-3* where the rosette habit was lost, GA-treated *35S::PIF4-HA* plants still formed a structure resembling a rosette (Fig. 3.8C-D, data not quantified). Taken together, these findings suggest that *ATH1* inhibits *PIF* function during rosette growth.

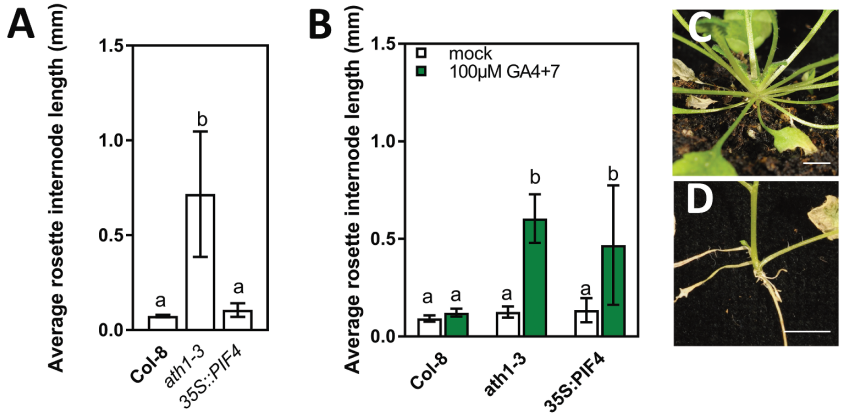


Fig. 3.8: GA-induced heterochronic bolting of 35S::HA-PIF4
A: Average rosette internode elongation of Col-8, *ath1-3* and *35S::PIF4-HA* plants grown at 27°C (n=6, 7, 7).
B: Average rosette internode elongation of Col-8, *ath1-3* and *35S::PIF4-HA* plants grown in FR conditions, treated with 0.1% ethanol (mock) or 100 µM GA4+7 (n=10, 8, 10, 10, 10, 10).
C-D: 79-day old SD-grown *35S::HA-PIF4* plants treated with 0.1% ethanol (C) or 100 µM GA4+7 (D)
Scale bar represents 5 mm. Letters denote statistically homogeneous subsets as defined by 1-way ANOVA followed by Tukey's post hoc test (α=0.05). For outcome of tests, see Supplemental Table 3.S1.

Discussion

ATH1 represses brassinosteroid and auxin function, partially through *LOB*

Elongation of the hypocotyl, non-elongation of rosette internodes and elongation of the stem internodes are contrasting growth processes tailored to functionally contrasting developmental stages. Previous research has shown that *ATH1* is a key factor in preventing elongation of rosette internodes. Loss of *ATH1* is correlated with the onset of bolting (Chapter 2). *LOB* is a negative regulator of BR levels (Bell et al., 2012), and a direct target of *ATH1*. Heterochronic bolting was also observed in GA-treated *lob* mutants (Chapter 2). Thus, we examined the role of brassinosteroids in the regulation of heterochronic bolting. We showed that chemical inhibition of BR biosynthesis through BRZ treatment repressed heterochronic bolting in *ath1-3*, while constitutive activation of BR signalling by *bzr1-1D* strongly enhanced heterochronic bolting in *ath1-3* (Fig. 3.1C-E, Fig. 3.2).

While brassinosteroids are required for heterochronic bolting, exogenous application of BL only had a small effect on *ath1-3* and *lob-3* and failed to induce heterochronic bolting in wild-type plants (Fig. 3.1). We showed that the combined function of auxin and BR was required to enable elongation of rosette internodes, even in backgrounds carrying endogenous *ATH1* (Fig. 3.5). In *ath1-3*, *lob-3* and *bzr1-1D* mutants, exogenous application of picloram was sufficient to induce heterochronic bolting. The combined picloram and BL treatment had no additional effect on *ath1-3, lob-3* and *bzr1-1D,* suggesting a heightened BR status in *ath1-3* and *lob-3*.

In *ath1-3* mutants, *LOB* expression is diminished (Chapter 2). Both *ath1-3* and *lob-3* mutants are responsive to BL and picloram treatments (Fig. 3.1A, Fig. 3.5A), which indicates that ATH1 requires LOB function for repression of rosette internode elongation. Auxin signalling and transport components have previously been listed as directly repressed by *LOB* (Bell et al., 2012), although the biological relevance of this interaction has not been studied. Nevertheless, *LOB* is likely only part of the story as rosette internodes of *lob-3* mutants were always less elongated than those of *ath1-3*. This suggests that *ATH1* regulates the auxin-BR response through other components than *LOB*. *ATH1* probably acts through other transcriptional targets as well, such as the boundary genes *BLADE ON PETIOLE 1* (*BOP1*) and *BOP2,* which are direct targets of *ATH1* (Chapter 2). Like *LOB*, ectopic expression of *BOP1* or *BOP2* repressed bolting, and it has been shown that BOP1 directly antagonizes BR signalling by binding BZR1, preventing BRZ1 from entering the nucleus (Norberg,

2005; Shimada et al., 2015). Alternatively, *ATH1* might directly regulate BR levels and/or signalling. Regulation of BR levels by KNOTTED1-LIKE HOMEOBOX (KNOX) family transcription factors has been observed in rice and maize. KNOX transcription factors belong to the TALE homeodomain transcription factor class, together with the BEL1-LIKE HOMEODOMAIN (BLH) transcription factor family, of which ATH1 is a member. As a BLH transcription factor, ATH1 interacts with KNOX transcription factors like *SHOOTMERISTEMLESS* (*STM*) (Cole et al., 2006). The rice orthologue of *STM*, *ORYZA SATIVA HOMEOBOX1* (*OsOSH1*), was shown to directly repress homologues of *BAS1* in the shoot apex, while the maize *STM* orthologue KNOTTED1 (*ZmKN1*) binds the promoters of orthologues of *BAS1* and several BR biosynthesis genes (Bolduc et al., 2012; Tsuda et al., 2014). Interestingly, loss of BR biosynthesis in maize, a stem plant, compromised internode elongation to such an extent that these plants strongly resembled a rosette. Upon flowering, tassels formed directly onto this rosette-like structure (Best et al., 2016, 2017). As *ATH1* and *STM* expression patterns are similar during vegetative growth, and *STM* function has been linked to boundary specification (Landrein et al., 2015; Scofield et al., 2018), ATH1-STM heterodimers might directly regulate brassinosteroid levels in Arabidopsis as well.

Less is known about the regulation of auxin by TALE homeobox transcription factors. We recently showed that repression of BR alone is sufficient to prevent auxin-induced heterochronic bolting in *ath1-3* (Chapter 4). However, it remains to be investigated whether ATH1 directly represses both auxin and BR levels or signalling during rosette growth.

Disruption of auxin, BR, GA and PIF interplay is necessary for formation of the rosette

Brassinosteroids, auxin and GA are necessary for cell elongation and cell division, and have overlapping functions in several processes, such as hypocotyl elongation and stem elongation (Bai et al., 2012b; Blázquez et al., 1998; Ibañez et al., 2018; Kauschmann et al., 1996; Li et al., 2012b; Oh et al., 2012; Peng et al., 1997; Timpte et al., 1992). Considerable crosstalk between signalling components of these hormones and PIF transcription factors has been observed, and it is proposed that these interactions form the BAP-D module for hypocotyl elongation (Oh et al., 2014). Our findings showed that heterochronic bolting requires BR, auxin, GA and PIF activity, and that combined BR and auxin application is sufficient to induce

heterochronic bolting in plants containing the endogenous, wild-type *ATH1* allele. This treatment reduces expression of *ATH1*, observed in *PATH1::GUS* seedlings. This explains why combined picloram-BL treatment did not induce heterochronic bolting or rescue stem elongation if ATH1 was expressed from the 35S promoter (Fig. 3.6). However, if repression of *ATH1* expression was the sole function of auxin and BR during internode elongation, then it would be expected that *ath1-3* mutants do not exhibit additional elongation in response to combined picloram-BL application, compared to wild-type plants. As *ath1-3* plants are more elongated than wild-type plants in these conditions (Fig. 3.5), auxin and BL likely promote internode elongation both upstream and downstream of *ATH1.*

In seedlings, auxin can induce BR biosynthesis and *vice versa*, but findings suggest that the auxin-BR synergism is achieved through regulation of shared target sets rather than regulation of biosynthesis (Nemhauser et al., 2004; Oh et al., 2014). This may also explain why activation of solely auxin or BR does not induce heterochronic bolting, while a combined application of these two hormones does induce heterochronic bolting. Previous studies have shown that BZR1 interacts with ARF6 and ARF8 proteins, and that BZR1-ARF6-PIF4 complexes co-regulate genes (Oh et al., 2014). Furthermore, it has also been shown that BES1 complex formation affects gene regulation— BES1-PIF4 complexes promote expression of *DWF4*, BES1 homodimers repress *DWF4* (Martínez et al., 2018). Probably, in the presence of both high BR and high auxin, complex formation of BZR1, PIFs and ARFs is altered, allowing regulation of a different set of targets than in the presence of auxin or BR alone.

Our data showed that repression of PIF4 and PIF7 action by ATH1 is necessary for maintaining the rosette habit. PIF4 was previously identified as a putative transcriptional target of *ATH1* (Chapter 2). Another direct target of ATH1, the boundary gene *BOP2,* has recently been shown to promote ubiquitination and degradation of PIF4 (Zhang et al., 2017a). Some elongation of rosette internodes was also observed in GA-treated *35S::PIF4-HA* overexpressors (Fig. 3.8) and it is likely that ATH1 represses PIF activity, either directly or indirectly. Interestingly, suppression of *ath1-3* by *pif4-2* and *pif7-1* appears to be condition-specific, with *pif7-1* completely repressing heterochronic bolting in *FR* only, while in 27°C conditions *pif4-2* showed (partial) suppression (Fig. 3.7A-B, D-E). High ambient temperature increases

expression of both *PIF4* and *PIF5* (Galvão et al., 2015) and possibly full repression of heterochronic bolting at 27°C requires loss of both of these *PIF*s. The fact that GA treatment restored heterochronic bolting of *ath1-3 pif7-1* (Fig. 3.7C) also suggests that multiple PIFs are involved in this process. PIF3 and PIF4 can be sequestered by RGA and GAI, the main DELLA proteins involved in stem elongation (Feng et al., 2008; King et al., 2001; de Lucas et al., 2008; Peng et al., 1997), thus GA-induced release of PIF3 and PIF4 can compensate for loss of *PIF7*. *PIF4-5* and *PIF7* also act upstream of auxin, promoting auxin biosynthesis (Franklin et al., 2011; Hornitschek et al., 2012; Li et al., 2012a), reflected in the retained sensitivity to picloram-induced heterochronic bolting of *ath1-3 pif7-1* (Fig. 3.7C). PIF4 is also negatively regulated by BIN2, and this link between BR and PIF4 is of particular interest in the context of the rosette habit, as the *pif41A* mutant, which cannot be phosphorylated by *BIN2,* exhibits heterochronic bolting (Bernardo-García et al., 2014).

Our findings show that major regulators of hypocotyl elongation, *PIFs* and *BZR1*, are required for heterochronic bolting. This raises the question whether ATH1-mediated repression of the hypocotyl elongation programme, e.g. the BAP-D module, is a prerequisite for the formation of a rosette. Further research is needed to investigate this question.

Does GA act in concert with or parallel to auxin and BR?

Although PIFs, GA, BR and auxin have all been linked to ATH1-mediated repression of internode elongation during rosette growth, the relationship between GA and auxin/BR during this process requires further investigation. As RGA and GAI are negative regulators of BZR1, BES1, ARF6 and PIFs (Feng et al., 2008; de Lucas et al., 2008; Oh et al., 2012, 2014), GA could potentially increase the levels of these proteins in the cell, thereby enhancing heterochronic bolting. If repression of heterochronic bolting fully depends on DELLA-BZR1 protein interactions, it would be expected that GA can enhance *bzr1-1D* mutants, or picloram or BL treated plants. However, we found that GA application or loss of DELLA did not enhance heterochronic bolting in any of these conditions, except in *ath1* backgrounds (Fig. 3.3, 3.4B, 3.5B). Thus, DELLA-mediated sequestering of BZR1, ARFs and PIFs does not appear to be a prerequisite for rosette growth, and GA might operate in a parallel pathway to BR/auxin to promote internode elongation. Possibly, GA has both auxin/BR-dependent and independent functions during internode elongation.

Similar results have been found in hypocotyls, where auxin, BR and GA promote hypocotyl elongation both dependently and independently of each other. In hypocotyls GA can promote elongation independently of auxin and BR, although some GA-BR synergism has also been observed (Li et al., 2012b; Stewart Lilley et al., 2013; Tanaka et al., 2003). Furthermore, promotion of hypocotyl elongation by either GA, BR or auxin could be negated by the reduction of biosynthesis or signalling of one the other two hormones (Chapman et al., 2012; Frigerio et al., 2006; Gallego-Bartolomé et al., 2012; Li et al., 2012b; Stavang et al., 2009). This mirrors our findings that BRZ and PAC treatment both repress heterochronic bolting (Fig. 3.1B-C, 3.2C). The observations that *ath1-3* mutants, but not *bzr1-1D,* strongly respond to GA and consistently are more elongated than other genotypes regardless of treatment, are in favour of a parallel GA route blocked by ATH1.

ATH1 and GA both regulate elongation, but appear to act as two independent but converging pathways (Chapter 2). It is likely that these two pathways converge downstream of DELLA, as loss of DELLA has no effect on rosette internodes as long as *ATH1* is present (Chapter 2), even in picloram-BL treated plants (Fig. 3.5B). Possibly, ATH1 blocks genes regulating cell division or cell elongation that are controlled by GA and not by auxin or BR.

Materials and methods

Plant material and growth conditions

Arabidopsis Col-8 and Ler were used as wild-type controls. Previously described mutants were used: the *ath1-3* (Proveniers et al., 2007), *bzr1-1D* (Wang et al., 2002) and *lob-3* (Bell et al., 2012)*, pif4-2* and *pif7-1* (Leivar et al., 2008) mutants, *PATH1::GUS* ($ATH1_{pro}$:GUS) reporter line (Proveniers et al., 2007) and *35S::PIF4-HA* (Nozue et al., 2007) and *35S::ATH1-HBD* (*35Spro:ATH1-HBD*)(Rutjens et al., 2009) overexpressors are in the Col-8 background. The global *della* mutant (Feng et al., 2008) is in the Ler background. To generate double mutants, *ath1-3* was crossed with *bzr1-1D, pif4-2* or *pif7-1,* and F_2 plants were genotyped to detect mutant alleles as described previously (Leivar et al., 2008; Park et al., 2010; Proveniers et al., 2007). Double homozygous mutants were used for subsequent experiments.

Plants were grown on soil or MS agar plates (0.8% plant agar, pH=6.0) and stratified for two days at 4°C in darkness before transferred to the light. Plants were grown

in long-day (LD; 16 hours light, 8 hours dark) or short-day conditions (8 hours light, 16 hours darkness) at 22°C or 27°C under white fluorescent light (Sylvania Luxline Plus Cool White) or in far-red enriched conditions (FR; LD, 22°C, red, blue and far-red LED light, R:FR ratio 1.34). All plants were grown under 120 µmol/m^2/s light, 70% relative humidity. Plants grown at 27°C were germinated for 2 days at 22°C before transfer to 27°C.

Hormone treatments

In experiments where the effects of single hormones were tested, seeds were sown onto MS agar plates supplemented with a mock solution (0.1% ethanol or 0.1% DMSO), picloram (0.1, 0.5, 1 or 5 µM; Sigma-Aldrich), 100 µM GA4+7 (Duchefa), 1 µM brassinazole (BRZ; TCI Europe) or 1 µM epi-brassinolide (BL; Sigma-Aldrich). Paclobutrazol (PAC; Duchefa) treatment was performed as described in Chapter 2. All plate-grown seedlings were transferred to soil on day 7. GA and picloram treatment was performed by pipetting 1 µl of hormone or mock solution onto the shoot apex. BL, BRZ and PAC treatment was performed by spraying the entire shoot with mock or hormone solution.

In experiments where a combination of hormones was tested, seeds were planted directly onto soil and treatment was started on day 7. Combined GA and picloram treatment was performed by pipetting 1 µl of a mixture of 100 µM GA4+7 and 5 µM picloram onto the shoot apex. Combined picloram and BL treatment was performed by spraying the entire shoot with 1 µM BL and applying 1 µM of 1 µM picloram to the shoot apex. In this experiment control plants were sprayed with 0.1% DMSO and 1 µl of DMSO was applied to the shoot apex; picloram-treated plants were sprayed with 0.1% DMSO and 1 µl of 1 µM picloram was pipetted onto the shoot apex. To determine the effect of picloram and BL on *35S::ATH1-HBD*, the same treatment regime was used, but hormone solutions were supplemented with 10 µM dexamethasone (DEX; Siglma-Aldrich).

All hormone treatments were performed three times a week until the opening of the first flower, except in experiments with *35S::ATH1-HBD*, where treatment was stopped after ripening of the first silique. All hormone solutions were supplemented with 0.01% Silwet-L77 (Momentive).

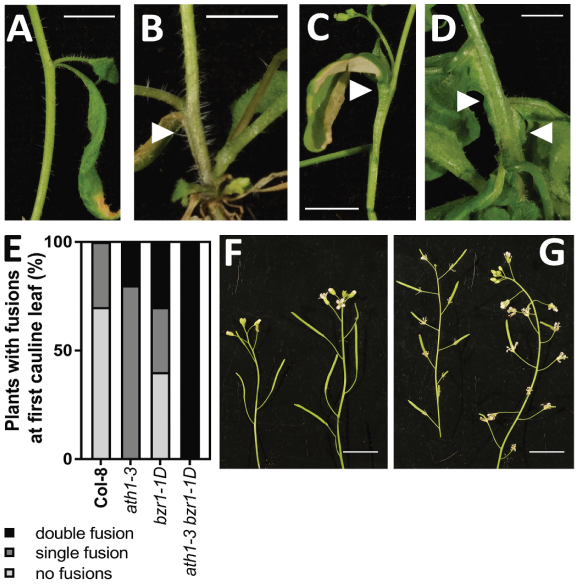
Phenotypic analyses

Average rosette internode elongation was determined by measuring the total elongation of the rosette, from the cotyledons to the last rosette leaf, divided by the number of rosette leaves. Ten plants were used for each genotype and treatment. To determine stem elongation the height of the first 10 internodes of the inflorescence stem were measured. Differences in average rosette internode elongation or stem elongation were determined using a 1-way ANOVA followed by Tukey's post-hoc test in IBM SPSS Statistics 24. To assess the effect of picloram concentration on rosette internode elongation, linear regression analysis was performed in SPSS 24. Stem-cauline leaf fusions were scored per individual cauline leaf (0: no fusions, 1: single fusion, 2: double fusion). Not all plants formed more than one cauline leaf, so data of only the first cauline leaf were used. For outcome of statistical tests, see Supplemental Table S3.1.

Histochemical analysis of GUS activity

Five-day old LD-grown seedlings were harvested directly into GUS staining buffer (50 mM sodium phosphate buffer (pH=7.2), 0.1% Triton X-100, 100 mM, potassium ferrocyanide, 100 mM, potassium ferricyanide, 2 mM X-glucoronide). Samples were vacuum infiltrated at room temperature for 10 minutes and incubated at 37°C for 16 hours. Seedlings were cleared in ethanol and imaged using a Nikon DXMI200 camera mounted to a Zeiss Stemi SV II stereo microscope.

Supplemental figures and tables



Supplemental Fig. 3.S1: Boundary defects of *ath1-3* are strongly enhanced by *bzr1-1D*
A-D: Stem-cauline leaf fusion phenotypes of LD-grown, 47-day old Col-8 (A), *ath1-3* (B), *bzr1-1D* (C) and *ath1-3 bzr1-1D* (D). Scale bar denotes 5 mm, arrowheads mark fused tissue.
E: Percentage of plants that have fusions (none, double or single) at the first cauline leaf. 10 plants were studied per line.
F: Floral organ abscission of 47-day old LD-grown Col-8 and *bzr1-1D* (F), *ath1-3* and *ath1-3 bzr1-1D* (G) plants. Scale bar represents 5 mm.

Supplemental Table 3.S1: Reporting of statistical tests

Figure	Statistical test	Outcome
Fig. 3.1A	1-way ANOVA	F(5;54)=42.67; p=1,4E-17
Fig. 3.1B	1-way ANOVA	F(5; 47)=26.76, p=1.1E-12
Fig. 3.1C	1-way ANOVA	F(5; 42)=23.60, $p=3.3 \cdot 10^{-11}$
Fig. 3.2B	1-way ANOVA	F(3; 35)=24.8; p=8,8E-9
Fig. 3.2C	1-way ANOVA	F(7; 71)=47.16; p=3.2E-24)
Fig. 3.3A	1-way ANOVA	F(5; 54)=62.75; p=2.9E-21
Fig. 3.3B	1-way ANOVA	F(3; 36)=12.437; p=1.0E-6
Fig. 3.4A	Linear regression F-test for *ath1-3*	Y=0.08852*X + 0.2243; R^2=0.95; F(1; 3) = 55.18, p=0.005;
Fig. 3.4A	Linear regression F-test for Col-8	Y=0.004791*X+0.09393; R^2=0.56; F(1; 3)=3.763; p=0.15;
Fig. 3.4B	1-way ANOVA	F(8; 81)=32.566; p=2.8E-22
Fig. 3.5A	1-way ANOVA	F (11; 108)=28.431; p=5.2E-27
Fig. 3.5A	1-way ANOVA	F(5; 54)=33.514; p=2.1E-15
Fig. 3.6A	1-way ANOVA	F(3; 137)=5.917; p=0.001
Fig. 3.6B	1-way ANOVA	F(3; 137)=6.740; p=2.81E-4
Fig. 3.7A	1-way ANOVA	F (3; 40) = 143.151; p=0.000
Fig. 3.7B	1-way ANOVA	F (3; 36)=47/629; p=1.5E-4
Fig. 3.7C	1-way ANOVA	F(7; 71)=82.592; p=1,5E-31
Fig. 3.7D	1-way ANOVA	F(3; 36)=4.744; p=9,1E-16
Fig. 3.7E	1-way ANOVA	F (3;36) = 218.495; p=3.8E-23
Fig. 3.8A	1-way ANOVA	F(2; 17)=22.858; p=1.5E-6
Fig. 3.8B	1-way ANOVA	F(5; 52)=24.094 ;p=1.9E-12

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CHAPTER 4

Identification of *suppressor of ath1-3 rosette internodes* mutants using EMS mutagenesis

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Abstract

The compact rosette and the inflorescence stem are two contrasting growth forms observed in rosette plants. In the rosette plant *Arabidopsis thaliana* perturbations in hormones or regulators of cell division and elongation affect leaf and inflorescence stem growth, but few mutations affect the growth of rosette internodes. Previously, we identified the transcription factor *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) as a key positive regulator of the rosette habit. In *ath1* mutants, rosette internodes are elongated (heterochronic bolting), which is exacerbated by growth at high ambient temperature, far-red light or by external application of auxin, gibberellins or brassinosteroids. *ATH1* function requires the boundary gene *LATERAL ORGAN BOUNDARIES* (*LOB*), the expression of which is severely reduced in *ath1* plants. *lob* mutants are also sensitive to hormonal induction of heterochronic bolting. However, little is known about other components that control this process. Here, we performed EMS mutagenesis on *ath1* mutants to identify suppressors and enhancers of heterochronic bolting. We selected 9 suppressor and 4 enhancer mutants, linking mutations in *WUSCHEL, DWARF1* and *BIG* to suppression of rosette internode elongation. Furthermore, we showed that *LOB* expression was restored to wild-type levels in the suppressor mutant *ath1-3 sri93,* which also was insensitive to both environmental and hormonal induction of heterochronic bolting. Together these findings suggest that *ATH1* may inhibit internode elongation during rosette growth through multiple routes.

Introduction

Vegetative development of *Arabidopsis thaliana* is characterized by the formation of a compact rosette of leaves. The rosette is a prevalent growth form, observed in several angiosperm species growing in a diverse range of climates (Martorell and Ezcurra, 2002; Tyler, 1902; Went, 1948). The compactness of the rosette protects the plant from high temperatures and herbivory and allows plants to overwinter beneath a snow cover in temperate and alpine climates (Fujita and Koda, 2015; Larcher et al., 2010; Martorell and Ezcurra, 2002). The start of the reproductive growth phase in rosette plants marks a stark change in growth habit by the formation of an elongated stem (bolting) and the formation of flowers (flowering). The reproductive phase change, the transition from vegetative to generative growth, has been well studied in Arabidopsis. This phase change is regulated by several environmental and internal signals. Inputs from light quality, photoperiod, ambient temperature, vernalization, age and levels of the hormone gibberellin (GA) are integrated at the shoot apical meristem (SAM), and initiate the phase change (Khan et al., 2014). However, much less is known about the factors that characterise the rosette phase, where elongation of the stem is inhibited.

Development of the rosette is spearheaded by the SAM and rib zone (RZ), situated in the shoot apex. The SAM carries a central population of stem cells (central zone; CZ), which are gradually displaced into a peripheral zone (PZ) where they differentiate into primordia (Reddy et al., 2004). These primordia develop into rosette leaves during vegetative growth, and into floral meristems during reproductive growth. The CZ stem cell population is maintained through two regulatory mechanisms. The first mechanism, the *WUSCHEL-CLAVATA3* (*WUS-CLV3)* feedback loop, maintains stem cell identity. *WUS* is expressed in the organising centre (OC), situated basally to the CZ, but above the RZ. WUS protein can move to adjacent cell layers, where WUS induces expression of the CLAVATA 3 (CLV3) peptide. *CLV3* expression marks stem cells, but CLV3 peptide also diffuses to neighbouring cells, repressing *WUS* through a signalling cascade involving the *CLV1-CLV2* receptor complex. Cells further away from the OC thus lose stem cell identity and start to differentiate (Daum et al., 2014; Lenhard and Laux, 2003; Schoof et al., 2000). Imbalances in WUS and CLV3 levels therefore have prominent effects on meristem size. In parallel, the three amino acid loop extension (TALE) homeodomain transcription factor SHOOT MERISTEMLESS

(STM) maintains the SAM by locally controlling the levels of the hormones GA and cytokinin (CK) and thereby repressing differentiation and promoting *WUS* expression, respectively (Endrizzi et al., 1996; Gordon et al., 2009; Hay et al., 2002; Jasinski et al., 2005).

The PZ is separated from primordia by a small strip of non-dividing cells, known as the meristem-organ boundary (Žádníková and Simon, 2014). The meristem-organ boundary enforces the balance between stem cell identity and differentiation and is marked by low auxin and brassinosteroid (BR) phytohormone levels. Cell division in the boundary is repressed by transcription factors CUP-SHAPED COTYLEDON 1 (CUC1), CUC2, CUC3, LATERAL ORGAN FUSION 1 (LOF1), LOF2, LIGHT SENSITIVE HYPOCOTYL 3 (LSH3) and LSH4 (Žádníková and Simon, 2014). The boundary-expressed LATERAL ORGAN BOUNDARIES (LOB) transcription factor promotes BR breakdown in the boundary (Bell et al., 2012), while the primordia-expressed transcription factors JAGGED LATERAL ORGANS (JLO), ASYMMETRIC LEAVES 1 (AS1) and AS2 indirectly promote auxin transport away from the boundary and towards the primordium (Borghi et al., 2007; Gendron et al., 2012; Rast and Simon, 2012). Other important organ expressed factors affecting boundary formation include the *BLADE ON PETIOLE 1* (*BOP1*) and *BOP2* genes, which indirectly promote expression of *LOB, JLO* and *AS2* (Ha et al., 2007; Jun et al., 2010; Norberg, 2005).

Beneath the SAM lies the RZ, which is inactive during vegetative growth. As a consequence, stem growth is virtually arrested, resulting in the formation of a compact rosette of leaves. During reproductive growth, cells in the RZ divide longitudinally and are displaced downwards to form stem tissue, thus leading to bolting (Bencivenga et al., 2016; Hempel and Feldman, 1994; Jacqmard et al., 2003; Metzger and Dusbabek, 1991; Peterson and Yeung, 1972; Talon et al., 1991). During this process, cells from the overlying CZ and PZ are supplied to the RZ (Bencivenga et al., 2016). Thus, during reproductive growth, cell proliferation occurs both towards the flanks and the base of the shoot apex. Although it is known that GA promotes RZ activity and GA, auxin and BR promote stem elongation (Kauschmann et al., 1996; Peterson and Yeung, 1972; Timpte et al., 1992; Wilson et al., 1992), very little is known about the mechanisms involved that keep the RZ inactive during rosette growth.

Recently, we showed that the BEL1-LIKE HOMEODOMAIN (BLH)-type TALE homeodomain transcription factor *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*)

is a key factor in promoting rosette habit. *ATH1* is expressed in the SAM and RZ during vegetative growth and is downregulated prior to bolting, coinciding with elongation of the RZ. The RZ of *ath1-3* mutants already adopts this morphology during vegetative growth, which results in the formation of elongated rosette internodes, which we named heterochronic bolting (Chapter 2). Previously, we showed that heterochronic bolting of *ath1-3* mutants is enhanced by high ambient temperature, far-red (FR) light-enriched conditions, auxin, BR and GA (Chapter 2, 3). Interestingly, GA, auxin and BR are needed for both lateral organ primordia and stem development (Hay et al., 2002; Heisler et al., 2005; Jasinski et al., 2005; Kauschmann et al., 1996; Peterson and Yeung, 1972; Timpte et al., 1992; Wilson et al., 1992). Additionally, heterochronic bolting can be induced in *lob* mutants (Chapter 2, 3), and ectopic expression of *LOB* strongly represses stem elongation (Shuai et al., 2002). This raises the possibility that the regulation of cell proliferation and differentiation between the RZ and stem is analogous to the regulation of cell proliferation and differentiation in the between the SAM and lateral organ primordia.

To gain better insight into the regulatory mechanisms that control RZ activity, we performed EMS mutagenesis on *ath1* loss-of-function mutants to identify second-site mutations that either restored wild-type rosette habit or enhanced heterochronic bolting in an *ath1* mutant background. After several rounds of re-screening this left us with nine suppressor mutants and four enhancer mutants . In enhancer mutants, heterochronic bolting was enhanced, as well as additional phenotypes associated to loss of ATH1, but up to now causal mutations could not be identified. In the suppressor mutants, named *suppressor of ath1-3 rosette internodes* (*sri*), specifically heterochronic bolting was suppressed. Suppression of heterochronic bolting could be linked to mutations in *WUS, BIG* and *DWARF1* (*DWF1*). In the latter case, heterochronic bolting could be rescued by exogenous BR application. However, rosette internode elongation in these plants was insensitive to GA or auxin, unless simultaneously treated with BL. Two of the *sri* mutants carried a dominant mutation. Although these have not been identified yet, one of them likely affects organ boundary gene function, as mutant plants strongly resembled plants ectopically expressing boundary genes. Furthermore, compared to *ath1-3, LOB* and *CUC3* expression was (partially) restored in this mutant, and *AS2* expression was weakly elevated compared to wild-type plants. Taken together, our results suggest that *ATH1* may maintain the rosette through multiple pathways, where boundary

gene function and strong interdependency between GA, auxin and BL play major roles.

Results

Identification of EMS-induced suppressor mutants of *ath1-3* heterochronic bolting

Previously, we showed that heterochronic bolting of *ath1-3* could be partially suppressed through loss of function of *PHYTOCHROME INTERACTING FACTOR* 7 (PIF7) or *PIF4* (Chapter 3). However, this effect was specific to environmental conditions: *pif7-1* only suppressed *ath1-3* in FR-enriched conditions, while *pif4-2* only partially suppressed *ath1-3*, most strongly at 27°C. To further dissect the role of *ATH1* in rosette development, we conducted an EMS suppressor screen on *ath1-3* and selected for plants that no longer exhibited heterochronic bolting at both 27°C and in FR-enriched conditions (Fig. 4.1). Initial screening resulted in the selection of 140 putative M2 *suppressor of ath1-3 rosette internodes* (*sri*) mutants. Plants that were non-sterile (96) were selfed and their M3 progeny was re-screened, leaving nine *ath1-3 sri* mutants that robustly maintained their suppressor phenotype (Table 4.1; Fig. 4.2). The suppressor phenotype of two of these M3 mutants (*ath1-3 sri93* and *ath1-3 sri104*) segregated in a 3:1 ratio of restored rosette habit versus heterochronic bolting, suggesting that these mutants were derived from heterozygous M2 parents carrying a dominant causal *sri* mutation.

Phenotypically, most of the *ath1-3 sri* mutant lines (*ath1-3 sri312*, *ath1-3 sri52, ath1-3 sri93*, *ath1-3 sri104*, *ath1-3 sri113*) exhibited general growth inhibition, resulting in smaller leaves, short petioles and/or shorter inflorescence stems. These defects were not seen in *ath1-3 sri43, ath1-3 sri46* and *ath1-3 sri47*, which exhibited identical phenotypes. Initially, these three mutants formed 2-4 rosette leaves with long petioles and small, oblong-shaped leaves, before the SAM arrested and ceased forming leaves (Fig. 4.2). Often this arrest was preceded by the emergence of a single central leaf, suggesting complete differentiation of the SAM stem cell pool. Following developmental arrest, these plants were capable of shoot re-initiation from axillary meristems from which the plants were able to bolt and flower. As only some flowers formed siliques, seed set was reduced in these plants, (Fig. 4.3E, not quantified).

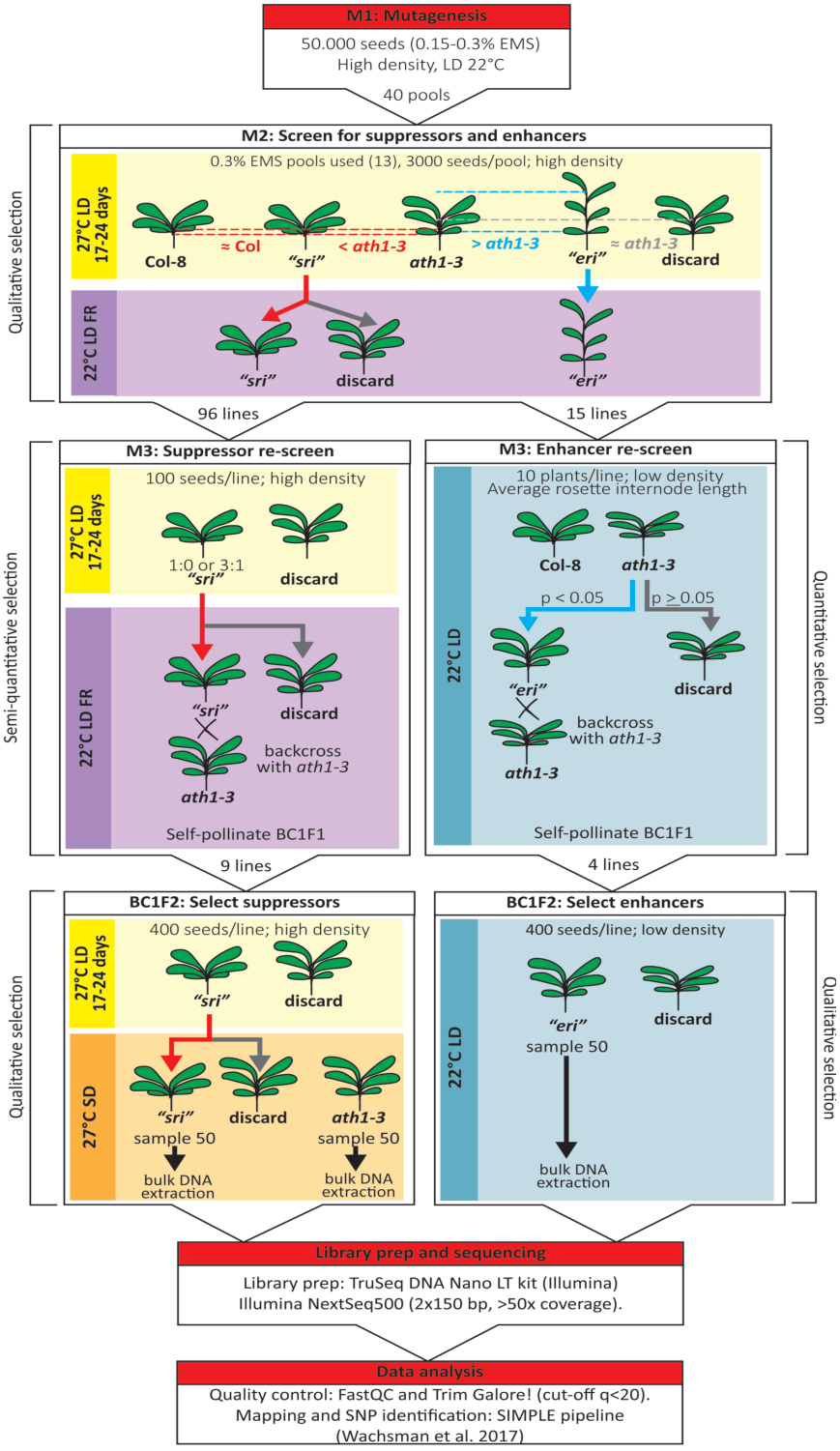

Fig. 4.1: Workflow of EMS suppressor and enhancer screens (For figure legend, see next page)

Growth of the other mutants was already inhibited from early development (Fig. 4.2A-B). In addition, leaf shape was affected in *ath1-3 sri311, ath1-3 sri312* and *ath1-3 sri52*. In Col-8 and *ath1-3* leaf shape shifts from round to spatulate during development. In *ath1-3 sri311* and *ath1-3 sri312* all rosette leaves adopted a round leaf shape. In *ath1-3 sri52* round leaves were formed for a longer period than in Col-8 and *ath1-3,* but later in development *ath1-3 sri52* plants also switched to form spatulate leaves. The petioles of these leaves did not elongate and overall plants formed more leaves before bolting compared to wild type and *ath1-3* (not quantified, Fig. 4.2B-C).

Apart from this, other growth defects were observed in *ath1-3 sri* mutants. The *ath1-3 sri311* and *ath1-3 sri312* mutants formed very small rosettes, the latter consisting of yellowish-green leaves containing lighter, yellow blotches of colour. The *ath1-3 sri113, ath1-3 sri93* and *ath1-3 sri104* also exhibited additional growth defects. After bolting, *ath1-3 sri113* mutants formed shorter inflorescence stems, and more secondary shoots (Fig. 4.3A). The *ath1-3 sri93* and *ath1-3 sri104* mutants formed small, compact rosettes (Fig 4.3). The rosette leaves of *ath1-3 sri104* were small, and after bolting plants formed a severely dwarfed primary inflorescence stem, though occasionally new stems formed from axial meristems that elongated normally (Fig 4.3A-C). Plants did flower, but siliques exhibited septum defects (Fig. 4.3D). Rosette leaves of *ath1-3 sri93* were dark green and curled inwards over the proximodistal axis (Fig. 4.2C). Plants developed slowly but did eventually form an elongated stem (Fig 4.3F) bearing folded cauline leaves in clusters along the stem (Fig 4.3G). The first formed flowers did not form siliques, but newer flowers did (Fig 4.3H-I).

Interestingly, we also observed strong growth defects in 33 M2 lines that did not exhibit suppression of heterochronic bolting. These defects included the formation of small rosettes, non-elongated petioles or short inflorescence stems (data not shown). Thus, general inhibition of growth alone does not appear to be the sole criterion for suppression of heterochronic bolting.

Fig. 4.1 (see previous page): Initial (M2) selection of *sri* and *eri* mutants was on a qualitative basis in conditions that strongly induce heterochronic bolting (high density (5.3 seeds/cm^2) at 27°C LD and transfer to FR conditions). M3 *sri* lines that, when retested in these conditions, exhibited the *sri* phenotype in a 1:0 or 3:1 ratio were selected. M3 *eri* lines were retested in conditions that do not strongly induce heterochronic bolting (low density (0.1 seeds/cm^2), 22°C LD). A quantitative selection (average rosette internode elongation of 10 plants) was used to select *eri* lines that were significantly more elongated than *ath1-3*. Selected *sri* and *eri* plants were back-crossed to *ath1-3* and BC1F2 plants were used for bulk segregant analysis.

To identify the causal mutations in the suppressor lines, we used whole genome sequencing and bulked segregant analysis on M3 mutants back-crossed once to *ath1-3* (BC1F2). Genomic DNA was pooled from 50 BC1F2 individuals per line and from non-mutagenized *ath1-3.* Suppressor phenotypes of the seven *ath1-3 sri* BC1F2 populations (*sri311, sri312, sri43, sri46, sri7* and *sri113*) segregated in a 1:3 suppressor versus non-suppressor ratio, indicating that these phenotypes were caused by recessive mutations. Mapping and SNP analysis was performed using the SIMPLE pipeline (Wachsman et al., 2017). Analysis revealed candidate causal mutations for 6 mutant lines (Table 1; Fig. 4.4). The causal *sri43, sri46* and *sri47* mutations were mapped to the same G to A substitution in *WUS*, which results in a Leu-49 → Ser mutation, situated in the homeodomain of WUS (Fig 4.4D). As such, we renamed these mutants *sri4*. Similar to *ath1-3 sri4* mutants (Fig. 4.2, 4.3E), plants carrying weak *wus* alleles such as *wus-3* and *wus-6* arrest growth after 2-9 rosette leaves due to SAM arrest and bolt and flower from subsequently formed adventitious meristems (Hamada et al., 2000; Mayer et al., 1998). This phenotypic similarity combined with the fact that sequencing of three BC1F2 populations uncovered identical mutations points to *wus* as the causal mutation of the *ath1-3 sri4* phenotype. However, this needs confirmation through complementation analysis. In contrast to *ath1-3 sri4* plants, that can form fertile siliques(Fig. 4.3E), published *wus* plants often exhibit floral defects and are sterile, indicating that the sri4 mutation corresponds with a weak *wus* allele.

The *sri52* mutation was mapped to a C to T substitution in exon 12 of the coding sequence of the gene encoding the calossin-like protein BIG. This point mutation in *BIG* was predicted to cause the formation of a premature stop codon at amino acid position 4416 thereby truncating the BIG protein by 682 amino acids (Fig. 4.4E). Loss of *BIG* results in the formation of a compact rosette containing leaves bearing unelongated petioles and these *big* mutants are late flowering (Gil et al., 2001; Kanyuka et al., 2003; Yamaguchi et al., 2007), similar to *ath1-3 sri52* (Fig. 4.2B-C). After bolting, mutants carrying strong *big* alleles, such as *tir3,* form dwarfed inflorescences with smaller siliques (Gil et al., 2001), while weaker alleles of *big*, such as *doc1-1*, display a more wild-type stature (Yamaguchi et al., 2007). As *ath1-3 sri52* closely resembles the latter, the *sri52* mutation most likely represents a weak allele of *big*.

Table 4.1: List of selected ath1-3 suppressor and enhancer mutants
Scoring of phenotypes: suppressed (S), enhanced (E), unchanged from *ath1-3* (U).

