Control of Plant Architecture by Distinctive *TALE* Homeobox Gene Interactions

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Regulatie van plantenarchitectuur door middel van specifieke *TALE* homeobox transcriptiefactor interacties
(met een samenvatting in het Nederlands)

Proefschrift

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Learning without thought is labor lost; thought without learning is perilous.

-Confucius (551 BC - 479 BC)

We next went to the school of languages, where three professors sat in consultation upon impoving that of their own country.
The first project was to shorten discourse by cutting polysyllables into one, and leaving out verbs and participles, because in reality all things imaginable are but nouns.
The other project was a scheme for entirely abolishing all words whatsoever; and this was urged as a great advantage in point of health as well as brevity.

-Jonathan Swift, Gullivers Travels (1726)

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Chapter 1
General Introduction

Plant Architecture

A striking feature of plants is the huge variety of plant forms that can be found in nature. This enormous diversity is due to variation in the shape, size, proportion and relative position of the different organs in the aerial part of the plant. Evolutionary changes in the three-dimensional organization, or architecture, of plant shoots have played a central role in the morphological diversification of plant species (Sussex and Kerk, 2001). Moreover, plant architecture is a determining factor in the agronomic performance of crop plants. As a result, the exploitation of natural genetic variation in plant architecture has played an important role in the domestication of crop plants (reviewed in Reinhardt and Kuhlemeier, 2002; Wang and Li, 2008).

Higher plant architecture mainly comprises the overall size and branching pattern of the shoot. Other major components of architectural variations in the plant kingdom are the number, shape, and position of shoot lateral organs, such as leaves and flowers. Plant height is a decisive factor for the size of plants and is determined by the total number of internodes and the length of each individual internode. Although the architecture of some vascular plants only consists of a single vegetative axis during their entire lifespan, most plants have a more complex architecture with additional axes in a repetitive fashion, a process known as shoot branching. Characteristic of shoot branching are type and degree, or pattern, and angle, which determines the amount of space occupied by the branches.

Plant architecture shows high plasticity in response to environmental cues. However, most of its features remain remarkably consistent within species. This low intraspecific variation of plant architecture suggests that it is subject to strict intrinsic genetic programs. In recent years, studies on the model plants *Arabidopsis thaliana* and snapdragon (*Antirrhinum majus*), as well as on crop plants such as rice (*Oryza sativa*), maize (*Zea mays*), and tomato (*Solanum lycopersicum*) have

begun to reveal the mechanisms that underlie the control of plant height, branching, leaf size and form, and floral transition.

The shoot apical meristem as ultimate source of all above-ground plant parts

In plants most of the adult body is formed post-embryonically by the continuous activity of indefinite pluripotent stem cell pools: the shoot and root apical meristems. The main activities of the SAM throughout plant development are to maintain the pluripotent stem cells and to form lateral organs and stems, by which the diverse architecture of different plant species is established.

Both apical meristems are established during plant embryogenesis and together with cotyledons, hypocotyl and embryonic root they make up the basic body plan. The apical-basal and the radial axes of the plant body are defined at the globular stage of Arabidopsis embryo development. Cells at the apical layer will produce cotyledons and the shoot apical meristem (SAM), whereas the basal layer will give rise to the hypocotyl and root apical meristem (RAM). Histologically, the first sign of the formation of the embryonic SAM is the outgrowth of the cotyledonary primordia from the flanks of the late globular embryo. However, gene expression studies revealed that onset of shoot apex development is earlier and includes three steps: specification of the apical domain, initiation of the stem cell niche, and central-peripheral patterning into shoot meristem and cotyledonary primordia (reviewed in Laux et al., 2004). Next, the two cotyledons develop, provascular tissues are enlarged and the embryo changes to a heart-shaped embryo. The heart-shaped embryo elongates to form the torpedo-stage embryo while retaining the same pattern of tissues and organs. Finally, characterized by rounds of rapid cell divisions and cotyledon expansion, the embryo fills the seed and completes its growth and morphogenesis (reviewed in Laux et al., 2004; Weijers and Jurgens, 2005; Jenik et al., 2007; Park and Harada, 2008).