Mutant name	Heterochronic bolting	Stem-cauline fusions	Floral organ attachment	Other phenotypes	M3 ratio	Candidate gene	Mutation	Effect
Suppressors								
ath1-3 sri311	S	U	U	Round leaves, short petioles	1:0	-	-	-
ath1-3 sri312	S	U	U	Small, yellowish, blotchy leaves	1:0	-	-	-
ath1-3 sri43	S	U	U	Meristem terminates, long leaves, long petioles, bolts from axillary meristems	1:0	*WUS*	Chr2:7810527 G>A	Leu49Phe
ath1-3 sri46	S	U	U	Meristem terminates, long leaves, long petioles, bolts from axillary meristems	1:0	*WUS*	Chr2:7810527 G>A	Leu49Phe
ath1-3 sri47	S	U	U	Meristem terminates, long leaves, long petioles, bolts from axillary meristems	1:0	*WUS*	Chr2:7810527 G>A	Leu49Phe
ath1-3 sri52	S	U	U	Oldest leaves are rounded with short petioles, older leaves are oblong but do not have petioles, flat rosette, shorter inflorescence, reduced apical dominance	1:0	*BIG*	Chr3:433482 C>T	Trp4416*
ath1-3 sri93	S	U	U	Small, folded, dark green leaves, slow growing, short petioles, oldest flowers do not develop siliques	3:1	-	-	-
ath1-3 sri104	S	U	U	Very compact rosette, leaves and petioles are small, poor germination, primary stem is very short, axillary stems are long	3:1	-	-	-
ath1-3 sri113	S	U	U	Small, rounded leaves, short petioles, short stem, reduced apical dominance	1:0	*DWF1*	Chr3:688117 C>T	Gly167Glu
Enhancers								
ath1-3 eri14	E	E	U	Large rosettes, form more rosette leaves	1:0	-	-	-
ath1-3 eri15	E	E	U	Large rosettes, form more rosette leaves	1:0	-	-	-
ath1-3 eri31	E	E	E	-	1:0	-	-	-
ath1-3 eri81	E	E	E	Short stem, reduced apical dominance, not all flowers form carpels	1:0	-	-	-

In case of *ath1-3 sri113*, phenotypically linked SNPs mapped to a single large peak on chromosome 3 (Fig 4.4C). Within this peak we identified a G to A point mutation in exon 4 of the *DWF1* gene, causing an Gly-167 → Glu amino acid change (Fig 4.4F). Gly167 is a semi-conserved residue in the DWF1 FAD-binding domain that is believed to be critical for DWF1 function. The *dwf1-3* mutant, which also carries a missense mutation in the DWF1 FAD binding domain, at amino acid position 164, phenotypically resembles the *ath1-3 sri113* mutant (Fig. 4.4F) (Choe et al., 1999). Like *ath1-3 sri113*, *dwf1* mutants form short petioles, rounded leaves and multiple, dwarfed inflorescences arising from the rosette (Kauschmann et al., 1996; Takahashi et al., 1995).

Identification of the causal mutations of the remaining suppressor mutants was less straightforward, as mapping of SNPs yielded multiple peaks on multiple chromosomes (Supplemental Fig 4.S1). Further fine-mapping of these mutations may be needed before causal mutations can be confirmed.

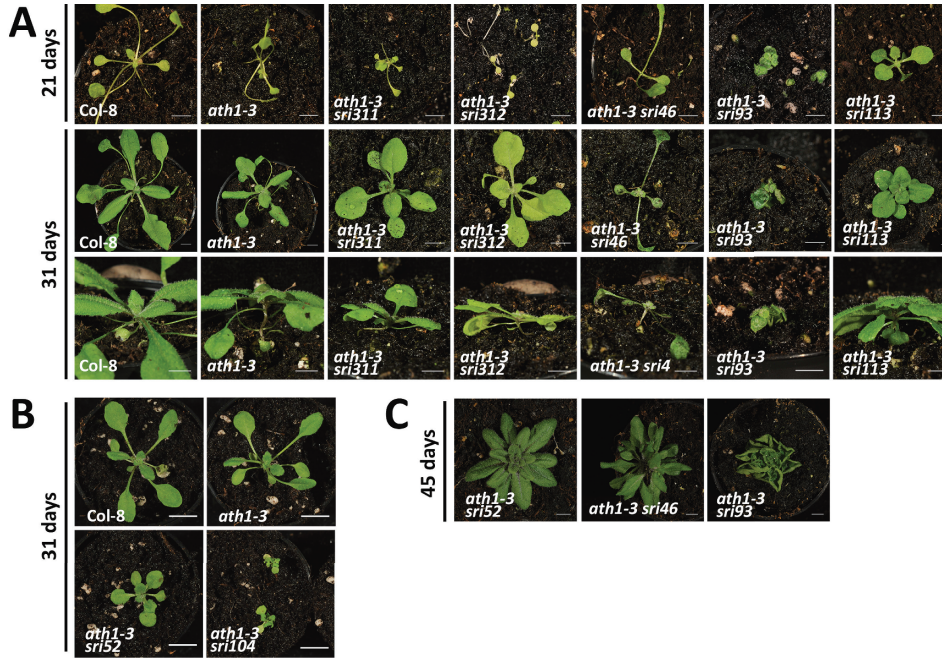

Fig. 4.2: Vegetative phenotypes of *ath1-3* suppressor mutants
A: 21 and 31-day old Col-8 and *ath1-3,* and *ath1-3 sri311, ath1-3 sri312, ath1-3 sri46, ath1-3 sri93* and *ath1-3 sri113* BC1F3 plants grown in LD at 27°C. Top and side views are shown for 31-day old plants.
B: 24-day old Col-8, *ath1-3,* and *ath1-3 sri52* and *ath1-3 sri104* M3 plants, grown for 17 days in LD at 27°C and then transferred to FR conditions.
C: 45-day old *ath1-3 sri52*, *ath1-3 sri4*, *ath1-3 sri93* BC1F3 rosettes grown in LD at 27°C
Scale bar represents 5 mm.

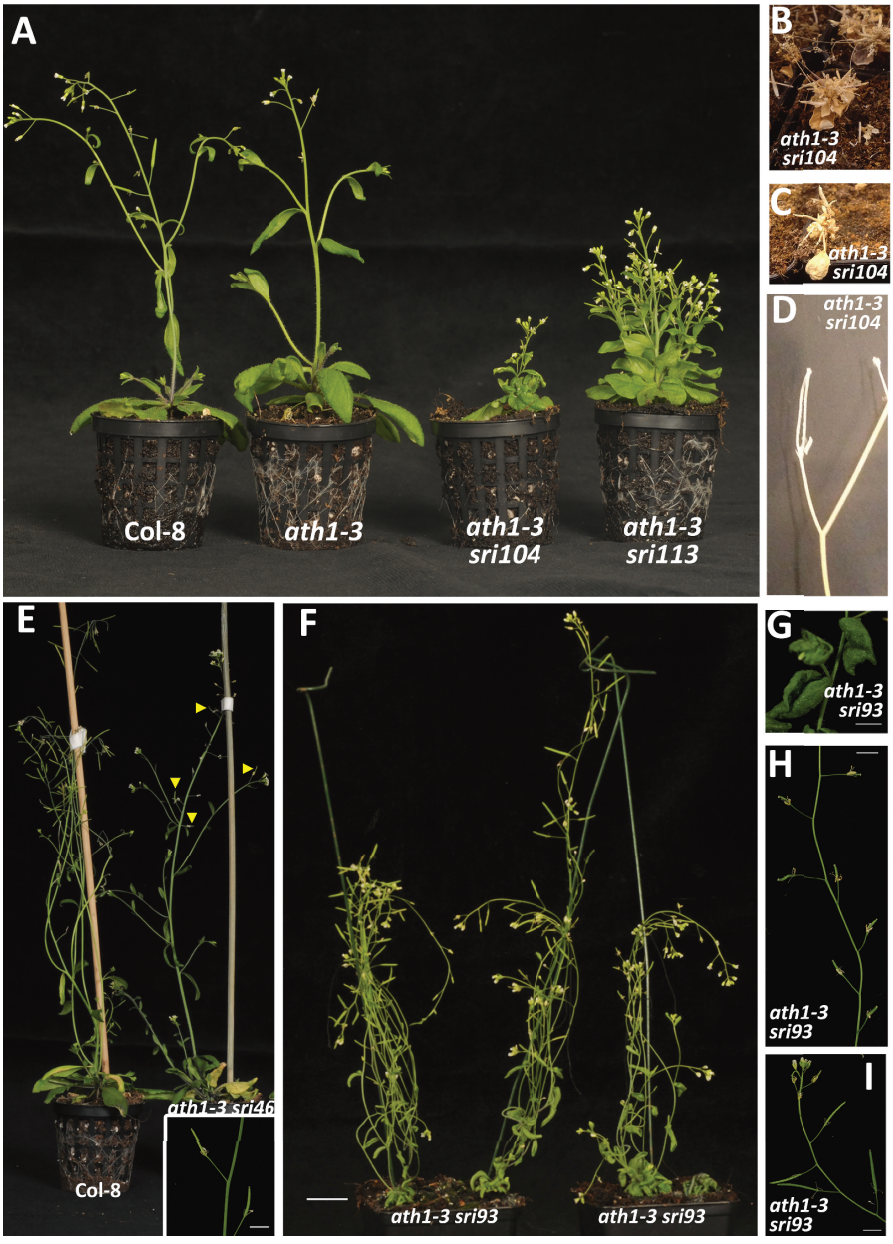

Fig. 4.3: Phenotypes of selected *ath1-3* suppressor mutants during the reproductive growth phase
A: 45-day old Col-8, *ath1-3,* and *ath1-3 sri104* and *ath1-3 sri113* B1F3 plants grown in LD at 27°C.
B-C: Non-bolting *ath1-3 sri104* BC1F2 mutants, with elongated axillary stems formed from axillary meristems (C).
D: Septum defects in *ath1-3 sri104* siliques formed on secondary stems.
E: 60-day old Col-8 (left) and *ath1-3 sri4* (right). Arrowheads mark developing siliques. Inset shows closeup of *ath1-3 sri4* siliques.
F-I: Phenotypes of 66-day old *ath1-3 sri93* mutants grown in LD at 27°C. Whole inflorescence of four *ath1-3 sri93* plants (I), folded and clustered cauline leaves (G), oldest flowers (H) bearing no siliques and youngest siliques (I) with normal seed set.
Scale bar represents 5 mm (E, G-I) or 2 cm (F).

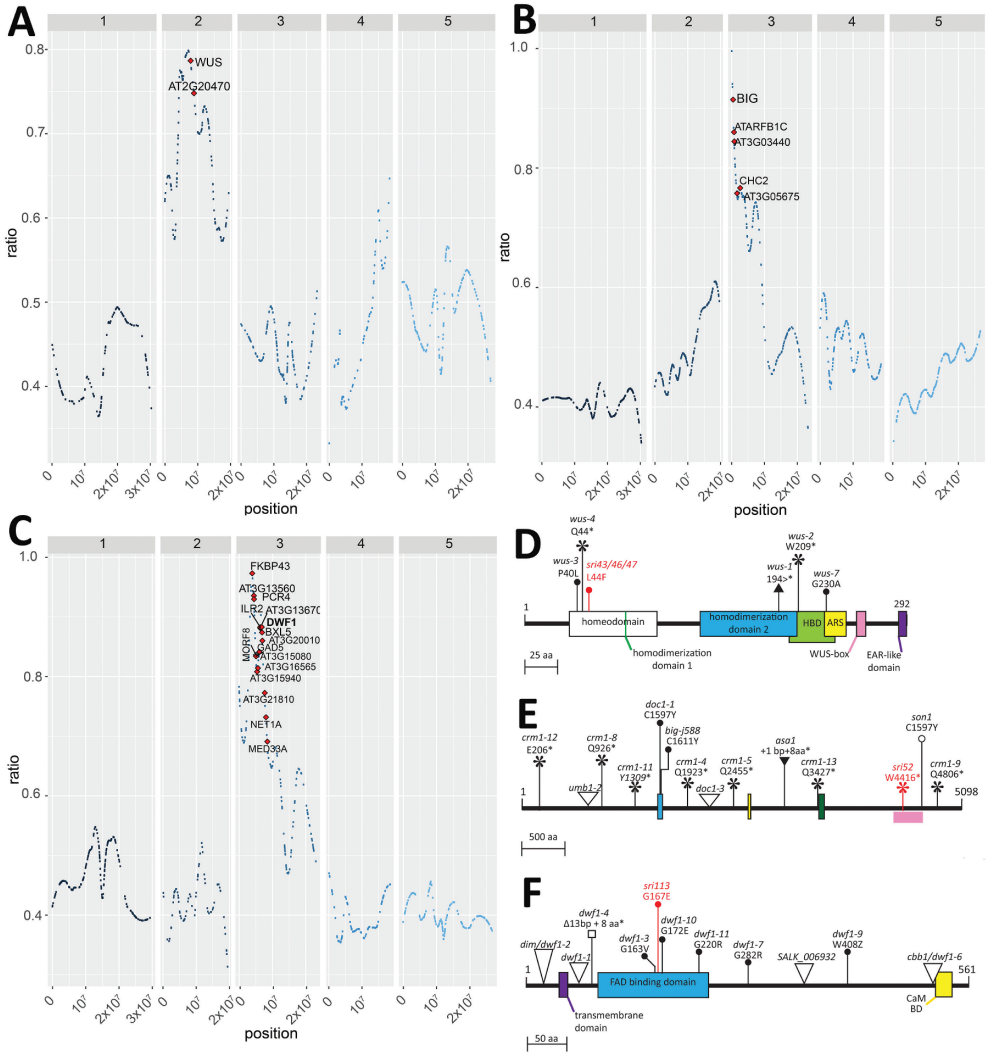

Fig. 4.4: Identification of causal mutations in *ath1-3* suppressor mutants

A-C: LOESS-fitted curves of the ratio variable of SNPs between *ath1-3* and suppressor mutants computed with SIMPLE pipeline for *ath1-3 sri46* (A), *ath1-3 sri52* (B), and *ath1-3 sri113* (C). SNPs with a ratio below 0.3 are not displayed. X-axis: chromosomal number (top), and chromosomal location in bp (bottom); y-axis: ratio variable.

D-F: Schematic representation of the WUS, BIG and DWF1 protein. Markers indicate location of missense (black circle), premature stop (asterisk), or exon-intron site (white circle) point mutations. Lines with open squares or black triangles depict small deletions or insertions, respectively. White triangles mark sites of T-DNA or transposable element insertion. Red line marks site of mutation identified in this study

D: Schematic representation of the WUS protein annotated with known *wus* alleles and functional protein domains (homeodomain, homodimerization domain 1; homodimerization domain 2, HAIRY MERISTEM binding domain (HBD), acidic residue stretch (ARS), WUS-box, EAR-like domain (Rodriguez et al., 2016; Snipes et al., 2018).

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Selection of EMS *ath1-3* enhancer mutants

In parallel to screening for EMS suppressor mutants, we also screened for mutations that enhanced heterochronic bolting in *ath1-3*. To this end we selected 17 M2 mutants with rosette internodes that appeared more elongated than *ath1-3* control plants and dubbed these *enhancer of ath1-3 rosette internodes* (*eri*) mutants. M3 progeny of non-sterile *eri* mutants (15) were grown in 22°C conditions, a condition that does not strongly induce heterochronic bolting in *ath1-3*, thereby facilitating the selection of plants with enhanced elongation of rosette internodes. Per line ten M3 individuals were grown and average rosette internode elongation was compared to *ath1-3* mutants. Four lines were identified that were significantly more elongated than *ath1-3* (Figure 4.5A; Table 4.1): *ath1-3 eri14*, *ath1-3 eri15*, *ath1-3 eri31* and *ath1-3 eri81*. Remarkably, while rosette internodes of *ath1-3 eri81* mutants were more elongated than *ath1-3*, inflorescence stems of *ath1-3 eri81* were short (Fig. 4.5D.

In addition to heterochronic bolting, *ath1-3* mutants are characterized by formation of fused cauline leaf tissue to the stem and reduced abscission of anthers post-fertilization (Bao, 2009; Gómez-Mena and Sablowski, 2008). These phenotypes were also enhanced in *ath1-3 eri* mutants. In the *ath1-3 eri14*, *ath1-3 eri15* and *ath1-3 eri31* mutants, fusions were observed on more cauline leaves than in *ath1-3* single mutants and often a larger part of the stem was fused to cauline leaves, which in some cases resulted in bending of the stem itself (Figure 4.5C-E, G, data not quantified). In addition, fusions were also clearly visible on rosette internodes of *ath1-3 eri14, ath1-3 eri15* and *ath1-3 eri81* mutants, a phenotype that is not seen in LD-grown *ath1-3* mutants. Floral organ abscission was enhanced in *ath1-3 eri31* (Fig 4.5H) and *ath1-3 eri81* mutants, where in addition to anthers, petals also remained attached to siliques.

Fig. 4.4 (Continued from previous page):

E: Schematic representation of the BIG protein annotated with a selection of known mutations in *BIG*, and functional protein domains (cysteine-rich domain-1/ubiquitin protein ligase E3 component N-recognin box (CRD-1/UBR-box), ZZ-type zinc finger domain (ZZ), cysteine-rich domain-2 (CRD-2)).

Pink box marks region skipped in the *son1* mutant (Gil et al., 2001; Guo et al., 2013; Hearn et al., 2018b; Yamaguchi et al., 2007).

F: Schematic representation of the DWF1 protein, marking known *dwf1* alleles and functional domains (putative membrane domain, FAD binding domain, calmodulin binding domain (CaMBD) (Choe et al., 1999; Du and Poovaiah, 2005; Klahre et al., 1998)).

Apart from these organ boundary-related defects, two of the *eri* mutants, *ath1-3 eri14* and *ath1-3 eri15*, were late bolting and on average formed over two times more rosette leaves than *ath1-3* (10.5±1.6 vs. 24.5±1.8 or 28.2±1.6, respectively, Fig 4.5B).

To identify causal mutations, a similar approach was taken as for the *sri* mutants, however, candidates for causal mutations could not be identified as mapping of SNPs gave multiple peaks of equal height for every line (Supplemental Fig. 4.S2). Selecting for an enhanced heterochronic bolting phenotype is less black and white than selecting for suppression of this phenotype and, possibly, false positive BC1F2 plants could have been selected and sequenced, thereby increasing noise during calculation of allelic ratios.

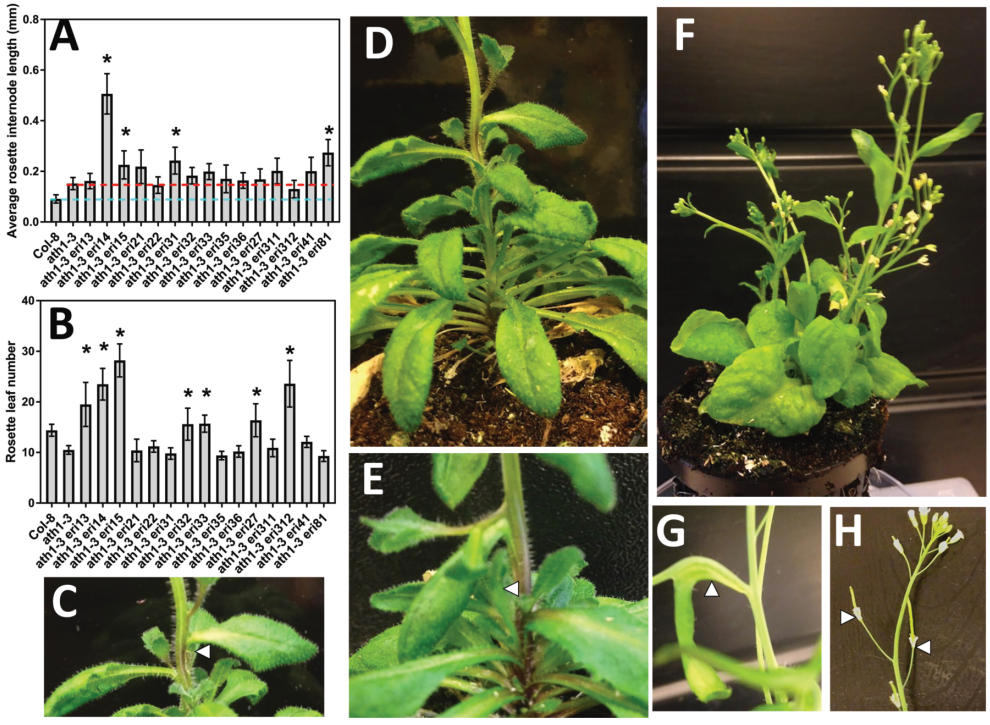


Fig. 4.5: *ath1-3 eri* mutants exhibit enhanced rosette internode elongation and boundary defects
A-B: Average internode elongation (A) and rosette leaf number (B) of Col-8, *ath1-3* and *ath1-3 eri* M3 mutants grown in LD at 22°C (n=10, except *ath1-3 sri37*, where n=8). Asterisks denote lines that were significantly different to *ath1-3,* tested through 1-way ANOVA followed by Tukey's post-hoc test (α=0.05; see Supplemental Table 4.S1). Dashed lines represent average elongation of Col-8 (blue) and *ath1-3* (red). Error bars represent standard deviation of the mean.
C-H: Phenotypes observed in *ath1-3 eri14* (C-D), *ath1-3 eri15* (E), *ath1-3 eri81* (F) and *ath1-3 eri31* (G-H) M3 enhancer mutants, such as strong cauline-leaf fusions (C, E, G; arrowheads), loss of abscission of petals and stamens post-fertilization (H; arrowheads) and short stature (F).

Heterochronic bolting of *ath1-3 sri113* is restored through BR application

As mentioned, the *ath1-3 sri113* mutant carries a Gly167 → Glu mutation in the key BR biosynthesis gene *DWF1*. DWF1 acts early during BR biosynthesis by catalysing the conversion of 24-methylene cholesterol to 24-campesterol and *dwf1* mutants can therefore be rescued by BR application (Choe et al., 1999; Kauschmann et al., 1996; Klahre et al., 1998; Youn et al., 2018). Thus, *sri113* plants are likely BR-deficient. As heterochronic bolting in *ath1-3* requires brassinosteroids (Chapter 3), we tested whether restoration of a wild-type rosette habit in *ath1-3 sri113* mutants is caused by BR deficiency. For this, *ath1-3 sri113* BC_1F_3 plants were grown at 22°C and 27°C and treated three times a week with 1 µM epi-brassinolide (BL) until anthesis. In both conditions BL treatment was sufficient to restore heterochronic bolting in *ath1-3 sri113* (Figure 4.6A-B, E), strongly indicating that the observed mutation in *DWF1* is the causal mutation for suppression of rosette internode elongation in *ath1-3* mutants.

Apart from BL, both auxin and GA are known to enhance heterochronic bolting in *ath1-3*, while chemical inhibition of GA inhibits this process, even in the presence of constitutive BR signalling (Chapter 3). However, it is not known if the opposite is also true, i.e. whether BR is essential for GA or auxin-mediated elongation of rosette internodes. To test this, *ath1-3 sri113* BC_1F_3 plants were grown at 22°C and treated with 100 µM GA4+7 or 5 µM of the synthetic auxin picloram until anthesis (Fig 4.6B-D). Neither picloram or GA could induce heterochronic bolting in *ath1-3 sri113*, while these treatments did induce heterochronic bolting in *ath1-3* plants. When plants were treated with a mixture of GA and BL or picloram and BL, *ath1-3 sri113* mutants responded the same as *ath1-3* mutants. These results suggest that a complex, cooperative, and interdependent relationship exists between GA, auxin and BR in inducing internode elongation.

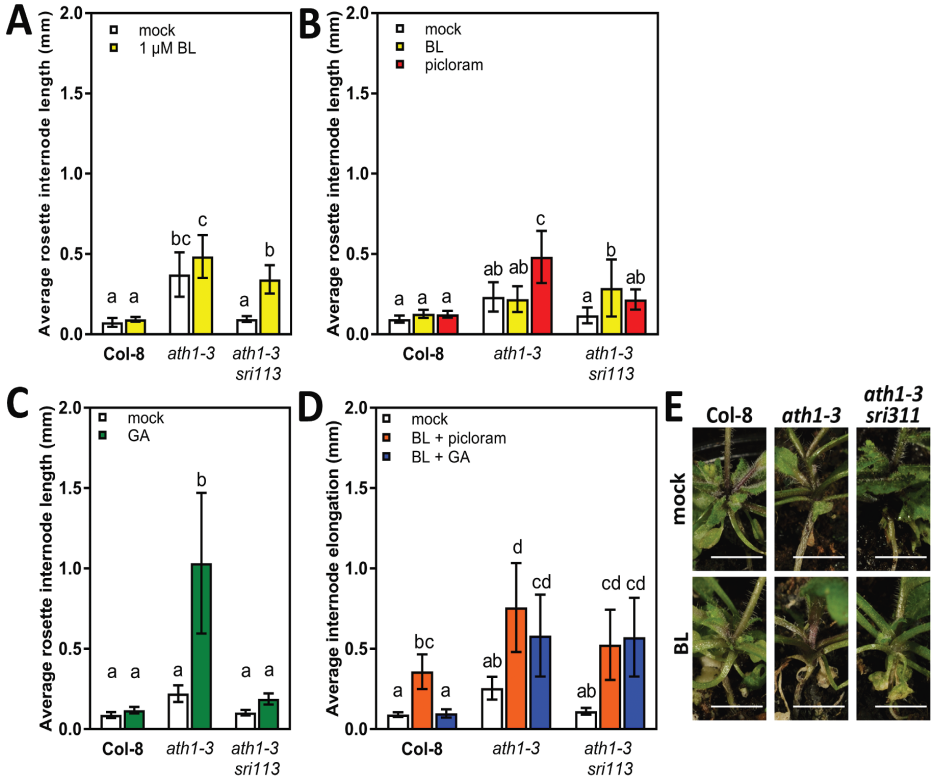


Fig. 4.6: The *ath1-3 sri113* mutant can be rescued with applied BL, but not with GA4+7 or picloram alone

A: Average internode elongation of Col-8, *ath1-3* and *ath1-3 sri113* plants (n=10) grown at 27°C.
B-D: Average rosette internode of Col-8, *ath1-3* and *ath1-3 sri113* plants grown at 22°C and treated with 5 µM picloram or 1 µM BL (B; n=10), 100 µM GA4+7 (C; n=10, 10, 10, 10, 10, 6) or a combination of 1 µM BL with 1 µM picloram or 1 µM BL with 100 µM GA4+7 (D; n=10, 10, 10, 10, 8, 10, 10, 6, 10).
E: Representative phenotypes of plants depicted in A. Plants are 28 days old. Scale bar represents 5 mm.
Error bars represent standard a of the mean, lowercase letters denote statistically homogeneous subsets as defined by 1-way ANOVA followed by Tukey's post-hoc test (see Supplemental Table 4.S1)

The *sri93* mutation affects boundary gene expression and inhibits hormonal induction of heterochronic bolting in *ath1-3*

Although no candidate causal mutation could be identified for *sri93*, the compact rosette with inward curled leaves phenotype of *ath1-3 sri93* mutants strongly resembles the vegetative phenotype of plants that ectopically overexpress or carry gain-of-function alleles of boundary genes, such as AS2, BOP1, BOP2, CUC3, LOB, and LOF1 (Jun et al., 2010; Li et al., 2010; Nakazawa et al., 2003; Shuai et al., 2002). Plants ectopically expressing the boundary gene *JLO* also form compact rosettes,

but do not form folded leaves (Borghi et al., 2007). The perturbation of boundary gene function can affect rosette internode elongation (Chapter 2, 3) (Supplemental fig. 4.S3) and *BOP1*, *BOP2*, and *LOB* have been identified as (direct) transcriptional targets of ATH1 (R. Sablowski, personal communication; Chapter 2). Therefore, this raises the possiblilty that the *ath1-3 sri93* phenotype is caused by ectopic boundary gene expression. To test this, we compared *LOB, AS1, AS2, BOP1, BOP2, CUC3, JLO* and *LOF1* expression in 7-day- old *ath1-3 sri93* seedlings to that of Col-8 and *ath1-3* seedlings of the same age. z Taken together, the characteristic plant morphology of *ath1-3 sri93* plants and the effect of the sri93 mutation on the expression of a specific subset of boundary genes, suggests that the causal mutation in this line is within an organ boundary regulator. However, as expression of several of the boundary genes analysed is affected but for none of them to substantially higher levels than in control plants, it remains to be seen whether the causal mutation in this line corresponds to one of the investigated loci (Fig. 4.7A-I). It is possible that restoration of *LOB* expression in *ath1-3 sri93* plants underlies gain of rosette habit in these plants. This could be the result of either a gain-of-function mutation in *LOB* itself, causing only a subtle and/or very localized increase in expression, or an indirect effect of perturbation of boundary formation due to the *sri93* mutation. Given that *LOB* expression is not solely controlled by *ATH1* (Chapter 2), but is also under the control of by the boundary genes *AS1*, *AS2*, *BOP1* and *BOP2* (Bell et al., 2012; Byrne et al., 2002; Ha et al., 2007), the second alternative seems the most likely of the two. If the *sri93* mutation affects protein function or stability, instead of gene expression, we therefore cannot rule out any of the boundary genes investigated so far as candidate locus for causal mutation.

The meristem-organ boundary is a region of low auxin, low BR and low cell proliferation (Bell et al., 2012; Gendron et al., 2012; Rast and Simon, 2012). The vegetative RZ is also a site of low cell proliferation, and reporter gene analysis suggests that it also is a site of low auxin and BR (Brunoud et al., 2012; Metzger and Dusbabek, 1991; Sachs et al., 1959a; Sandhu et al., 2012). We previously showed that BR and auxin partly act upstream of *ATH1,* as combined application of BR and auxin reduces *ATH1* reporter expression and induces heterochronic bolting in plants carrying a wild-type *ATH1,* but not in plants expressing *ATH1* from a 35S promoter (Chapter 3). As *ath1-3 sri93* mutants lack *ATH1*, but appear to have restored

expression of at least one boundary gene functioning downstream of *ATH1, i.e. LOB*, it is possible that *ath1-3 sri93* mutants are insensitive to hormonal induction of heterochronic bolting. Indeed, unlike in Col-8 and *ath1-3*, rosette internodes of *ath1-3 sri93* mutants did not elongate in response to combined picloram and BL treatment (Fig. 4.8), nor in response to singular hormone treatment using picloram, GA or BL (data not shown). These findings strongly suggest that the *sri93* mutation reinitiates the block on elongation normally enforced by *ATH1*, possibly by acting on boundary genes and auxin/BL-induced targets downstream of *ATH1.* Furthermore, these data point towards an essential role for meristem-organ boundaries in the maintenance of the rosette habit

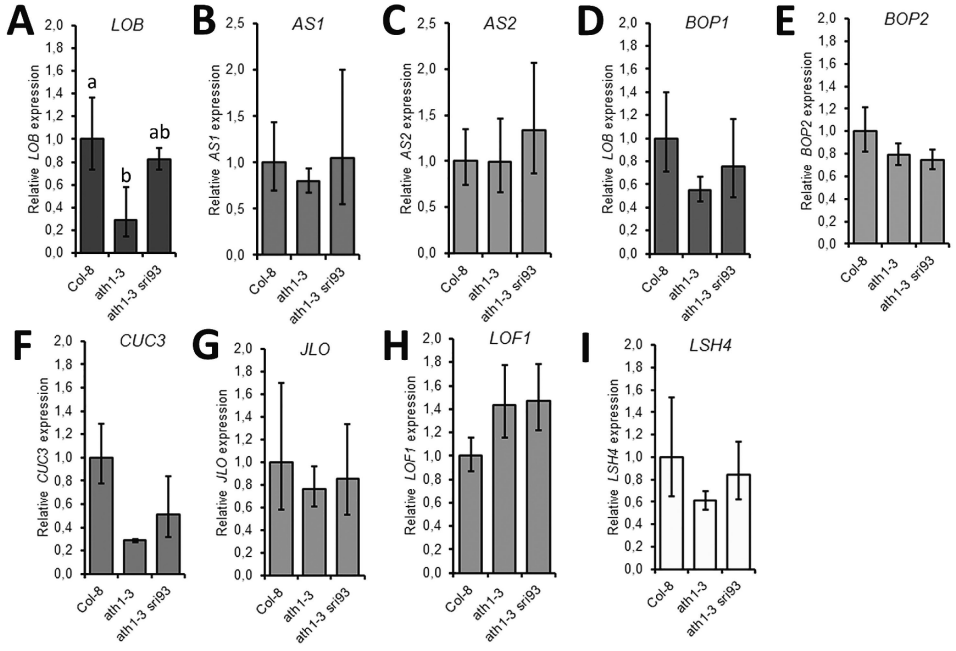


Fig. 4.7: Boundary gene expression in Col-8, *ath1-3* and *ath1-3 sri93*
Expression of *LOB* (A), *AS1* (B), *AS2* (C), *BOP1* (D), *BOP2* (E), *CUC3* (F), *JLO* (G), *LOF1* (H), *LSH4* (I), in 7-day old seedlings grown in LD conditions at 22°C.
Expression is relative to *At5g15400*. Error bars represent standard deviation calculated from the ΔCT mean. Lowercase letters denote statistically homogeneous subsets as defined by 1-way ANOVA followed by Tukey's post-hoc test (see Supplemental Table 4.S1).

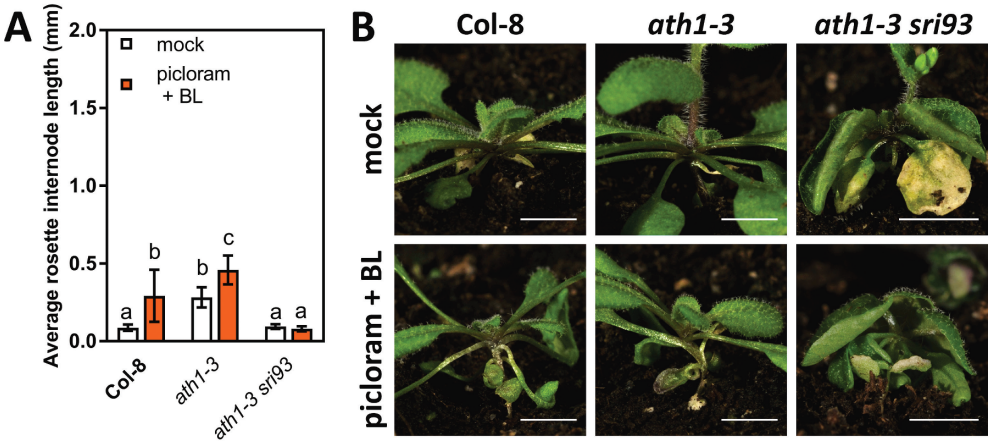


Fig. 4.8: Combined picloram and BL treatment cannot induce heterochronic bolting in *ath1-3 sri93*
A: Average rosette internode elongation of Col-8, *ath1-3* and *ath1-3 sri93* plants grown at 22°C and treated with a mock solution or 1 µM picloram and 1 µM BL. Lowercase letters denote statistically homogeneous subsets as defined by 1-way ANOVA followed by Tukey's post-hoc test (n= 10, 11, 13, 7, 11, 12; see Supplemental Table 4.S1). Error bars represent standard deviation of the mean.
B: Representative phenotypes of the plants from A. Plants are 25 days old, scale bar represents 5 mm.

Discussion

The *ath1-3 sri113* and *ath1-3 sri52* mutants validate the link between phytohormones and heterochronic bolting in *ath1-3*

Initiation of bolting and subsequent stem elongation requires the function of several hormones, primarily GA, auxin and BR, which can stimulate cell division and cell elongation. These hormones are subject to a complex web of inter-regulation and, as such, disruption of a single of these hormonal components can strongly repress elongation of the inflorescence stem. For formation of the rosette, where internodes do not elongate, it therefore follows that the responses to these hormones must be locally blocked. We previously showed that *ath1-3* mutants exhibit heterochronic bolting upon treatment with auxin, GA or BR, while heterochronic bolting was repressed through chemical inhibition of BR or GA (Chapter 2, 3). While complementation analysis of *ath1-3 sri113* is still needed to confirm that this mutation is the causal mutation of *sri113*, the most likely candidate for the *sri113* mutation is situated at a conserved residue in the DWF1 FAD-domain, a domain required for the catalytic activity of DWF1 (Choe et al., 1999; Klahre et al., 1998). Phenotypically, *ath1-3 sri113* mutants (Fig. 4.2, 4.3A) strongly resemble *dwf1* mutants that have strongly reduced BR biosynthesis (Choe et al., 1999; Du and Poovaiah, 2005), and heterochronic bolting of *ath1-3 sri113* could be restored

through BR application (Fig 4.6A, E). Most strikingly, heterochronic bolting could not be induced with GA or auxin in *ath1-3 sri113*, unless BR was applied (Fig 4.6). This demonstrates an interdependency of GA, auxin and BR during heterochronic bolting, showing that loss of a single hormone may be sufficient to restore rosette formation in *ath1-3*.

The *ath1-3 sri52* suppressor mutant ties into this. The candidate mutation for *sri52* is situated in the *BIG* gene, which encodes a 560 kD protein of unknown function. The reduced height and rosette leaf shape of *ath1-3 sri52* resembles the phenotypes of other known *big* mutants, including the *corymbosa1-9* (*crm1-9*) mutant, which also is predicted to form a slightly truncated BIG protein (Yamaguchi et al., 2007), like *sri52*. Mutations in *BIG* affect several auxin-regulated processes, and while the precise function of *BIG* is unclear, it is thought that it stabilizes PIN-FORMED 1 (PIN1) auxin efflux transporters at the plasma membrane, promoting auxin flow. BIG protein has been detected in microsomal fractions containing PIN1, PIN2 and the ATP-binding cassette auxin efflux transporter ABCB19/PGP19, which is also linked to PIN membrane stabilization (Gil et al., 2001; Titapiwatanakun et al., 2009; Yamaguchi et al., 2007). Auxin transport is also strongly reduced in *big* inflorescence stems and these mutants form shorter inflorescence stems (Gil et al., 2001; Guo et al., 2013; Ruegger et al., 1997). This reduction in height has been correlated with a reduction in both cell length and cell number during later stages of internode elongation, which was similar to internode cell lengths and cell number measured in auxin signalling mutants (Yamaguchi and Komeda, 2013).

To confirm that the *ath1-3 sri52* phenotype is indeed caused by a mutation in *big*, complementation analysis must be performed. However, due to the phenotypical similarities between *ath1-3 sri52* and other *big* mutants, it is very likely that this is the causal mutation. Furthermore, *BIG* has been previously linked to heterochronic bolting. The *big* mutant allele *attenuated shade avoidance 1* (*asa1*), was shown to repress all phenotypes of the *phytochrome A* (*phyA*) *phyB* double mutant, including elongation of the rosette internodes (Kanyuka et al., 2003). This is similar to our findings that loss of *BIG* suppresses heterochronic bolting of *ath1-3.* As auxin is required for shade-induced and temperature-induced elongation, which both are phytochrome regulated processes (Franklin et al., 2011; Jung et al., 2016; Tao et al., 2008) and auxin strongly induces heterochronic bolting in *ath1-3*, reduced auxin

function through loss of *BIG* may explain suppression of heterochronic bolting observed in *ath1-3 sri52* mutants. However, it should be noted that mutations in *big* also affect the sensitivity to, and biosynthesis of other hormones, including GA, cytokinins and ethylene while *BIG* itself is repressed by jasmonic acid (Desgagné-Penix et al., 2005; Kanyuka et al., 2003; Sponsel et al., 1997; Zhang et al., 2019). Thus, it is possible that sensitivity to multiple hormones is lost in *ath1-3 sri52*. To fully understand the role of *BIG* downstream of *ATH1*, a more comprehensive analysis of *sri52* and its effects on hormone sensitivity in *ath1-3* is necessary. For example, analysis of *ath1-3 sri113* has shown the interdependence of auxin, GA and BR for heterochronic bolting. Thus it would be very informative to whether test whether RZ-specific biosynthesis of auxin restores heterochronic bolting in *ath1-3 sri52*, or whether auxin alone is insufficient.

Boundary gene function contributes to formation of compact rosette internodes

Tight regulation auxin and BR levels is also required for organ boundary formation (Bell et al., 2012; Gendron et al., 2012; Rast and Simon, 2012). Our findings show that boundary gene function is needed for rosette habit formation. This raises the question whether a boundary programme is active in the vegetative RZ. If this is the case, possibly the maintenance of the rosette habit, and by extension the repression of bolting by boundary genes (and ATH1) is simply caused by the reduction of auxin and BR in the RZ, similar to what is observed in meristem-organ boundaries.

The combined application of BR and auxin is sufficient to induce heterochronic bolting in plants carrying a wild-type *ATH1* allele, most likely through downregulation of *ATH1* (Chapter 3), which suggests that the vegetative RZ is a region of low auxin and BR. Similarly, the combination of low auxin and low BR characterizes meristem-organ boundaries (Bell et al., 2012; Gendron et al., 2012; Rast and Simon, 2012), a region of very low cell proliferation. *BOP1* and *LOB* both antagonise BR, while *JLO*, *AS1* and *AS2* restrict auxin in the boundary through regulation of auxin transport (Bell et al., 2012; Rast and Simon, 2012; Shimada et al., 2015). When expressed ectopically, several boundary genes inhibit internode elongation. These genes include *AS2, BOP1, BOP2, CUC3, JLO, LOB, LOF1* and *LOF2* (Borghi et al., 2007; Lee et al., 2009; Li et al., 2010; Nakazawa et al., 2003; Norberg, 2005; Shuai et al., 2002; Vroemen et al., 2003). Misexpression of *LSH4* in plants lacking the BLH-type transcription factor *PENNYWISE* (*PNY*; *BELLRINGER*; *REPLUMLESS*; *VAAMANA*;

LARSON; *BEL1-LIKE HOMEODOMAIN 9*) also represses stem elongation (Bencivenga et al., 2016). The *ath1-3 sri93* mutant closely resembles plants ectopically expressing boundary genes. While the causal mutation of *ath1-3 sri93* could not be identified, expression analysis revealed that *LOB* expression is restored to wild-type levels in *ath1-3 sri93* (Fig. 4.7). *LOB, BOP1, BOP2* and *LSH4* were recently identified as targets of ATH1 through ChIP-seq analysis (R. Sablowski, personal communication). ATH1 induces expression of BOP1 and LSH4, and expression of *LOB, BOP1* and *LSH4* is reduced in *ath1-3* seedlings (Chapter 2). *CUC3* expression was also reduced in *ath1-3,* but was slightly increased in *ath1-3 sri93,* compared to *ath1-3* (Fig. 4.7). Boundary formation is disrupted in *ath1-3* mutants, which results in stem-cauline leaf fusions and reduced floral organ abscission (Bao, 2009; Gómez-Mena and Sablowski, 2008). We showed previously that GA or auxin application induces heterochronic bolting in *lob* mutants (Chapter 2, 3). Heterochronic bolting could also be induced using GA or auxin treatments in *bop1-3 bop2-1*, *as1-1* and *as2-2* mutants (Supplementary Fig. 4.S3). Although *lof1-1*, *lof2-1*, *lsh3-1* and *lsh4-1* did not respond to hormone treatments mutants, it is possible that effects are only seen in higher order mutants as these genes belong to two large gene families in which functional redundancy may occur. Taken together, these findings highlight the importance of boundary genes in maintaining a compact rosette habit.

If a boundary programme prevents internode elongation in the rosette, the expectation is that boundary genes are expressed in the vegetative RZ. However, thus far, *LSH3* and *LSH4* are the only boundary genes whose expression has been detected in the RZ (Takeda et al., 2011). A target of *LOB* and key BR catabolic enzyme— *PHYB ACTIVATION TAGGED SUPPRESSOR 1* (*BAS1*), is expressed in the RZ. Moreover, *BAS1* expression in the RZ is limited to the vegetative growth phase, and is not expressed in the RZ during reproductive growth (Bell et al., 2012; Sandhu et al., 2012). Studies of auxin reporter lines suggest that auxin levels in the RZ are also low during vegetative growth and high during reproductive growth (Brunoud et al., 2012; Shi et al., 2018). In addition, auxin rapidly induces bolting in lettuce, where a sharp peak in stem tip auxin levels precedes bolting (Hao et al., 2018; Liu et al., 2018b). Therefore, it would be informative to study changes in auxin and BR signalling in the RZ during the induction of bolting in wild-type plants, *ath1-3* or boundary gene mutants to elucidate this mode of regulation.

Enhancers of *ath1-3* heterochronic bolting also enhance boundary defects

In addition to mutants that no longer exhibited heterochronic bolting, four EMS mutants that exhibited increased heterochronic bolting were selected. Analysis of *ath1-3 eri* mutant phenotypes reinforced the link between the rosette habit and organ boundaries. Besides enhanced heterochronic bolting, *ath1* mutants exhibit boundary defects as cauline leaves of *ath1* are (partially) fused to inflorescence stems, and stamens no longer abscise from flowers after pollination. In *ath1 eri* mutants, both heterochronic bolting and boundary defects are enhanced compared to *ath1-3* (Fig.4.5, Table 4.1).

Interestingly, while *ath1-3 sri* mutants did not exhibit heterochronic bolting, they all still exhibited stem-cauline leaf fusions and reduced abscission of stamens after fertilization (Table 4.1). This is also the case in *ath1-3 pif7-1* mutants, where heterochronic bolting is suppressed in FR conditions, but other *ath1-3* defects remained present (Chapter 3, data not quantified). In contrast, two of the *ath1-3* enhancer mutants, *ath1-3 eri31* and *ath1-3 eri81* mutants not only exhibited enhanced heterochronic bolting, but also increased stem-cauline leaf fusion and floral organ abscission defects (Fig. 4.5). Similarly, mutations in *PNY* or the ATH1 interaction partners KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2 (KNAT2) or KNAT6 enhance several phenotypes of *ath1-3* besides heterochronic bolting (Chapter 6, (Bao, 2009)). Likewise, elevated BR signalling enhances heterochronic bolting and boundary defects in *ath1-3* (Chapter 3). Possibly, disruption of more general processes, such as hormone function, may be enough to exacerbate *ath1-3* developmental defects that lie at the basis of heterochronic bolting, formation of fused stem-cauline leaf tissue and loss of floral abscission. This would explain why enhancing heterochronic bolting of *ath1-3* often also enhances floral abscission defects and stem-cauline leaf fusions. On the other hand, the probability of finding a single mutation that completely restores three different developmental defects is much lower. Targets of *ATH1* that affect the elongation of internodes also do not affect both stem-cauline fusion and abscission. Loss of *LOB* or *BOP1* and *BOP2* makes plants sensitive to hormonal induction of heterochronic bolting (Chapter 2, Chapter 3, Supplementary fig. 4.S7). However, *lob-3* mutants exhibit stem-cauline leaf fusions, but do not exhibit abscission defects, while the opposite is true for *bop1-3 bop2-1* mutants (Bell et al., 2012; Norberg, 2005; Shuai et al., 2002). This indicates that *LOB* and *BOP1/2* do not act in all *ATH1*-regulated processes.

The *ath1-3 eri14* and *ath1-3 eri15* mutants adopt similar phenotypes, exhibiting both enhanced heterochronic bolting and stronger stem-cauline leaf fusions (Fig 4.5). Interestingly, these plants were also late bolting, forming over two times more rosette leaves compared to *ath1-3*. This increase in leaf number is reminiscent of plants grown in conditions that are non-inductive to flowering, e.g. short-day (SD) conditions. In SD conditions, total rosette internode elongation and stem-cauline leaf fusions of *ath1-3* mutants are enhanced, but floral organ abscission is unchanged compared to LD-grown *ath1-3* (M. Proveniers, unpublished data). SD-grown *ath1-3* form more than 30 rosette leaves ((Rutjens, 2007), Chapter 6), but otherwise mirror the phenotypes of *ath1-3 eri14* and *ath1-3 eri15*. Growth conditions that delay the reproductive phase change, such as SD photoperiods and low light conditions, also enhance heterochronic bolting and stem-cauline leaf fusions, but do not affect floral organ abscission (M. Proveniers, unpublished data). Therefore, it is quite likely that the causal mutation in these lines enhances *ath1-3* phenotypes by delaying the reproductive phase change.

Mutations in *WUS* may repress heterochronic bolting

The formation of Arabidopsis shoot tissue relies on a delicate balance of stem cell maintenance in the SAM and differentiation towards organ primordia or the RZ (Gaillochet et al., 2015). Meristem-organ boundaries enforce the balance between SAM and organ primordia (Žádníková and Simon, 2014) and likely also affect the RZ, as discussed previously. Stem cell maintenance in the shoot is regulated by a molecular negative feedback loop formed by the CLAVATA (CLV)-WUSCHEL (WUS) pathway (Lenhard and Laux, 2003; Schoof et al., 2000). Balanced signalling between WUS and CLV3 is fundamental to positioning of meristem-organ boundaries (Müller et al., 2006). Given that organ boundary genes are relevant to the control of RZ function (Bencivenga et al., 2016) (Supplementary Fig. 4.S3), this might shed light on the identification of a point mutation in the homeodomain domain of *WUS* as causal mutation in the *ath1-3 sri4* suppressor mutant. As *sri4* represents a loss of function allele, in *ath1-3* mutants, *WUS* might be ectopically expressed, thereby stimulating elongation of rosette internodes. The *wus* gain of function mutant *stem ectopic flowers* (*sef*), which expresses ectopic *WUS* in the hypocotyl, shows increased hypocotyl growth. Interestingly, while hypocotyl growth is normally achieved through cell elongation alone, ectopic WUS in *sef* causes hypocotyl growth

by promoting cell division in the hypocotyl (Zhang et al., 2013).

Effects of ectopic *WUS* on the RZ have been observed in the meristems of *clv3* mutants, where *WUS* expression is strongly increased. The *clv3* SAM and IM are greatly enlarged along the apical-basal axis (Mandel et al., 2016; Schoof et al., 2000). The RZ of vegetative *clv3* mutants is also significantly larger than that of wild-type plants, and *clv1* and *clv3* IMs contain large, vacuolated cells in the L3 (Mandel et al., 2016; Schoof et al., 2000). Furthermore, a genetic link between bolting, the *WUS-CLV3* loop and the BLH-type transcription factors *PNY* and *POUND-FOOLISH* (*PNF*; *BLH8*) has been reported previously. Unlike *ATH1*, *PNY* and *PNF* are classified as positive regulators of bolting and flowering, as *pny pnf* double mutants do not bolt or flower. During vegetative growth, *pny pnf* plants form a smaller SAM, which does not transition to an IM after exposure to floral inductive signals (Smith et al., 2004; Ung et al., 2011). The RZ of these plants remains inactive and the SAM continues to form leaf primordia (Smith et al., 2004). The expression domain of *CLV3* in *pny pnf* is much broader than that of wild-type plants, while the expression of *WUS* is decreased, which could explain their smaller meristems. However, this change in expression also appears to be important for RZ activity as *WUS* expression, bolting, and flowering is restored in *pny pnf clv3* triple mutants. Subsequent loss of *WUS* reverts these plants back to non-bolting and non-flowering (Ung et al., 2011). This suggests that the effects of *WUS* on the RZ are biologically relevant for (heterochronic) bolting. Inflorescence internode elongation is also affected in *CLV3* overexpressors, which can bolt but form aerial rosettes: areas where internode elongation between cauline leaves is temporarily repressed (Brand et al., 2000). Together, these findings suggest that *WUS* function may be an important factor in the regulation of internode elongation, possibly by regulating RZ cell number or cell allocation to the RZ.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Col-8 was used as a wild type. Mutants used were *ath1-3* (Proveniers et al., 2007), *bop1-3 bop2-1* (Norberg, 2005), *cuc1-1* (Aida et al., 1997), *lsh4-1* (Takeda et al., 2011), *jlo-1*, *lof1-1* (Lee et al., 2009), *lof2-1* (Lee et al., 2009), *as1-1* and *as2-2* (Ori et al., 2000) were described previously. Plants are in the Col background, with the exception of *cuc1-1*, *as1-1* and *as2-2*, which are in the Ler,

Ler/Col and ER backgrounds respectively. For *LSH3,* a T-DNA insertion line with an insertion in the promoter of *LSH3* (SALK_123953C) was used, hereafter termed *lsh3-1.*

Seeds were stratified for 2 days at 4°C on soil or on Murashige and Skoog medium (Duchefa) agar plates unless otherwise specified. Plants were grown at 22°C or 27°C, in long-day (LD; 16h light, 8h dark) or short-day (SD; 8h light, 16h dark) photoperiods under white light (Sylvania, Luxline Plus, Cool White; 120 µmol/m^2/s) or at LD 22°C under red, blue and far-red LED light (FR; 120 µmol/m^2/s; FR ratio 1.34). Relative humidity was 70%. Plants grown at 27°C were always first placed in LD 22°C conditions for 2 days to induce germination before they were transferred to 27°C for further growth. On soil, plants were grown at a density of 0.1 seeds/cm^2 unless specified otherwise.

EMS treatment of *ath1-3* seeds and mutant selection

Approximately 50.000 *ath1-3* seeds (2g) were divided into three batches and stratified overnight at 4°C in water, then incubated overnight in 0.3%, 0.4% or 0.6% v/v ethyl methanesulfonate (EMS; Sigma-Aldrich) at room temperature, under gentle shaking. M1 seeds were thoroughly washed to remove residual EMS and grown in LD conditions. M1 plants were split into 40 pools for M2 seed harvesting. M2 seeds from the 0.3% EMS-treated M1 batches were suspended in 0.1% agarose and planted onto soil at high density (approximately 5.3 seeds/cm^2), and grown for 17-24 days at 27°C, LD. Plants that no longer exhibited heterochronic bolting (*sri* mutants) and plants that appeared more elongated than neighbouring plants (*eri* mutants) were transferred to fresh soil and placed in FR conditions. If *ath1-3 sri* mutants exhibited heterochronic bolting under these conditions, these lines were discarded. M3 seeds were harvested from individual plants.

M3 *ath1-3 sri* mutants were screened again under the conditions of the initial M2 screen to identify true suppressors, which were back-crossed to *ath1-3* (BC1F1), and selfed (BC1F2). BC1F2 seeds were grown at 27°C in LD for 15 days at medium density (0.49 seeds/cm^2). Plants exhibiting heterochronic bolting were removed and remaining plants were grown for an additional 15 days at 27°C in SD conditions to increase biomass. To select enhancers, 10 mutants per M3 *ath1-3 eri* mutant line were grown at standard density in 22°C LD conditions until flowering. Average internode elongation of *ath1-3 eri* mutants was measured and compared to *ath1-*

3 (1-way ANOVA, Tukey's post hoc test). Mutant lines that were significantly more elongated than *ath1-3* were back-crossed to *ath1-3*. BC1F2 seeds were grown at 22°C in LD conditions at standard density.

From each BC1F2 *ath1-3 sri* and *ath1-3 eri* line, leaf material was harvested and pooled from at least 50 individuals exhibiting the suppressor or enhancer phenotype, and from 50 parental *ath1-3* mutants. Genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN).

Sequencing and identification of causal mutations

Library prep and sequencing was performed at the Utrecht Sequencing Facility (USEQ; www.useq.nl). Libraries of genomic DNA were made from 500 ng of genomic DNA using the TruSeq DNA Nano LT kit (Illumina) and sequenced using the Illumina NextSeq500 sequencers, 2x150 bp, >50x coverage. Quality of raw reads was assessed using FastQC version 0.11.8 (www.bioinformatics.bbsrc.ac.uk/projects/fastqc), reads with a Phred score under 20 were trimmed using Trim Galore! version 0.6.0 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The SIMPLE version 1.8.1 bioinformatic pipeline (Wachsman et al., 2017) was used to map reads to the Arabidopsis TAIR10 reference genome, SNP calling and identification of putative causal mutations.

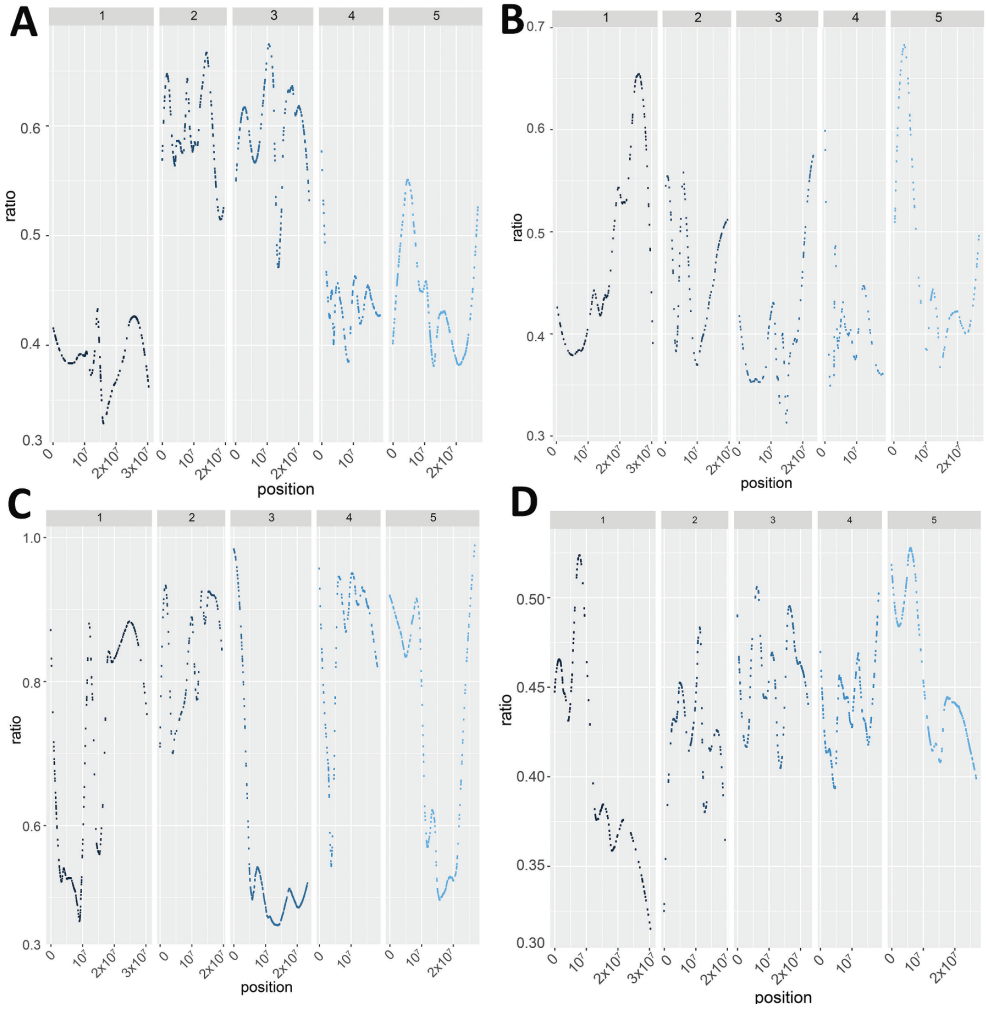
Phenotypic analyses

Average rosette internode elongation was determined by measuring the total rosette internode height from the cotyledons to the last rosette leaf, using a digital calliper, and dividing this by the number of rosette leaves. Hormone treatments were performed by growing plants for 7 days on MS agar plates supplemented with 0.1% DMSO, 5 µM picloram (Sigma-Aldrich), 1 µM epi-brassionolide (BL, Sigma-Aldrich) or 100 µM GA4+7 (Duchefa). On day 7, plants were transferred to soil. Hormone treatment was continued by pipetting 1 µl of 0.1% DMSO, 5 µM picloram or 100 µM GA4+7, or by spaying the rosette with 1 µM BL. Hormone solutions were supplemented with 0.01% Silwet-L55 (Momentive). Hormone treatments were performed three times a week, until opening of the first flower. Statistical analyses were performed using 1-way ANOVA followed by Tukey's post-hoc test (α=0.05) in IBM SPSS Statistics 24, for statistical reporting see Supplemental Table 4.S1.

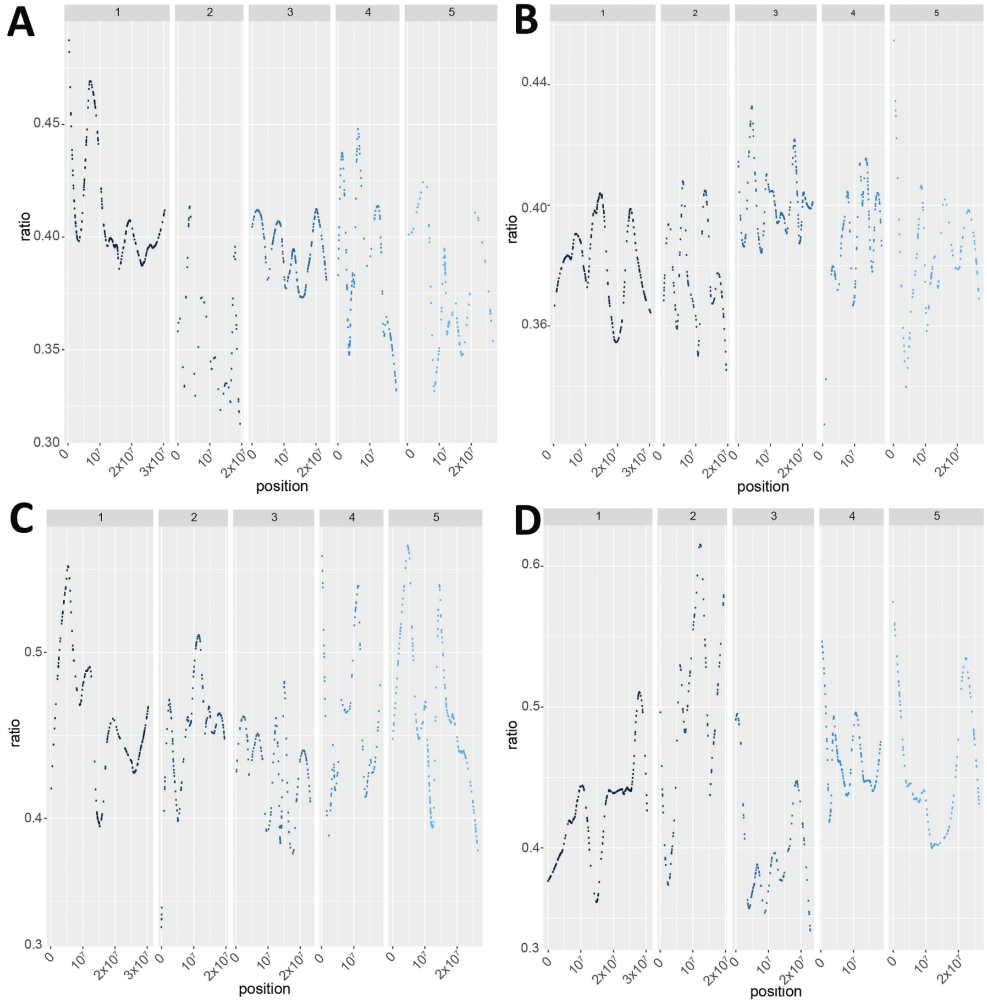
Analysis of gene expression

Boundary gene expression was determined through qRT-PCR analysis. Total RNA was extracted from 7-day old Col-8, *ath1-3* and *ath1-3 sri93* seedlings grown on plate in LD conditions at 22°C as described previously (Oñate-Sánchez and Vicente-Carbajosa, 2008). RNA was treated with DNAseI (Thermo-Fisher) and used for cDNA synthesis using RevertAid Reverse Transcriptase and Ribolock RNAse inhibitor (Thermo-Fisher), according to manufacturer's instructions. cDNA was used for qRT-PCR using the Viia7 system (Thermo-Fisher). qRT-PCR was performed for *AS1, AS2, BOP1, BOP2, CUC3, DOF5.1, JLO, LOB*, *LOF1, LSH4* and *MYB33* using At5g15400 as a control. For primer sequences see Supplemental Table 4.S2.

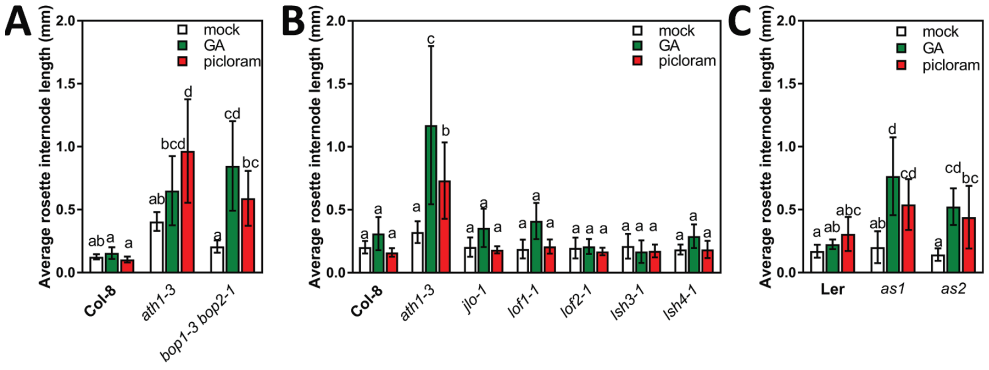
Supplemental figures and tables



Supplemental Fig. 4.S1: LOESS-fitted curve of allelic ratios for *ath1-3 sri311, ath1-3 sri312, ath1-3 sri93* and *ath1-3 sri104* SNPs
Ratio variable of SNPs between *ath1-3* and suppressor mutants computed with SIMPLE pipeline for *ath1-3 sri311* (A), *ath1-3 sri312* (B), *ath1-3 sri93* (C) and *ath1-3 sri104* (D). SNPs with a ratio under 0.3 are not displayed. X-axis: chromosomal number (top), and chromosomal location in bp (bottom); y-axis displays ratio variable.



Supplemental Fig. 4.S2: LOESS-fitted curve of allelic ratios of SNPs for sequenced enhancer mutants
Ratio variable of SNPs between *ath1-3* and suppressor mutants computed with SIMPLE pipeline for *ath1-3 eri14* (A), *ath1-3 eri15* (B), *ath1-3 eri31* (C), *ath1-3 eri81* (E). SNPs with a ratio under 0.3 are not displayed. X-axis: chromosomal number (top), and chromosomal location in bp (bottom); y-axis displays ratio variable.



Supplemental Fig. 4.S3: Heterochronic bolting can be induced with GA4+7 or picloram in boundary mutants grown in LD at 22°C

A: Average rosette internode elongation of Col-8, *ath1-3*, *bop1-3 bop2-1* (n=10, 10, 9, 10, 9, 10, 10, 10, 10)

B: Average rosette internode elongation of Col-8, *ath1-3, jlo-1, lof1-1, lof2-1, lsh3-1* and *lsh4-1* (n=10, except *lof2* GA and *lsh3* mock, where n=9).

C: Average rosette internode elongation of Ler, *as1-1*, *as2-2*, (n=9, 10, 9, 9, 11, 9, 10, 10, 10)

Plants were treated with a mock solution, 100 µM GA4+7 or 5 µM picloram. Error bars represent standard deviation of the mean, lowercase letters denote statistically homogenous groups as defined by 1-way ANOVA and Tukey's post-hoc test analysis (α=-0.05, see Supplemental Table 4.S1).

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Supplemental Table 4.S1: Reporting of statistical tests from phenotypic analyses

Figure	Statistical test	Outcome
Fig. 4.5A	1-way ANOVA	F(16; 151)=37.25; p=4.0E-44
Fig. 4.5B	1-way ANOVA	F (16; 151) = 57.1; p=9.9E-55
Fig. 4.6A	1-way ANOVA	F (5; 54) = 41.254; p=2.9E-17
Fig. 4.6B	1-way ANOVA	F (8; 78) = 15.642; p=1.7E-13
Fig. 4.6C	1-way ANOVA	F(5; 50)=37.781; p=7.5E-16
Fig. 4.6D	1-way ANOVA	F(8; 75)=20.490; p=4,6E-16
Fig. 4.7A	1-way ANOVA	F(2; 6)=6.790; p=0.029
Fig. 4.7B	1-way ANOVA	F(2; 6) = 0.335; p=0.728
Fig. 4.7C	1-way ANOVA	F(2; 6) = 0.609; p=0.669
Fig. 4.7D	1-way ANOVA	F(2; 6) = 2.364; p=0.175
Fig. 4.7E	1-way ANOVA	F(2; 6) = 3.270; p=0.110
Fig. 4.7F	1-way ANOVA	F(2; 6) = 5.846; p=(0.065)
Fig. 4.7G	1-way ANOVA	F(2; 5) = 0.224; p=0.807
Fig. 4.7H	1-way ANOVA	F(2; 5) = 4.023; p=0.078
Fig. 4.7I	1-way ANOVA	F(2; 5) = 1.295; p=0.352
Fig. 4.8A	1-way ANOVA	F(5; 60)=53.018; p=9.4E-21;
Fig. 4.S3A	1-way ANOVA	F(8; 78)=22.075; p=1.3E-17
Fig. 4.S3B	1-way ANOVA	F(20; 187)=17.818; p=2.8E-33
Fig. 4.S3C	1-way ANOVA	F(8; 78)=15.029; p=4.3E-13

Supplemental table 4.S2: List of primer sequences used in this chapter

Name	Sequence	Description
At5g15400 QPCR-F	GGGCACTCAAGTATCTTGTTAGC	qPCR primer used as endogenous control
At5g15400 QPCR-R	TGCTGCCCAACATCAGGTT	qPCR primer used as endogenous control
AS1-qF	ACAGCCTGAGAGAGCAGAGAAC	qPCR primer for *AS1*
AS1-qR	TCGCTACTCCCACTACAAGACG	qPCR primer for *AS1*
AS2-qF	ATGGTCGCCGTACTGTTGATCC	qPCR primer for *AS2*
AS2-qR	ATCACCAAGCGATCGACGAAGATG	qPCR primer for *AS2*
BOP1 AT3G57130.1_QF	AGCTTGGAGCAGCTGATGTGAAC	qPCR primer for *BOP1*
BOP1 AT3G57130.1_QR	ACCATTTCAGCCGCAATGTGAAG	qPCR primer for *BOP1*
BOP2-qF2	CTTCAAGGAAGATCTGGGAAGAGC	qPCR primer for *BOP2*
BOP2-qR2	AAGCCTATGTGCTTGCATCAGC	qPCR primer for *BOP2*
CUC3QPCR-FWD	GATCCACACCAAAACTACAA	qPCR primer used for *CUC3*
CUC3QPCR-REV	TGTGAAGAGGTCCAGGAGAA	qPCR primer used for *CUC3*
JLO-qF	GACGCCGTCGTTTCAATCTG	qPCR primer used for *JLO*
JLO-qR	GCAAACTTACCACCTGTTGC	qPCR primer used for *JLO*
LOB QF2	TGCGTCGGAGCCATCTCTTATC	qPCR primer for *LOB*
LOB QR2	AGTCAGCATTAGCTGCGTCGAG	qPCR primer for *LOB*
LOF1-qF2	CTTCAAGGAAGATCTGGGAAGAGC	qPCR primer for *LOF1*
LOF1-qR2	AAGCCTATGTGCTTGCATCAGC	qPCR primer for *LOF1*
LSH4-qF	ACCAATTCGGCAAGACTAAGGTTC	qPCR primer for *LSH4*
LSH4-qR	AGCAGCTCTAAGACGGCCAATG	qPCR primer for *LSH4*

CHAPTER 5

A TALE of opposition and coalition—BEL1-LIKE HOMEODOMAIN and KNOTTED1-LIKE HOMEOBOX transcription factors control rosette habit and stem elongation

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Abstract

In plants, three-amino-acid-loop-extension (TALE) homeobox transcription factors belonging to the BEL1-LIKE HOMEODOMAIN (BLH) and KNOTTED1-LIKE HOMEOBOX (KNOX) families control a vast array of developmental processes, including stem elongation. BLH-KNOX heterodimerization facilitates transcriptional regulation by BLH and KNOX proteins, which highlights the need to study their function in the context of functional BLH-KNOX heterodimers. The BLH transcription factor ARABIDOPSIS THALIANA 1 (ATH1) promotes rosette growth and represses bolting. ATH1 interacts with class I KNOX transcription factors, including KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2 (KNAT2) and KNAT6. The BLH transcription factor PENNYWISE (PNY), on the other hand, is reported to promote stem elongation, acting antagonistically to ATH1, KNAT2 and KNAT6. Here, we investigated the role of class I KNOX transcription factors and ATH1-PNY antagonism in ATH1-mediated repression of internode elongation. We show that ATH1 requires both KNAT2 and KNAT6 to repress stem elongation. Loss of KNAT2 or KNAT6 was sufficient to restore stem elongation in plants constitutively expressing *ATH1* and *knat2 knat6* double mutants formed elongated rosette internodes (heterochronic bolting) when treated with exogenous gibberellins. Additionally, heterochronic bolting could be induced in the *shoot meristemless* (*stm*) mutant *bum1-1*, suggesting that *STM* also contributes to repression of internode elongation during rosette growth. Strikingly, we uncover a novel role for *PNY* in regulating internode elongation, contrary to its previously published function in the generative phase. Loss of *PNY* enhanced heterochronic bolting of *ath1,* and *pny*^{40126} single mutants exhibited heterochronic bolting when treated with exogenous gibberellin. Furthermore, ectopic expression of *PNY* repressed stem elongation in a *KNAT6*-dependent manner. *PNY* acts both antagonistically to and in concert with *ATH1* to control internode elongation. Our findings suggest that complex interplay of TALE homeobox transcription factors in the shoot apex balances rosette versus stem formation, where *PNY* plays a multifaceted role.

Introduction

Coordination of developmental processes is essential for all multicellular organisms. Central to the coordination of development lie the three-amino-acid-loop-extension (TALE) homeodomain transcription factors. These transcription factors are found across eukaryotes and control many developmental processes, such as the haploid to diploid transition in algae and fungi and body plan specification in animals (Furumizu et al., 2015; Kues and Ton, 1992; Lee et al., 2008; Moens and Selleri, 2006). In plants the TALE homeodomain transcription factor superclass is subdivided into the KNOTTED-LIKE HOMEOBOX (KNOX) and BEL1-LIKE HOMEOBOX (BLH) families. In *Arabidopsis thaliana*, these families consist of eight and thirteen members, respectively, which are involved in the regulation of virtually all developmental processes from embryonic to gametophyte development. This includes, but is not limited to, germination, stem cell maintenance, development of the shoot, inflorescence, leaves, vascular tissue and floral organs, and regulation of the reproductive phase change (Alonso-Cantabrana et al., 2007; Bao, 2009; Endrizzi et al., 1996; Kanrar et al., 2008; Khan et al., 2015; Kim et al., 2013; Kumar et al., 2007; Liu et al., 2014; Long et al., 1996; Pagnussat et al., 2007; Proveniers et al., 2007; Rutjens et al., 2009; Smith et al., 2004).

BLH and KNOX proteins form selective BLH-KNOX heterodimers. This interaction enables stable nuclear localization, and enhances binding affinity to target DNA (Bellaoui et al., 2001; Cole et al., 2006; Rutjens et al., 2009; Viola and Gonzalez, 2009). Therefore, heterodimerization is important for transcriptional regulation by BLH and KNOX proteins. BLH and KNOX proteins preferentially bind target DNA containing a TCAG core, but the sequence surrounding this core can vary between plant TALE members (Bencivenga et al., 2016; Smith et al., 2002; Viola and Gonzalez, 2006). Therefore, it is likely that heterodimerization with different KNOX proteins changes the transcriptional output of a given BLH protein. Thus, to gain a comprehensive understanding of the role of a BLH protein in a given process, it is necessary to study it in the context of its KNOX partners, and *vice versa*.

Post-embryonic shoot tissue is formed by the shoot apical meristem (SAM), which also acts as a major signalling centre for the coordination of developmental transitions. The SAM contains a central population of stem cells (central zone; CZ), flanked by proliferating cells (peripheral zone; PZ) that undergo differentiation into

organ primordia. The PZ is separated from the primordia by the meristem-organ boundary (Gaillochet et al., 2015; Poethig, 2003). Beneath the SAM lies the rib zone (RZ), which is responsible for stem formation (Bencivenga et al., 2016; Jacqmard et al., 2003). During vegetative growth of rosette plants such as *Arabidopsis thaliana*, the SAM produces leaf primordia and the RZ remains compact and mitotically inactive. This leads to the formation of a rosette of leaves. During reproductive growth the SAM undergoes a fate switch (reproductive phase change) and becomes an inflorescence meristem (IM), which produces floral primordia instead of leaf primordia, while the cells in the RZ proliferate to form an elongated stem (Hempel and Feldman, 1994; Jacqmard et al., 2003; Kwiatkowska, 2008).

To coordinate growth and phase transitions throughout development, a delicate balance must be maintained between stem cell maintenance and differentiation, while ensuring the correct developmental programme is maintained. Unsurprisingly, TALE homeobox transcription factors are closely intertwined with these processes. The KNOX transcription factor *SHOOT MERISTEMLESS* (*STM*) is vital for repressing differentiation of stem cells. *STM* promotes biosynthesis of the hormone cytokinin (CK), which is necessary for stem cell identity, and represses gibberellin (GA), a hormone that antagonizes CK in the shoot apex (Bolduc and Hake, 2009; Jasinski et al., 2005; Yanai et al., 2005). *STM* acts redundantly with the KNOX transcription factor *BREVIPEDICELLUS* (*BP*), and requires the function of BLH transcription factor partners *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*), *PENNYWISE* (*PNY*; *BELLRINGER; REPLUMLESS; VAAMANA; LARSON; BEL1-LIKE HOMEODOMAIN 9*) and *POUND-FOOLISH* (*PNF; BEL1-LIKE HOMEODOMAIN 8*) (Bhatt et al., 2004; Byrne et al., 2002, 2003; Rutjens et al., 2009; Ung et al., 2011). *STM, ATH1*, *PNY* and *PNF*, together with the KNOX factors *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*) and *KNAT6,* also affect formation of meristem-organ boundaries, which are also essential for maintaining the balance between stem cells and differentiation (Bao, 2009; Belles-boix et al., 2006; Gómez-Mena and Sablowski, 2008; Kanrar et al., 2006; Landrein et al., 2015; Ung et al., 2011).

BLH and KNOX transcription factors are important regulators of vegetative versus generative development. *ATH1* is key in promoting the rosette habit and repressing bolting (Chapter 2), and is a negative regulator of the reproductive phase change (Proveniers et al., 2007). Downregulation of ATH1 in the meristem is correlated

with the onset of bolting in wild-type plants. In *ath1-3* loss of function mutants, the vegetative RZ strongly resembles the RZ of bolting wild-type plants, resulting in the formation of elongated rosette internodes (heterochronic bolting). Furthermore, ectopic expression of *ATH1* represses bolting (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009).

PNY and *PNF* are positive regulators of stem elongation and are antagonists of *ATH1*. In *pny* loss of function mutants, stem height is reduced, due to disruption of mitotic angle of cells in the RZ (Bencivenga et al., 2016; Bhatt et al., 2004; Byrne et al., 2003; Smith and Hake, 2003). Moreover, *pny pnf* double mutants neither bolt nor flower. No mitotic activity is detected in the RZ of *pny pnf*, nor do these plants initiate expression of floral meristem identity genes, needed to specify flowers (Smith et al., 2004). Loss of *ATH1* restores plant height of *pny,* and restores bolting and flowering in *pny pnf* (Bao, 2009; Khan et al., 2015).

ATH1, PNY and PNF can form heterodimers with the class I KNOX proteins STM, KNAT2, KNAT6 and BREVIPEDICELLUS (BP; KNAT1) (Bao, 2009; Bhatt et al., 2004; Byrne et al., 2003; Kanrar et al., 2006; Li et al., 2012c; Rutjens et al., 2009). Expression patterns of these KNOX genes partially overlap with *ATH1*, *PNY* and *PNF* during development, and class I KNOX mutant phenotypes suggest KNAT2, KNAT6 and BP to regulate internode elongation together with ATH1, PNY and PNF. ATH1 is expressed throughout the SAM, particularly at the lateral boundaries and RZ (Chapter 2), but is downregulated during the reproductive phase change (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007). In the vegetative SAM, KNAT2 is expressed in the RZ and KNAT6 is expressed in organ boundaries, while they are absent from inflorescence meristems— a pattern that overlaps with ATH1 expression (Bao, 2009; Belles-boix et al., 2006; Ragni et al., 2008).

BP is expressed in the SAM, the flanks of the IM and the RZ, and is a positive regulator of stem elongation, like *PNY and PNF* (Lincoln et al., 1994; Venglat et al., 2002). *BP* expression partly overlaps with *PNY* and *PNF,* as *PNY* is expressed throughout the SAM and IM, and *PNF* is expressed in the shoot apex during reproductive growth only (Andrés et al., 2015; Lincoln et al., 1994; Rutjens, 2007; Smith and Hake, 2003). Stem height is reduced in *bp* mutants, and *bp* enhances the *pny* phenotype. Both PNY and BP act antagonistically to *KNAT6*, and to a lesser extent *KNAT2* (Bhatt et al., 2004; Li et al., 2012c; Ragni et al., 2011; Smith and Hake, 2003; Venglat et al., 2002). Furthermore, bolting and flowering of *pny pnf* is also restored through loss of

KNAT6 (Khan et al., 2015). These phenotypes suggest that multiple TALE homeobox transcription factors opposingly control stem elongation during generative growth. However, it is not known whether other TALE homeodomain transcription factors besides ATH1 regulate elongation during vegetative growth. It is also not known whether ATH1-mediated repression of internode elongation requires class I KNOX factors.

Here, we investigate the role of class I KNOX transcription factors in ATH1-mediated repression of internode elongation, and the role of *ATH1-PNY* antagonism during rosette growth. We show that repression of rosette internode elongation during vegetative growth requires the function of the ATH1 interaction partners KNAT2, KNAT6 and STM. GA induced heterochronic bolting in *knat2-5 knat6-2* mutants, while the *stm* mutant *bum1-1* exhibited heterochronic bolting without hormone application, like *ath1-3*. Additionally, we show that repression of elongation by ATH1 requires KNAT2 and KNAT6 function.

Strikingly, we reveal a novel role for PNY as a repressor of bolting. This is in contrast with its established role as a positive regulator of stem elongation. Loss of *PNY* enhanced heterochronic bolting and reduced bolting time in *ath1-3* loss-of-function mutants, while ectopic expression of *PNY* strongly repressed bolting in a *KNAT6*-dependent manner. In addition, GA application induced heterochronic bolting in *pny*^{40126} mutants. On the other hand, *ath1-3* rescues *pny*^{40126} inflorescence internode elongation defects (Bao, 2009; Khan et al., 2012a), which suggests that *PNY* acts both in tandem with and antagonistically to *ATH1* to regulate internode elongation. Our findings show that rosette growth versus stem growth is controlled by opposing clusters of BLH and KNOX factors, where *PNY* plays a complex, multifaceted role.

Results

Maintaining *ATH1* expression upon bolting induction represses stem elongation

Previously, we showed that *ATH1* represses internode elongation during rosette growth, thereby promoting rosette growth and preventing bolting. *ATH1* is expressed in the shoot apex post-germination until the end of the vegetative growth phase (Chapter 2)(Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007; Quaedvlieg et al., 1995). Downregulation of *ATH1* coincides with activation of the RZ (Chapter 2). Previously, it was shown that ectopic expression of *ATH1* strongly represses bolting (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009). In these studies, *ATH1* was overexpressed from germination onward, therefore it is unknown if the non-bolting phenotype of *35S::ATH1* is caused by early developmental defects or by *ATH1* expression during the reproductive phase.

This was investigated using dexamethasone (DEX) inducible 35S::ATH1-HBD plants grown for five weeks in non-inducing short-day (SD) conditions. DEX treatment, to induce nuclear expression of ATH1, was either started from germination onwards, or after 1, 2, 3, 4, or 5 weeks of growth in SD. After 5 weeks of growth, plants were transferred to inductive conditions to induce bolting and flowering, and plant total height was measured. All DEX treatments resulted in repression of stem elongation in *35S::ATH1-HBD*, and no significant differences in final plant height were observed between the *35S::ATH1-HBD* DEX treatment groups (Fig. 5.1), including the one that started directly upon bolting induction. This suggests that the repression of internode elongation observed in *35S::ATH1-HBD* is solely caused by ectopic *ATH1* expression during the generative phase, and is not the result of permanent changes that occurred during earlier development.

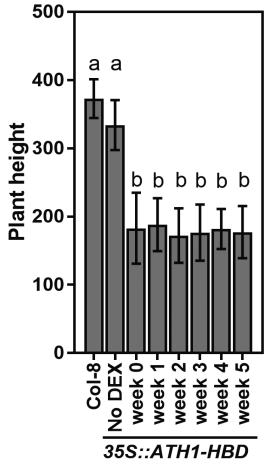


Fig. 5.1 Ectopic expression of *ATH1* during generative growth represses stem elongation
Total heights of primary inflorescences of Col-8 plants, or *35S::ATH1-HBD* plants grown in SD for 5 weeks and then transferred to inductive conditions.
Plants were treated with 0.1% ethanol (No DEX), or with 10 µM from week 0, 1, 2, 3, 4 or 5 onwards (n=17, 19, 21, 17, 13, 14, 20, 20).
Error bars denote standard deviation of the mean, letters denote statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post hoc test (See Supplemental Table 5.S2).