At the completion of embryogenesis the apical meristems are quiescent, but become reactivated after seed germination. The SAM starts as a cluster of about one hundred cells that retain organogenic potential throughout the plant lifecycle. Via the formation of organ primordia at its flanks, the SAM gives rise to the aerial portion of the plant.

In flowering plants, post-embryonic development can be divided in two major phases reflecting the identity of the SAM: a vegetative phase and a reproductive phase (Figure 1.1). During the vegetative phase of plant development, the Arabidopsis SAM generates leaf primordia in a spiral phyllotaxy to form a basal rosette with each leaf having a quiescent axillary meristem (AM) in its axil. The number of leaves and the leaf morphology, i.e. the shape, the size and leaf angle are important determinants of plant architecture as different species exhibit

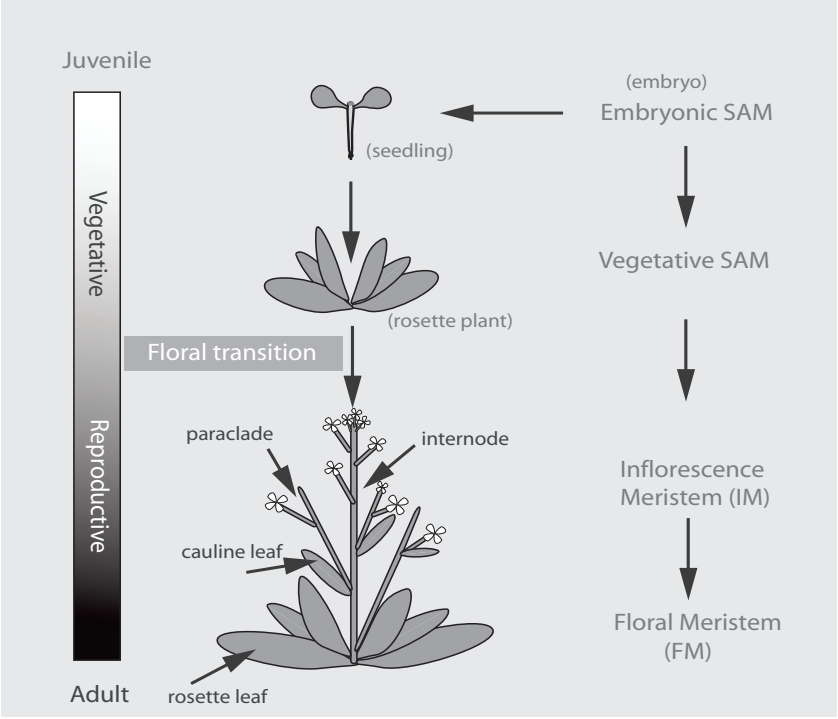

Figure 1.1 Developmental stages of Arabidopsis.
Schematic representation of the distinctive Arabidopsis developmental stages. After germination, Arabidopsis develops into a seedling, starting the juvenile phase. During vegetative stage, a rosette-like statue is formed. After floral evocation (see text), the vegetative shoot apical meristem (SAM) becomes an inflorescence meristem (IM) which can produce floral meritem (FM) that gives rise to flower. The stem elongates (bolting process) and the basal nodes of the bolting stem carry cauline leaves. The more apical nodes of the stem produce solitary flowers. The axillary SAMs in the axils of rosette and cauline leaves can grow into a secondary inflorescence, called paraclade (for a detailed description see the text).

remarkable diversity in nature. Leaf form can be either simple, as in Arabidopsis, tobacco, maize and rice, species in which the leaf blade is undivided, or more complex as in tomato and pea, species that have compound leafs where the blade is divided into distinct leaflets. In the latter case, a leaf primordium branches one or more times before initiating leaflets to produce secondary outgrowths along their edges. These outgrowths follow a developmental pattern similar to that of a simple leaf (reviewed in Goliber et al., 1999; Sinha, 1999; Champagne and Sinha, 2004). Additionally, margins of both simple and compound leaves can elaborate less-pronounced incisions such as serrations or lobes.