ATH1 requires *KNAT2* and *KNAT6* for repression of internode elongation

In heterologous systems , ATH1 interacts with the class I KNOX transcription factors STM, BP, KNAT2 and KNAT6 (Cole et al., 2006; Li et al., 2012c; Rutjens et al., 2009). The biological relevance of the ATH1-STM heterodimer has been explored previously, but the three remaining ATH1-KNOX heterodimers have received less attention. Similar to ATH1, *KNAT2* and *KNAT6* have been linked to boundary formation and repression of stem elongation (Bao, 2009; Belles-boix et al., 2006; Ragni et al., 2008) (Bao, 2009; Gómez-Mena and Sablowski, 2008). Therefore, stem elongation might require combined ATH1-KNAT2/KNAT6 function. This was investigated by generating *knat2-5 35S::ATH1-HBD, knat6-2 35S::ATH1-HBD* and *knat2-5 knat6-1 35S::ATH1-HBD* plants. Inflorescence internode elongation in LD conditions was established and loss of either *KNAT2 or KNAT6* was sufficient to restore internode elongation in DEX-induced *ATH1* overexpressors (Fig. 5.2A-D), indicating that both KNAT2 and KNAT6 are necessary for ATH1-mediated repression of inflorescence internode elongation. To determine whether *KNAT2* and *KNAT6* are also required for *ATH1*-mediated repression of internode elongation during rosette growth, we grew Col-8, *ath1-3*, *knat2-5, knat6-1* and *knat6-2* mutants and mutant combinations and measured elongation of the first five rosette internodes (Fig. 5.2E). Whereas no rosette internode defects were observed in *knat 2*, *knat6*, and *knat2 knat6* single or double mutant plants, loss of *KNAT6* enhanced heterochronic bolting of *ath1-3*, but loss of *KNAT2* did not. Taken together, these data show that *KNAT6,* and to a lesser extent *KNAT2*, are involved in ATH1-mediated repression of internode elongation.

The *stm bum1-1* mutant exhibits heterochronic bolting

We showed that KNAT6, and possibly KNAT2, act as partners of ATH1 in repressing internode elongation. However, while *ath1-3* mutants display heterochronic bolting, this phenotype is absent in *knat2-5 knat6-2* plants, which suggests that additional KNOX factors are involved in this response. ATH1 interacts with all class I KNOX factors (Rutjens et al., 2009), so it is possible that BP or STM are involved in regulating internode elongation together with ATH1. *BP* and *ATH1* are expressed in overlapping domains during vegetative growth, but BP promotes stem elongation during generative growth and is an antagonist of ATH1 and KNAT6 during pedicel development (Gómez-Mena and Sablowski, 2008; Li et al., 2012c; Lincoln et al., 1994; Proveniers et al., 2007; Venglat et al., 2002). To determine whether ATH1 and

BP are antagonists in regulating internode elongation, we measured heterochronic bolting in Col-8, *ath1-3, bp-11* and *ath1-3 bp-11* mutants grown at 27°C in LD conditions. These conditions enhance heterochronic bolting in *ath1-3* (Chapter 2). In addition, we grew these mutants in standard LD conditions and measured total plant height. In *ath1-3 bp-11* double mutants, heterochronic bolting was significantly

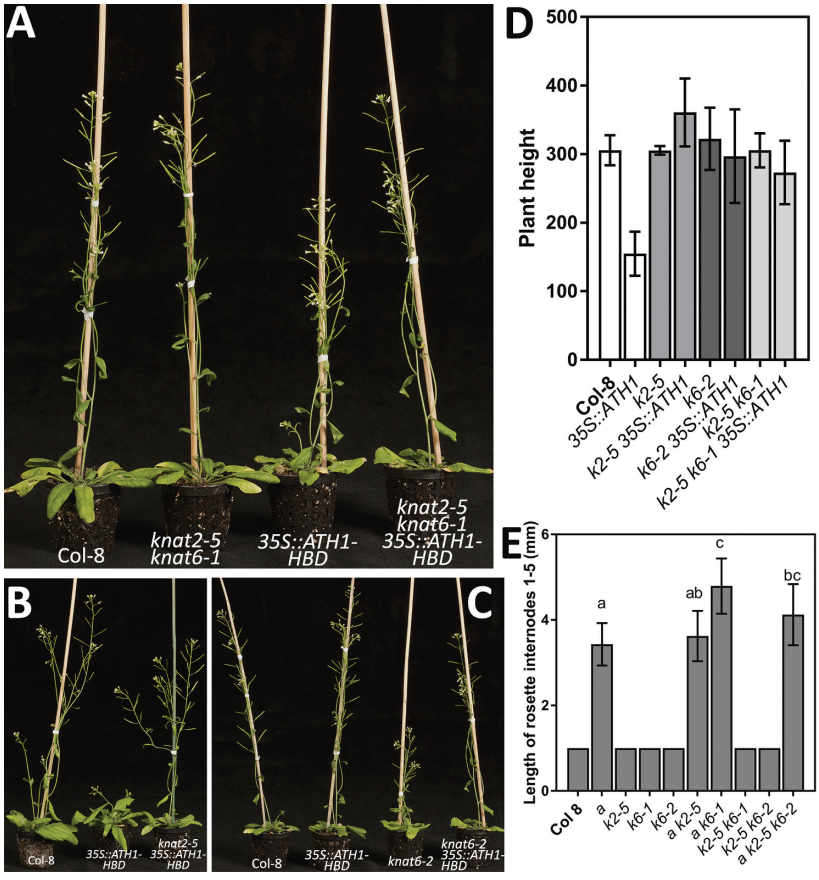


Fig. 5.2: *ATH1-mediated* repression of internode elongation requires *KNAT2* and *KNAT6*

A-C: Phenotypes of 44-day old Col-8, *knat2-5 knat6-1*, *35S::ATH1-HBD*, *knat2-5 knat6-1 35S::ATH1-HBD* (A), Col-8, *knat2-5*, *35S::ATH1-HBD* and *knat2-5 35S::ATH1-HBD* (B), Col-8, *knat2-5*, *35S::ATH1-HBD*, *knat2-5 35S::ATH1-HBD* (C).

D: Length of primary inflorescence in Col-8, *35S::ATH1-HBD* (*35S::ATH1*), *knat2-5* (*k2-5*), *knat2-5 35S::ATH1-HBD* (*k2-5 35S::ATH1*), *knat6-2* (*k6-2*), *knat6-2 35S::ATH1-HBD* (*k6-2 35S::ATH1*), *knat2-5 knat6-1* (*k2-5 k6*-1) and *knat2-5 knat6-1 35S::ATH1-HBD* (*k2-4 k6-1 35S::ATH1*) (n=9, 12, 2, 28, 8, 28, 7, 30).

E: Length of first 5 rosette internodes of SD-grown Col-8, *ath1-3* (*a*), *knat2-5* (*k2-5*), *knat6-1* (*k6-1*), *knat6-2* (*k6-1*) single, double and triple mutants (n= 13, 13, 13, 13, 12, 11, 14, 13, 11). Total length of internodes in plants lacking *ath1-3* mutation was too small to be accurately quantified and was set as <1 mm. Letters denote statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post hoc test on *ath1-3, ath1-3 knat2-5, ath1-3 knat6-1* and *ath1-3 knat2-5 knat6-2* only (See Supplemental Table 5.S2).

Error bars denote standard deviation of the mean.

reduced compared to *ath1-3* single mutants, although not completely to the level as observed in wild-type plants (Fig. 5.3A). In a similar vein, loss of *ATH1* restored total height of *bp-11* to wild-type level (Fig 5.3B). Together, these findings show that ATH1 and BP act antagonistically during vegetative and generative internode development. It is therefore unlikely that the ATH1-BP heterodimer compensates for the loss of the ATH1-KNAT2 and ATH1-KNAT6 heterodimers in *knat2-5 knat6-2*.

The final candidate KNOX partner of ATH1 for repressing internode elongation is STM. STM is essential for stem cell maintenance in the SAM plants, therefore homozygous loss-of-function *stm* mutations fail to establish or maintain a functional SAM throughout the plant life cycle, which hampers functional characterization of STM at later stages during development. To test whether STM plays a role in rosette internode development, we grew Col-8, *ath1-3* and the weak *stm* loss of function mutant *bumbershoot1-1* (*bum1-1*) in FR-enriched conditions, which induce heterochronic bolting in *ath1-3*. In *bum1-1* mutants, developmental arrest due to termination of the SAM typically occurs after formation of 2-5 rosette leaves, with some plants surviving into the reproductive growth phase (Jasinski et al., 2005; Rutjens et al., 2009). Exogenous application of cytokinins can partially restore meristem defects of strong *stm* mutants (Yanai et al., 2005). Thus, to delay meristem arrest in *bum1-1*, all plants were treated 1 µM of the synthetic cytokinin 6-benzylaminopurine (BAP) or a mock solution. Elongation of rosette internodes of both the mock and BAP-treated *bum1-1* plants was similar to that of *ath1-3* (Fig. 5.4A-B). These findings suggest that *STM* represses internode elongation during the rosette phase, similar to *ATH1*.

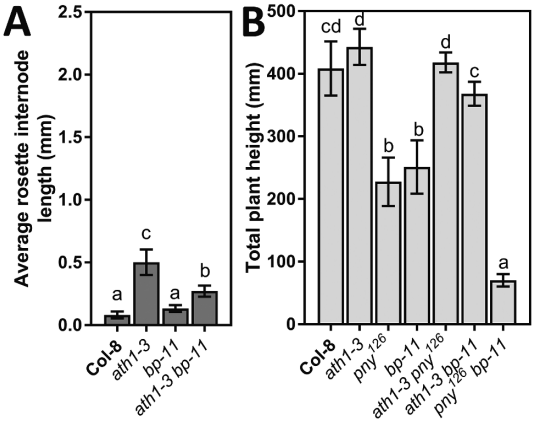


Fig. 5.3: Elongation of the primary inflorescence and rosette internodes in LD-grown *ath1-3, pny* and *bp-11* single and double mutants.
A: Average rosette internode elongation of Col-8, *ath1-3, bp-11* and *ath1-3 bp-11* grown at 27°C in LD conditions (n=9, 10, 10, 14).
B: Height of primary inflorescence in of plants grown in LD at 22°C (n=9, 10, 8, 10, 10, 10, 9). Error bars denote standard deviation from the mean, letters represent statistically homogeneous groups defined by 1-way ANOVA followed by Tukey's post-hoc test (See Supplemental Table 5.S2).

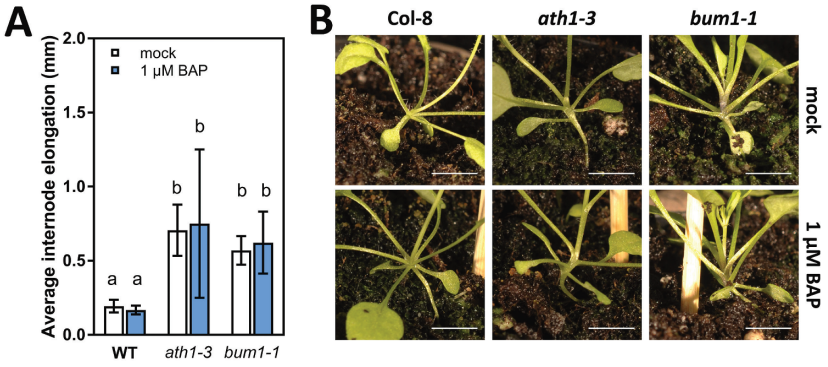


Fig. 5.4 *bum1-1* mutants exhibit heterochronic bolting in FR conditions
A: Average rosette internode elongation of Col-8, *ath1-3* and *bum1-1* plants grown in FR conditions (n=8, 10, 10, 9, 8, 26). Shoot apices were treated with 0.1% DMSO (mock) or 1 µM BAP. Error bars depict standard deviation of the mean. Letters denote statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test.
B: Photos of representative plants from A. Plants are 25 days old. Scale bar represents 5 mm.

PNY both promotes and represses internode elongation

Similar to *bp* mutants, *pny*^{40126} mutants are dwarfed and can be rescued through loss of *ATH1* (Fig. 5.3B) or *KNAT6,* but not *KNAT2* (Bao, 2009; Bhatt et al., 2004; Khan et al., 2015; Ragni et al., 2008; Smith and Hake, 2003; Venglat et al., 2002). As BP partially represses heterochronic bolting of *ath1-3* (Fig. 5.3A) and is an interaction partner of PNY, we tested if loss of *pny* suppressed heterochronic bolting of *ath1-3*. To this end, we grew *ath1-3,* and *pny*^{40126} single and double mutants and wild-type Col-8 plants grown at 27°C. In stark contrast to *ath1-3 bp*-11, heterochronic bolting of *ath1-3 pny*^{40126} double mutants was significantly enhanced compared to *ath1-3* single mutants (Fig 5.5A). *pny*^{40126} mutants did not exhibit heterochronic bolting in these conditions (data not shown). The effect of *pny*^{40126} on heterochronic bolting of *ath1-3* suggests that *PNY* acts in concert with *ATH1* to repress internode elongation during vegetative growth. This is opposite to the previous observed function of *PNY* in the inflorescence stem (Fig. 5.3B).

To further investigate the effect of *KNAT6* and *PNY* on heterochronic bolting, we scored *ath1-3, pny*^{40126}*,* and *knat6* single, double and triple mutant combinations. Also included were *pnf*^{96116} and *ath1-3 pny*^{40126} *pnf*^{96116} mutants, as PNF promotes inflorescence elongation in conjunction with PNY (Kanrar et al., 2008; Smith et al., 2004). Apart from *ath1-3,* none of the single mutants exhibited heterochronic bolting. In line with our previous results, loss of *PNY* or *KNAT6* enhanced heterochronic

bolting of *ath1-3* (Fig. 5.5B). However, heterochronic bolting was not observed in *pny*^{40126} *knat6-2* mutants. The *ath1-3 pny*^{40126} *knat6-1* triple mutant was significantly more elongated than *ath1-3 pny*^{40126} and *ath1-3 knat6-1*. On the other hand, *pnf*^{96116} reduced heterochronic bolting, as the *ath1-3 pny*^{40126} *pnf*^{96116} triple mutant was less elongated than the *ath1-3 pny*^{40126} double mutant. Together, these findings show that KNAT6 and PNY repress internode elongation during rosette growth, but ATH1 can compensate for the loss of *PNY* and *KNAT6.*

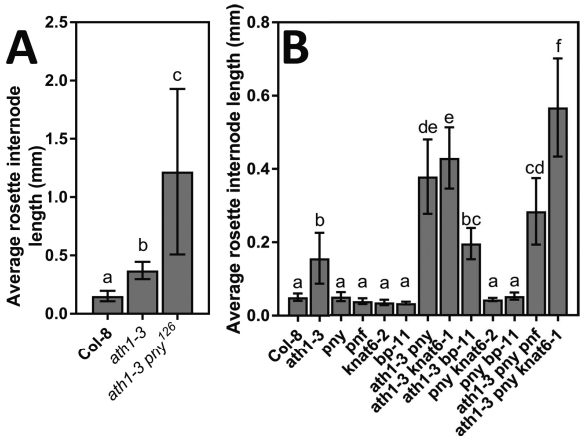


Fig. 5.5: Heterochronic bolting is affected in LD and SD-grown *BLH* and *KNOX* mutants
A: Average rosette internode elongation of Col-8, *ath1-3, pny* and *ath1-3 pny* plants grown at 27°C in LD conditions (n=14).
B: Average length of rosette internodes of SD-grown Col-8, *ath1-3*, *pny*^{40126}, *pnf*^{96116}, *knat6-2*, *bp-11*, *ath1-3 pny*^{40126}, *ath1-3 knat6-1, ath1-3 bp-11*, *pny*^{40126} *knat6-2, pny*^{40126} *bp-11* and *ath1-3 pny*^{40126} *pnf*^{96116} (n=9, 10, 9, 7, 10, 6, 10, 10, 10, 10, 7, 10, 7).
Error bars depict standard deviation of the mean, lowercase letters depict homogeneous subsets as defined by Tukey's post-hoc test (See Supplemental Table 5.S2).

In addition to these mutants, we also measured heterochronic bolting in *bp-11*, *ath1-3 bp-11* and *pny*^{40126} *bp-11* mutants (Fig. 5.5B). Heterochronic bolting was not observed in *bp-11* or *pny bp-11* mutants, but loss of *BP* did not reduce heterochronic bolting of *ath1-3* as was observed in 27°C LD conditions (Fig. 5.3A), which might indicate that *BP* has photoperiod or temperature dependent functions in the rosette.

Besides internode elongation, ATH1-PNY antagonism and ATH1-PNY synergism was also observed in other developmental processes. Both *ath1-3* and *pny*^{40126} are also early flowering in SD conditions (Proveniers et al., 2007; Rutjens, 2007). Rosette leaf number and days to bolting, both measures for bolting time, were significantly reduced in both *pny*^{40126} and *ath1-3* mutants, and rosette leaf number was further

reduced in *ath1-3 pny*^{40126} double mutants (Fig 5.S1A-B). In addition, loss of *PNY* also strongly enhanced stem-cauline leaf fusions of *ath1-3* (Fig. 5.S2A), while loss of *ATH1* restored apical dominance of *pny*^{40126} mutants (Fig. 5.S2B).

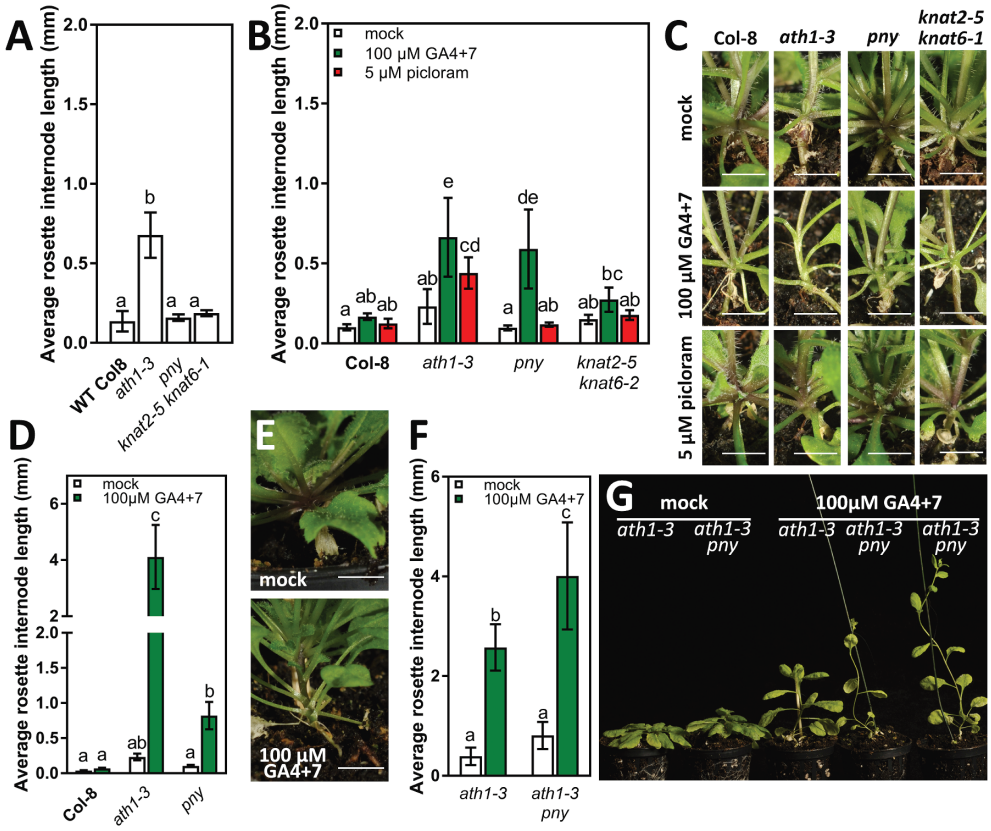


Fig. 5.6 GA-treatment of *knat2-5 knat6-1* and *pny* mutants induces heterochronic bolting
A: Average rosette internode elongation of Col-8, *ath1-3, pny*^{40126} and *knat2-5 knat6-1* grown in FR conditions (n=7, 10, 9, 10).
B: Average rosette internode elongation of Col-8, *ath1-3, pny*^{40126} and *knat2-5 knat6-1* (n=7, 10, 9, 10) of plants grown in standard LD conditions and treated with 0.1% DMSO (mock), 100 µM GA4+7 or 5 µM picloram (n=10, 10, 9, 10, 10, 10, 10, 10, 10, 9, 10, 10).
C: Photographs of representative 41-day old plants from B.
D: Average rosette internode elongation of SD-grown Col-8, *ath1-3* or *pny*^{40126} treated with 0.1% ethanol (mock) or 100 µM GA4+7 (n=9, 6, 10, 10, 10, 10). Data for Col-8 and *ath1-3* were also presented in Fig. 2.3 (Chapter 2).
E: Representative phenotypes of 53-day old mock and GA-treated *pny*^{40126} plants from D.
F: Average rosette internode elongation of SD-grown *ath1-3* and *ath1-3 pny*^{40126} plants treated with 0.1% EtOH (mock) or 100 µM GA4+7 (n=10).
G: Representative phenotypes of 55-day old plants from F.
Error bars depict standard deviation of the mean. Letters represent statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test (See Supplemental Table 5.S2). Scale bar represents 5 mm.

GA induces heterochronic bolting in *pny*^{40126} and *knat2-5 knat6-1* mutants

Our previous findings strongly implicate KNAT2, KNAT6 and PNY as repressors of internode elongation. However, although *knat6* and *pny*^{40126} mutations strongly enhance heterochronic bolting in *ath1-3* backgrounds, we did not observe heterochronic bolting in *knat6* or *pny*^{40126} mutants in the presence of a wild-type *ATH1* allele (Fig. 5.6E, 5.5B). Previously, similar observations have been made for *lob* and *bop1 bop2* mutations, and in these cases heterochronic bolting in the presence of a functional ATH1 allele could be induced when mutants were treated with GA4+7 or the synthetic auxin picloram (Chapter 2-4). Therefore, Col-8, *ath1-3, pny*^{40126} and *knat2-5 knat6-2* mutants were grown in both in FR-enriched conditions and in standard LD conditions and treated with GA4+7 or picloram. Unlike *ath1-3*, rosette internodes of *pny*^{40126} and *knat2-5 knat6-1* did not elongate in FR-enriched conditions or when treated with picloram. However, GA4+7 treatment had a small, but visible, effect on rosette internode elongation in *knat2-5 knat6-2* plants and a pronounced effect on *pny*^{40126} plants (Fig. 5.6A-C). Remarkably, in GA4+7-treated *pny*^{40126} mutants the internode following the second or third rosette leaf was strongly elongated, ranging between 1.38-5.88 mm in length, whereas all other internodes remained non-elongated. As such, total height of the rosette was attributed almost solely to this single elongated internode. (Fig. 5.6B-C). The observed phenotype is reminiscent of the internode patterning phenotype of *pny*^{40126} during reproductive growth, where extremely short internodes are interspersed with internodes that are longer than normal (Bhatt et al., 2004; Byrne et al., 2003; Smith and Hake, 2003). Possibly, the phenotype of GA4+7-treated *pny*^{40126} rosette internodes mirrors the phenotype of *pny*^{40126} stem internodes. Alternatively, the observed phenotype may be linked to the juvenile growth phase, as the elongated rosette internode always formed after rosette leaf 2 or 3, which are formed during juvenile growth. To discern between these two possibilities, Col-8, *ath1-3, pny*^{40126} and *ath1-3 pny*^{40126} mutants were grown in SD conditions and treated with GA4+7. In SD conditions, the vegetative growth phase is strongly extended and plants form more rosette leaves before bolting. However, the length of the juvenile growth phase remains the same. Therefore, if GA4+7-treated *pny*^{40126} mutants form only a single elongated rosette internode in SD, the phenotype is specific to the juvenile growth phase. If plants form multiple elongated and non-elongate internodes, the phenotype likely is related to the clustering phenotype of *pny*^{40126}. SD-grown, GA4+7-treated

pny^{40126} mutants formed multiple elongated internodes and multiple non-elongated internodes, resulting in clustering of rosette leaves along the vegetative "stem". GA4+7-treated *pny*^{40126} single mutants were less elongated than GA4+7-treated *ath1-3* single mutants. However, GA4+7-treated *ath1-3 pny*^{40126} mutants were significantly more elongated than GA4+7-treated *ath1-3* single mutants and barely any distinction could be made between rosette and stem internodes (Fig. 5.6D-F). This suggests that while *ATH1* is the dominant BLH-factor in repressing rosette internode elongation, subsequent loss of *pny* is required for total abolition of the rosette habit in response to GA. Interestingly, clustering of *pny*^{40126} rosette internodes was completely suppressed by *ath1-3*, as *ath1-3 pny*^{40126} only formed elongated rosette internodes (Fig. 5.6E). Suppression of clustering is also observed in the inflorescence stems of *ath1-3 pny*^{40126} (Khan et al., 2012a). This shows that, unlike for regulation of internode elongation, ATH1 and PNY are antagonists in the regulation of organ clustering during both the vegetative and reproductive phase.

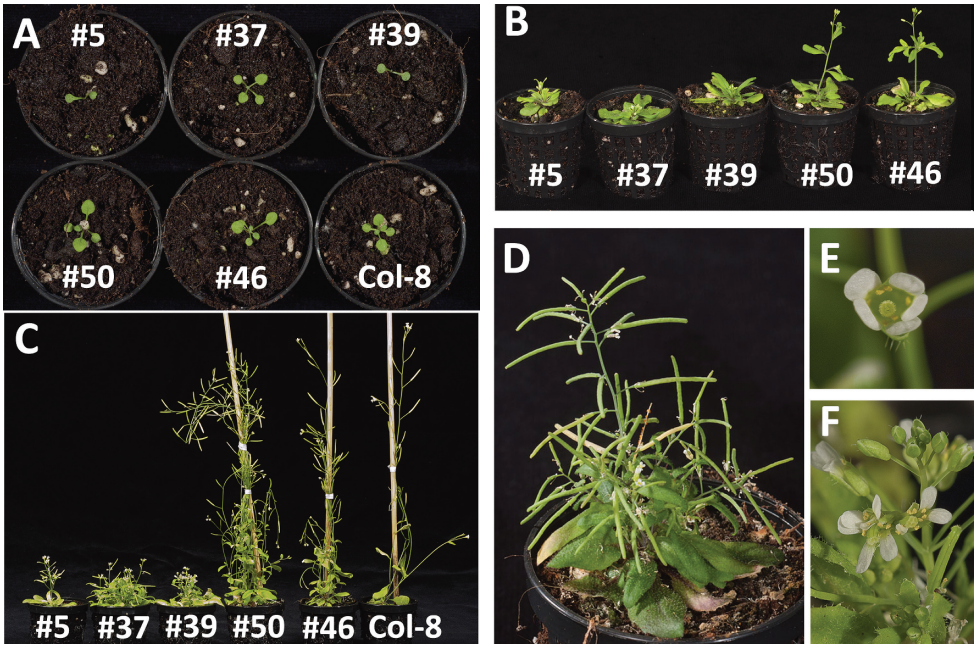


Fig. 5.7: Phenotypes of the *35S::HA-PNY* overexpressor
A-C: Phenotypes of T2 *35S::HA-PNY* overexpressor lines grown in LD conditions for 12 (A), 39 (B) or 55 (C) days.
D-F: Mature inflorescence (D), flower with supernumerary petals (E) and fused flowers (F) of T3 *35S::HA-PNY* overexpressor line #22.1.

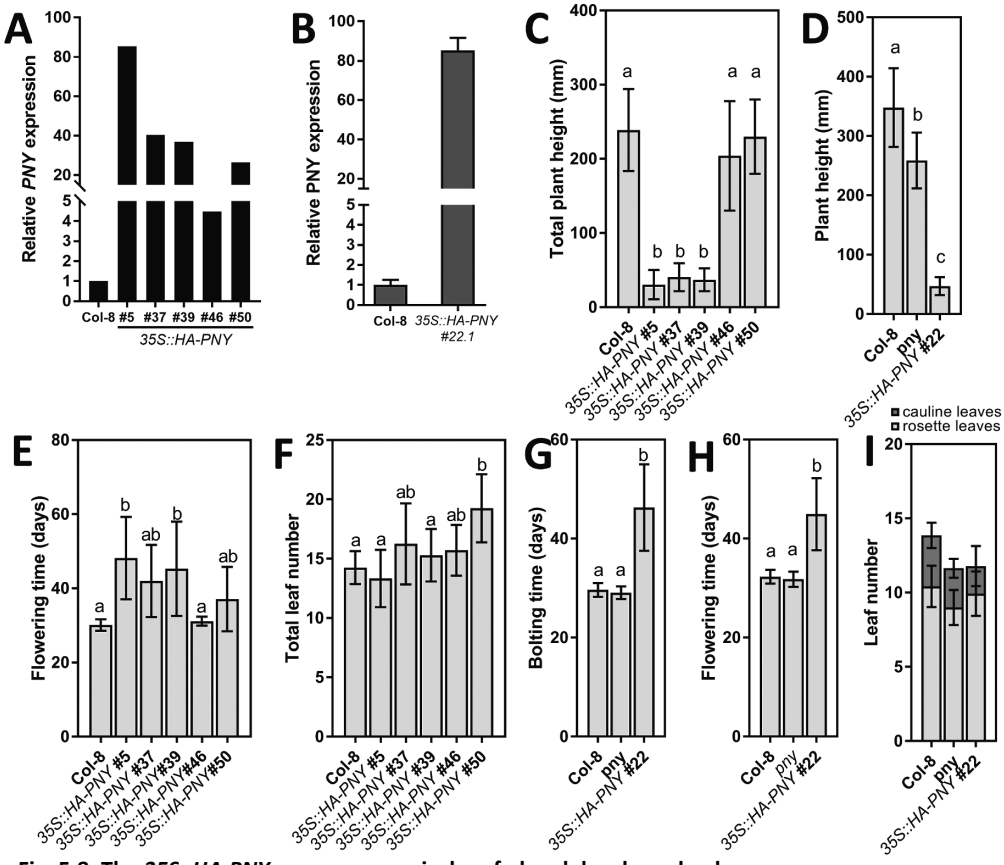


Fig. 5.8: The *35S::HA-PNY* overexpressor is dwarfed and develops slowly

A: Relative *PNY* expression from first cauline leaf tissue collected from individual, independent T1 *35S::HA-PNY* overexpressor plants, defined by qRT-PCR.

B: Relative *PNY* expression in meristem enriched tissue from Col-8 and *35S::HA-PNY* #21.1 homozygous T3 lines at the 2-leaf stage, defined by qRT-PCR.

C: Height of the primary inflorescences of LD grown Col-8 or *35S::HA-PNY* T2 overexpressors (n=8, 6, 8, 7, 7, 7)

D: Height of the primary inflorescences of LD grown Col-8 or *pny*^{40126}, and *35S::HA-PNY* #22.1 T3 overexpressor (n=14.)

E-F: Flowering time in days (E) and total number of leaves formed (F) measured in LD-grown Col-8 and *35S::HA-PNY* T2 plants (n=8, 6, 8, 7, 7, 8).

G-I Bolting time (G), flowering time (H) and the number of leaves formed (I) in LD-grown Col-8, *pny*^{40126} and the homozygous T3 line *35S::HA-PNY* #22 (n=14).

Error bars denote standard deviation of the mean. Letters represent statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test (See Supplemental Table 5.S2).

Ectopic expression of *PNY* represses stem elongation in a KNAT6-dependent manner

Our previous findings suggest that *PNY* acts both in concert with and antagonistically to *ATH1*. To further study this dual role of *PNY* we generated *35S::HA-PNY* overexpressors and examined the phenotypes of six independent overexpressor lines. Surprisingly, stem elongation was strongly reduced in plants that expressed high levels of *PNY* (Fig. 5.7C-D, 5.8A, E), a phenotype that is also observed in *pny* loss of function mutants (Bhatt et al., 2004; Byrne et al., 2003; Smith and Hake, 2003). Additionally, leaf initiation and development of strong *PNY* overexpressors was slower than Col-8 plants. In the strong *35S::HA-PNY* #22, #5, #37 and #39 lines, seedlings appeared to temporarily arrest after germination, and were delayed in initiating true leaves (Fig. 5.7A). Normally, the first two leaves of Arabidopsis formed post-germination emerge simultaneously as a pair. However, in the two strongest overexpressors (#5 and #22) a single large leaf was initiated first and the second leaf emerged later. Overexpressors also flowered and bolted significantly later than wild-type plants, but formed a similar number of leaves in total (Fig. 5.8C-G). These phenotypes are the opposites of *pny*^{40126} loss of function mutants, which bolt early in SD conditions (Fig. 5.S1B) and have a higher leaf initiation rate (Bao, 2009; Byrne et al., 2003; Rutjens, 2007). Occasionally, we also observed other inflorescence defects in strong overexpressors, such as supernumerary petals, fusion of flowers (Fig. 5.7E-F) and fasciation of stems (not pictured).

As *ATH1* represses stem elongation and is a direct target of PNY, and loss of *ATH1* rescues stem elongation of *pny*^{40126} (Bao, 2009; Bencivenga et al., 2016; Khan et al., 2012a), we tested if loss of *ATH1* also rescued *35S::HA-PNY*. To this end, we crossed *ath1-3* or *ath1-1* mutants with *35S::HA-PNY.* However, both *ath1-3* and *ath1-1* silenced expression of the *35S::HA-PNY* transgene and these lines could not be used for further analysis (data not shown). Instead, we tested if *ATH1* is ectopically expressed in *pny*^{40126} and *35S::HA-PNY*. We measured *ATH1* expression with qRT-PCR in meristem-enriched material harvested from LD-grown Col-8, *pny*^{40126} and *35S::HA-PNY* #22 at the 2-leaf and bolting stage. *ATH1* expression was increased in *pny*^{40126} and *35S::HA-PNY* plants at the 2-leaf stage, and in bolting *pny*^{40126} plants (Fig. 5.9A). This suggests that while reduced stem elongation of *pny*^{40126} might be caused by ectopic expression of *ATH1* during reproductive growth, this is not the

case in *35S::HA-PNY.* Additionally, our data shows that PNY has both a repressive and an inductive effect on *ATH1* expression. To determine whether this is also the case for other targets of *PNY,* we also tested expression of the boundary genes *LATERAL ORGAN BOUNDARIES* (*LOB*), *BLADE ON PETIOLE 1* (*BOP1*), *BOP2* and *LIGHT SENSITIVE HYPOCOTYL 4* (*LSH4*). Misexpression of these genes during reproductive growth represses stem elongation (Bencivenga et al., 2016; Norberg, 2005; Shuai et al., 2002), and these gene are targets of both *ATH1* (Chapter 2) and *PNY* (Bencivenga et al., 2016). In *35S::HA-PNY, LOB* and *BOP1* appeared to be upregulated, while *BOP2* showed slight upregulation in *pny*^{40126} only. *LSH4* expression was increased in both *pny*^{40126} and *35S::HA-PNY* at the bolting stage, but was also increased at the 2-leaf stage of *35S::HA-PNY* (Fig. 5.9B-E). These data suggest that *PNY* has a positive and negative effect on the expression of *ATH1, LOB, BOP1* and *LSH4*.

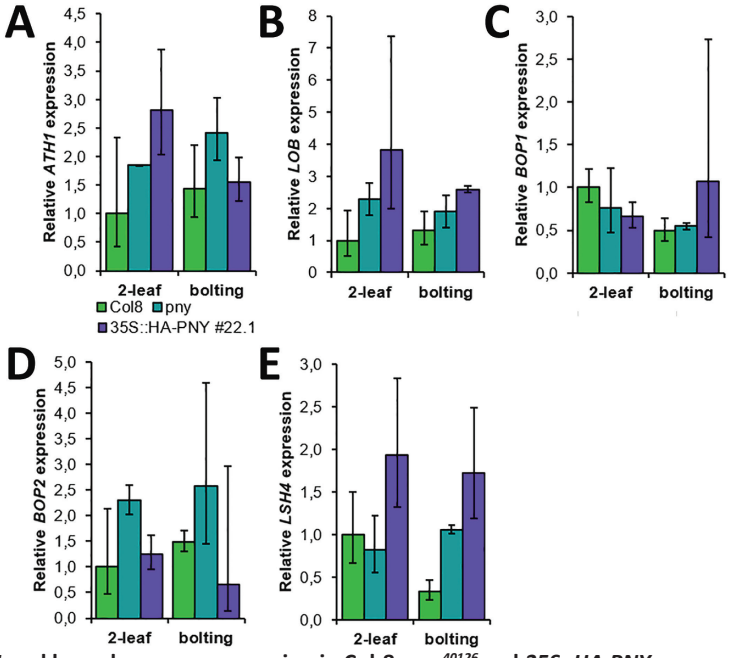


Fig. 5.9 *ATH1* and boundary gene expression in Col-8, *pny*^{40126} and *35S::HA-PNY*
Expression of *ATH1* (A), *LOB* (B), *BOP1* (C), *BOP2* (D) and *LSH4* (E) in shoot apices of Col-8, *pny*^{40126} and *35S::HA-PNY*. Expression was measured at the 2-leaf stage (vegetative) and in bolting plants. Error bars represent standard deviation of the ΔCT mean.

Like ATH1, PNY interacts with KNAT2 and KNAT6, which repress internode elongation (Bao, 2009; Bhatt et al., 2004; Ragni et al., 2008) together with ATH1 (Fig. 5.2). To determine whether PNY-KNAT2 or PNY-KNAT6 heterodimers also repress internode elongation, we crossed *35S::HA-PNY* #22 plants with *knat2-5* or *knat6-2* and

measured internode elongation in LD conditions. Loss of *KNAT2* or *KNAT6* was sufficient to restore slowed development of *35S::HA-PNY* Fig. 5.10B,D), but stem elongation was only restored in *knat6-2 35S::HA-PNY* (Fig. 5.10A-B, Supplemental Fig. 5.S3). Therefore, we conclude that that repression of stem elongation in *35S::HA-PNY* requires *KNAT6*, but not *KNAT2*. This is similar to *pny*^{40126} mutants, which can be rescued through loss of *KNAT6,* but not *KNAT2* (Ragni et al., 2008).

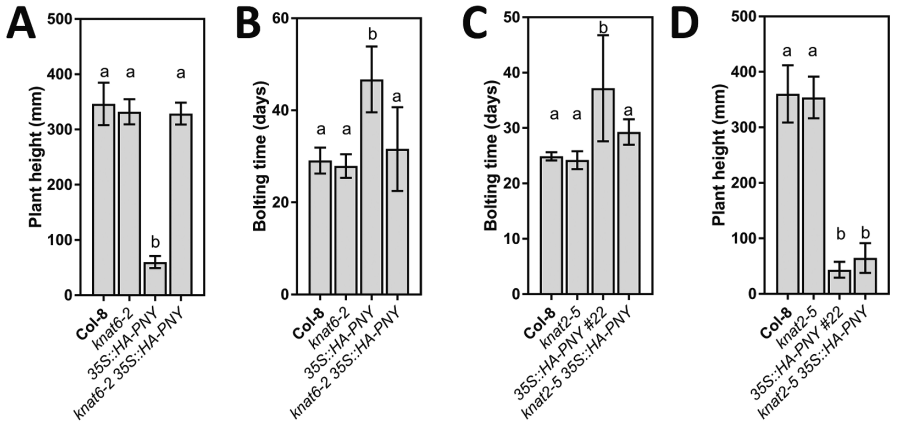


Fig. 5.10 Repression of stem elongation by *35S::HA-PNY* requires *KNAT6*
A-B: Length of the primary inflorescence (A) and bolting time (F) of LD-grown Col-8, *knat6-2, 35S::HA-PNY* and *knat6-2 35S::HA-PNY* (n=10, 10, 7, 10).
C-D: Length of the primary inflorescence (C) and bolting time (D) of LD-grown Col-8, *ath1-3*, *knat2-5, 35S::HA-PNY, ath1-3 35S::HA-PNY* and *knat2-5 35S::HA-PNY* (n=10, 10, 10, 10, 10, 7).
Letters denote homogeneous subsets defined by 1-way ANOVA followed by Tukey's post hoc test (See Supplemental Table 5.S2). Error bars represent standard deviation of the mean.

Discussion

ATH1-mediated repression of internode elongation requires *KNAT2*, *KNAT6* and *STM*

BLH and KNOX transcription factors control a wide array of developmental processes, including shoot architecture, stem cell maintenance and the transition to reproductive growth (Endrizzi et al., 1996; Proveniers et al., 2007; Ragni et al., 2008; Smith et al., 2004; Venglat et al., 2002). ATH1, a key repressor of internode elongation during rosette growth (Chapter 2), can heterodimerize with the class I KNOX transcription factors STM, BP and KNAT2, KNAT6. Together, ATH1 and these KNOX factors are expressed in the shoot apex (Belles-boix et al., 2006; Chuck et al., 1996; Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Li et al., 2012c; Proveniers et al., 2007; Rutjens et al., 2009; Smith and Hake, 2003). Here, we show

that repression of internode elongation by ATH1 requires the class I KNOX factors KNAT2, KNAT6 and STM, but is antagonized by BP.

For proper functioning of BLH and KNOX proteins, the formation of BLH-KNOX heterodimers is required, if not essential. Both BLH and KNOX proteins require heterodimerization to enable stable nuclear localization and BLH-KNOX heterodimerization enhances binding affinity to target DNA sequences (Bellaoui et al., 2001; Cole et al., 2006; Rutjens et al., 2009; Viola and Gonzalez, 2006, 2009). Stem cell maintenance in the SAM, for example, is controlled by STM in conjunction with its BLH partners ATH1, PNY and PNF. Knocking out these three partners phenocopies the *stm* meristem arrest phenotype, showing that without these partners, STM function is impaired (Rutjens et al., 2009). Similarly, loss of ATH1's partners, *KNAT2* and *KNAT6* or *STM*, enables heterochronic bolting (Fig. 5.3, 5.4, 5.6). STM might be the primary KNOX partner of ATH1 in this process, as *bum1-1* mutants exhibited a stronger heterochronic bolting phenotype than *knat2-5 knat6-1* mutants and thus were phenotypically closer to *ath1-3* (Fig. 5.4, 5.6). In addition, unlike *ath1* mutants, the RZ of *knat2 knat6* seedlings is compact (Bao, 2009; Rutjens et al., 2009), while the this region appears enlarged in *stm* mutants (Endrizzi et al., 1996).

However, unravelling the role of the ATH1-STM heterodimer within the context of the rosette habit and bolting is not straightforward as *STM* has an essential role in meristem maintenance. Even a weak *stm* allele has a pronounced effect on the SAM, which may mask RZ defects. For example, the enlarged subapical region of weak *stm* mutants has been attributed to the loss of stem cell maintenance and fusion of the cotyledons (Endrizzi et al., 1996). Additionally, meristem arrest of weak *stm* alleles can be strongly enhanced by loss of *ATH1* or increased GA signalling (Hay et al., 2002; Rutjens et al., 2009). It is likely that auxin treatment of *stm* mutants has a similar effect, as *stm* mutants are cytokinin deficient and the auxin-cytokinin balance is essential for SAM identity (Gaillochet et al., 2015; Jasinski et al., 2005; Yanai et al., 2005). Therefore, it will be challenging to examine heterochronic bolting phenotypes in *stm* backgrounds and to subsequently disentangle effects on the rosette internodes from defects in the SAM.

The switch from rosette growth to stem growth requires repression of internode elongation to be released. *ATH1* is downregulated in the shoot apex during the reproductive phase change (Gómez-Mena et al., 2005; Proveniers et al., 2007),

which is correlated with expansion of the RZ and the onset of bolting (Chapter 2). Like *ATH1, KNAT2* and *KNAT6* are downregulated prior to reproductive growth (Ragni et al., 2008). As KNAT2 and KNAT6 are required for ATH1 function (Fig 5.2) but also directly targeted by *ATH1* (Chapter 2), downregulation of *KNAT2* and *KNAT6* is likely linked to *ATH1* downregulation.

During vegetative growth, *KNAT6* is expressed at meristem-organ boundaries and the stipules, while *KNAT2* is expressed in the L3 and RZ of plants (Bao, 2009; Belles-boix et al., 2006). This expression is dependent on *ATH1* (Bao, 2009). During reproductive growth, *KNAT2* and *KNAT6* expression is lost from these regions (Ragni et al., 2008). Repression of *KNAT2* and *KNAT6* during reproductive growth requires *BP* (Ragni et al., 2008), which acts antagonistically to *ATH1* in stem elongation (Fig. 5.3). BP and KNAT2/6 are antagonists in several processes, including stem elongation, pedicel development and petal abscission (Li et al., 2012c; Ragni et al., 2008; Shi et al., 2011). During reproductive growth, BP directly represses *KNAT2* and *KNAT6* through the chromatin remodelling factor BRAHMA (BRM), which interacts with BP and deposits repressive H3K4me3 marks at the *KNAT2* and *KNAT6* promoter (Zhao et al., 2015). However, during vegetative growth *BP* is expressed in the shoot apex, yet so are *KNAT2* and *KNAT6* (Bao, 2009; Lincoln et al., 1994; Ragni et al., 2008). This suggests that BP function is limited by another factor. Negative BLH-KNOX interactions have been described, where BP is sequestered by the BLH transcription factors SAWTOOTH1 (SAW1) and SAW2 involved in leaf margin development (Kumar et al., 2007). As *ATH1* and *BP* are expressed in overlapping domains during vegetative growth, ATH1 might interact with BP and sequester BP in the RZ and boundary, preventing *KNAT2/6* repression. Downregulation of *ATH1* might therefore have a two-fold effect on *KNAT2/6* expression. On the one hand, ATH1 downregulation would lead to less induction of *KNAT2/6* expression, as fewer ATH1-STM/KNAT2/KNAT6 heterodimers can form. Concurrently, fewer ATH1-BP heterodimers form, allowing BP to repress *KNAT2/6*, possibly by interacting with other BLH factors present in the shoot apex, such as PNY or PNF.

ATH1 induces *KNAT2/6* expression, thus it is possible *KNAT2* and *KNAT6* are not downregulated in the shoot apices of *35S::ATH1-HBD* plants during reproductive growth, and are still expressed in the organ-boundaries and RZ, respectively. Loss of either *KNAT2* or *KNAT6* is sufficient to restore stem elongation of *35S::ATH1-HBD*, which suggests that activity of ATH1-KNAT2 and ATH1-KNAT6 heterodimers

at both the RZ and meristem-organ boundaries is essential for repression of internode elongation. The RZ is necessary for stem formation during reproductive growth (Bencivenga et al., 2016; Sachs, 1991) and disruption of lateral organ boundaries induces heterochronic bolting under certain conditions (Chapter 2-4). This also suggests that both the RZ and meristem-organ boundaries are important for regulating internode elongation. Therefore, it is possible that the combined expression domain of *KNAT2* and *KNAT6* in the vegetative shoot apex marks the cells that control internode elongation.

A dual role for PNY in the regulation of internode elongation

PNY has previously been defined as a positive regulator of internode elongation, but we show that PNY also represses internode elongation, marking it as both a positive and a negative regulator of bolting. Our data show that PNY represses internode elongation during rosette growth, in tandem with ATH1, *pny*^{40126} enhances heterochronic bolting in of *ath1-3* (Fig. 5.5) and GA-treated *pny*^{40126} single mutants exhibit heterochronic bolting (Fig. 5.6). Ectopic expression of *PNY* represses internode elongation during reproductive growth, in *KNAT6*-dependent manner (Fig. 5.10). In addition, *PNY* negatively affects the timing of bolting (Fig. 5.S1) and flowering (Rutjens, 2007). Together, these findings are in stark contrast with the previously established role of *PNY* as a positive regulator of bolting, flowering and internode elongation. Stems of *pny* single mutants are dwarfed, and *pny pnf* double mutants do not bolt or flower at all (Bhatt et al., 2004; Byrne et al., 2003; Smith and Hake, 2003; Smith et al., 2004). These phenotypes can be rescued through loss of *ATH1* or *KNAT6* (Bao, 2009; Khan et al., 2012a, 2015; Ragni et al., 2008). This shows that PNY acts both as an antagonist and a partner of ATH1 and KNAT6, depending on developmental stage, again highlighting the duality of PNY action.

A key question is: How does PNY both promote and repress bolting? The answer likely lies with the balance of BLH and KNOX transcription factors in the shoot apex. In animals, a single TALE homeobox protein can have opposing effects on transcriptional regulation of a single target gene depending on its interacting partners (Fig. 5.11A). The animal TALE homeodomain transcription factor superfamily consists of the Pbc and Meis/Prep families, which form selective heterodimers and regulate transcription, like their plant TALE counterparts. Animal TALE factors recruit additional transcription factors into large, multimeric complexes, which can have

repressive or activating effects on transcription depending on complex composition (Choe et al., 2014; Saleh et al., 2000). Composition of these complexes is affected by heterodimerization. For example, the zebrafish Pbc protein Pbx4 can form complexes with the Hox transcription factor PG1, with or without heterodimerizing with the Meis protein Meis3. In complexes lacking Meis3, expression of the transcription factor *Hoxb1a* is repressed. On the other hand, complexes containing PG1, Pb4 and Meis3 promote *Hoxb1a* expression. Pbx4-Meis3 heterodimerization prevents Pbx4 from interacting with histone deacetylases, thus changing the complex from repressor to an activator of transcription (Choe et al., 2009). As BLH-KNOX heterodimerization is required for stable nuclear localisation of both proteins (Bellaoui et al., 2001; Cole et al., 2006; Rutjens et al., 2009), it is unlikely that BLH proteins form complexes without KNOX proteins or *vice versa*. However, KNOX interactions with chromatin remodellers have been described, as BP interacts with BRM, a component of the SWI/SNF chromatin remodelling complex. This interaction facilitates *KNAT2* and *KNAT6* repression (Zhao et al., 2015). Thus, it is possible that changes in BLH-KNOX heterodimerization affects complex composition and thus on transcriptional regulation of target genes (Fig. 5.11B).

PNY is expressed in the SAM and RZ during vegetative and reproductive growth (Andrés et al., 2015). ATH1 and PNF are also expressed in the shoot apex, but their expression changes over time. During vegetative growth, *ATH1* is present in the SAM and RZ, but *ATH1* is downregulated during the reproductive phase change (Chapter 2). The spatiotemporal expression domain of *PNF* has not been studied, but micro-array expression data show that *PNF* is very lowly expressed in the shoot apex during vegetative growth, and upregulated during the reproductive phase change (Rutjens, 2007). *PNY* promotes bolting and flowering together with *PNF* (Smith et al., 2004). Loss of *PNF* enhances meristem arrest of *ath1-1 pny* mutants (Rutjens et al., 2009), but no other vegetative phenotypes have been attributed to *pnf* mutants. It is therefore unlikely that the low expression of *PNF* in the vegetative SAM has a (significant) effect on the rosette habit. This means that the vegetative SAM primarily expresses *ATH1* and *PNY*, which repress internode elongation, while the reproductive IM expresses only *PNY* and *PNF*, which promote internode elongation (Fig 5.11C).

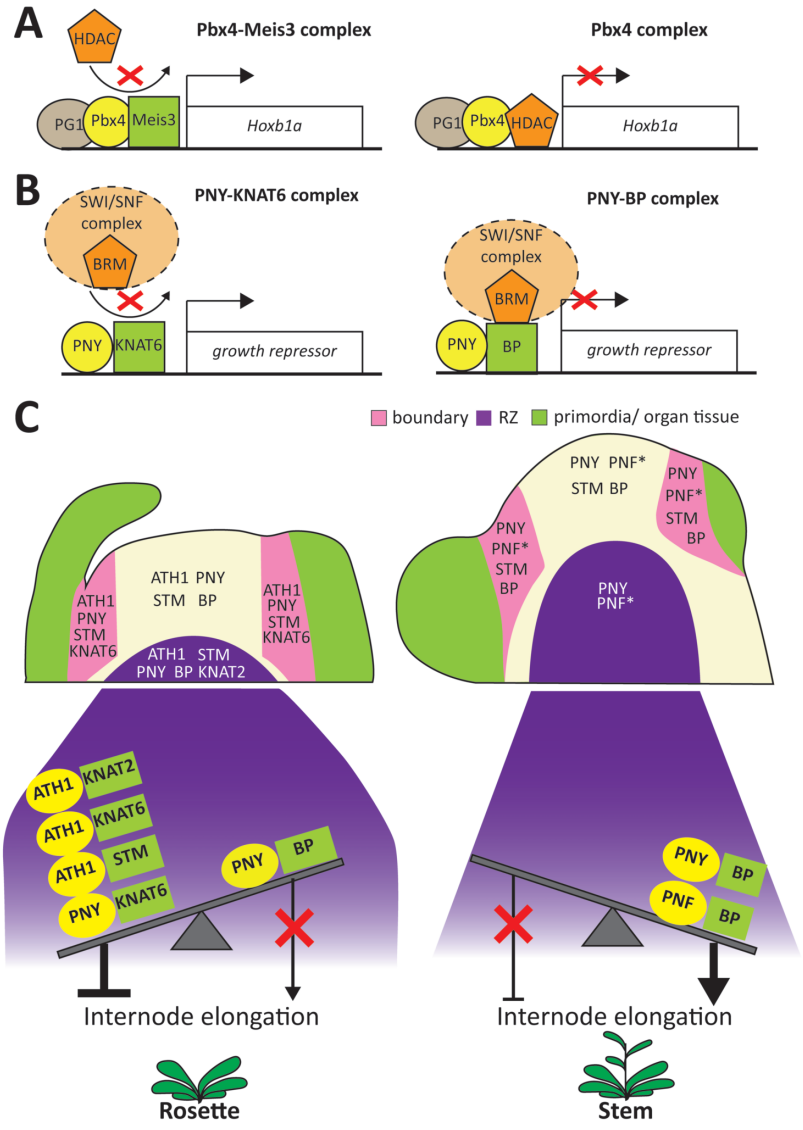


Fig. 5.11 PNY-KNOX heterodimer shift: A proposed mechanism for the dual role of *PNY*
A-B: A single TALE homeobox transcription factor can have opposing effects on expression of a single gene. In zebrafish (A), the TALE homeobox transcription factor Pbx4 opposingly regulates *Hoxb1a* expression. In PG1-Pbx4-Meis3 complexes, interaction between Pbx4 and Meis3 prevents binding of histone deacetylates (HDAC), leading to *Hoxb1a* upregulation. In absence of Meis3, PG1-Pbx4-HDAC complexes repress *Hox1ba*. In Arabidopsis (B), a similar mechanism could occur with PNY-KNAT6 and PNY-BP heterodimers, leading to opposing regulation of growth repressor target genes, e.g. *LSH4*. These changes in regulation might require the SWI/SNF chromatin remodeller complex, of which BRM interacts with BP.
C: Changes in BLH and KNOX transcription factor levels in the shoot apex during development affect heterodimer formation, which likely impacts internode elongation. Expression patterns are based on mRNA (*ATH1*, *PNY*, *STM*, *KNAT2, KNAT6*) or protein (ATH1, PNY) localization studies. As class I KNOX proteins can be trafficked to adjacent cell layers, it is possible that they are present outside the mRNA expression domain depicted here. Asterisk indicates that expression domain of *PNF* is extrapolated from mutant phenotypes and transcriptome data of meristem-enriched tissue.

ATH1, PNY and PNF share the class I KNOX proteins (STM, BP, KNAT2 and KNAT6) as interaction partners (Bao, 2009; Bhatt et al., 2004; Cole et al., 2006; Kanrar et al., 2006; Li et al., 2012c; Rutjens et al., 2009). *STM* and *BP* are expressed in the shoot apex throughout development, but K*NAT2* and *KNAT6* are only expressed in the shoot apex during vegetative growth (Chuck et al., 1996; Liu et al., 2018a; Ori et al., 2000; Ragni et al., 2008; Rast and Simon, 2012). Little is known about the spatiotemporal expression of class I KNOX proteins in the shoot apex, but it is known that class I KNOX proteins are mobile, and can move between cell layers of Arabidopsis, rice and maize meristems (Balkunde et al., 2017; Kuijt et al., 2004; Lucas et al., 1995). Thus, it should be noted that KNOX proteins expressed in the boundary or CZ, such as BP, could potentially diffuse to the RZ.

PNY-STM heterodimers likely regulate stem cell maintenance and boundary specification (Kanrar et al., 2006; Rutjens et al., 2009). The PNY-BP heterodimer promotes stem elongation redundantly with the PNF-BP heterodimer (Kanrar et al., 2006), while PNY-KNAT6 heterodimers likely repress internode elongation (Fig. 5.10). Thus, the PNY-BP and PNY-KNAT6 heterodimers have opposing effects on stem elongation. During vegetative growth, PNY can theoretically interact with all four class I KNOX proteins. During reproductive growth, PNY is limited to forming PNY-STM and PNY-BP heterodimers. Thus, a shift in PNY heterodimer formation likely occurs during the reproductive phase change (Fig. 5.11C), which may result in changes in transcriptional regulation by PNY.

PNY targets *ATH1,* but also *KNAT2, KNAT6, BOP1, BOP2, LOB* and *LSH4* (Andrés et al., 2015; Bencivenga et al., 2016), which are also targets of *ATH1* (Chapter 2). Ectopic expression of *BOP1, BOP2, LOB* and *LSH4* represses stem elongation (Bencivenga et al., 2016; Norberg, 2005; Shuai et al., 2002). Loss of *KNAT6, BOP1, BOP2*, *LSH4* or *ATH1* rescues stem elongation defects of *pny* mutants (Bencivenga et al., 2016; Khan et al., 2012b, 2012a; Ragni et al., 2008), and we showed that loss of *KNAT6* rescues stem elongation defects of *35S::HA-PNY* (Fig 5.10). Moreover, expression of *ATH1, LOB* and *LSH4* was elevated in *pny* and *35S::HA-PNY*, dependent on developmental phase (Fig. 5.9). In *pny* mutants, *LSH4* is expressed ectopically in the RZ during bolting. This ectopic expression of *LSH4* restricts stem elongation (Bencivenga et al., 2016). *LSH4* expression was elevated in apices of bolting *pny* plants and in apices of vegetative and bolting *35S::HA-PNY* plants (Fig. 5.9). The elevated *LSH4* expression in bolting *35S::HA-PNY*, enabled by an increase in PNY-KNAT6 heterodimer availability

could explain why stem elongation is reduced in these plants.

The change in KNOX partner availability and the overlap of ATH1 and PNY targets very likely lies at the heart of the dual function of *PNY.* The transcriptional activity of PNY might be changed by switching from PNY-KNAT6 heterodimers to PNY-BP heterodimers, resulting in opposing regulation of target genes (Fig 5.11). Further investigation of PNY interactors and shared targets at different stages of development is likely to shed light on the complex relationship between PNY, the rosette habit and bolting.

Materials and methods

Plant materials and growth conditions

All plant lines used were in the Col-8 background.

The *ath1-3* (Proveniers et al., 2007), *pny*^{40126} (Smith and Hake, 2003), *pnf*^{96116} (Smith et al., 2004), *ath1-3 pny*^{40126} (Khan et al., 2012a)*, ath1-3 pny*^{40126} *pnf*^{96116} (Khan et al., 2015)*, 35S::ATH1-HBD* (*35Spro:ATH1-HBD*) (Rutjens et al., 2009*), knat2-5, knat2-5 knat6-2, knat6-1, knat6-2* (Belles-boix et al., 2006; Ragni et al., 2008) *bum1-1* (Jasinski et al., 2005) and *bp-11* (dela Paz et al., 2012) mutants have been described previously. Double and triple mutants were created by crossing and were selected through genotyping (For primer sequences see Supplemental Table 5.S1). Seeds were sown on soil or Murashige and Skoog medium (pH=6.0, 0.8% plant agar; Duchefa) and stratified for two days in darkness at 4°C, then transferred to the light. Plants were grown in long-day (LD; 16 hours light, 8 hours darkness) conditions at 22°C or 27°C, short-day (SD; 8 hours light; 8 hours dark) at 22°C under white fluorescent light (Sylvania Luxline Plus Cool White) conditions, or in LD under LED light in far-red enriched conditions (FR; 22°C, red, blue and far-red LED light, R:FR ratio 1.34). All plants were grown at 120 µmol/m^2/s light, 70% relative humidity. Plants grown at 27°C were germinated for 2 days at 22°C in LD prior to transfer to 27°C.

Phenotypic analyses

Heterochronic bolting was assayed by measuring the total height of the rosette, from cotyledons to the last rosette leaf, and dividing this by the total number of primary rosette leaves formed. Bolting time was scored as the number of days until the emergence of a visible inflorescence stem (> 5 mm) and flowering time was

scored as the number of days until the opening of the first flower. In addition, the number of primary rosette leaves was used as a measure for bolting time, and the total number of primary leaves was used as a measure for flowering time. Studies that included bolting time measurements for *ath1-3* (Fig. 5.5A, 5.S1) were conducted in SD conditions. Statistical analyses were performed in IBM SPSS 24, α=0.05 (For outcome of statistical tests, see Supplemental Table 5.S2).

For GA or picloram treatments, plants were grown for 1 week on MS agar plates supplemented with 0.1% DMSO (mock), 100 µM GA4+7 (Duchefa) or 5 µM picloram (Sigma-Aldrich) and transferred to soil. Hormone treatment was continued three times a week until opening of the first flower, by pipetting 1 µM of mock or hormone solution, supplemented with 0.01% v/v Silwet L-55 (Momentive), onto the shoot apex. Cytokinin treatments were performed with 1 µM 6-benzylaminopurine (BAP; Duchefa) solution supplemented with 0.01% v/v Silwet-L55 (Momentive). Plants were germinated on soil, and treated three times a week with 1 µl of BAP solution pipetted onto shoot apices from germination until opening of the first flower.

Dexamethasone (DEX; Sigma-Aldrich) were performed by spraying plants with 3 times a week with a 10 µM DEX + 0.01% Silwet-L55 from week 1, 2, 3, 4 or 5 onwards until senescence. For continuous DEX treatment, seeds were first stratified for 2 days in DEX solution, sown onto soil and sprayed 3 times a week from germination until senescence.

Analysis of gene expression

For qRT-PCR analysis of gene expression plant material was snap-frozen in liquid N_2 and RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich) according to manufacturer's instructions. RNA was treated with DNAseI (Thermo-Fisher) and used for cDNA synthesis using RevertAid Reverse Transcriptase and Ribolock RNAse inhibitor (Thermo-Fisher), according to manufacturer's instructions. cDNA was used for qRT-PCR using the Viia7 system (Thermo-Fisher).

Overexpression of *PNY* was determined by harvesting leaf material from the first cauline leaf of Col-8 or *35S::HA-PNY* T1 overexpressor plants, or from shoot apex enriched material of Col-8 or *35S::HA-PNY* #22 plants at the 2-leaf stage. For *ATH1* and boundary gene expression, Col-8, *pny*^{40126} and *35S::HA-PNY* #22 were grown in LD conditions and sampled at the 2-leaf and bolting stage. Due to differences in

growth rates, Col-8 and *pny*^{40126} were sampled at day 9 and 31, while *35S::HA-PNY* #22 plants were sampled on day 19 and 40. qRT-PCR was performed for *PNY, ATH1, LOB, BOP1, BOP2* and *LSH4,* using *At5g15400* as an endogenous control. For primer sequences see Supplemental table 5.S1.

Generation of *35S::HA-PNY* overexpressors

Full-length cDNA of *PNY,* flanked by Gateway linkers was amplified from Col-8 (for primer sequences see Supplemental Table 5.S1). PCR product was integrated into the pDONR201 vector (Thermo-Fisher) and subsequently into the pAlligator2 vector (Bensmihen et al., 2004) using the Gateway cloning system (Thermo-Fisher), according to manufacturer's instructions. The vector was transformed into Col-8 with *Agrobacterium tumefaciens* C48, using the floral dip method. T1 seeds were screened for GFP fluorescence and planted on soil for further analysis.

Supplemental figures and tables

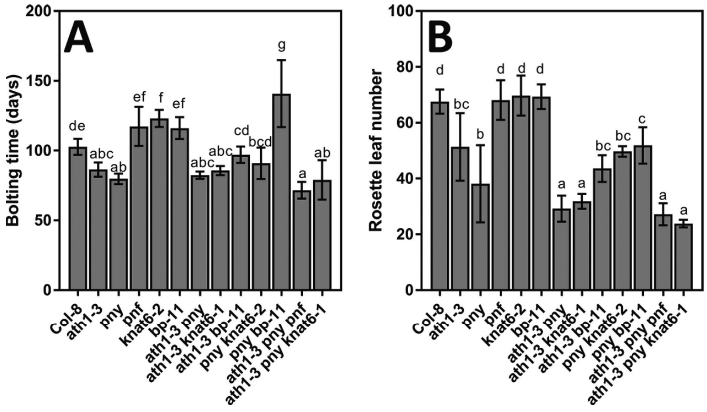
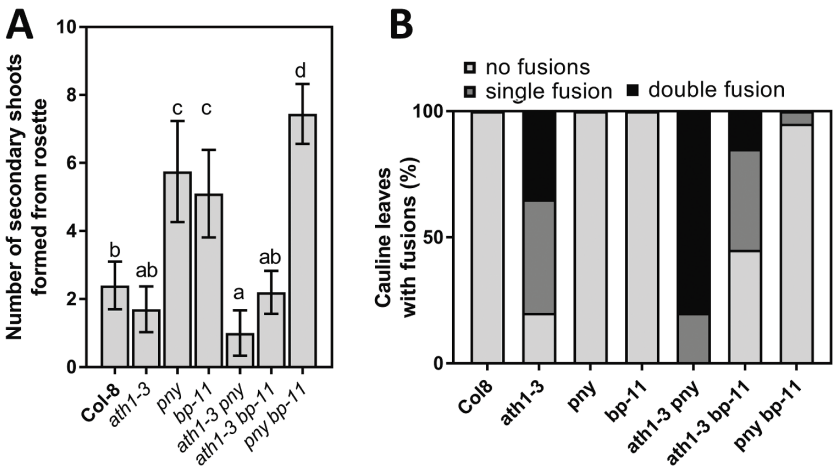


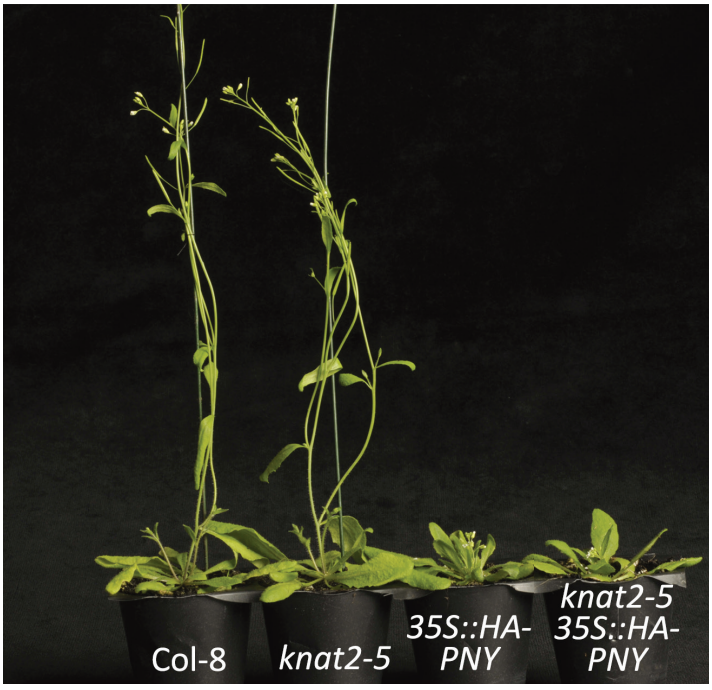
Fig. 5.S1: Bolting time is affected in SD-grown *BLH* and *KNOX* mutants
SD-grown Col-8, *ath1-3*, *pny*^{40126}, *pnf*^{96116}, *knat6-2*, *bp-11*, *ath1-3 pny*^{40126}, *ath1-3 knat6-1, ath1-3 bp-11*, *pny*^{40126} *knat6-2, pny*^{40126} *bp-11* and *ath1-3 pny*^{40126} *pnf*^{96116} phenotyped for:
B: Bolting time (n=9, 9, 9, 8, 10, 6, 10, 10, 10, 10, 7, 10, 8)
C: Number of rosette leaves (n=9, 9, 10, 8, 10, 6, 10, 10, 10, 10, 7, 10, 7)
Error bars depict standard deviation of the mean, lowercase letters depict homogeneous subsets as defined by Tukey's post-hoc test (See Supplemental Table 5.S2).



Supplemental Fig. 5.S2: Stem-cauline leaf fusion and apical dominance phenotypes in LD-grown Col-8, *ath1-3*, *pny*^{40126}, *bp-11*, *ath1-3 pny*^{40126} and *ath1-3 bp-11* mutants

A: Number of secondary shoots formed from the rosette. Error bars represent standard deviation, letters denote statistically homogeneous subsets defined by 1-way ANOVA followed by Tukey's post-hoc test (n=10, 10, 8, 10, 10, 10, 10).

B: Percentage of cauline leaves with single, double or no cauline leaf-stem fusions. First and second cauline leaves were measured (n=20, 20 16, 10, 10, 10, 10).



Supplemental Fig. 5.S3: Phenotypes of 38-day old LD-grown Col-8, *knat2-2, 35S::HA-PNY* and *knat2-5 35S::HA-PNY*

Supplemental Table 5.S1: Primer sequences

Name	Sequence	Type	Description
113353-F	TTTGTAGTTCAAGAGAAAAGCTTGA	genotyping	Genotyping *ath1-3* (F/R, F/LBb1)
113353-R	GGCGGGTTTTCGGATCTACATT	genotyping	
040126-LPn	TTGGAACCAAGTTCAAACTCG	genotyping	Genotyping *pny* (LPn/RPn, LBb1/RPn)
040126 RPn	ATGTTCACAGTTTTTGGTCGG	genotyping	
4K1	CGCTTCTCATCCTTTGTATC	genotyping	Genotyping *knat2-5* (4K1/K11, LBa1/K11)
K11	TACCCATGAGTCTCTTAATG	genotyping	
k6-41	GCTACCAATCATTTTTCAGAAA	genotyping	Genotyping *knat6-1*
k6-15	TAAGTCGGTTCTGATGATG	genotyping	(k6-15/k6-41; LBa1/k6-41)
k6-03	GAAGATAAACCCTAGCTACAAG	genotyping	Genotyping *knat6-2*
k6-04	AACCTGCATCGATCTATTTTC	genotyping	(k6-03/k6-04, LBa1/k6-04)
137958.48.30LP	CTGTTGTCGAGCCTCAAAGTC	genotyping	Genotyping *bp-11* (LP/RP, LBb1/RP)
137958.48.30RP	AACGACGTCTTGTCAATCCAC	genotyping	
35S-mini	CTGCAGCAAGACCCTTCC	genotyping	Detecting 35S-ATH1
ATH1CDS-R	CCGAGCATTCATCAGAAACA	genotyping	(35s-mini/ATH1CDS-R)
LBa1	TGGTTCACGTAGTGGGCCATC	Genotyping	Insertion primer for genotyping SALK mutants
LBb1	GCGTGGACCGCTTGCTGCAAC	Genotyping	Insertion primer for genotyping SALK mutants
At5g15400 QPCR-F	GGGCACTCAAGTATCTTGTTAGC	qPCR	qRT-PCR primers used as endogenous control
At5g15400 QPCR-R	TGCTGCCCAACATCAGGTT	qPCR	
ATH1_NCON-QPCR_F	TCCTCCACTTCATCCTTTGG	qPCR	qRT-PCR primers for *ATH1*
ATH1_NCON-QPCR_R	TCACTTGGACCAACTACACCTG	qPCR	
PNY Q-PCR F	TGTAAACCTCGTCGAGCATGGAGA	qPCR	qRT-PCR primers for *PNY*
PNY Q-PCR R	GCGGCGGAGGAGATAATGGAAAG	qPCR	
LOB QF2	TGCGTCGGAGCCATCTCTTATC	qPCR	qRT-PCR primers for *LOB*
LOB QR2	AGTCAGCATTAGCTGCGTCGAG	qPCR	
BOP1 AT3G57130.1_QF	AGCTTGGAGCAGCTGATGTGAAC	qPCR	qRT-PCR primers for *BOP1*
BOP1 AT3G57130.1_QR	ACCATTTCAGCCGCAATGTGAAG	qPCR	
BOP2-qF	GGAAGGTATGAGTCGGCATC	qPCR	qRT-PCR primers for *BOP2*
BOP2-qR	TGCATGCCCCTCTTCTTAAT	qPCR	
LSH4-qF	ACCAATTCGGCAAGACTAAGGTTC	qPCR	qRT-PCR primers for *LSH4*
LSH4-qR	AGCAGCTCTAAGACGGCCAATG	qPCR	
PNY GW-FWD	GGGGACAAGTTTGTACAAAAAAGCAGG CTTCATGGCTGATGCATACGAGCCT	cloning	For amplification of PNY full-length cDNA flanked by gateway attB1 and attB2 sequences.
PNY GW-REV	GGGGACCACTTTGTACAAGAAAGCTGG GTCTCAACCTACAAAATCATGTAG	cloning	

Supplemental Table 5.S2: Outcomes of statistical testing

Figure	Statistical test	Outcome
Fig. 5.1A	1-way ANOVA	F(7; 133)=76.7; p=1.3E-43
Fig. 5.2E	1-way ANOVA	F(3; 43)=11.5; p=1.2E-5
Fig. 5.3A	1-way ANOVA	F(3; 39)=103.8; p=1.2E-18
Fig. 5.3B	1-way ANOVA	F(6; 56)=179.6; p=5.4E-36
Fig. 5.4A	1-way ANOVA	F(5; 65)=12.0; p=2.9E-8
Fig. 5.5A	1-way ANOVA	F(2; 35) = 52,6 , p=4.7E-10
Fig. 5.5B	1-way ANOVA	F(12; 101)=73.4; p=2.9E-44
Fig. 5.6A	1-way ANOVA	F(3; 33)=98.5; p=1.5E-16
Fig. 5.6B	1-way ANOVA	F(11; 106)=29.5; p=2.2E-27
Fig. 5.6D	1-way ANOVA	F(5; 49)=100.9; p=1,4E-24
Fig. 5.6E	1-way ANOVA	F(3; 36)=75.6; p=1,3E-15
Fig. 5.8C	1-way ANOVA	F(5; 37)=38.2; p=1.3E-13
Fig. 5.8D	1-way ANOVA	F (5; 39) = 146.4; 7.4E-19
Fig. 5.8E	1-way ANOVA	F (5; 38) = 5.2; p=9.3E-5
Fig. 5.8F	1-way ANOVA	F (5; 38) = 4.9; p=1.4E-4
Fig. 5.8G	1-way ANOVA	F(2; 39)= 50.1; p=1.7E-11
Fig. 5.8H	1-way ANOVA	F(2; 39)=40.8; p=2.7E-10
Fig. 5.10A	1-way ANOVA	F(3; 33)=214.7; p=1.0E-21
Fig. 5.10B	1-way ANOVA	F(3; 33)=16.3; p=1.1E-06
Fig. 5.10C	1-way ANOVA	F(3; 33)=13.41; p=6,5E-6
Fig. 5.10D	1-way ANOVA	F(3; 32)=210.331; p=3,9E-21
Fig. 5.S1A	1-way ANOVA	F(12; 103)=39.0; p=7.8E-33
Fig. 5.S1B	1-way ANOVA	F(12; 102)=77.1; p=1.6E-45
Fig. 5.S2A	1-way ANOVA	F(6; 60)=62.6; p=5.6E-24

CHAPTER 6

Summarizing discussion

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The rosette habit i**s** widely adopted by plants throughout Angiosperm lineages, both in monocot and dicot species, and is prevalent in several different kinds of environments, including alpine, desert, temperate and tropical areas (Cohen, 2011; Givnish et al., 1999; Hao et al., 2017; Larcher et al., 2010; Marks et al., 2011; Martorell and Ezcurra, 2002). A key fundamental event during the life cycle of rosette plants is the switch from rosette to stem habit: bolting. Architecturally, the rosette, where internode elongation is repressed, and the stem, where internode elongation is promoted, are opposites. These contrasting growth habits contribute to plant fitness in different ways. The rosette offers protection against (a)biotic stresses during vegetative growth, while the stem facilitates pollination and seed dispersal during reproductive growth (Fujita and Koda, 2015; Larcher et al., 2010). To ensure that the growth habit matches the changing functional demands resulting from the life cycle of a rosette plant, the switch from rosette to stem growth needs to be tightly controlled.

Besides being a fundamental developmental process of rosette plants, bolting is also agriculturally relevant. Bolting has a strong effect on plant architecture, but also on metabolite content and resource allocation of tissues produced during rosette growth, such as rosette leaves and tubers (Hoffmann and Kluge-Severin, 2011; Mathieu et al., 2018; Sessa et al., 2000). Rosette plants make up a significant part of cultivated vegetable crops (Table 1.1), and their consumable tissues are often produced and harvested during the rosette phase. As such, (premature) bolting has a negative effect on yield of these crops. Study of the rosette habit and understanding the initiation of bolting is therefore highly relevant in both a developmental and agricultural context. However, very little is known about factors that control rosette formation and the initiation of bolting.

Induction of bolting requires the hormone gibberellin (GA). In lettuce and *Arabidopsis thaliana* sharp increases in GA levels precede bolting, and GA is essential for bolting in several species, including Arabidopsis (Eriksson et al., 2006; Fukuda et al., 2009; Umetsu et al., 2011; Wilson et al., 1992; Xu et al., 1997). The hormones auxin and brassinosteroids (BR) are also required for stem elongation: stem elongation in auxin or BR biosynthesis or signalling mutants is severely reduced (Kauschmann et al., 1996; Timpte et al., 1992). Despite this, increased GA, BR or auxin activity alone is insufficient to disrupt the rosette habit during vegetative growth of Arabidopsis,

which suggests that a regulatory mechanism exists during rosette growth that overrides these elongation signals.

In this thesis we identified the *BEL1-LIKE HOMEODOMAIN* (*BLH*)-type transcription factor *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) as a key regulator of rosette growth. We showed that *ATH1* instils a robust block on multiple elongation signals that would otherwise enable internode elongation during rosette growth. In Chapter 2 we showed that *ATH1* is expressed in the shoot apex during vegetative growth, including the rib zone (RZ). The RZ is inactive during vegetative growth and is responsible for stem growth during bolting (Bencivenga et al., 2016; Jacqmard et al., 2003). Loss of *ATH1* during vegetative growth causes plants to precociously adopt an elongated RZ, causing internode elongation to occur already between rosette leaves (heterochronic bolting, Chapter 2). Heterochronic bolting of *ath1-3* is enhanced by high temperature, far-red light (FR), auxin, BR and GA (Chapter 2, 3). Individually, none of these signals cause heterochronic bolting in wild-type plants (Chapter 2, 3), which suggests that their outputs are all blocked by ATH1. Furthermore, each of the elongation routes blocked by *ATH1* is essential for internode elongation. Mutations in factors promoting tissue elongation, hormone function or cell division, i.e. *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*), *PIF7*, *DWARF1* (*DWF1*), *BIG* and *WUSCHEL* (*WUS*), all suppress heterochronic bolting of *ath1-3* mutants (Chapter 3, 4). In addition, inhibition of GA or BR production represses heterochronic bolting induced by high temperature, FR light (Chapter 2, 3) or phytohormone treatment (BR, or auxin and GA, respectively; Chapter 3, 4). Similarly, ectopic expression of *ATH1* during reproductive growth represses bolting (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007), even if ectopic *ATH1* is induced immediately prior to induction of the reproductive phase change (Chapter 5). Like wild-type rosette internodes, GA, auxin or BR application also do not induce stem internode elongation in *ATH1* overexpressors (Chapter 2, 3). Together this shows that the block on elongation instilled by *ATH1* cannot be bypassed, and is only released once *ATH1* is repressed.

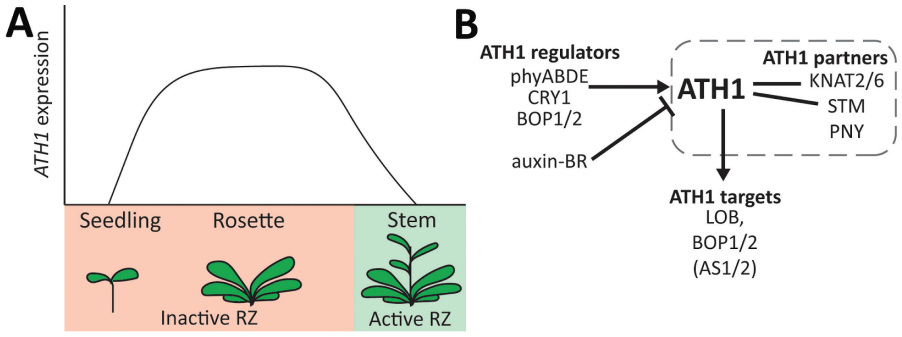

Fig. 6.1: ATH1 function is correlated to the rosette
A: Expression of *ATH1* in the shoot meristem coincides with the entire rosette phase, adapted from (Proveniers et al., 2007).
B: All known mutants exhibiting heterochronic bolting can be linked to ATH1 function. Disruption of any of the depicted factors can result in heterochronic bolting. Arrows, bars and simple lines represent (indirect) positive regulation, negative regulation and heterodimerization. Dashed box depicts possible ATH1-protein complex. *AS1* and *AS2* are targets of *PNY* (Bencivenga et al., 2016).

6.1 ATH1: La vie en rosette

What makes Arabidopsis form a rosette? Our data show that ATH1 is crucial for the formation of a compact rosette in Arabidopsis, thus shoot apex expression of *ATH1* itself may be the defining factor of the Arabidopsis rosette phase. *ATH1* expression in the SAM and RZ coincides with the entire rosette phase (Fig. 6.1). *ATH1* is not expressed in the SAM/RZ prior to the initiation of the first internodes, *i.e.* in embryos or in etiolated seedlings (Gómez-Mena and Sablowski, 2008; Proveniers, 2000; Quaedvlieg et al., 1995). In light-grown plants, *ATH1* expression is induced after germination and is maintained until the reproductive phase change, when rosette growth ends (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007; Quaedvlieg et al., 1995). Downregulation of *ATH1* in the SAM and RZ coincides with the elongation of the RZ and bolting and if *ATH1* is absent during vegetative growth plants lose the compactness of their rosette (Chapter 2).

ATH1 function is restricted to internode tissue, which sets it apart from general regulators of growth, and makes *ATH1* a specific regulator of the rosette habit. As mentioned previously, *ATH1* prevents elongation in response to several elongation signals (Chapter 2-3). Despite instilling such a broad brake on elongation, this effect of *ATH1* is specific to internode tissue. *ath1-3* mutants do not form larger rosettes but only elongate internodes. Similarly, *35S::ATH1* stem internodes, but not leaves, are reduced in size (Bao, 2009; Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009). In contrast, disrupting processes downstream of ATH1

has systemic effects on growth: plants ectopically expressing *LOB*, *BOP1* and *BOP2* exhibit general inhibition of growth, as do plants that are deficient in GA, auxin or BR (Blázquez et al., 1998; Kauschmann et al., 1996; Norberg, 2005; Shuai et al., 2002; Timpte et al., 1992; Wilson et al., 1992). It is likely that local inhibition of elongation by *ATH1* is caused by a combination of the *ATH1* expression domain and the availability of KNOTTED1-LIKE HOMEOBOX (KNOX) transcription factors, which interact with ATH1 and are required for *ATH1* function (Chapter 5).

Another point suggesting that *ATH1* lies at the heart of rosette habit control, is that all other known mutants that exhibit heterochronic bolting can be related back to *ATH1* function and none of these mutants exhibit phenotypes as severe as *ath1-3.* Repression of internode elongation by ATH1 requires the function of its KNOX interaction partners *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*), *KNAT6* and *SHOOT MERISTEMLESS,* and is enhanced by the *BLH* transcription factor *PENNYWISE* (*PNY; BELLRINGER; REPLUMLESS; VAAMANA; LARSON; BEL1-LIKE HOMEODOMAIN 9)* (Chapter 5). Plants mutated in any of these genes exhibit heterochronic bolting (Chapter 6). This is also observed in plants lacking targets of ATH1, the boundary genes *LATERAL ORGAN BOUNDARIES* (*LOB*) and *BLADE ON PETIOLE 1* (*BOP1*), *BOP2* (Chapter 2-4) (Zhang et al., 2017a), or the boundary genes ASYMMETRIC LEAVES1 (AS1) or AS2 (Chapter 4), which are targets of PNY (Bencivenga et al., 2016). Mutants in upstream regulators of *ATH1* also exhibit this phenotype. Combined auxin and BR treatment reduces *ATH1* expression and induces heterochronic bolting in wild-type plants, and auxin alone induces heterochronic bolting in the gain of function BR signalling mutant *brassinazole insensitive 1-1D* (*bzr1-1D*) (Chapter 3). Other regulators of *ATH1* include *BOP1, BOP2* and light (Khan et al., 2015; Quaedvlieg et al., 1995). *ATH1* is induced by light and repressed in darkness. Repression of *ATH1* in darkness requires CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), which in turn is repressed by the phytochrome A (phyA) and phyB red/FR light receptors, and the CRYPTOCHROME 1 (CRY1) blue light receptor (Liu et al., 2011; Lu et al., 2015; Quaedvlieg et al., 1995; Sheerin et al., 2015). Loss of multiple light receptors from the phytochrome and cryptochrome families induces heterochronic bolting, which can be enhanced by FR light and high ambient temperature (Devlin, 1998; Mazzella et al., 2000), like in *ath1-3.* In addition, heterochronic bolting of *phyB* is enhanced through loss of *BOP1* or *BOP2* (Zhang et al., 2017a). Thus, it is likely that the heterochronic bolting phenotype of

light receptor mutants is caused by loss of *ATH1* expression.