In Arabidopsis, internodes are initiated during vegetative development with little or no elongation and, consequently, the shoot displays a compact rosette stature (Figure 1.1). Upon a signal to flower, the vegetative SAM undergoes

a corresponding phase change, and becomes converted into an inflorescence meristem (IM). The inflorescence constitutes the major part of the shoot and thus contributes significantly to the overall shoot architecture. The IM retains its indeterminate characteristics and continues to give rise to lateral organ primordia during generative development. The onset of reproductive growth is generally characterized by a rapid increase in stem internode elongation, a process named bolting. Each basal node of the bolting stem carries a petiole-less cauline leaf in the axils of which AMs give rise to indeterminate secondary inflorescences. At the younger nodes, determinate, solitary floral meristems (FMs) are formed that directly grow into flowers.

Research on plant architecture has mainly focused on fundamental questions relating to meristem functions, such as how a plant maintains the activity of its SAM and initiates AMs, when AMs become active, how floral transition is timed, and when shoots start to elongate and stop again.

SAM organization

The SAM is a highly organized, dome-shaped structure which can be arbitrarily divided into cytological layers and functionally distinct domains. In most dicotyledons, including Arabidopsis, the meristem surface, or tunica, usually comprises two separate layers, known as L1 and L2 (Figure 1.2), whereas cells in deeper regions of the meristem are more randomly positioned, forming the corpus or L3 layer. Most cells in L1 and L2 undergo anticlinal division to maintain individual layers. Occasionally, periclinal cell divisions occur in these layers, that is, the progeny of the cell will move to a new layer from which new cell fates will be adopted. Genetic chimeras have shown that the position of a cell in the SAM is more important to determine its fate than its clonal lineage (Poethig, 1989; Irish, 1991). The L1 and L2 layers contribute differentially to organ formation. In dicots, leaf epidermis develops from the L1, and inner cell types are progenies from L2 and L3. Cells in L3 can divide in various planes. They connect the SAM with the vascular system.

In addition to the cytological zonation model, the SAM can also be classified into three radial zones, based on distinct functions: the central zone (CZ) with slowly dividing cells, the peripheral zone (PZ) with rapidly dividing smaller cells and the rib zone (RZ). The CZ spans the tunica and corpus from the apex to the base of the dome of the SAM (Figure 1.2). Cells in the CZ are of great importance because they act as the initial reservoir of pluripotent stem cells, replenishing the PZ and RZ whilst maintaining the integrity of the CZ itself. The PZ surrounds the CZ and reiteratively initiates leaves, axillary buds and the outer layers of the stem. The RZ lies at the base of the CZ and gives rise to the stem pith and petioles. Interestingly, a set of small densely cytoplasmic cells beneath the CZ was origi-