6.2 Stemming elongation: Does ATH1 induction interrupt an ongoing elongation programme?

In essence, the rosette is a non-elongated growth stage sandwiched between two stages of elongation growth (Fig. 6.1A). This begs the question whether the rosette phase simply signifies a temporary brake on the same elongation programme. It is possible that part of the hypocotyl elongation programme is reactivated during bolting. Both hypocotyl and stem elongation are stimulated by similar signals, such as GA, BR and auxin. Additionally, FR light, ambient temperature and photoperiod promote hypocotyl elongation as well as the reproductive phase change, which initiates bolting (Blázquez et al., 1998; Chapman et al., 2012; Fukuda et al., 2009; Hornitschek et al., 2012; Jacqmard et al., 2003; Kauschmann et al., 1996; Nemhauser et al., 2004; Oh et al., 2014; Stavang et al., 2009; Timpte et al., 1992). These factors all promote heterochronic bolting as well (Chapter 2-4). In addition, *PIF* transcription factors, which are major positive regulators of hypocotyl elongation, also have a positive effect on heterochronic bolting (Chapter 3), and have been linked to internode elongation (Brock et al., 2010). However, the hypocotyl programme alone cannot account for all the elongation of the stem. Bolting requires both cell division and cell elongation, orchestrated by the RZ, while hypocotyls elongate through cell elongation of existing tissue alone (Gonzalez et al., 2010; Sachs and Long, 1957; Sachs et al., 1959a; Timpte et al., 1992 (Bencivenga et al., 2016; Gendreau et al., 1997; Kauschmann et al., 1996; Sachs, 1991; Sachs and Long, 1957; Sachs et al., 1959a; Timpte et al., 1992). Heterochronic bolting is affected by similar signals as hypocotyl elongation and internode elongation, but it is not known whether heterochronic bolting occurs through a combination of cell division and elongation, or whether these internodes grow through cell elongation alone, like hypocotyl tissue. In the stem, loss of important multiple important regulators of hypocotyl elongation, such as *PIFs*, reduces stem internode elongation but do not abolish it (our unpublished observations). The overlap between regulators of hypocotyl and internode elongation might be part of a general tissue elongation programme. The BAP-D module, a genetic circuit linking hormonal and environmental elongation signals controlling hypocotyl elongation, was recently proposed as a universal growth promoting complex across several developmental processes (Bai et al.,

2012; Bouré et al., 2019; Oh et al., 2012, 2014), which might also include internode elongation.

To understand how a temporary brake on a shared elongation programme is locally instilled during rosette growth, it is necessary to take a closer look at RZ regulation during the earliest stages of growth. After germination, light is necessary for photomorphogenesis, which involves a developmental phase change at the SAM, changing the inactive embryonic SAM to an active vegetative SAM. As discussed, initial induction of *ATH1* in the shoot apex is induced by light, two days after germination (Quaedvlieg et al., 1995), coinciding with photomorphogenesis and marking the start of the rosette phase. Unlike the SAM, the RZ remains inactive during photomorphogenesis. As ATH1 is crucial for the rosette habit, this early, light-dependent induction of *ATH1* might function to prevent RZ activation separating it from SAM activation during photomorphogenesis. During germination and seedling growth in darkness, the meristem is inactive and *ATH1* is not expressed (Pfeiffer et al., 2016; Quaedvlieg et al., 1995). Despite the absence of *ATH1,* the RZ is mitotically inactive, but this is likely a consequence of general SAM inactivity in the dark, as the RZ relies on supply of cells from the overlying meristem (Bencivenga et al., 2016; Roldan et al., 1999; Sachs, 1991). SAM activation can be forced in dark-grown plants by supplying the meristem with sucrose or glucose (Pfeiffer et al., 2016; Roldan et al., 1999). The RZ is also activated by sugars and elongated internodes form, akin to heterochronic bolting (Mohammed et al., 2018; Roldan et al., 1999). *ATH1* is not expressed in these conditions (Gómez-Mena and Sablowski, 2008), which suggests that that the default state of the vegetative RZ is an active state, and the initial induction of *ATH1* is required to overrule this state during rosette growth.

6.3 Making a bolt for it: leaving behind the rosette phase

As ATH1 enforces a block on internode elongation during vegetative growth that cannot be bypassed, downregulation of *ATH1* from the SAM and RZ is necessary to enable bolting. *ATH1* is downregulated prior to the reproductive phase change (Gómez-Mena and Sablowski, 2008; Proveniers et al., 2007). We showed that in plants transferred to floral inductive conditions, this downregulation coincides with expansion of the RZ and occurs rapidly after transfer (Chapter 2). Therefore, *ATH1* likely is under control of key regulators of the reproductive phase change, *i.e.* the floral pathway integrators.

Three floral promotive pathways (GA, photoperiod, light quality) and four floral enabling pathways (temperature, vernalization, developmental age, autonomous) (Chapter 1.3) regulate the reproductive phase change by activating the floral pathway integrators *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FRUITFULL* (*FUL*) (Khan et al., 2014). Expression of floral pathway integrators marks the start of the reproductive phase change. SOC1 directly regulates gene expression, while FT and TSF regulate transcription by interacting with the bZIP transcription factors FLOWERING LOCUS D (FD) and FD PARALOG (FDP) (Abe et al., 2005; Immink et al., 2012; Tao et al., 2012). The floral pathway integrator genes SOC1 and FT likely act upstream of ATH1. In *constans* (*co*) and *ft* mutants, which do not transition to reproductive growth when transferred from short-day (SD) to long-day (LD) conditions, downregulation of *ATH1* does not occur. *ATH1* downregulation also does not occur in *soc1* and *fd* mutants (Fig 6.2) (Leal Valentim et al., 2015; Schmid et al., 2003). A negative effect of *SOC1* on *ATH1* expression has been observed in 7-day old seedlings. Additionally, ChIP-seq analysis revealed that the *ATH1* promoter is bound by *SOC1* during the reproductive phase change in two out of three biological replicates (Immink et al., 2012; Tao et al., 2012). This suggests that SOC1 directly represses *ATH1*. In addition, we showed that combined auxin and BR application represses *ATH1* expression (Chapter 3). FD and SOC1 targets also include genes related to auxin function (Collani et al., 2019; Immink et al., 2012; Tao et al., 2012). Auxin is necessary for stem elongation and, in lettuce, a subapical auxin peak precedes bolting (Hao et al., 2018; Timpte et al., 1992). In line with this, hormone reporter studies suggest that the auxin and BR state of the RZ is low during vegetative growth and high during reproductive growth (Brunoud et al., 2012; Sandhu et al., 2012; Shi et al., 2018).

Thus, the downregulation of *ATH1* during the reproductive phase change could be caused by (in)direct repression by floral pathway integrators, releasing the ATH1-instilled block on elongation.

Parallels can be drawn between the relationship of ATH1, floral pathway integrators and promotive elongation signals, and the interplay between floral repressors, enablers and promoters in regulating the reproductive phase change (Fig. 6.3). For example, repression of the floral repressor *FLOWERING LOCUS C* (*FLC*) by vernalization, a floral enabler, releases a brake on floral pathway integrators. Once this brake is released, floral promotive pathways are able to induce expression of these genes and initiate the reproductive phase change. Similarly, only once *ATH1* is lost from the SAM and RZ, signals promoting internode elongation can take effect. Here, *ATH1* acts as a bolting repressor, with the floral pathway integrators possibly acting as bolting enablers. GA likely acts as a bolting promotive signal, as it induces cell division in the RZ and is essential for bolting (Metzger and Dusbabek, 1991; Moon et al., 2005; Sachs et al., 1959b; Wilson et al., 1992), but has no effect in the presence of ATH1 (Chapter 2).

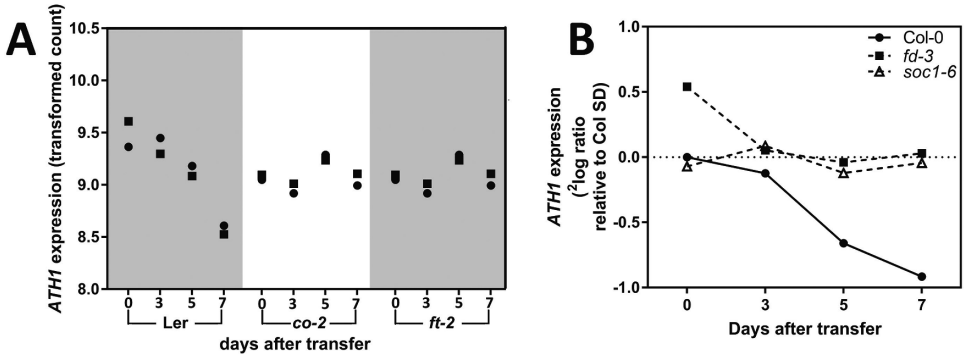

Fig. 6.2: Expression of *ATH1* during induction of the reproductive phase change
Expression levels of *ATH1* in meristem-enriched tissue harvested during induction of the reproductive phase change obtained from public micro-array datasets. Plants were grown for 30 days in non-inductive SD conditions and sampled 0, 3, 5, and 7 days after transfer to inductive LD conditions.
A: Transformed count data of two biological replicates of Ler, *ft-2* and *co-2* plants (Schmid et al., 2005). Data were obtained from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo).
B: Relative expression of *ATH1* in Col-0, *fd-3* and *soc1-6* mutants (Leal Valentim et al., 2015). Data are relative to non-induced Col-0 samples and were obtained via Genevestigator (Zimmermann et al., 2004).

Floral pathway integrator control of *ATH1*, a factor that affects bolting but has no effect on flowering in inductive conditions, shows that bolting is induced in parallel to flowering. Indeed, disruption of floral pathway integrators or the floral pathways that regulate them, affects both bolting and flowering (Cerdán and Chory, 2003; Devlin, 1998; Ferrándiz et al., 2000; Lee et al., 2013; Liu et al., 2008; Macknight et al., 1997; Moon et al., 2005; Posé et al., 2013; Simpson et al., 2003; Suárez-López et al., 2001; Wahl et al., 2013; Yamaguchi et al., 2005). The induction of flowering requires initial expression of the floral meristem identity genes, *LEAFY* (*LFY*) and *APETALA1* (*AP1*), which orchestrate a cell fate switch in organ primordia cells needed for floral development and do not affect bolting (Eriksson et al., 2006; Liljegren et al., 1999; Wigge et al., 2005). A similar set of genes has thus far not been described for coordinating the fate switch of the RZ between vegetative growth and bolting. If, as proposed in Section 6.2, the default state of the RZ is active, but is actively repressed by *ATH1* during rosette growth, it is likely the RZ is controlled by so-called "rib zone inactivity genes". ATH1 is a prime candidate for this group.

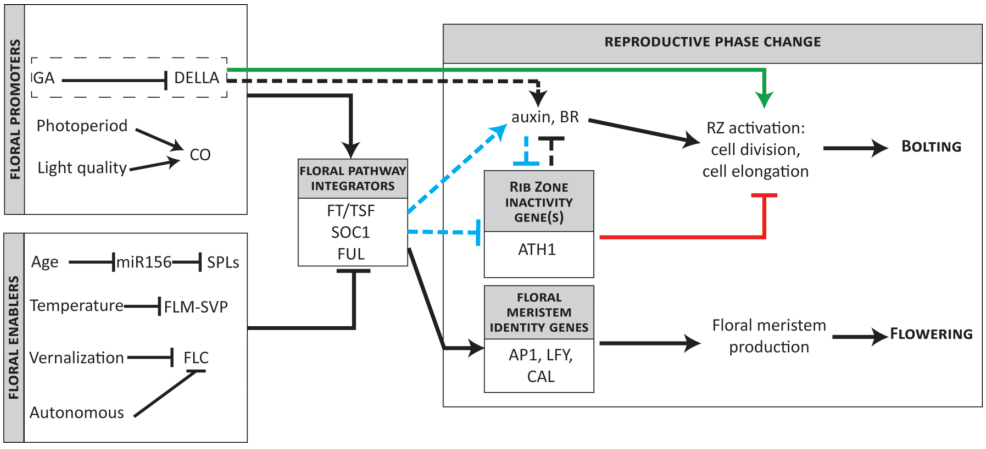

Fig. 6.3: Proposed model for the induction of bolting and flowering in Arabidopsis
Floral promotive and floral enabling pathways can each induce expression of floral pathway integrator genes. Expression of floral pathway integrator genes (*FT*, *TSF, SOC1, FUL*) marks the start of the reproductive phase change, consisting of the induction of bolting and flowering. Floral pathway integrator genes induce expression of floral meristem identity genes (*AP1*, *LFY* and *CAL*) to induce flowering. Floral pathway integrators repress "RZ inactivity gene(s)" such as *ATH1* to induce bolting, releasing an *ATH1*-mediated brake on rib zone cell division and cell elongation. In parallel, GA stimulates RZ cell division and elongation and promotes auxin and BR function. Bolting promoting, enabling and repressing regulation is depicted in green, blue and red, respectively. Dashed lines depict hypothesized regulatory pathways. Crosstalk between floral inductive pathways is not depicted.

6.4 Beating bolting

Premature bolting has a significant negative effect on the yield of rosette crops, affecting both quality and quantity of harvestable tissues. Current bolting resistant crop varieties have been generated through selection and breeding of late-bolting individuals, often resulting in the incorporation of vernalization requirement or reducing sensitivity to inductive environmental signals (Chapter 1.3, Table 1.1). Current agricultural practice to prevent premature bolting is focused mainly on restriction of the growing season to avoid crop exposure to environmental conditions that could trigger the reproductive phase change, e.g. prolonged cold or increased ambient temperature. There are a number of drawbacks associated with these strategies. Firstly, current bolting resistant varieties can still bolt if exposed to inductive environmental cues. Growth season restriction does not guarantee that these environmental cues can be avoided, due to weather unpredictability and climate change. Furthermore, some crops, such as sugar beet, are planted increasingly earlier in the growing season, when the probability of exposure to prolonged cold is higher (Jaggard et al., 2009). Secondly, restricting growth seasons also restricts the potential of crops. Earlier sowing of sugar beet can increase biomass and sugar yield. The cultivation of sugar beet sown in autumn and grown for a full year before harvest (winter beets), could theoretically have a 26% higher sugar yield than spring-sown sugar beet (Hoffmann and Kluge-Severin, 2010; Jaggard et al., 2009). However, as exposure to prolonged cold during the winter will induce bolting before harvest, neither winter beets nor sugar beet sown early in the season can be cultivated in temperate climates without large bolting-incurred yield losses.

These problems can be circumvented by generating plants that maintain high levels of bolting repressors regardless of environmental conditions. *ATH1* is a very promising breeding target for creating the next generations of bolting resistant rosette crops. Ectopic expression of *ATH1* represses bolting in LD conditions (Cole et al., 2006; Gómez-Mena and Sablowski, 2008; Rutjens et al., 2009) and FR conditions (Chapter 5), and cannot be rescued using GA (Gómez-Mena and Sablowski, 2008), BR or auxin (Chapter 3). As discussed above, *ATH1* likely acts downstream of the floral pathway integrators. Therefore, plants retaining a constant, high level of *ATH1* in the shoot apex throughout growth likely will be insensitive to both hormonal and environmental bolting signals. Ectopic expression of *ATH1* does not cause general

growth defects, in contrast to other non-bolting mutants, thus crops that maintain high *ATH1* levels can potentially repress bolting without affecting crop growth.

Orthologues of *ATH1* have been identified in genomes of both monocots and dicots, including rosette crops such as sugar beet, lettuce, radish, and multiple *Brassica rapa* and *B. oleracea* derived rosette crops (Hearn et al., 2018; Mukherjee et al., 2009; Mutasa-Göttgens et al., 2012; Wang et al., 2017; Zhang et al., 2017b). In addition, expression of *ATH1* orthologues was detected in shoot apices of sugar beet, *B. rapa* and *B. oleraceae* (Hearn et al., 2018; Mutasa-Göttgens et al., 2010). However, the function of these *ATH1* orthologues has not been studied thus far. Constitutive expression of Arabidopsis *ATH1* in tobacco, a stem plant, can repress internode elongation, resulting in rosette formation (M. Proveniers, unpublished data). On the other hand, bolting can be induced in lettuce through ectopic expression of Arabidopsis *BP* (Frugis et al., 2001). In Arabidopsis, *BP* acts antagonistically to *ATH1* in internode elongation (Chapter 6). The phenotypes of *ATH1* and *BP* overexpressors in tobacco and lettuce indicate that control of rosette habit and internode elongation by TALE homeobox transcription factors may be conserved. Studying the effects of (ectopic) expression of *ATH1* on bolting of rosette crops would be a good first step in utilizing the knowledge generated in this thesis for the development of new, bolting-resistant crops.

6.5 Future perspectives

The findings presented in this thesis contribute to understanding the rosette habit in Arabidopsis, which has been poorly studied until now. We focused specifically on bolting and not on flowering, unlike most other studies related to the reproductive phase change. Our understanding of the molecular control of the rosette habit and bolting is only beginning, and the findings from this thesis have opened up new questions to study these processes in a targeted manner.

First, understanding the downregulation of *ATH1* during the reproductive phase change is crucial to understand the earliest stages of bolting induction. In this thesis, we have considerably increased understanding of the components that act downstream of *ATH1* but concrete knowledge of the molecular factors that control *ATH1* downregulation is still lacking. Analysis of the *ATH1* promoter, to identify

regulatory elements that are necessary for *ATH1* induction or repression, will help to identify regulators of *ATH1* during the reproductive phase change. Promoter bashing of *ATH1* will help to identify these regulatory elements through the analysis of lines that no longer downregulate *ATH1.* These plants can be selected on the basis of reporter gene expression or by selecting plants with (heterochronic) bolting defects. In parallel, yeast-one-hybrid screening can be used to find transcription factors that bind to the *ATH1* promoter, and downregulation of *ATH1* can be tested after disrupting their binding sites.

Second, the interplay of GA, auxin and BR in the RZ warrants further investigation. We showed that restriction of auxin, BR and GA function by *ATH1* is necessary for the formation of a compact rosette, but their contributions to RZ activity are still largely unknown. GA induces cell division in the RZ of several rosette plants, but does not have a significant effect on cell elongation in the inflorescence stem (Sachs and Long, 1957; Serrano-Mislata et al., 2017; Talon et al., 1991). On the other hand, the dwarfed stature of the BR-deficient *dwarf4* (*dwf4*) mutant and the auxin-signalling mutant *auxin resistant 2* (*axr2*) is linked to a reduction in cell size and not cell number (Azpiroz et al., 1998; Timpte et al., 1992). Tracking cell division and elongation during heterochronic bolting under normal conditions and hormone deficient conditions will help elucidate the roles of these three hormones in internode elongation. In line with this, it is worth investigating the GA, auxin and BR state in the RZ during vegetative growth and bolting, and whether this state is disrupted in *ath1-3* mutants. To investigate this, it would be informative to study hormonal changes in the RZ during the induction of bolting and in vegetative *ath1-3,* through the use of sensitive hormone reporter lines, such as the R2D2 auxin reporter, GPS1 GA sensor or the auxin and GA Cas9-based HACR hormone sensors (Khakhar et al., 2018; Liao et al., 2015; Rizza et al., 2017).

Lastly, as ATH1 is crucial for the Arabidopsis rosette habit, it is tempting to speculate whether *ATH1* function underlies rosette habit evolution in Angiosperms. The rosette habit is found throughout Angiosperm lineages, and has been gained and lost several times throughout evolution, even occurring multiple times within the same tribe (Cohen, 2011; Givnish et al., 1999; Hao et al., 2017; Marks et al., 2011).

In some cases, plants adopting stem habits and plants adopting rosette habits are observed within the same species, depending on environmental conditions (Chase et al., 2018). In other rosette plants, GA application can directly induce cell division in the RZ and induce bolting in growth conditions that normally promote rosette growth (Achard et al., 2009; Gonzalez et al., 2010; Metzger and Dusbabek, 1991; Sachs and Long, 1957; Talon et al., 1991). In Arabidopsis, this does not occur in the presence of *ATH1* (Chapter 2), which raises the question whether *ATH1* is not expressed or is bypassed by GA in certain rosette species. Little is known about the evolutionary history of *ATH1* in Angiosperms, let alone rosette plants. Thus, to investigate whether changes in *ATH1* are linked to growth habit, it would be informative to compare *ATH1* loci and *ATH1* expression patterns in rosette and stem plants, particularly in tribes with known switches between rosette and stem, or in rosette plants where GA induces internode elongation in non-inductive conditions. Investigating the conservation of *ATH1* function across rosette plants will greatly contribute to understanding of the emergence and fundamental development of rosette plants.

References

Abe, E., Fujino, K., Masuda, K., and Yamaguchi, Y. (2014). Isolation and Expression Profiling of a CONSTANS-Like Gene and Two FLOWERING LOCUS T-Like Genes from Spinacia oleracea L. Am. J. Plant Sci. 05, 4018–4028.

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science 309, 1052–1056.

Achard, P., Baghour, M., Chapple, A., Hedden, P., Van Der Straeten, D., Genschik, P., Moritz, T., and Harberd, N.P. (2007). The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. Proc. Natl. Acad. Sci. U. S. A. 104, 6484–6489.

Achard, P., Gusti, A., Cheminant, S., Alioua, M., Dhondt, S., Coppens, F., Beemster, G.T.S., and Genschik, P. (2009). Gibberellin Signaling Controls Cell Proliferation Rate in Arabidopsis. Curr. Biol. 19, 1188–1193.

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H., and Tasaka, M. (1997). Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. Plant Cell 9, 841–857.

Alexandratos, N., and Bruinsma, J. (2012). World agriculture towards 2030/2050: the 2012 revision.

Alonso-Cantabrana, H., Ripoll, J.J., Ochando, I., Vera, A., Ferrándiz, C., and Martínez-Laborda, A. (2007). Common regulatory networks in leaf and fruit patterning revealed by mutations in the Arabidopsis ASYMMETRIC LEAVES1 gene. Development 134, 2663–2671.

Andrés, F., Porri, A., Torti, S., Mateos, J., Romera-Branchat, M., García-Martínez, J.L., Fornara, F., Gregis, V., Kater, M.M., and Coupland, G. (2014). SHORT VEGETATIVE PHASE reduces gibberellin biosynthesis at the Arabidopsis shoot apex to regulate the floral transition. Proc. Natl. Acad. Sci. 111, E2760-9.

Andrés, F., Romera-Branchat, M., Martínez-Gallegos, R., Patel, V., Schneeberger, K., Jang, S., Altmüller, J., Nürnberg, P., and Coupland, G. (2015). Floral induction in Arabidopsis thaliana by FLOWERING LOCUS T requires direct repression of BLADE-ON-PETIOLE genes by homeodomain protein PENNYWISE. Plant Physiol. 169, pp.00960.2015.

Angel, A., Song, J., Dean, C., and Howard, M. (2011). A Polycomb-based switch underlying quantitative epigenetic memory. Nature 476, 105–108.

Ariizumi, T., Lawrence, P.K., and Steber, C.M. (2011). The role of two F-box proteins, SLEEPY1 and SNEEZY, in Arabidopsis gibberellin signaling. Plant Physiol. 155, 765–775.

Aukerman, M.J., and Sakai, H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell 15, 2730–2741.

Azpiroz, R., Wu, Y., LoCascio, J.C., and Feldmann, K.A. (1998). An Arabidopsis brassinosteroid-dependent mutant is blocked in cell elongation. Plant Cell 10, 219–230.

Bai, M.-Y., Shang, J.-X., Oh, E., Fan, M., Bai, Y., Zentella, R., Sun, T.-P., and Wang, Z.-Y. (2012a). Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in Arabidopsis. Nat. Cell Biol. 14, 810–817.

Bai, M.-Y., Fan, M., Oh, E., and Wang, Z.-Y. (2012b). A Triple Helix-Loop-Helix/Basic Helix-Loop-Helix Cascade Controls Cell Elongation Downstream of Multiple Hormonal and Environmental Signaling Pathways in Arabidopsis. Plant Cell 24, 4917–4929.

Balasubramanian, S., Sureshkumar, S., Lempe, J., and Weigel, D. (2006). Potent Induction of Arabidopsis thaliana Flowering by Elevated Growth Temperature. PLoS Genet. 2, e106.

Balkunde, R., Kitagawa, M., Xu, X.M., Wang, J., and Jackson, D. (2017). SHOOT MERISTEMLESS trafficking controls axillary meristem formation, meristem size and organ boundaries in Arabidopsis. Plant J. 90, 435–446.

Bao, D. (2009). Control of Plant Architecture by Distinctive Homeobox Gene Interactions. Utrecht University.

Bell, E.M., Lin, W.W. -c., Husbands, A.Y., Yu, L., Jaganatha, V., Jablonska, B., Mangeon, A., Neff, M.M., Girke, T., and Springer, P.S. (2012). Arabidopsis lateral organ boundaries negatively regulates brassinosteroid accumulation to limit growth in organ boundaries. Proc. Natl. Acad. Sci. 109, 21146–21151.

Bellaoui, M., Pidkowich, M.S., Samach, A., Kushalappa, K., Kohalmi, S.E., Modrusan, Z., Crosby, W.L., and Haughn, G.W. (2001). The Arabidopsis BELL1 and KNOX TALE Homeodomain Proteins Interact through a Domain Conserved between Plants and Animals. Plant Cell 13, 2455–2470.

Belles-boix, E., Hamant, O., Witiak, S.M., Morin, H., and Traas, J. (2006). KNAT6 : An Arabidopsis Homeobox Gene Involved in Meristem Activity and Organ Separation. Plant Cell 18, 1900–1907.

de Bello, F., Lepš, J., and Sebastià, M.-T. (2005). Predictive value of plant traits to grazing along a climatic gradient in the Mediterranean. J. Appl. Ecol. 42, 824–833.

Bencivenga, S., Serrano-Mislata, A., Bush, M., Fox, S., and Sablowski, R. (2016). Control of Oriented Tissue Growth through Repression of Organ Boundary Genes Promotes Stem Morphogenesis. Dev. Cell 39, 198–208.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. Cell 115, 591–602.

Bensmihen, S., To, A., Lambert, G., Kroj, T., Giraudat, J., and Parcy, F. (2004). Analysis of an activated ABI5 allele using a new selection method for transgenic Arabidopsis seeds. FEBS Lett. 561, 127–131.

Bernardo-García, S., de Lucas, M., Martínez, C., Espinosa-Ruiz, A., Davière, J.-M., and Prat, S. (2014). BR-dependent phosphorylation modulates PIF4 transcriptional activity and shapes diurnal hypocotyl growth. Genes Dev. 28, 1681–1694.

Bernier, G., Kinet, J.M., Jacqmard, A., Havelange, A., and Bodson, M. (1977). Cytokinin as a Possible Component of the Floral Stimulus in Sinapis alba. Plant Physiol. 60, 282–285.

Best, N.B., Hartwig, T., Budka, J., Fujioka, S., Johal, G., Schulz, B., and Dilkes, B.P. (2016). nana plant2 Encodes a Maize Ortholog of the Arabidopsis Brassinosteroid Biosynthesis Gene DWARF1, Identifying Developmental Interactions between Brassinosteroids and Gibberellins. Plant Physiol. 171, 2633–2647.

Best, N.B., Johal, G., and Dilkes, B.P. (2017). Phytohormone inhibitor treatments phenocopy brassinosteroid-gibberellin dwarf mutant interactions in maize. Plant Direct 1.

Bhatt, A.M., Etchells, J.P., Canales, C., Lagodienko, A., and Dickinson, H. (2004). VAAMANA--a BEL1-like homeodomain protein, interacts with KNOX proteins BP and STM and regulates inflorescence stem growth in Arabidopsis. Gene 328, 103–111.

Blázquez, M.A., Green, R., Nilsson, O., Sussmann, M.R., and Weigel, D. (1998). Gibberellins Promote Flowering of Arabidopsis by Activating the LEAFY Promoter. Plant Cell 10, 791–800.

Bolduc, N., and Hake, S. (2009). The maize transcription factor KNOTTED1 directly regulates the gibberellin catabolism gene ga2ox1. Plant Cell 21, 1647–1658.

Bolduc, N., Yilmaz, A., Mejia-Guerra, M.K., Morohashi, K., O'Connor, D., Grotewold, E., and Hake, S. (2012). Unraveling the KNOTTED1 regulatory network in maize meristems. Genes Dev. 26, 1685–1690.

Booij, R., and Meurs, E.J.J. (1995). Effect of photoperiod on flower stalk elongation in celeriac (Apium graveolens L. var. rapaceum (Mill.) DC.). Sci. Hortic. (Amsterdam). 63, 143–154.

Borghi, L., Bureau, M., and Simon, R. (2007). Arabidopsis JAGGED LATERAL ORGANS is expressed in boundaries and coordinates KNOX and PIN activity. Plant Cell 19, 1795–1808.

Boscá, S., Knauer, S., and Laux, T. (2011). Embryonic development in Arabidopsis thaliana: from the zygote division to the shoot meristem. Front. Plant Sci. 2, 93.

Bouré, N., Kumar, S.V., and Arnaud, N. (2019). The BAP Module: A Multisignal Integrator Orchestrating Growth. Trends Plant Sci. 24, 602–610.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of Stem Cell Fate in Arabidopsis on a Feedback Loop Regulated by CLV3 Activity. Science (80-.). 289, 617–619.

Brock, M.T., Maloof, J.N., and Weinig, C. (2010). Genes underlying quantitative variation in ecologically important traits: PIF4 (PHYTOCHROME INTERACTING FACTOR 4) is associated with variation in internode length, flowering time, and fruit set in Arabidopsis thaliana. Mol. Ecol. 19, 1187–1199.

Brunoud, G., Wells, D.M., Oliva, M., Larrieu, A., Mirabet, V., Burrow, A.H., Beeckman, T., Kepinski, S., Traas, J., Bennett, M.J., et al. (2012). A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature 482, 103–106.

Bürglin, T.R. (1997). Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. Nucleic Acids Res. 25, 4173–4180.

Byrne, M.E., Simorowski, J., and Martienssen, R.A. (2002). ASYMMETRIC LEAVES1 reveals knox gene redundancy in Arabidopsis. Development 129, 1957–1965.

Byrne, M.E., Simorowski, J., Martienssen, R.A., Groover, A.T., Fontana, J.R., Martienssen, R.A., Morin, H., Fournet, F., Belcram, K., Gillet, F., et al. (2003). Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. Development 130, 3941–3950.

Cazzonelli, C.I., Vanstraelen, M., Simon, S., Yin, K., Carron-Arthur, A., Nisar, N., Tarle, G., Cuttriss, A.J., Searle, I.R., Benková, E., et al. (2013). Role of the Arabidopsis PIN6 Auxin Transporter in Auxin Homeostasis and Auxin-Mediated Development. PLoS One 8, e70069.

Cerdán, P.D., and Chory, J. (2003). Regulation of flowering time by light quality. Nature 423, 881–885.

Chapman, E.J., Greenham, K., Castillejo, C., Sartor, R., Bialy, A., Sun, T., and Estelle, M. (2012). Hypocotyl Transcriptome Reveals Auxin Regulation of Growth-Promoting Genes through GA-Dependent and -Independent Pathways. PLoS One 7, e36210.

Chase, M.W., Christenhusz, M.J.M., Conran, J.G., Dodsworth, S., Medeiros de Assis, F.N., Felix, L.P., and Fay, M.F. (2018). Unexpected diversity of Australian tobacco species (Nicotiana section Suaveolentes, Solanaceae). Curtis's Bot. Mag. 35, 212–227.

Chen, Z., Han, Y., Ning, K., Ding, Y., Zhao, W., Yan, S., Luo, C., Jiang, X., Ge, D., Liu, R., et al. (2018a). Inflorescence Development and the Role of LsFT in Regulating Bolting in Lettuce (Lactuca sativa L.). Front. Plant Sci. 8, 1–10.

Chen, Z., Zhao, W., Ge, D., Han, Y., Ning, K., Luo, C., Wang, S., Liu, R., Zhang, X., and Wang, Q. (2018b). LCM-seq reveals the crucial role of LsSOC1 in heat-promoted bolting of lettuce (Lactuca sativa L.). Plant J. 95, 516–528.

Chiurugwi, T., Holmes, H.F., Qi, A., Chia, T.Y.P., Hedden, P., and Mutasa-Göttgens, E.S. (2013). Development of new quantitative physiological and molecular breeding parameters based on the sugar-beet vernalization intensity model. J. Agric. Sci. 151, 492–505.

Choe, S.-K., Lu, P., Nakamura, M., Lee, J., and Sagerström, C.G. (2009). Meis cofactors control HDAC and CBP accessibility at Hox-regulated promoters during zebrafish embryogenesis. Dev. Cell 17, 561–567.

Choe, S.-K., Ladam, F., and Sagerström, C.G. (2014). TALE Factors Poise Promoters for Activation by Hox Proteins. Dev. Cell 28, 203–211.

Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Yuan, H., Noguchi, T., Fujioka, S., Takatsuto, S., Tanaka, A., Yoshida, S., et al. (1999). The Arabidopsis dwarf1 mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. Plant Physiol. 119, 897–907.

Chuck, G., Lincoln, C., and Hake, S. (1996). KNAT1 induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. Plant Cell 8, 1277–1289.

Chung, Y., Maharjan, P.M., Lee, O., Fujioka, S., Jang, S., Kim, B., Takatsuto, S., Tsujimoto, M., Kim, H., Cho, S., et al. (2011). Auxin stimulates DWARF4 expression and brassinosteroid biosynthesis in Arabidopsis. Plant J. 66, 564–578.

Cleland, C.F., and Zeevaart, J.A.D. (1970). Gibberellins in Relation to Flowering and Stem Elongation in the Long Day Plant Silene armeria. Plant Physiol. 46, 392–400.

Cohen, J.I. (2011). A phylogenetic analysis of morphological and molecular characters of Lithospermum L. (Boraginaceae) and related taxa: evolutionary relationships and character evolution. Cladistics 27, 559–580.

Cole, M., Nolte, C., and Werr, W. (2006). Nuclear import of the transcription factor SHOOT MERISTEMLESS depends on heterodimerization with BLH proteins expressed in discrete sub-domains of the shoot apical meristem of Arabidopsis thaliana. Nucleic Acids Res. 34, 1281–1292.

Coles, J.P., Phillips, A.L., Croker, S.J., García-Lepe, R., Lewis, M.J., and Hedden, P. (1999). Modification of gibberellin production and plant development in Arabidopsis by sense and antisense expression of gibberellin 20-oxidase genes. Plant J. 17, 547–556.

Collani, S., Neumann, M., Yant, L., and Schmid, M. (2019). FT Modulates Genome-Wide DNA-Binding of the bZIP Transcription Factor FD. Plant Physiol. 180, 367–380.

Corbesier, L., Prinsen, E., Jacqmard, A., Lejeune, P., Onckelen, H. Van, Pe, C., and Tilman, B.S. (2003). Cytokinin levels in leaves , leaf exudate and shoot apical meristem of Arabidopsis thaliana during floral transition. 54, 2511–2517.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., et al. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science (80-.). 316, 1030–1033.

Dally, N., Xiao, K., Holtgrawe, D., and Jung, C. (2014). The B2 flowering time locus of beet encodes a zinc finger transcription factor. Proc. Natl. Acad. Sci. 111, 10365–10370.

Daum, G., Medzihradszky, A., Suzaki, T., and Lohmann, J.U. (2014). A mechanistic framework for noncell autonomous stem cell induction in Arabidopsis. Proc. Natl. Acad. Sci. 111, 14619–14624.

Desgagné-Penix, I., Eakanunkul, S., Coles, J.P., Phillips, A.L., Hedden, P., and Sponsel, V.M. (2005). The auxin transport inhibitor response 3 (tir3) allele of BIG and auxin transport inhibitors affect the gibberellin status of Arabidopsis. Plant J. 41, 231–242.

Devlin, P.F. (1998). Phytochrome E Influences Internode Elongation and Flowering Time in Arabidopsis. Plant Cell 10, 1479–1488.

Devlin, P.F., Halliday, K.J., Harberd, N.P., and Whitelam, G. (1996). The rosette habit of Arabidopsis thaliana is dependent upon phytochrome action: novel phytochromes control internode elongation and flowering time. Plant J. 10, 1127–1134.

Devlin, P.F., Somers, D.E., Quail, P.H., and Whitelam, G.C. (1997). The Brassica rapa elongated internode (EIN) gene encodes phytochrome B. Plant Mol. Biol. 34, 537–547.

Dill, A., and Sun, T. (2001). Synergistic derepression of gibberellin signaling by removing RGA and GAI function in Arabidopsis thaliana. Genetics 159, 777–785.

Dill, A., Thomas, S.G., Hu, J., Steber, C.M., and Sun, T.-P. (2004). The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell 16, 1392–1405.

Ditengou, F.A., Gomes, D., Nziengui, H., Kochersperger, P., Lasok, H., Medeiros, V., Paponov, I.A., Nagy, S.K., Nádai, T.V., Mészáros, T., et al. (2018). Characterization of auxin transporter PIN6 plasma membrane targeting reveals a function for PIN6 in plant bolting. New Phytol. 217, 1610–1624.

Du, L., and Poovaiah, B.W. (2005). Ca2+/calmodulin is critical for brassinosteroid biosynthesis and plant growth. Nature 437, 741–745.

Endrizzi, K., Moussaian, B., Haecker, A., Levin, J.Z., and Laux, T. (1996). The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. Plant J. 10, 967–979.

Eriksson, S., Böhlenius, H., Moritz, T., and Nilsson, O. (2006). GA4 Is the Active Gibberellin in the Regulation of LEAFY Transcription and Arabidopsis Floral Initiation. Plant Cell 18, 2172–2181.

Feng, S., Martínez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J.M., Kircher, S., et al. (2008). Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. Nature 451, 475–479.

Ferrándiz, C., Gu, Q., Martienssen, R., and Yanofsky, M.F. (2000). Redundant regulation of meristem identity and plant architecture by FRUITFULL, APETALA1 and CAULIFLOWER. Development 127, 725–734.

Food and Agriculture Organization of the United Nations (2017). Crops (Production).

Franklin, K.A., Lee, S.H., Patel, D., Kumar, S.V., Spartz, A.K., Gu, C., Ye, S., Yu, P., Breen, G., Cohen, J.D., et al. (2011). Phytochrome-interacting factor 4 (PIF4) regulates auxin biosynthesis at high temperature. Proc. Natl. Acad. Sci. U. S. A. 108, 20231–20235.

Frerichmann, S.L.M., Kirchhoff, M., Müller, A.E., Scheidig, A.J., Jung, C., and Kopisch-obuch, F.J. (2013). EcoTILLING in Beta vulgaris reveals polymorphisms in the FLC-like gene BvFL1 that are associated with annuality and winter hardiness. BMC Plant Biol. 13.

Frigerio, M., Alabadí, D., Pérez-Gómez, J., García-Cárcel, L., Phillips, A.L., Hedden, P., and Blázquez, M.A. (2006). Transcriptional regulation of gibberellin metabolism genes by auxin signaling in Arabidopsis. Plant Physiol. 142, 553–563.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B.F., Ljung, K., Sandberg, G., et al. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science 306, 862–865.

Frugis, G., Giannino, D., Mele, G., Nicolodi, C., Chiappetta, A., Bitonti, M.B., Innocenti, A.M., Dewitte, W., Van Onckelen, H., and Mariotti, D. (2001). Overexpression of KNAT1 in lettuce shifts leaf determinate growth to a shoot-like indeterminate growth associated with an accumulation of isopentenyl-type cytokinins. Plant Physiol. 126, 1370–1380.

Fujita, N., and Koda, R. (2015). Capitulum and rosette leaf avoidance from grazing by large herbivores in Taraxacum. Ecol. Res. 30, 517–525.

Fukazawa, J., Teramura, H., Murakoshi, S., Nasuno, K., Nishida, N., Ito, T., Yoshida, M., Kamiya, Y., Yamaguchi, S., and Takahashi, Y. (2014). DELLAs Function as Coactivators of GAI-ASSOCIATED FACTOR1 in Regulation of Gibberellin Homeostasis and Signaling in Arabidopsis. Plant Cell 26, 2920–2938.

Fukuda, M., Matsuo, S., Kikuchi, K., Mitsuhashi, W., Toyomasu, T., and Honda, I. (2009). The endogenous level of GA1 is upregulated by high temperature during stem elongation in lettuce through LsGA3ox1 expression. J. Plant Physiol. 166, 2077–2084.

Fukuda, M., Matsuo, S., Kikuchi, K., Kawazu, Y., Fujiyama, R., and Honda, I. (2011). Isolation and functional characterization of the FLOWERING LOCUS T homolog, the LsFT gene, in lettuce. J. Plant Physiol. 168, 1602–1607.

Furumizu, C., Alvarez, J.P., Sakakibara, K., and Bowman, J.L. (2015). Antagonistic Roles for KNOX1 and KNOX2 Genes in Patterning the Land Plant Body Plan Following an Ancient Gene Duplication. PLoS Genet. 11, 1–24.

Gaillochet, C., Daum, G., and Lohmann, J.U. (2015). O Cell, Where Art Thou? The mechanisms of shoot meristem patterning. Curr. Opin. Plant Biol. 23, 91–97.

Gallego-Bartolomé, J., Minguet, E.G., Grau-Enguix, F., Abbas, M., Locascio, A., Thomas, S.G., Alabadí, D., and Blázquez, M.A. (2012). Molecular mechanism for the interaction between gibberellin and brassinosteroid signaling pathways in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 109, 13446–13451.

Galvão, V.C., Horrer, D., Kuttner, F., and Schmid, M. (2012). Spatial control of flowering by DELLA proteins in Arabidopsis thaliana. Development 139, 4072–4082.

Galvão, V.C., Collani, S., Horrer, D., and Schmid, M. (2015). Gibberellic acid signaling is required for ambient temperature-mediated induction of flowering in Arabidopsis thaliana. Plant J. 84, 949–962.

Gendreau, E., Traas, J., Desnos, T., Grandjean, O., Caboche, M., and Höfte, H. (1997). Cellular basis of hypocotyl growth in Arabidopsis thaliana. Plant Physiol. 114, 295–305.

Gendron, J.M., Liu, J., Fan, M., Bai, M.-Y., Wenkel, S., Springer, P.S., Barton, M.K., and Wang, Z.-Y. (2012). Brassinosteroids regulate organ boundary formation in the shoot apical meristem of Arabidopsis. Proc. Natl. Acad. Sci. 109, 21152–21157.

Gianquinto, G. (1997). Morphological and physiological aspects of phase transition in radicchio (Cichorium intybus L. var. silvestre Bisch.): influence of daylength and its interaction with low temperature. Sci. Hortic. (Amsterdam). 71, 13–26.

Gil, P., Dewey, E., Friml, J., Zhao, Y., Snowden, K.C., Putterill, J., Palme, K., Estelle, M., and Chory, J. (2001). BIG: a calossin-like protein required for polar auxin transport in Arabidopsis. Genes Dev. 15, 1985–1997.

Givnish, T.J., Evans, T.M., Pires, J.C., and Sytsma, K.J. (1999). Polyphyly and Convergent Morphological Evolution in Commelinales and Commelinidae: Evidence from rbcL Sequence Data. Mol. Phylogenet. Evol. 12, 360–385.

Gómez-Mena, C., and Sablowski, R. (2008). ARABIDOPSIS THALIANA HOMEOBOX GENE1 establishes the basal boundaries of shoot organs and controls stem growth. Plant Cell 20, 2059–2072.

Gómez-Mena, C., de Folter, S., Costa, M.M.R., Angenent, G.C., and Sablowski, R. (2005). Transcriptional program controlled by the floral homeotic gene AGAMOUS during early organogenesis. Development 132, 429–438.

Gonzalez, N., Bodt, S. De, Sulpice, R., Jikumaru, Y., Chae, E., Dhondt, S., Daele, T. Van, Milde, L. De, Weigel, D., Kamiya, Y., et al. (2010). Increased Leaf Size: Different Means to an End. Plant Physiol. 153, 1261–1279.

Gordon, S.P., Chickarmane, V.S., Ohno, C., and Meyerowitz, E.M. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. Proc. Natl. Acad. Sci. 106, 16529–16534.

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z.-L., Powers, S.J., Gong, F., Phillips, A.L., Hedden, P., Sun, T., et al. (2006). Genetic Characterization and Functional Analysis of the GID1 Gibberellin Receptors in Arabidopsis. Plant Cell 18, 3399–3414.

Guo, D.P., Ali Shah, G., Zeng, G.W., and Zheng, S.J. (2004). The interaction of plant growth regulators and vernalization on the growth and flowering of cauliflower (Brassica oleracea var. botrytis). Plant Growth Regul. 43, 163–171.

Guo, Q., Yoshida, Y., Major, I.T., Wang, K., Sugimoto, K., Kapali, G., Havko, N.E., Benning, C., and Howe, G.A. (2018). JAZ repressors of metabolic defense promote growth and reproductive fitness in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 115, E10768–E10777.

Guo, X., Lu, W., Ma, Y., Qin, Q., and Hou, S. (2013). The BIG gene is required for auxin-mediated organ growth in Arabidopsis. Planta 237, 1135–1147.

Guttormsen, G., and Moe, R. (1985). Effect of plant age and temperature on bolting in Chinese cabbage. Sci. Hortic. (Amsterdam). 25, 217–224.

Ha, C.M., Jun, J.H., Nam, H.G., and Fletcher, J.C. (2007). BLADE-ON-PETIOLE1 and 2 Control Arabidopsis Lateral Organ Fate through Regulation of LOB Domain and Adaxial-Abaxial Polarity Genes. Plant Cell 19, 1809–1825.

Halliday, K.J., and Whitelam, G.C. (2003). Changes in photoperiod or temperature alter the functional relationships between phytochromes and reveal roles for phyD and phyE. Plant Physiol. 131, 1913–1920.

Halliday, K.J., Salter, M.G., Thingnaes, E., and Whitelam, G.C. (2003). Phytochrome control of flowering is temperature sensitive and correlates with expression of the floral integrator FT. Plant J. 33, 875–885.

Hamada, S., Onouchi, H., Tanaka, H., Kudo, M., Liu, Y.-G., Shibata, D., Machida, C., and Machida, Y. (2000). Mutations in the WUSCHEL gene of Arabidopsis thaliana result in the development of shoots without juvenile leaves. Plant J. 24, 91–101.

Hamano, M., Yamato, Y., Yamazaki, H., and Miura, H. (2002). Endogenous gibberellins and their effects on flowering and stem elongation in cabbage (Brassica oleracea var. capitata). J. Hortic. Sci. Biotechnol. 77, 220–225.

Han, Y., Chen, Z., Lv, S., Ning, K., Ji, X., Liu, X., Wang, Q., Liu, R., Fan, S., and Zhang, X. (2016). MADS-Box Genes and Gibberellins Regulate Bolting in Lettuce (Lactuca sativa L.). Front. Plant Sci. 7, 1–14.

Hanisova, A., and Krekule, J. (1975). Treatments to shorten the development period of celery (Apium graveolens L.). J. Hortic. Sci. 50, 97–104.

Hao, G., Al-Shehbaz, I.A., Ahani, H., Liang, Q., Mao, K., Wang, Q., and Liu, J. (2017). An integrative study of evolutionary diversification of Eutrema (Eutremeae, Brassicaceae). Bot. J. Linn. Soc. 184, 204–223.

Hao, J.-H., Zhang, L.-L., Li, P.-P., Sun, Y.-C., Li, J.-K., Qin, X.-X., Wang, L., Qi, Z.-Y., Xiao, S., Han, Y.-Y., et al. (2018). Quantitative Proteomics Analysis of Lettuce (Lactuca sativa L.) Reveals Molecular Basis-Associated Auxin and Photosynthesis with Bolting Induced by High Temperature. Int. J. Mol. Sci. 19, 2967.

Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S., and Tsiantis, M. (2002). The Gibberellin Pathway Mediates KNOTTED1-Type Homeobox Function in Plants with Different Body Plans. Curr. Biol. 12, 1557–1565.

He, Y.-W., and Loh, C.-S. (2002). Induction of early bolting in Arabidopsis thaliana by triacontanol, cerium and lanthanum is correlated with increased endogenous concentration of isopentenyl adenosine (iPAdos). J. Exp. Bot. 53, 505–512.

He, J.-X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.-Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 99, 10185–10190.

Hearn, D.J., O'Brien, P., and Poulsen, T.M. (2018a). Comparative transcriptomics reveals shared gene expression changes during independent evolutionary origins of stem and hypocotyl/root tubers in Brassica (Brassicaceae). PLoS One 13, e0197166.

Hearn, T.J., Marti Ruiz, M.C., Abdul-Awal, S.M., Wimalasekera, R., Stanton, C.R., Haydon, M.J., Theodoulou, F.L., Hannah, M.A., and Webb, A.A.R. (2018b). BIG Regulates Dynamic Adjustment of Circadian Period in Arabidopsis thaliana. Plant Physiol. 178, 358–371.

Hébrard, C., Trap-Gentil, M.-V., Lafon-Placette, C., Delaunay, A., Joseph, C., Lefèbvre, M., Barnes, S., and Maury, S. (2013). Identification of differentially methylated regions during vernalization revealed a role for RNA methyltransferases in bolting. J. Exp. Bot. 64, 651–663.

Hébrard, C., Peterson, D.G., Willems, G., Delaunay, A., Jesson, B., Lefèbvre, M., Barnes, S., and Maury, S. (2015). Epigenomics and bolting tolerance in sugar beet genotypes. J. Exp. Bot. erv449.

Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy, G. V, Long, J.A., and Meyerowitz, E.M. (2005). Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. Curr. Biol. 15, 1899–1911.

Heisler, M.G., Hamant, O., Krupinski, P., Uyttewaal, M., Ohno, C., Jönsson, H., Traas, J., and Meyerowitz, E.M. (2010). Alignment between PIN1 Polarity and Microtubule Orientation in the Shoot Apical Meristem Reveals a Tight Coupling between Morphogenesis and Auxin Transport. PLoS Biol. 8, e1000516.

Helliwell, C.A., Wood, C.C., Robertson, M., James Peacock, W., and Dennis, E.S. (2006). The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. Plant J. 46, 183–192.

Hempel, F.D., and Feldman, L.J. (1994). Bi-directional inflorescence development in Arabidopsis thaliana: Acropetal initiation of flowers and basipetal initiation of paraclades. Planta 192, 276–286.

Hibara, K., Karim, M.R., Takada, S., Taoka, K., Furutani, M., Aida, M., and Tasaka, M. (2006). Arabidopsis CUP-SHAPED COTYLEDON3 Regulates Postembryonic Shoot Meristem and

Organ Boundary Formation. Plant Cell 18, 2946–2957.

Hisamatsu, T., King, R.W., Helliwell, C.A., and Koshioka, M. (2005). The Involvement of Gibberellin 20-Oxidase Genes in Phytochrome-Regulated Petiole Elongation of Arabidopsis. Plant Physiol. 138, 1106–1116.

Hoffmann, C.M., and Kluge-Severin, S. (2010). Light absorption and radiation use efficiency of autumn and spring sown sugar beets. F. Crop. Res. 119, 238–244.

Hoffmann, C.M., and Kluge-Severin, S. (2011). Growth analysis of autumn and spring sown sugar beet. Eur. J. Agron. 34, 1–9.

Höft, N., Dally, N., and Jung, C. (2018). Sequence variation in the bolting time regulator BTC1 changes the life cycle regime in sugar beet. Plant Breed. 137, 412–422.

Hornitschek, P., Kohnen, M. V., Lorrain, S., Rougemont, J., Ljung, K., López-Vidriero, I., Franco-Zorrilla, J.M., Solano, R., Trevisan, M., Pradervand, S., et al. (2012). Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. Plant J. 71, 699–711.

Hou, X., Hu, W.-W., Shen, L., Lee, L.Y.C., Tao, Z., Han, J.-H., and Yu, H. (2008). Global identification of DELLA target genes during Arabidopsis flower development. Plant Physiol. 147, 1126–1142.

Hou, X., Lee, L.Y.C., Xia, K., Yan, Y., and Yu, H. (2010). DELLAs Modulate Jasmonate Signaling via Competitive Binding to JAZs. Dev. Cell 19, 884–894.

Huala, E., and Sussex, I.M. (1992). LEAFY Interacts with Floral Homeotic Genes to Regulate Arabidopsis Floral Development. Plant Cell 4, 901–913.

Hyun, Y., Richter, R., Vincent, C., Martinez-Gallegos, R., Porri, A., and Coupland, G. (2016). Multi-layered Regulation of SPL15 and Cooperation with SOC1 Integrate Endogenous Flowering Pathways at the Arabidopsis Shoot Meristem. Dev. Cell 37, 254–266.

Ibañez, C., Delker, C., Martínez, C., Bürstenbinder, K., Janitza, P., Lippmann, R., Ludwig, W., Sun, H., James, G.V., Klecker, M., et al. (2018). Brassinosteroids Dominate Hormonal Regulation of Plant Thermomorphogenesis via BZR1. Curr. Biol. 28, 303–310.e3.

Immink, R.G.H., Posé, D., Ferrario, S., Ott, F., Kaufmann, K., Valentim, F.L., de Folter, S., van der Wal, F., van Dijk, A.D.J., Schmid, M., et al. (2012). Characterization of SOC1's central role in flowering by the identification of its upstream and downstream regulators. Plant Physiol. 160, 433–449.

Jacqmard, A., Gadisseur, I., and Bernier, G. (2003). Cell Division and Morphological Changes in the Shoot Apex of Arabidopsis thaliana during Floral Transition. Ann. Bot. 91, 571–576.

Jaeger, K.E., and Wigge, P.A. (2007). FT Protein Acts as a Long-Range Signal in Arabidopsis. Curr. Biol. 17, 1050–1054.

Jaggard, K.W., Wickens, R., Webb, D.J., and Scott, R.K. (1983). Effects of sowing date on plant establishment and bolting and the influence of these factors on yields of sugar beet. J. Agric. Sci. 101, 147–161.

Jaggard, K.W., Qi, A., and Ober, E.S. (2009). Capture and use of solar radiation, water, and nitrogen by sugar beet (Beta vulgaris L.). J. Exp. Bot. 60, 1919–1925.

Janick, J., and Leopold, A.C. (1961). A distinction between Bolting and Flowering effects on Senescence. Nature 192, 887–888.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P., and Tsiantis, M. (2005). KNOX action in Arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr. Biol. 15, 1560–1565.

Jun, J.H., Ha, C.M., and Fletcher, J.C. (2010). BLADE-ON-PETIOLE1 Coordinates Organ Determinacy and Axial Polarity in Arabidopsis by Directly Activating ASYMMETRIC LEAVES2. Plant Cell 22, 62–76.

Jung, J.-H., Seo, P.J., Kang, S.K., and Park, C.-M. (2011). miR172 signals are incorporated into the miR156 signaling pathway at the SPL3/4/5 genes in Arabidopsis developmental transitions. Plant Mol. Biol. 76, 35–45.

Jung, J.-H., Domijan, M., Klose, C., Biswas, S., Ezer, D., Gao, M., Khattak, A.K., Box, M.S., Charoensawan, V., Cortijo, S., et al. (2016). Phytochromes function as thermosensors in Arabidopsis. Science (80-.). 354, 886–889.

Kakizaki, T., Kato, T., Fukino, N., Ishida, M., Hatakeyama, K., and Matsumoto, S. (2011). Identification of quantitative trait loci controlling late bolting in Chinese cabbage (Brassica rapa L.) parental line Nou 6 gou. Breed. Sci. 61, 151–159.

Kanrar, S., Onguka, O., and Smith, H.M.S. (2006). Arabidopsis inflorescence architecture requires the activities of KNOX-BELL homeodomain heterodimers. Planta 224, 1163–1173.

Kanrar, S., Bhattacharya, M., Arthur, B., Courtier, J., and Smith, H.M.S. (2008). Regulatory networks that function to specify flower meristems require the function of homeobox genes PENNYWISE and POUND-FOOLISH in Arabidopsis. Plant J. 54, 924–937.

Kanyuka, K., Praekelt, U., Franklin, K.A., Billingham, O.E., Hooley, R., Whitelam, G.C., and Halliday, K.J. (2003). Mutations in the huge Arabidopsis gene BIG affect a range of hormone and light responses. Plant J. 35, 57–70.

Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L., and Altmann, T. (1996). Genetic evidence for an essential role of brassinosteroids in plant development. Plant J. 9, 701–713.

Khakhar, A., Leydon, A.R., Lemmex, A.C., Klavins, E., and Nemhauser, J.L. (2018). Synthetic hormone-responsive transcription factors can monitor and re-program plant development. Elife 7.

Khan, M., Tabb, P., and Hepworth, S.R. (2012a). BLADE-ON-PETIOLE1 and 2 regulate Arabidopsis inflorescence architecture in conjunction with homeobox genes KNAT6 and ATH1. Plant Signal. Behav. 7, 788–792.

Khan, M., Xu, M., Murmu, J., Tabb, P., Liu, Y., Storey, K., McKim, S.M., Douglas, C.J., and Hepworth, S.R. (2012b). Antagonistic interaction of BLADE-ON-PETIOLE1 and 2 with BREVIPEDICELLUS and PENNYWISE regulates Arabidopsis inflorescence architecture. Plant Physiol. 158, 946–960.

Khan, M., Ragni, L., Tabb, P., Salasini, B.C., Chatfield, S., Datla, R., Lock, J., Kuai, X., Després, C., Proveniers, M.C.G., et al. (2015). Repression of Lateral Organ Boundary Genes by PENNYWISE and POUND-FOOLISH Is Essential for Meristem Maintenance and Flowering in Arabidopsis. Plant Physiol. 169, 2166–2186.

Khan, M.R.G., Ai, X.-Y., and Zhang, J.-Z. (2014). Genetic regulation of flowering time in annual and perennial plants. Wiley Interdiscip. Rev. RNA 5, 347–359.

Kim, D., Cho, Y., Ryu, H., Kim, Y., Kim, T.-H., and Hwang, I. (2013). BLH1 and KNAT3 modulate ABA responses during germination and early seedling development in Arabidopsis. Plant J. 75, 755–766.

King, K.E., Moritz, T., and Harberd, N.P. (2001). Gibberellins are not required for normal stem growth in Arabidopsis thaliana in the absence of GAI and RGA. Genetics 159, 767–776.

Kitamoto, N., Yui, S., Nishikawa, K., Takahata, Y., and Yokoi, S. (2014). A naturally occurring long insertion in the first intron in the Brassica rapa FLC2 gene causes delayed bolting. Euphytica 196, 213–223.

Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, S., Yokota, T., Nomura, T., Yoshida, S., and Chua, N.H. (1998). The Arabidopsis DIMINUTO/DWARF1 gene encodes a protein involved in steroid synthesis. Plant Cell 10, 1677–1690.

Koda, Y., Ohkawa-Takahashi, K., and Kikuta, Y. (2001). Stimulation of root thickening and inhibition of bolting by jasmonic acid in beet plants. Plant Prod. Sci. 4, 131–135.

Koini, M.A., Alvey, L., Allen, T., Tilley, C.A., Harberd, N.P., Whitelam, G.C., and Franklin, K.A. (2009). High Temperature-Mediated Adaptations in Plant Architecture Require the bHLH Transcription Factor PIF4. Curr. Biol. 19, 408–413.

Kozuka, T., Kobayashi, J., Horiguchi, G., Demura, T., Sakakibara, H., Tsukaya, H., and Nagatani, A. (2010). Involvement of auxin and brassinosteroid in the regulation of petiole elongation under the shade. Plant Physiol. 153, 1608–1618.

Kues, U., and Ton, L.A.C. (1992). Fungal mating type genes - regulators of sexual development. Mycol. Res. 96, 993–1006.

Kuijt, S.J.H., Lamers, G.E.M., Rueb, S., Scarpella, E., Ouwerkerk, P.B.F., Spaink, H.P., and Meijer, A.H. (2004). Different subcellular localization and trafficking properties of KNOX class 1 homeodomain proteins from rice. Plant Mol. Biol. 55, 781–796.

Kumar, R., Kushalappa, K., Godt, D., Pidkowich, M.S., Pastorelli, S., Hepworth, S.R., and Haughn, G.W. (2007). The Arabidopsis BEL1-LIKE HOMEODOMAIN proteins SAW1 and SAW2 act redundantly to regulate KNOX expression spatially in leaf margins. Plant Cell 19, 2719–2735.

Kurihara, D., Mizuta, Y., Sato, Y., and Higashiyama, T. (2015). ClearSee: a rapid optical clearing reagent for whole-plant fluorescence imaging. Development 142, 4168–4179.

Kwiatkowska, D. (2008). Flowering and apical meristem growth dynamics. J. Exp. Bot. 59, 187–201.

Landrein, B., Kiss, A., Sassi, M., Chauvet, A., Das, P., Cortizo, M., Laufs, P., Takeda, S., Aida, M., Traas, J., et al. (2015). Mechanical stress contributes to the expression of the STM homeobox gene in Arabidopsis shoot meristems. Elife 4.

Larcher, W., Kainmüller, C., and Wagner, J. (2010). Survival types of high mountain plants under extreme temperatures. Flora - Morphol. Distrib. Funct. Ecol. Plants 205, 3–18.

Leal Valentim, F., Mourik, S. van, Posé, D., Kim, M.C., Schmid, M., van Ham, R.C.H.J., Busscher, M., Sanchez-Perez, G.F., Molenaar, J., Angenent, G.C., et al. (2015). A Quantitative and Dynamic Model of the Arabidopsis Flowering Time Gene Regulatory Network. PLoS One 10, e0116973.

Lee, D.-K., Geisler, M., and Springer, P.S. (2009). LATERAL ORGAN FUSION1 and LATERAL ORGAN FUSION2 function in lateral organ separation and axillary meristem formation in Arabidopsis. Development 136, 2423–2432.

Lee, J.-H., Lin, H., Joo, S., and Goodenough, U. (2008). Early sexual origins of homeoprotein heterodimerization and evolution of the plant KNOX/BELL family. Cell 133, 829–840.

Lee, J.H., Ryu, H.-S., Chung, S.K., Posé, D., Kim, S., Schmid, M., and Ahn, J.H. (2013). Regulation of Temperature-Responsive Flowering by MADS-Box Transcription Factor Repressors. Science (80-.). 341, 530–534.

Legris, M., Klose, C., Burgie, E.S., Rojas, C.C.R., Neme, M., Hiltbrunner, A., Wigge, P.A., Schäfer, E., Vierstra, R.D., and Casal, J.J. (2016). Phytochrome B integrates light and temperature signals in Arabidopsis. Science (80-.). 354, 897–900.

Leivar, P., Monte, E., Al-Sady, B., Carle, C., Storer, A., Alonso, J.M., Ecker, J.R., and Quail, P.H. (2008). The Arabidopsis phytochrome-interacting factor PIF7, together with PIF3 and PIF4, regulates responses to prolonged red light by modulating phyB levels. Plant Cell 20, 337–352.

Lenhard, M., and Laux, T. (2003). Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. Development 130, 3163–3173.

Li, C., Zheng, L., Wang, X., Hu, Z., Zheng, Y., Chen, Q., Hao, X., Xiao, X., Wang, X., Wang, G., et al. (2019). Comprehensive expression analysis of Arabidopsis GA2-oxidase genes and their functional insights. Plant Sci. 285, 1–13.

Li, K., Yu, R., Fan, L.-M., Wei, N., Chen, H., and Deng, X.W. (2016a). DELLA-mediated PIF degradation contributes to coordination of light and gibberellin signalling in Arabidopsis. Nat. Commun. 7, 11868.

Li, L., Ljung, K., Breton, G., Schmitz, R.J., Pruneda-Paz, J., Cowing-Zitron, C., Cole, B.J., Ivans, L.J., Pedmale, U. V, Jung, H.-S., et al. (2012a). Linking photoreceptor excitation to changes in plant architecture. Genes Dev. 26, 785–790.

Li, M., An, F., Li, W., Ma, M., Feng, Y., Zhang, X., and Guo, H. (2016b). DELLA proteins interact with FLC to repress flowering transition. J. Integr. Plant Biol. 58, 642–655.

Li, Q.F., Wang, C., Jiang, L., Li, S., Sun, S.S.M., and He, J.X. (2012b). An interaction between BZR1 and DELLAs mediates direct signaling crosstalk between brassinosteroids and gibberellins in Arabidopsis. Sci. Signal. 5, 1–12.

Li, X.G., Su, Y.H., Zhao, X.Y., Li, W., Gao, X.Q., and Zhang, X.S. (2010). Cytokinin overproduction-caused alteration of flower development is partially mediated by CUC2 and CUC3 in Arabidopsis. Gene 450, 109–120.

Li, Y., Pi, L., Huang, H., and Xu, L. (2012c). ATH1 and KNAT2 proteins act together in regulation of plant inflorescence architecture. J. Exp. Bot. 63, 1423–1433.

Liao, C.-Y., Smet, W., Brunoud, G., Yoshida, S., Vernoux, T., and Weijers, D. (2015). Reporters for sensitive and quantitative measurement of auxin response. Nat. Methods 12, 207–210.

Liljegren, S.J., Gustafson-Brown, C., Pinyopich, A., Ditta, G.S., and Yanofsky, M.F. (1999). Interactions among APETALA1, LEAFY, and TERMINAL FLOWER1 specify meristem fate. Plant Cell 11, 1007–1018.

Lin, W., Shuai, B., and Springer, P.S. (2003). The Arabidopsis LATERAL ORGAN BOUNDARIES – Domain Gene ASYMMETRIC LEAVES2 Functions in the Repression of KNOX Gene Expression and in Adaxial-Abaxial Patterning. Plant Cell 15, 2241–2252.

Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K., and Hake, S. (1994). A knotted1-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell 6, 1859–1876.

Liu, Y., and Douglas, C.J. (2015). A role for OVATE FAMILY PROTEIN1 (OFP1) and OFP4 in a BLH6-KNAT7 multi-protein complex regulating secondary cell wall formation in Arabidopsis thaliana. Plant Signal. Behav. 00–00.

Liu, B., Zuo, Z., Liu, H., Liu, X., and Lin, C. (2011). Arabidopsis cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. Genes Dev. 25, 1029–1034.

Liu, C., Chen, H., Er, H.L., Soo, H.M., Kumar, P.P., Han, J.-H., Liou, Y.C., and Yu, H. (2008). Direct interaction of AGL24 and SOC1 integrates flowering signals in Arabidopsis. Development 135, 1481–1491.

Liu, F., Quesada, V., Crevillén, P., Bäurle, I., Swiezewski, S., and Dean, C. (2007). The Arabidopsis RNA-Binding Protein FCA Requires a Lysine-Specific Demethylase 1 Homolog to Downregulate FLC. Mol. Cell 28, 398–407.

Liu, F., Marquardt, S., Lister, C., Swiezewski, S., and Dean, C. (2010). Targeted 3' Processing of Antisense Transcripts Triggers Arabidopsis FLC Chromatin Silencing. Science (80-.). 327, 94–98.

Liu, L., Li, C., Song, S., Teo, Z.W.N., Shen, L., Wang, Y., Jackson, D., and Yu, H. (2018a). FTIP-Dependent STM Trafficking Regulates Shoot Meristem Development in Arabidopsis. Cell Rep. 23, 1879–1890.

Liu, X., Lv, S., Liu, R., Fan, S., Liu, C., Liu, R., and Han, Y. (2018b). Transcriptomic analysis reveals the roles of gibberellin-regulated genes and transcription factors in regulating bolting in lettuce (Lactuca sativa L.). PLoS One 13, e0191518.

Liu, Y., You, S., Taylor-Teeples, M., Li, W.L., Schuetz, M., Brady, S.M., and Douglas, C.J. (2014). BEL1-LIKE HOMEODOMAIN6 and KNOTTED ARABIDOPSIS THALIANA7 Interact and Regulate Secondary Cell Wall Formation via Repression of REVOLUTA. Plant Cell 26, tpc.114.128322-.

Locascio, A., Blázquez, M.A., and Alabadí, D. (2013). Dynamic Regulation of Cortical Microtubule Organization through Prefoldin-DELLA Interaction. Curr. Biol. 23, 804–809.

Lodha, M., Marco, C.F., and Timmermans, M.C.P. (2013). The ASYMMETRIC LEAVES complex maintains repression of KNOX homeobox genes via direct recruitment of Polycomb-repressive complex2. Genes Dev. 27, 596–601.

Löfke, C., Zwiewka, M., Heilmann, I., Montagu, M.C.E. Van, Teichmann, T., and Friml, J. (2013). Asymmetric gibberellin signaling regulates vacuolar trafficking of PIN auxin transporters during root gravitropism. Proc. Natl. Acad. Sci. 110, 3627–3632.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature 379, 66–69.

Longden, P.C. (1989). Effect of increasing weed-beet density on sugar-beet yield and quality. Ann. Appl. Biol. 114, 527–532.

Longden, P.C., Scott, R.K., and Tyldesley, B. (1975). Bolting of sugar beet grown In England. Outlook Agric. 8, 188–193.

Lu, X.-D., Zhou, C.-M., Xu, P.-B., Luo, Q., Lian, H.-L., and Yang, H.-Q. (2015). Red-Light-Dependent Interaction of phyB with SPA1 Promotes COP1–SPA1 Dissociation and Photomorphogenic Development in Arabidopsis. Mol. Plant 8, 467–478.

Lucas, W.J., Bouché-Pillon, S., Jackson, D.P., Nguyen, L., Baker, L., Ding, B., and Hake, S. (1995). Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science 270, 1980–1983.

de Lucas, M., Davière, J.-M., Rodríguez-Falcón, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blázquez, M.A., Titarenko, E., and Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. Nature 451, 480–484.

Luo, M., Tai, R., Yu, C.-W., Yang, S., Chen, C.-Y., Lin, W.-D., Schmidt, W., and Wu, K. (2015). Regulation of flowering time by the histone deacetylase HDA5 in Arabidopsis. Plant J. 82, 925–936.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., et al. (1997). FCA, a Gene Controlling Flowering Time in Arabidopsis, Encodes a Protein Containing RNA-Binding Domains. Cell 89, 737–745.

Mandel, T., Candela, H., Landau, U., Asis, L., Zelinger, E., Carles, C.C., and Williams, L.E. (2016). Differential regulation of meristem size, morphology and organization by the ERECTA, CLAVATA and class III HD-ZIP pathways. Development 143, 1612–1622.

Marks, C.E., Newbigin, E., and Ladiges, P.Y. (2011). Comparative morphology and phylogeny of Nicotiana section Suaveolentes (Solanaceae) in Australia and the South Pacific. Aust. Syst. Bot. 24, 61.

Martínez, C., Espinosa-Ruíz, A., de Lucas, M., Bernardo-García, S., Franco-Zorrilla, J.M., and Prat, S. (2018). PIF4-induced BR synthesis is critical to diurnal and thermomorphogenic growth. EMBO J. 37, e99552.

Martorell, C., and Ezcurra, E. (2002). Rosette scrub occurrence and fog availability in arid mountains of Mexico. J. Veg. Sci. 13, 651–662.

Mathieu, A.-S., Lutts, S., Vandoorne, B., Descamps, C., Périlleux, C., Dielen, V., Van Herck, J.C., and Quinet, M. (2014). High temperatures limit plant growth but hasten flowering in root chicory (Cichorium intybus) independently of vernalisation. J. Plant Physiol. 171, 109–118.

Mathieu, A.-S., Tinel, C., Dailly, H., Quinet, M., and Lutts, S. (2018). Impact of high temperature on sucrose translocation, sugar content and inulin yield in Cichorium intybus L. var. sativum. Plant Soil 432, 273–288.

Matschi, S., Werner, S., Schulze, W.X., Legen, J., Hilger, H.H., and Romeis, T. (2013). Function of calcium-dependent protein kinase CPK28 of Arabidopsis thaliana in plant stem elongation and vascular development. Plant J. 73, 883–896.

Matschi, S., Hake, K., Herde, M., Hause, B., and Romeis, T. (2015). The Calcium-Dependent Protein Kinase CPK28 Regulates Development by Inducing Growth Phase-Specific, Spatially Restricted Alterations in Jasmonic Acid Levels Independent of Defense Responses in Arabidopsis. Plant Cell 27, 591–606.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux, T. (1998). Role of WUSCHEL in Regulating Stem Cell Fate in the Arabidopsis Shoot Meristem. Cell 95, 805–815.

Mazzella, M. a, Bertero, D., and Casal, J.J. (2000). Temperature-dependent internode elongation in vegetative plants of Arabidopsis thaliana lacking phytochrome B and cryptochrome 1. Planta 210, 497–501.

McKim, S.M., Stenvik, G.-E., Butenko, M.A., Kristiansen, W., Cho, S.K., Hepworth, S.R., Aalen, R.B., and Haughn, G.W. (2008). The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis. Development 135, 1537–1546.

Medford, J.I. (1992). Vegetative Apical Meristems. Plant Cell 4, 1029–1039.

Metzger, J.D., and Dusbabek, K. (1991). Determination of the Cellular Mechanisms Regulating Thermo-Induced Stem Growth in Thlaspi arvense L. Plant Physiol. 97, 630–637.

Moens, C.B., and Selleri, L. (2006). Hox cofactors in vertebrate development. Dev. Biol. 291, 193–206.

Mohammed, B., Bilooei, S.F., Dóczi, R., Grove, E., Railo, S., Palme, K., Ditengou, F.A., Bögre, L., and López-Juez, E. (2018). Converging Light, Energy and Hormonal Signaling Control Meristem Activity, Leaf Initiation, and Growth. Plant Physiol. 176, 1365–1381.

Moon, J., Suh, S.-S., Lee, H., Choi, K.-R., Hong, C.B., Paek, N.-C., Kim, S.-G., and Lee, I. (2003). The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant J. 35, 613–623.

Moon, J., Lee, H., Kim, M., and Lee, I. (2005). Analysis of Flowering Pathway Integrators in Arabidopsis. Plant Cell Physiol. 46, 292–299.

Mukherjee, K., Brocchieri, L., and Bürglin, T.R. (2009). A comprehensive classification and evolutionary analysis of plant homeobox genes. Mol. Biol. Evol. 26, 2775–2794.

Müller, R., Borghi, L., Kwiatkowska, D., Laufs, P., and Simon, R. (2006). Dynamic and compensatory responses of Arabidopsis shoot and floral meristems to CLV3 signaling. Plant Cell 18, 1188–1198.

Murase, K., Hirano, Y., Sun, T., and Hakoshima, T. (2008). Gibberellin-induced DELLA recognition by the gibberellin receptor GID1. Nat. 2008 4567221 456, 459.

Mutasa-Göttgens, E.S., Qi, A., Zhang, W., Schulze-Buxloh, G., Jennings, A., Hohmann, U., Müller, A.E., and Hedden, P. (2010). Bolting and flowering control in sugar beet: relationships and effects of gibberellin, the bolting gene B and vernalization. AoB Plants 2010, plq012.

Mutasa-Göttgens, E.S., Joshi, A., Holmes, H.F., Hedden, P., and Göttgens, B. (2012). A new RNASeq-based reference transcriptome for sugar beet and its application in transcriptome-scale analysis of vernalization and gibberellin responses. BMC Genomics 13, 99.

Nakazawa, M., Ichikawa, T., Ishikawa, A., Kobayashi, H., Tsuhara, Y., Kawashima, M., Suzuki, K., Muto, S., and Matsui, M. (2003). Activation tagging, a novel tool to dissect the functions of a gene family. Plant J. 34, 741–750.

Nemhauser, J.L., Mockler, T.C., and Chory, J. (2004). Interdependency of Brassinosteroid and Auxin Signaling in Arabidopsis. PLoS Biol. 2, e258.

Nieuwhof, M. (1984). Effect of Gibberellic Acid on Bolting and Flowering of Carrot (Daucus carota L.). Sci. Signal. 24, 211–219.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine Kinase Homologs That Act as Cytokinin Receptors Possess Overlapping Functions in the Regulation of Shoot and Root Growth in Arabidopsis. Plant Cell 16, 1365–1377.

Norberg, M. (2005). The BLADE ON PETIOLE genes act redundantly to control the growth and development of lateral organs. Development 132, 2203–2213.

Nozue, K., Covington, M.F., Duek, P.D., Lorrain, S., Fankhauser, C., Harmer, S.L., and Maloof, J.N. (2007). Rhythmic growth explained by coincidence between internal and external cues. Nature 448, 358–361.

Ogawara, T., Higashi, K., Kamada, H., and Ezura, H. (2003). Ethylene advances the transition from vegetative growth to flowering inArabidopsis thaliana. J. Plant Physiol. 160, 1335–1340.

Oh, E., Zhu, J.-Y., and Wang, Z.-Y. (2012). Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. Nat. Cell Biol. 14, 802–809.

Oh, E., Zhu, J.-Y., Bai, M.-Y., Arenhart, R.A., Sun, Y., and Wang, Z.Y. (2014). Cell elongation is regulated through a central circuit of interacting transcription factors in the Arabidopsis hypocotyl. Elife 2014, 1–19.

Oñate-Sánchez, L., and Vicente-Carbajosa, J. (2008). DNA-free RNA isolation protocols for Arabidopsis thaliana, including seeds and siliques. BMC Res. Notes 1, 1–7.

Ori, N., Eshed, Y., Chuck, G., Bowman, J.L., and Hake, S. (2000). Mechanisms that control knox gene expression in the Arabidopsis shoot. Development 127.

Pagnussat, G.C., Yu, H.-J., and Sundaresan, V. (2007). Cell-fate switch of synergid to egg cell in Arabidopsis eostre mutant embryo sacs arises from misexpression of the BEL1-like homeodomain gene BLH1. Plant Cell 19, 3578–3592.

Paik, I., Kathare, P.K., Kim, J.-I., and Huq, E. (2017). Expanding Roles of PIFs in Signal Integration from Multiple Processes. Mol. Plant 10, 1035–1046.

Park, C.H., Kim, T.-W., Son, S.-H., Hwang, J.-Y., Lee, S.C., Chang, S.C., Kim, S.-H., Kim, S.W., and Kim, S.-K. (2010). Brassinosteroids control AtEXPA5 gene expression in Arabidopsis thaliana. Phytochemistry 71, 380–387.

Park, J., Nguyen, K.T., Park, E., Jeon, J.-S., and Choi, G. (2013). DELLA Proteins and Their Interacting RING Finger Proteins Repress Gibberellin Responses by Binding to the Promoters of a Subset of Gibberellin-Responsive Genes in Arabidopsis C W. Plant Cell 25, 927–943.

dela Paz, J.S., Stronghill, P.E., Douglas, S.J., Saravia, S., Hasenkampf, C.A., and Riggs, C.D. (2012). Chromosome Fragile Sites in Arabidopsis Harbor Matrix Attachment Regions That May Be Associated with Ancestral Chromosome Rearrangement Events. PLoS Genet. 8, e1003136.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The Arabidopsis GAI gene defines a signaling pathway that negatively regulates gibberellin responses . Genes Dev. 11, 3194–3205.

Peterson, R.L., and Yeung, E.C. (1972). Effect of Two Gibberellins on Species of the Rosette Plant Hieracium. Bot. Gaz. 133, 190–198.

Pfeiffer, A., Janocha, D., Dong, Y., Medzihradszky, A., Schöne, S., Daum, G., Suzaki, T., Forner, J., Langenecker, T., Rempel, E., et al. (2016). Integration of light and metabolic signals for stem cell activation at the shoot apical meristem. Elife 5.

Pin, P.A., Benlloch, R., Bonnet, D., Wremerth-Weich, E., Kraft, T., Gielen, J.J.L., and Nilsson, O. (2010). An antagonistic pair of FT homologs mediates the control of flowering time in sugar beet. Science (80-.). 330, 1397–1400.

Pin, P.A., Zhang, W., Vogt, S.H., Dally, N., Büttner, B., Schulze-Buxloh, G., Jelly, N.S., Chia, T.Y.P., Mutasa-Göttgens, E.S., Dohm, J.C., et al. (2012). The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. Curr. Biol. 22, 1095–1101.

Poethig, R.S. (2003). Phase change and the regulation of developmental timing in plants. Science (80-.). 301, 334–336.

Posé, D., Verhage, L., Ott, F., Yant, L., Mathieu, J., Angenent, G.C., Immink, R.G.H., and Schmid, M. (2013). Temperature-dependent regulation of flowering by antagonistic FLM variants. Nature 503, 414–417.

Pouteau, S., and Albertini, C. (2011). An assessment of morphogenetic fluctuation during reproductive phase change in Arabidopsis. Ann. Bot. 107, 1017–1027.

Proveniers, M. (2000). Light control of plant development. A functional analysis of the Arabidopsis thaliana ATH1 gene. Utrecht University.

Proveniers, M.C.G., Rutjens, B., Brand, M., and Smeekens, S. (2007). The Arabidopsis TALE homeobox gene ATH1 controls floral competency through positive regulation of FLC. Plant J. 52, 899–913.

Quaedvlieg, N., Dockx, J., Rook, F., Weisbeek, P., and Smeekens, S. (1995). The homeobox gene ATH1 of Arabidopsis is derepressed in the photomorphogenic mutants cop1 and det1. Plant Cell 7, 117–129.

Ragni, L., Belles-Boix, E., Günl, M., and Pautot, V. (2008). Interaction of KNAT6 and KNAT2 with BREVIPEDICELLUS and PENNYWISE in Arabidopsis inflorescences. Plant Cell 20, 888–900.

Ragni, L., Nieminen, K., Pacheco-Villalobos, D., Sibout, R., Schwechheimer, C., and Hardtke, C.S. (2011). Mobile Gibberellin Directly Stimulates Arabidopsis Hypocotyl Xylem Expansion. Plant Cell 23, 1322–1336.

Rast, M.I., and Simon, R. (2012). Arabidopsis JAGGED LATERAL ORGANS acts with ASYMMETRIC LEAVES2 to coordinate KNOX and PIN expression in shoot and root meristems. Plant Cell 24, 2917–2933.

Reddy, G.V., Heisler, M.G., Ehrhardt, D.W., and Meyerowitz, E.M. (2004). Real-time lineage analysis reveals oriented cell divisions associated with morphogenesis at the shoot apex of Arabidopsis thaliana. Development 131, 4225–4237.

Reeves, P.A., He, Y., Schmitz, R.J., Amasino, R.M., Panella, L.W., and Richards, C.M. (2007). Evolutionary conservation of the FLOWERING LOCUS C-mediated vernalization response: Evidence from the sugar beet (Beta vulgaris). Genetics 176, 295–307.

Regnault, T., Davière, J.-M., Heintz, D., Lange, T., and Achard, P. (2014). The gibberellin biosynthetic genes AtKAO1 and AtKAO2 have overlapping roles throughout Arabidopsis development. Plant J. 80, 462–474.

Regnault, T., Davière, J.-M., Wild, M., Sakvarelidze-Achard, L., Heintz, D., Carrera Bergua, E., Lopez Diaz, I., Gong, F., Hedden, P., and Achard, P. (2015). The gibberellin precursor GA12 acts as a long-distance growth signal in Arabidopsis. Nat. Plants 1, 15073.

Reinhardt, D., Pesce, E.-R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. Nature 426, 255–260.

Rieu, I., Eriksson, S., Powers, S.J., Gong, F., Griffiths, J., Woolley, L., Benlloch, R., Nilsson, O., Thomas, S.G., Hedden, P., et al. (2008). Genetic Analysis Reveals That C19-GA 2-Oxidation Is a Major Gibberellin Inactivation Pathway in Arabidopsis. Plant Cell 20, 2420–2436.

Rizza, A., Walia, A., Lanquar, V., Frommer, W.B., and Jones, A.M. (2017). In vivo gibberellin gradients visualized in rapidly elongating tissues. Nat. Plants 3, 803–813.

Rodriguez, K., Perales, M., Snipes, S., Yadav, R.K., Diaz-Mendoza, M., and Reddy, G.V. (2016). DNA-dependent homodimerization, sub-cellular partitioning, and protein destabilization control WUSCHEL levels and spatial patterning. Proc. Natl. Acad. Sci. 113, E6307–E6315.

Roldan, M., Gomez-Mena, C., Ruiz-Garcia, L., Salinas, J., and Martinez-Zapater, J.M. (1999). Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of Arabidopsis in the dark. Plant J. 20, 581–590.

Rood, S.B., Zanewich, K.P., and Williams, P.H. (1990). Growth and development of Brassica genotypes differing in endogenous gibberellin content. II. Gibberellin content, growth analyses and cell size. Physiol. Plant. 79, 679–685.

Ruegger, M., Dewey, E., Hobbie, L., Brown, D., Bernasconi, P., Turner, J., Muday, G., and Estelle, M. (1997). Reduced naphthylphthalamic acid binding in the tir3 mutant of Arabidopsis is associated with a reduction in polar auxin transport and diverse morphological defects. Plant Cell 9, 745–757.

R**uiz-García, L., Madueño, F., Wilkinson, M., Haughn, G., Salinas, J., and Martínez-Zapater, J.M.** (1997). Different roles of flowering-time genes in the activation of floral initiation genes in Arabidopsis. Plant Cell 9, 1921–1934.

Ruonala, R., Rinne, P.L.H., Kangasjärvi, J., and van der Schoot, C. (2008). CENL1 expression in the rib meristem affects stem elongation and the transition to dormancy in Populus. Plant Cell 20, 59–74.

Rutjens, B. (2007). Control of Plant Architecture by the TALE Homeobox Genes ATH1 and PENNYWISE. Utrecht University.

Rutjens, B., Bao, D., van Eck-Stouten, E., Brand, M., Smeekens, S., and Proveniers, M.C.G. (2009). Shoot apical meristem function in Arabidopsis requires the combined activities of three BEL1-like homeodomain proteins. Plant J. 58, 641–654.

R**yu, H., Kim, K., Cho, H., Park, J., Choe, S., and Hwang, I.** (2007). Nucleocytoplasmic shuttling of BZR1 mediated by phosphorylation is essential in Arabidopsis brassinosteroid signaling. Plant Cell 19, 2749–2762.

Ryu, H., Kim, K., Cho, H., and Hwang, I. (2010). Predominant actions of cytosolic BSU1 and nuclear BIN2 regulate subcellular localization of BES1 in brassinosteroid signaling. Mol. Cells 29, 291–296.

Sachs, R.M. (1991). Stem Elongation. Annu. Rev. Plant Physiol. 16, 73–96.

Sachs, R.M., and Long, A. (1957). Effect of Gibberellin on Cell Division in Hyoscyamus. Science (80-.). 125, 1144–1145.

Sachs, R.M., Bretz, C., and Lang, A. (1959b). Cell division and gibberellic acid. Exp. Cell Res. 18, 230–244.

Sachs, R.M., Bretz, C.F.C.F., and Lang, A. (1959a). Shoot Histogenesis : The Early Effects of Gibberellin Upon Stem Elongation in Two Rosette Plants. Am. J. Bot. 46, 376–384.

Salanenka, Y., Verstraeten, I., Löfke, C., Tabata, K., Naramoto, S., Glanc, M., and Friml, J. (2018). Gibberellin DELLA signaling targets the retromer complex to redirect protein trafficking to the plasma membrane. Proc. Natl. Acad. Sci. U. S. A. 115, 3716–3721.

Saleh, M., Rambaldi, I., Yang, X.-J., and Featherstone, M.S. (2000). Cell Signaling Switches HOX-PBX Complexes from Repressors to Activators of Transcription Mediated by Histone Deacetylases and Histone Acetyltransferases. Mol. Cell. Biol. 20, 8623–8633.

Sandhu, K.S., Hagely, K., and Neff, M.M. (2012). Genetic interactions between brassinosteroid-inactivating P450s and photomorphogenic photoreceptors in Arabidopsis thaliana. G3 Genes, Genomes, Genet. 2, 1585–1593.

Sarojam, R., Sappl, P.G., Goldshmidt, A., Efroni, I., Floyd, S.K., Eshed, Y., and Bowman, J.L. (2010). Differentiating Arabidopsis Shoots from Leaves by Combined YABBY Activities. Plant Cell 22, 2113–2130.

Schaffer, W.M., and Schaffer, M.V. (1979). The Adaptive Significance of Variations in Reproductive Habit in the Agavaceae II: Pollinator Foraging Behavior and Selection for Increased Reproductive Expenditure. Ecology 60, 1051–1069.

Schittenhelm, S. (2001). Effect of sowing date on the performance of root chicory. Eur. J. Agron. 15, 209–220.

Schmid, M., Uhlenhaut, N.H., Godard, F., Demar, M., Bressan, R., Weigel, D., and Lohmann, J.U. (2003). Dissection of floral induction pathways using global expression analysis. Development 130, 6001–6012.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D., and Lohmann, J.U. (2005). A gene expression map of Arabidopsis thaliana development. Nat. Genet. 37, 501–506.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F.X., Jürgens, G., and Laux, T. (2000). The Stem Cell Population of Arabidopsis Shoot Meristems Is Maintained by a Regulatory Loop between the CLAVATA and WUSCHEL Genes. Cell 100, 635–644.

Scofield, S., Murison, A., Jones, A., Fozard, J., Aida, M., Band, L.R., Bennett, M., and Murray, J.A.H. (2018). Coordination of meristem and boundary functions by transcription factors in the SHOOT MERISTEMLESS regulatory network. Development 145, dev157081.

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Kröber, S., Amasino, R.A., and Coupland, G. (2006). The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes Dev. 20, 898–912.

Serrano-Mislata, A., Bencivenga, S., Bush, M., Schiessl, K., Boden, S., and Sablowski, R. (2017). DELLA genes restrict inflorescence meristem function independently of plant height. Nat. Plants 3, 749–754.

Sessa, R. a., Bennett, M.H., Lewis, M.J., Mansfield, J.W., and Beale, M.H. (2000). Metabolite Profiling of Sesquiterpene Lactones from Lactuca Species. J. Biol. Chem. 275, 26877–26884.

Sheerin, D.J., Menon, C., zur Oven-Krockhaus, S., Enderle, B., Zhu, L., Johnen, P., Schleifenbaum, F., Stierhof, Y.-D., Huq, E., and Hiltbrunner, A. (2015). Light-activated phytochrome A and B interact with members of the SPA family to promote photomorphogenesis in Arabidopsis by reorganizing the COP1/SPA complex. Plant Cell 27, 189–201.

Sheldon, C.C., Conn, A.B., Dennis, E.S., and Peacock, W.J. (2002). Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. Plant Cell 14, 2527–2537.

Shi, B., Guo, X., Wang, Y., Xiong, Y., Wang, J., Hayashi, K.-I., Lei, J., Zhang, L., and Jiao, Y. (2018). Feedback from Lateral Organs Controls Shoot Apical Meristem Growth by Modulating Auxin Transport. Dev. Cell 44, 204–216.e6.

Shi, C.-L., Stenvik, G.-E., Vie, A.K., Bones, A.M., Pautot, V., Proveniers, M.C.G., Aalen, R.B., and Butenko, M.A. (2011). Arabidopsis class I KNOTTED-like homeobox proteins act downstream in the IDA-HAE/HSL2 floral abscission signaling pathway. Plant Cell 23, 2553–2567.

Shimada, S., Komatsu, T., Yamagami, A., Nakazawa, M., Matsui, M., Kawaide, H., Natsume, M., Osada, H., Asami, T., and Nakano, T. (2015). Formation and Dissociation of the BSS1 Protein Complex Regulates Plant Development via Brassinosteroid Signaling. Plant Cell 27, 375–390.

Shuai, B., Reynaga-pen, C.G., and Springer, P.S. (2002). The LATERAL ORGAN BOUNDARIES Gene Defines a Novel, Plant-Specific Gene Family. Plant Physiol. 129, 747–761.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., and Dean, C. (2003). FY Is an RNA 3′ End-Processing Factor that Interacts with FCA to Control the Arabidopsis Floral Transition. Cell 113, 777–787.

Smith, H.M.S., and Hake, S. (2003). The Interaction of Two Homeobox Genes, BREVIPEDICELLUS and PENNYWISE, Regulates Internode Patterning in the Arabidopsis Inflorescence. Plant Cell 15, 1717–1727.

Smith, H.M.S., Boschke, I., and Hake, S. (2002). Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. Proc. Natl. Acad. Sci. 99, 9579.

Smith, H.M.S., Campbell, B.C., and Hake, S. (2004). Competence to Respond to Floral Inductive Signals Requires the Homeobox Genes PENNYWISE and POUND-FOOLISH. Curr. Biol. 14, 812–817.

Snipes, S.A., Rodriguez, K., DeVries, A.E., Miyawaki, K.N., Perales, M., Xie, M., and Reddy, G.V. (2018). Cytokinin stabilizes WUSCHEL by acting on the protein domains required for nuclear enrichment and transcription. PLOS Genet. 14, e1007351.

Soons, M.B., Heil, G.W., Nathan, R., and Katul, G.G. (2004). DETERMINANTS OF LONG-DISTANCE SEED DISPERSAL BY WIND IN GRASSLANDS. Ecology 85, 3056–3068.

Spinelli, S. V, Martin, A.P., Viola, I.L., Gonzalez, D.H., and Palatnik, J.F. (2011). A mechanistic link between STM and CUC1 during Arabidopsis development. Plant Physiol. 156, 1894–1904.

Sponsel, V.M., Schmidt, F.W., Porter, S.G., Nakayama, M., Kohlstruk, S., and Estelle, M. (1997). Characterization of New Gibberellin-Responsive Semidwarf Mutants of Arabidopsis. Plant Physiol. 115, 1009–1020.

Stavang, J.A., Gallego-Bartolomé, J., Gómez, M.D., Yoshida, S., Asami, T., Olsen, J.E., García-Martínez, J.L., Alabadí, D., and Blázquez, M.A. (2009). Hormonal regulation of temperature-induced growth in Arabidopsis. Plant J. 60, 589–601.

Stewart Lilley, J.L., Gan, Y., Graham, I.A., and Nemhauser, J.L. (2013). The effects of DELLA s on growth change with developmental stage and brassinosteroid levels. Plant J. 76, n/a-n/a.

Suárez-López, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature 410, 1116–1120.

Suge, H., and Rappaport, L. (1968). Role of gibberellins in stem elongation and flowering in radish. Plant Physiol. 43, 1208–1214.

Sung, S., He, Y., Eshoo, T.W., Tamada, Y., Johnson, L., Nakahigashi, K., Goto, K., Jacobsen, S.E., and Amasino, R.M. (2006). Epigenetic maintenance of the vernalized state in Arabidopsis thaliana requires LIKE HETEROCHROMATIN PROTEIN 1. Nat. Genet. 38, 706–710.

Sureshkumar, S., Dent, C., Seleznev, A., Tasset, C., and Balasubramanian, S. (2016). Nonsense-mediated mRNA decay modulates FLM-dependent thermosensory flowering response in Arabidopsis. Nat. Plants 2, 16055.

Takada, N., Wakita, E., Yamazaki, E., Hashimoto, M., and Koda, Y. (2013). Methyl 3-iso-cucurbate, a potential bolting inhibitor to Brassica rapa. Tetrahedron 69, 5426–5430.

Takahashi, H., Kimura, M., Suge, H., and Saito, T. (1994). Interaction between Vernalization and Photoperiod on the Flowering and Bolting of Different Turnip Varieties. J. Japanese Soc. Hortic. Sci. 63, 99–108.

Takahashi, T., Gasch, A., Nishizawa, N., and Chua, N.H. (1995). The DIMINUTO gene of Arabidopsis is involved in regulating cell elongation. Genes Dev. 9, 97–107.

Takeda, S., Hanano, K., Kariya, A., Shimizu, S., Zhao, L., Matsui, M., Tasaka, M., and Aida, M. (2011). CUP-SHAPED COTYLEDON1 transcription factor activates the expression of LSH4 and LSH3, two members of the ALOG gene family, in shoot organ boundary cells. Plant J. 66, 1066–1077.

Talon, M., Tadeo, F.R., and Zeevaart, J.A.D. (1991). Cellular changes induced by exogenous and endogenous gibberellins in shoot tips of the long-day plant Silene armeria. Planta 185, 487–493.

Tanaka, K., Nakamura, Y., Asami, T., Yoshida, S., Matsuo, T., and Okamoto, S. (2003). Physiological Roles of Brassinosteroids in Early Growth of Arabidopsis: Brassinosteroids Have a Synergistic Relationship with Gibberellin as well as Auxin in Light-Grown Hypocotyl Elongation. J. Plant Growth Regul. 22, 259–271.

Tao, Y., Ferrer, J.-L., Ljung, K., Pojer, F., Hong, F., Long, J.A., Li, L., Moreno, J.E., Bowman, M.E., Ivans, L.J., et al. (2008). Rapid Synthesis of Auxin via a New Tryptophan-Dependent Pathway Is Required for Shade Avoidance in Plants. Cell 133, 164–176.

Tao, Z., Shen, L., Liu, C., Liu, L., Yan, Y., and Yu, H. (2012). Genome-wide identification of SOC1 and SVP targets during the floral transition in Arabidopsis. Plant J. 70, 549–561.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. Nature 448, 661–665.

Thomson, F.J., Moles, A.T., Auld, T.D., and Kingsford, R.T. (2011). Seed dispersal distance is more strongly correlated with plant height than with seed mass. J. Ecol. 99, 1299–1307.

Timpte, C.S., Wilson, A., and Estelle, M. (1992). Effects of the axr2 mutation of Arabidopsis on cell shape in hypocotyl and inflorescence. Planta 188, 271–278.

Titapiwatanakun, B., Blakeslee, J.J., Bandyopadhyay, A., Yang, H., Mravec, J., Sauer, M., Cheng, Y., Adamec, J., Nagashima, A., Geisler, M., et al. (2009). ABCB19/PGP19 stabilises PIN1 in membrane microdomains in Arabidopsis. Plant J. 57, 27–44.

Tooke, F., Ordidge, M., Chiurugwi, T., and Battey, N. (2005). Mechanisms and function of flower and inflorescence reversion. J. Exp. Bot. 56, 2587–2599.

Trap-Gentil, M.-V., Hébrard, C., Lafon-Placette, C., Delaunay, A., Hagège, D., Joseph, C., Brignolas, F., Lefebvre, M., Barnes, S., and Maury, S. (2011). Time course and amplitude of DNA methylation in the shoot apical meristem are critical points for bolting induction in sugar beet and bolting tolerance between genotypes. J. Exp. Bot. 62, 2585–2597.

Truernit, E., Siemering, K.R., Hodge, S., Grbic, V., and Haseloff, J. (2006). A map of KNAT gene expression in the Arabidopsis root. Plant Mol. Biol. 60, 1–20.

Tsuda, K., Kurata, N., Ohyanagi, H., and Hake, S. (2014). Genome-Wide Study of KNOX Regulatory Network Reveals Brassinosteroid Catabolic Genes Important for Shoot Meristem Function in Rice. Plant Cell 26, 3488–3500.

Turk, E.M., Fujioka, S., Seto, H., Shimada, Y., Takatsuto, S., Yoshida, S., Wang, H., Torres, Q.I., Ward, J.M., Murthy, G., et al. (2005). BAS1 and SOB7 act redundantly to modulate Arabidopsis photomorphogenesis via unique brassinosteroid inactivation mechanisms. Plant J. 42, 23–34.

Tyler, F.J. (1902). Rosette Plants of Ohio. O. S. U. Nat. 2, 290–294.

Umetsu, A., Sawada, Y., Mitsuhashi, W., Mazier, M., and Toyomasu, T. (2011). Characterization of a Loss-ofFunction Mutant of Gibberellin Biosynthetic Gene LsGA2ox1 in Lettuce. Biosci. Biotechnol. Biochem. 75, 2398–2400.

Ung, N., Lal, S., and Smith, H.M.S. (2011). The role of PENNYWISE and POUND-FOOLISH in the maintenance of the shoot apical meristem in Arabidopsis. Plant Physiol. 156, 605–614.

Unterholzner, S.J., Rozhon, W., Papacek, M., Ciomas, J., Lange, T., Kugler, K.G., Mayer, K.F.X., Sieberer, T., and Poppenberger, B. (2015). Brassinosteroids Are Master Regulators of Gibberellin Biosynthesis in Arabidopsis. Plant Cell 27, 2261–2272.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science 303, 1003–1006.

Venglat, S.P., Dumonceaux, T., Rozwadowski, K., Parnell, L., Babic, V., Keller, W., Martienssen, R., Selvaraj, G., and Datla, R. (2002). The homeobox gene BREVIPEDICELLUS is a key regulator of inflorescence architecture in Arabidopsis. Proc. Natl. Acad. Sci. 99, 4730.

Vert, G., Walcher, C.L., Chory, J., and Nemhauser, J.L. (2008). Integration of auxin and brassinosteroid pathways by Auxin Response Factor 2. Proc. Natl. Acad. Sci. 105, 9829–9834.

Vineyard, L., Elliott, A., Dhingra, S., Lucas, J.R., and Shaw, S.L. (2013). Progressive Transverse Microtubule Array Organization in Hormone-Induced Arabidopsis Hypocotyl Cells. Plant Cell 25, 662–676.

Viola, I.L., and Gonzalez, D.H. (2006). Interaction of the BELL-like protein ATH1 with DNA: role of homeodomain residue 54 in specifying the different binding properties of BELL and KNOX proteins. Biol. Chem. 387, 31–40.

Viola, I.L., and Gonzalez, D.H. (2009). Binding properties of the complex formed by the Arabidopsis TALE homeodomain proteins STM and BLH3 to DNA containing single and double target sites. Biochimie 91, 974–981.

Vogt, S.H., Weyens, G., Lefèbvre, M., Bork, B., Schechert, A., and Müller, A.E. (2014). The FLC-like gene BvFL1 is not a major regulator of vernalization response in biennial beets. Front. Plant Sci. 5, 1–12.

Vroemen, C.W., Mordhorst, A.P., Albrecht, C., Kwaaitaal, M.A.C.J., and de Vries, S.C. (2003). The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. Plant Cell 15, 1563.

Wachsman, G., Modliszewski, J.L., Valdes, M., and Benfey, P.N. (2017). A SIMPLE Pipeline for Mapping Point Mutations. Plant Physiol. 174, 1307–1313.

Wahl, V., Ponnu, J., Schlereth, A., Arrivault, S., Langenecker, T., Franke, A., Feil, R., Lunn, J.E., Stitt, M., and Schmid, M. (2013). Regulation of flowering by trehalose-6-phosphate signaling in Arabidopsis thaliana. Science 339, 704–707.

Wang, H., and Wang, H. (2015). Phytochrome Signaling: Time to Tighten up the Loose Ends. Mol. Plant 8, 540–551.

Wang, R., and Estelle, M. (2014). Diversity and specificity: auxin perception and signaling through the TIR1/AFB pathway. Curr. Opin. Plant Biol. 21, 51–58.

Wang, J.-W., Czech, B., and Weigel, D. (2009). miR156-Regulated SPL Transcription Factors Define an Endogenous Flowering Pathway in Arabidopsis thaliana. Cell 138, 738–749.

Wang, J., Qiu, Y., Cheng, F., Chen, X., Zhang, X., Wang, H., Song, J., Duan, M., Yang, H., and Li, X. (2017). Genome-wide identification, characterization, and evolutionary analysis of flowering genes in radish (Raphanus sativus L.). BMC Genomics 18, 1–10.

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., Huber, S.C., and Clouse, S.D. (2008). Sequential Transphosphorylation of the BRI1/BAK1 Receptor Kinase Complex Impacts Early Events in Brassinosteroid Signaling. Dev. Cell 15, 220–235.

Wang, Z.-Y., Nakano, T., Gendron, J.M., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., et al. (2002). Nuclear-Localized BZR1 Mediates Brassinosteroid-Induced Growth and Feedback Suppression of Brassinosteroid Biosynthesis. Dev. Cell 2, 505–513.

Went, F.W. (1948). Some parallels between desert and alpine flora in California. Madroño 9, 241–249.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., and Weigel, D. (2005). Integration of Spatial and Temporal Information During Floral Induction in Arabidopsis. Science (80-.). 309, 1056–1059.

Willige, B.C., Isono, E., Richter, R., Zourelidou, M., and Schwechheimer, C. (2011). Gibberellin regulates PIN-FORMED abundance and is required for auxin transport-dependent growth and development in Arabidopsis thaliana. Plant Cell 23, 2184–2195.

Wittwer, S.H., and Bukovac, M.J. (1957). Gibberellin Effects on Temperature and Photoperiodic Requirements for Flowering of Some Plants. Science (80-.). 126, 30–31.

Wollenberg, A.C., Strasser, B., Cerdán, P.D., and Amasino, R.M. (2008). Acceleration of Flowering during Shade Avoidance in Arabidopsis Alters the Balance between FLOWERING LOCUS C-Mediated Repression and Photoperiodic Induction of Flowering. Plant Physiol. 148, 1681–1694.

Wood, D.W., and Scott, R.K. (1975). Sowing sugar beet in autumn in England. J. Agric. Sci. 84, 97–108.

Wu, C., Wang, M., Cheng, Z., and Meng, H. (2016). Response of garlic (Allium sativum L.) bolting and bulbing to temperature and photoperiod treatments. Biol. Open 5, 507–518.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.-W., Weigel, D., and Poethig, R.S. (2009). The Sequential Action of miR156 and miR172 Regulates Developmental Timing in Arabidopsis. Cell 138, 750–759.

Xu, Y.-L., Gage, D.A., and Zeevaart, J.A.D. (1997). Gibberellins and Stem Growth in Arabidopsis thaliana: Effects of Photoperiod on Expression of the GA4 and GA5 Loci.

Yadav, R.K., Girke, T., Pasala, S., Xie, M., and Reddy, G.V. (2009). Gene expression map of the Arabidopsis shoot apical meristem stem cell niche. Proc. Natl. Acad. Sci. 106, 4941–4946.

Wilson, R.N., Heckman, J.W., and Somerville, C.R. (1992). Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. Plant Physiol. 100, 403–408.

Yadav, R.K., Tavakkoli, M., Xie, M., Girke, T., and Reddy, G. V. (2014). A high-resolution gene expression map of the Arabidopsis shoot meristem stem cell niche. Development 141, 2735–2744.

Yamaguchi, N., and Komeda, Y. (2013). The role of CORYMBOSA1/BIG and auxin in the growth of Arabidopsis pedicel and internode. Plant Sci. 209, 64–74.

Y**amaguchi, A., Kobayashi, Y., Goto, K., Abe, M., and Araki, T.** (2005). TWIN SISTER OF FT (TSF) Acts as a Floral Pathway Integrator Redundantly with FT. Plant Cell Physiol. 46, 1175–1189.

Yamaguchi, N., Winter, C.M., Wu, M.-F.M.-F., Kanno, Y., Yamaguchi, A., Seo, M., and Wagner, D. (2014). Gibberellin Acts Positively Then Negatively to Control Onset of Flower Formation in Arabidopsis. Science (80-.). 344, 638–641.

Yamasaki, A., Miura, H., and Tanaka, K. (2000). Effect of photoperiods before, during and after vernalization on flower initiation and development and its varietal difference in Japanese bunching onion (Allium fistulosum L.). J. Hortic. Sci. Biotechnol. 75, 645–650.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A., and Ori, N. (2005). Arabidopsis KNOXI proteins activate cytokinin biosynthesis. Curr. Biol. 15, 1566–1571.

Yang, D.-L., Yao, J., Mei, C.-S., Tong, X.-H., Zeng, L.-J., Li, Q., Xiao, L.-T., Sun, T., Li, J., Deng, X.-W., et al. (2012). Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proc. Natl. Acad. Sci. U. S. A. 109, E1192-200.

Yin, Y., Wang, Z.-Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., and Chory, J. (2002). BES1 Accumulates in the Nucleus in Response to Brassinosteroids to Regulate Gene Expression and Promote Stem Elongation. Cell 109, 181–191.

Yoshida, Y., Takada, N., and Koda, Y. (2010). Isolation and identification of an anti-bolting compound, hexadecatrienoic acid monoglyceride, responsible for inhibition of bolting and maintenance of the leaf rosette in radish plants. Plant Cell Physiol. 51, 1341–1349.

Youn, J.H., Kim, T.-W., Joo, S.-H., Son, S.-H., Roh, J., Kim, S., Kim, T.-W., and Kim, S.-K. (2018). Function and molecular regulation of DWARF1 as a C-24 reductase in brassinosteroid biosynthesis in Arabidopsis. J. Exp. Bot. 69, 1873–1886.

Yu, C.-W., Liu, X., Luo, M., Chen, C., Lin, X., Tian, G., Lu, Q., Cui, Y., and Wu, K. (2011). HISTONE DEACETYLASE6 interacts with FLOWERING LOCUS D and regulates flowering in Arabidopsis. Plant Physiol. 156, 173–184.

Yu, S., Galvão, V.C., Zhang, Y.-C., Horrer, D., Zhang, T.-Q., Hao, Y.-H., Feng, Y.-Q., Wang, S., Schmid, M., and Wang, J.-W. (2012). Gibberellin regulates the Arabidopsis floral transition through miR156-targeted SQUAMOSA promoter binding-like transcription factors. Plant Cell 24, 3320–3332.

Žádníková, P., and Simon, R. (2014). How boundaries control plant development. Curr. Opin. Plant Biol. 17, 116–125.

Zanewich, K.P., Rood, S.B., and Williams, P.H. (1990). Growth and development of Brassica genotypes differing in endogenous gibberellin content. I. Leaf and reproductive development. Physiol. Plant. 79, 673–678.

Zeevaart, J.A.D. (1971). Effects of photoperiod on growth rate and endogenous gibberellins in the long-day rosette plant spinach. Plant Physiol. 47, 821–827.

Z**hai, Q., Zhang, X., Wu, F., Feng, H., Deng, L., Xu, L., Zhang, M., Wang, Q., and Li, C.** (2015). Transcriptional Mechanism of Jasmonate Receptor COI1-Mediated Delay of Flowering Time in Arabidopsis. Plant Cell 27, 2814–2828.

Zhang, B., Holmlund, M., Lorrain, S., Norberg, M., Bakó, L., Fankhauser, C., and Nilsson, O. (2017a). BLADE-ON-PETIOLE proteins act in an E3 ubiquitin ligase complex to regulate PHYTOCHROME INTERACTING FACTOR 4 abundance. Elife 6. 136, 2621–2632.

Zhang, D., Wang, X., Wang, M., Li, J., Guo, X., Chong, K., and Xu, Y. (2013). Ectopic Expression of WUS in Hypocotyl Promotes Cell Division via GRP23 in Arabidopsis. PLoS One 8, e75773.

Zhang, L., Su, W., Tao, R., Zhang, W., Chen, J., Wu, P., Yan, C., Jia, Y., Larkin, R.M., Lavelle, D., et al. (2017b). RNA sequencing provides insights into the evolution of lettuce and the regulation of flavonoid biosynthesis. Nat. Commun. 8, 2264.

Zhang, R., Ge, S., He, J., Li, S., Hao, Y., Du, H., Liu, Z., Cheng, R., Feng, Y., Xiong, L., et al. (2019). BIG regulates stomatal immunity and jasmonate production in Arabidopsis. New Phytol. 222, 335–348.

Zhang, S., Yang, C., Peng, J., Sun, S., and Wang, X. (2009). GASA5, a regulator of flowering time and stem growth in Arabidopsis thaliana. Plant Mol. Biol. 69, 745–759.

Zhao, M., Yang, S., Chen, C.-Y., Li, C., Shan, W., Lu, W., Cui, Y., Liu, X., and Wu, K. (2015). Arabidopsis BREVIPEDICELLUS Interacts with the SWI2/SNF2 Chromatin Remodeling ATPase BRAHMA to Regulate KNAT2 and KNAT6 Expression in Control of Inflorescence Architecture. PLoS Genet. 11, e1005125.

Zhou, Y., Liu, X., Engstrom, E.M., Nimchuk, Z.L., Pruneda-Paz, J.L., Tarr, P.T., Yan, A., Kay, S.A., and Meyerowitz, E.M. (2015). Control of plant stem cell function by conserved interacting transcriptional regulators. Nature 517, 377–380.

Zimmermann, P., Hirsch-hoffmann, M., Hennig, L., and Gruissem, W. (2004) GENEVESTIGATOR . Arabidopsis Microarray Database and Analysis Toolbox. Bioinformatics

Nederlandse samenvatting

Dankzij millennia aan plantenveredeling beschikken wij over hoogproductieve voedselgewassen. Echter moet de opbrengstcapaciteit van onze gewassen de komende jaren fors omhoog doordat het beschikbare landbouwareaal zal afnemen, terwijl de wereldbevolking blijft groeien. Tijdens de Groene Revolutie in de jaren ’70 is een forse verhoging van de oogst van o.a. tarwe, rijst en mais bewerkstelligd, maar blad-, wortel- en knolgewassen zoals sla, suikerbiet en radijs zijn hierbij achtergebleven. Deze groentes worden geoogst van rozetplanten. Rozetplanten kenmerken zich door het vormen van een compacte rozet van bladeren tijdens hun vegetatieve groeifase, gevolgd door het vormen van een bloeistengel (doorschieten) en bloemen (bloei) tijdens hun generatieve groeifase. De overgang van een compacte rozet naar een geëlongeerde stengel is een fundamenteel ontwikkelingsproces in rozetplanten. Vroegtijdig doorschieten van rozetgewassen heeft een zeer nadelig effect op kwaliteit en kwantiteit van het te oogsten product, met als gevolg een verminderde opbrengst. Desondanks is er echter weinig bekend over hoe het proces van doorschieten wordt gereguleerd en hoe rozetgroei tijdens de vegetatieve fase in stand wordt gehouden.

De rozet en de stengel worden beide gevormd vanuit de scheutapex. In deze regio bevindt zich het scheut apicale meristeem (SAM) en de onderliggende rib zone (RZ). Het SAM bevat stamcellen van waaruit tijdens vegetatieve groei bladeren en tijdens generatieve groei bloemen worden gevormd. De RZ vormt stengelweefsel tussen twee opeenvolgende bladeren of bloemen (internodiën) en is tijdens de vegetatieve groei inactief in rozetplanten. Het plantenhormoon gibberelline (GA) is van essentieel belang voor doorschieten en de plantenhormonen auxine en brassinosteroïde (BR) hebben een positief effect op stengelgroei.

De rozetplant *Arabidopsis thaliana* (zandraket) is een veelgebruikte modelplant binnen de plantenbiologie en is daardoor uitermate geschikt voor het onderzoeken van doorschieten. Eerder onderzoek heeft aangetoond dat de Arabidopsis transcriptiefactor ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1) elongatie van de stengel remt. ATH1 is onder meer actief in de scheutapex en de aanwezigheid van dit regulatoire eiwit in dit weefsel valt geheel samen met de vegetatieve rozetfase van *Arabidopsis thaliana*. Verlies van ATH1 tijdens deze groeifase leidt tot inductie van rozetinternodiën, terwijl het artificieel verhogen van *ATH1*-niveaus tijdens

generatieve groei doorschieten onderdrukt. Deze planten bloeien daarentegen normaal. ATH1 is dus een belangrijke regulator van rozetvorming en doorschieten. Het is echter niet bekend hoe ATH1 dit doet.

In dit proefschrift is de rol van *ATH1* tijdens rozetvorming en doorschieten in *Arabidopsis thaliana* onderzocht.

In **hoofdstuk 2** worden microscopische analyses en farmacologische experimenten gecombineerd om aan te tonen dat *ATH1* cruciaal is voor het behoud van de rozetvorm. Microscopische analyse laat zien dat het ATH1 eiwit uit het SAM en RZ verdwijnt tijdens de inductie van doorschieten. Dit gaat gepaard met een vergroting van de RZ. In mutanten die geen werkend *ATH1* produceren (*ath1*), vindt deze vergroting van de RZ al plaats tijdens vegetatieve groei en vormen *ath1* planten geëlongeerde rozetinternodiën. Dit fenotype is heterochronisch doorschieten genoemd. In *ath1* planten wordt heterochronisch doorschieten versterkt door omgevingsfactoren (warme omgevingstemperatuur, verrood licht) en GA. Extern toedienen van GA aan *ath1* planten kan de rozetvorm zelfs grotendeels opheffen. ATH1 en GA hebben dus een tegenovergesteld effect op elongatie van internodiën. De rozetten van wildtype planten blijven in alle bovengenoemde condities compact, wat duidt op een cruciale rol van *ATH1* bij het onderdrukken van elongatie tijdens rozetgroei, zelfs in aanwezigheid van het stimulerende hormoon GA.

ATH1 stimuleert de activiteit (expressie) van zogenaamde *boundary* genen, waaronder het gen *LATERAL ORGAN BOUNDARIES* (*LOB*). *Boundary* genen zijn genen die betrokken zijn bij het vormen van grensweefsel tussen stamcellen en differentiërend weefsel. *LOB* expressie is bijna volledig afhankelijk van ATH1. Net als in *ath1* mutanten kan in *lob* mutanten heterochronisch doorschieten worden geïnduceerd met behulp van GA, maar in mindere mate dan in het eerste geval. Deze resultaten laten zien dat ATH1 stengelelongatie deels controleert door middel van *LOB* regulatie. Omdat de activiteit van *LOB* en *ATH1* beide niet onder controle van GA staat, duidt dit er op dat de antagonistische rollen van GA en ATH1 grotendeels onafhankelijk van elkaar verlopen.

In **hoofdstuk 3** wordt het effect van auxine en BR op heterochronisch doorschieten onderzocht. Zowel auxine als BR induceert heterochronisch doorschieten in *ath1* en *lob*, maar niet in wildtype planten. Een gecombineerde auxine/BR behandeling verlaagt *ATH1* expressie en leidt tot heterochronisch doorschieten in wildtype planten. In planten waar *ATH1* niveaus artificieel hoog gehouden worden, zijn zowel rozet- en stengelinternodiën ongevoelig voor deze behandeling.

De *PHYTOCHROME INTERACTING FACTOR* (*PIF*) genfamilie is een belangrijke regulator van strekking van de embryonale stam en integreert signalen van meerdere hormonen, waaronder GA, BR en auxine. Verlies van *PIF4* of *PIF7*, twee van de leden van de PIF genfamilie, heft heterochronisch doorschieten (deels) op in *ath1* planten. Dit zou er op kunnen wijzen dat één van de functies van ATH1 is om het voorgaande elongatieprogramma, ingezet tijdens de ontwikkeling van de embryonale stam, te termineren en zodoende de vorming van een rozet mogelijk te maken. Het effect van *pif4* en *pif7* mutaties op *ath1* rozetmorfologie is niet volledig en is afhankelijk van gebruikte groeicondities. Dit betekent waarschijnlijk dat meerdere leden uit de PIF genfamilie betrokken zijn bij heterochronisch doorschieten.

Om meer inzicht te krijgen in factoren die belangrijk zijn voor RZ activiteit is in **hoofdstuk 4** een mutantscreening uitgevoerd. Willekeurige mutaties zijn aangebracht in het genoom van *ath1* mutanten en mutanten zijn vervolgens geselecteerd op het vormen van wederom compacte of juist sterker geëlongeerde rozetinternodiën, respectievelijk *suppressor of ath1-3 internodes* (*sri*) of *enhancer of ath1-3 rosette internodes* (*eri*) genoemd. Negen *ath1 sri* mutanten en vier *ath1 eri* mutanten zijn geselecteerd. Mutaties in het stamcelgen *WUSCHEL* (*sri4*), auxine-gerelateerde *BIG* (*sri52*) en het *DWARF1* (*sri113*) BR biosynthese gen onderdrukken heterochronisch doorschieten van *ath1*. Bovendien zijn *ath1 sri113* mutanten in afwezigheid van BR ongevoelig voor GA en auxine, wat duidt op een sterke onderlinge afhankelijkheid van GA, auxine en BR tijdens heterochronisch doorschieten. De *ath1 sri93* mutant lijkt op mutanten die *boundary* genen verhoogd tot expressie brengen en is ongevoelig voor GA, BR en auxine. Daarentegen zijn planten die gemuteerd zijn in de *boundary* genen *ASYMMETRIC LEAVES1* (*AS1*), *AS2* of *BLADE ON PETIOLE1* (*BOP1*) en *BOP2* juist gevoelig voor GA en auxine. De uitkomst van deze mutantscreen, in combinatie met de bevindingen zoals beschreven in hoofdstukken 3 en 4, versterkt de eerder verkregen indruk dat *ATH1* de compactheid van de rozet behoudt door

meerdere, uiteenlopende, maar onderling afhankelijke groeiregulatoire processen te onderdrukken.

ATH1 is een BEL1-LIKE HOMEODOMAIN (BLH) transcriptiefactor. Tot deze familie hoort ook de transcriptiefactor PENNYWISE (PNY), een positieve regulator van stengelelongatie. ATH1 en PNY kunnen binden aan de transcriptiefactoren SHOOT MERISTEMLESS (STM), KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2 (KNAT2), KNAT6 en BREVIPEDICELLUS (BP), allen behorend tot de KNOTTED1-LIKE HOMEOBOX (KNOX)-klasse. Uit eerder onderzoek is gebleken dat interacties tussen BLH en KNOX eiwitten belangrijk zijn voor het functioneren van deze eiwitten. In **hoofdstuk 5** wordt de rol van deze eiwitpartners van ATH1 tijdens rozetvorming en doorschieten onder de loep genomen. We laten zien dat KNAT2 en KNAT6 nodig zijn voor *ATH1* functie: elongatie van de bloeistengel van *35S:ATH1* planten wordt hersteld door verlies van *KNAT2* of *KNAT6*, terwijl GA heterochronisch doorschieten induceert in *knat2 knat6* mutanten. Ook *stm* mutanten vertonen heterochronisch doorschieten, zelfs zonder toevoeging van hormonen, zoals *ath1.* Het is dus zeer aannemelijk dat ATH1 voor het compact houden van de rozet, functionele dimeren vormt met *KNAT2, KNAT6* en *STM*. Gezien het sterke fenotype van *stm* mutanten, lijkt STM van deze drie hierbij de meest prominente rol te vervullen.

Naast de functionele interactie tussen ATH1 en KNOX eiwitten, hebben we ook gekeken naar de antagonistische interactie tussen de BLH eiwitten ATH1 en PNY. Uit de literatuur is bekend dat stengelelongatie verlaagd is in *pny* mutanten en eerder onderzoek heeft laten zien dat een *ath1* mutatie dit defect kan onderdrukken. Wij laten zien dat *PNY*, net als *ATH1,* ook een onderdrukkend effect heeft op internodiënelongatie tijdens de vegetatieve groeifase. Dit is een opvallende bevinding in het licht van de reeds bekende rol van *PNY*. Verlies van *PNY* versterkt heterochronisch doorschieten van *ath1* en met behulp van GA kan heterochronisch doorschieten worden geïnduceerd in *pny* mutanten. Daarnaast laten we zien dat ook planten die *PNY* verhoogd tot expressie brengen (*35S:PNY*) korte bloeistengels vormen, net als *pny* mutanten, en dat dit fenotype afhankelijk is van *KNAT6* functie. Dit betekent dat PNY zowel belangrijk is voor het onderdrukken van stengelgroei tijdens de vegetatieve groeifase als voor het induceren van stengelgroei tijdens de generatieve fase. Deze dubbele, ogenschijnlijk tegenstrijdige rol van *PNY* speelt mogelijk een grote rol bij een snelle en effectieve omschakeling van rozet- naar stengelgroei.

De aan- of afwezigheid van *ATH1* heeft een uitgesproken effect heeft op internodiënelongatie, dus het is zeer aannemelijk dat *ATH1* een cruciale en definiërende factor is voor de vorming van een (compacte) rozet. *ATH1* lijkt de vorming van geëlongeerde rozetinternodiën te voorkomen via het inactiveren van meerdere nauw met elkaar verweven groeiroutes. Orthologen van het *ATH1* gen zijn aanwezig in zowel monocotyle en dicotyle planten en deze zijn daardoor een zeer interessant richtpunt voor de ontwikkeling van nieuwe vormen van resistentie tegen doorschieten in rozetgewassen. De bevindingen uit dit proefschrift dragen bij aan een groter begrip van rozetvorming en doorschieten, twee processen die zowel fundamenteel als landbouwkundig zeer relevant, desondanks onderbelicht, zijn.

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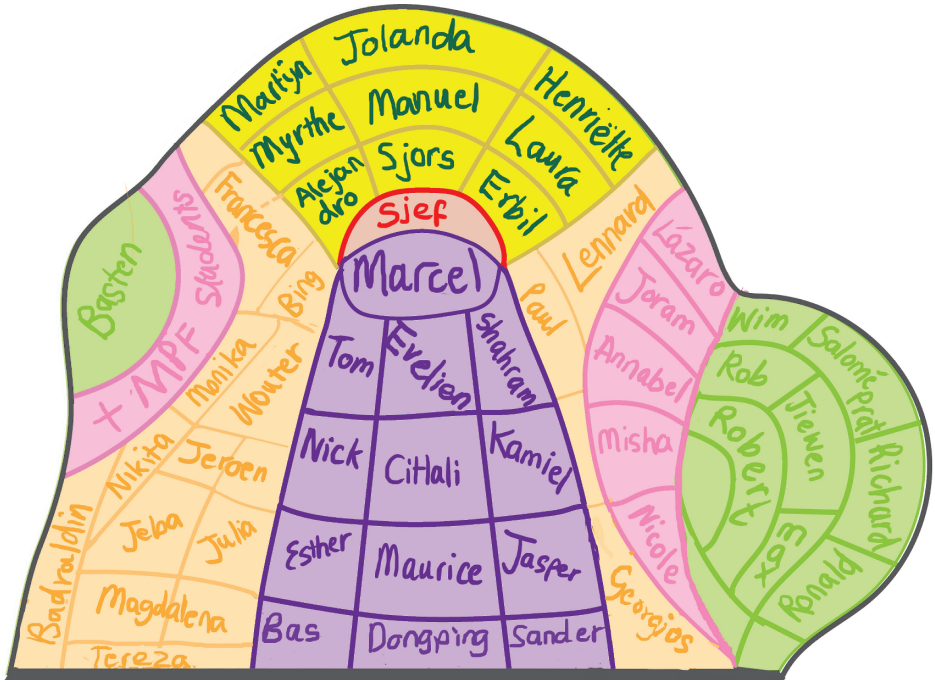

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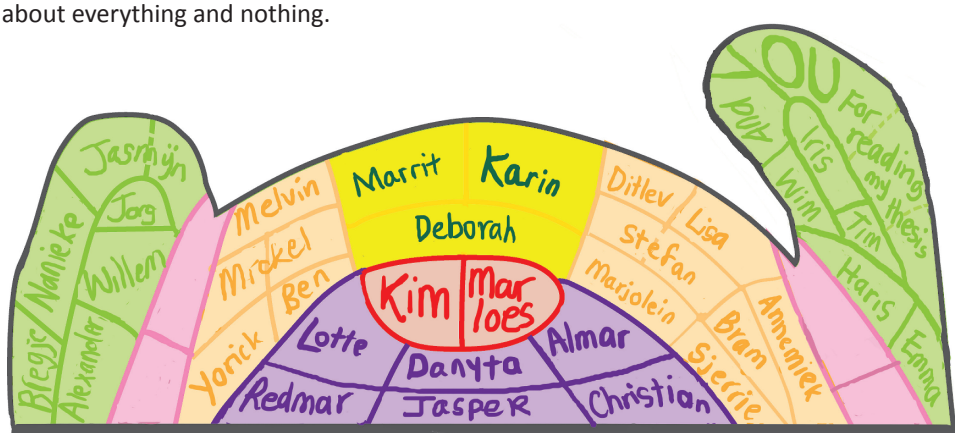

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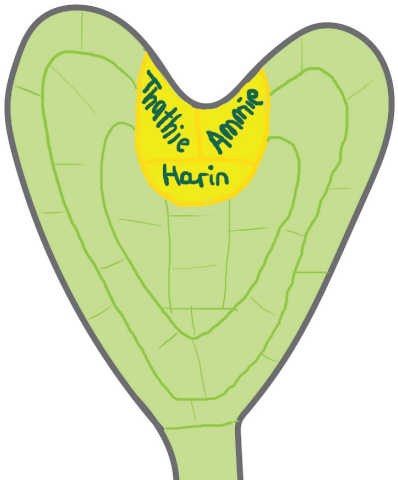

Curriculum Vitae

Savani Sarasini Silva was born on the 29th of April 1989 in Hillingdon, the United Kingdom. In 2007 she obtained her VWO Bilingual Atheneum diploma *cum laude* at Anna van Rijn College in Nieuwegein, the Netherlands. Subsequently, she started studying BSc Biology at Utrecht University. As a final project, she wrote a Bachelor thesis under the supervision of Dr. Marcel Proveniers titled "Knotted together in the meristem: An overview of the interplay between KNOX and BEL in the framework of meristem function". Savani completed her Bachelor degree *cum laude* in 2010 and continued her education at Utrecht University by starting her MSc in Molecular and Cellular Life Sciences. During her MSc studies, she performed two research internships at Utrecht University. Her first internship was conducted at the Molecular Plant Physiology research group, under the supervision of Dr. Marcel Proveniers. During this 11-month internship, she worked on a project titled "Functional characterisation of the ATH1-KNOX heterodimers in *Arabidopsis thaliana* inflorescence development." Subsequently, she conducted a 7-month internship at the Microbiology research group, supervised by Dr. Luis Lugones. During this internship she worked on a project titled "The regulatory network of fructification surrounding the *Schizophyllum commune* transcription factor *hom2*". For her final thesis, she wrote about conditionally dispensable chromosomes in plant pathogenic fungi, under the Supervision of Prof. Dr. Guido van der Ackerveken, at the Plant-Microbe Interactions group at Utrecht University. After completing her MSc degree in 2013, Savani was accepted to the Experimental Plant Sciences (EPS) Graduate Programme. Here she wrote a research proposal under the supervision of Dr. Marcel Proveniers, based on the results of her first internship. This proposal was awarded with a personal PhD grant through the EPS Graduate School Starting Materials programme of the Dutch Organisation for Scientific Research (NWO). With this personal grant, Savani started working as PhD student in 2014 at the Molecular Plant Physiology group, supervised by Dr. Marcel Proveniers and Prof. Dr. Sjef Smeekens. The results of this project are described in this thesis.