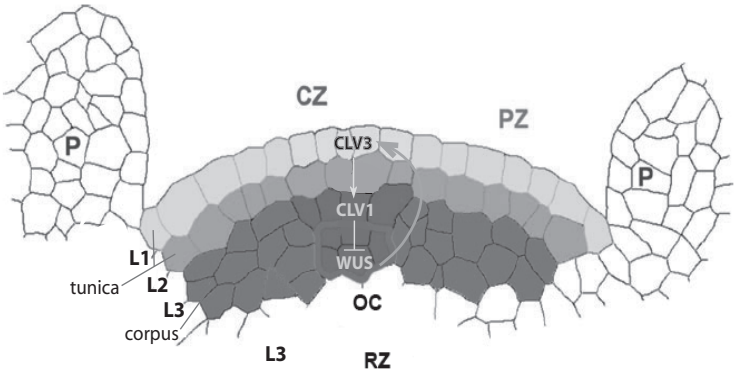

Figure 1.2 Structure of the shoot apical meristem (SAM) and the WUS-CLV loop.
The concentric L1-L3 cell layers, tunica and corpus areas are indicated by different shades of colour. The central zone (CZ) is marked in purple, the peripheral zone (PZ) is in green, and the position of the rib zone (RZ) is indicated below the meristem dome. Tunica and corpus are shown with their corresponding layers. P indicates organ primordia initiated on the flanks of the meristem. The region is circled in red makes up so-called organizing center (CZ) where cells express *WUSCHEL* (*WUS*). These cells produce a signal that promotes central zone identity and expression of *CLAVATA3* (*CLV3*) in the upper layer (tunica) cells of the CZ, where stem cells are positioned. The CLV3 signal peptide migrates to the underlying domain (corpus), activating the CLV1 receptor complex, resulting in the restriction of *WUS* expression. This establishes a feedback loop that maintains the size of the CZ (for a detailed description see the text). Arrows indicate positive regulation and line ending in a bar indicates negative regulation (adapted from Sablowski, 2004).

nally recognized as rib meristem (RM) due to their distinct morphogenetic duties. These cells are thought to produce vacuolated and elongated cells to load into the RZ; however, it is difficult to precisely delineate the dividing line between the RM and RZ. The divisions of SAM organization, that is, the longitudinal (L1-L3 layers) and radial (CZ, PZ and RZ) structures, overlap in such a way that the PZ and CZ each contain cells from both the tunica and corpus.

Organogenesis

One of the unique features of plant development is continuous organogenesis throughout its lifetime. To allow continuous shoot development, the SAM must maintain its size and organization whilst constituent cells divide and lateral organs are produced on its flanks. Therefore, a critical balance between the incorporation of stem cell daughters into developing primordia and proliferation of the central stem cell population is required for proper SAM function (Laufs et al., 1998).

In the SAM, cells leaving the CZ towards the PZ first lose stem-cell identity. Prior to visible histological characteristics, at a certain point cells cease to express meristem marker genes, marking the initial site of the lateral primordium. These cells give rise to primordium founder cells and initiate expression of genes that are required for the establishment of the primordium and its outgrowth. The upward growing primordium becomes histologically distinguishable due to the emergence of a cleft between the primordium and the SAM. During vegetative phase, the emerging primordium develops into a leaf and in its axil, an AM can be formed.

The transition of the SAM from a vegetative to an inflorescence meristem is accompanied by a dramatic increase in cell proliferation over the entire meristem, including the CZ where cells divided slowly before (reviewed in Blazquez et al., 2006). This causes both expansion of the SAM and a change in shape. The SAM often becomes flatter. As a result, the cytohistological zonation of the SAM becomes less distinct. Following the floral transition, the fate of the lateral primordia must be reprogrammed to acquire the identity of generative organs. The primordia can be floral meristems (FM), or leaf primordia that develop into leaves or bracts, in the axils of which either determinate FMs or indeterminate lateral inflorescence meristems will develop. In Arabidopsis, each FM develops into a single flower by specifying the floral organs which will form in concentric whorls, with four sepals in the outermost first whorl, followed by four petals, six stamens, and finally a central whorl of two fused carpels. Studies on flower development led to the proposal of the broadly accepted ABC model and recently, a revised version of this model called floral quartet model has been proposed (reviewed in Robles and Pelaz, 2005; Theissen and Melzer, 2007). In mature Arabidopsis flowers, carpels grow into the gynoecium of a plant. They typically consist of an ovary, a filament style and an apical sticky stigma. An additional set of complex structures, the ovules, develop within the carpels. Ovules are the site of processes essential for fertilization, seed development and female gametophyte, which is also called the embryo sac. Immediately after the gynoecium has been fertilized by pollen, cells of the ovary keep dividing and elongating until the fruit reaches its final length and thickness. A mature ovary then becomes a fruit. The Arabidopsis fruit, the silique, internally has two cavities, separated by a septum. The external of a silique is made up of three major parts: the valves, the replum and the valve margins. The valves are the peripheral walls of the silique and are connected on both sides to the middle ridge, the replum which creates the skeleton for seeds to attach to the plant. At the valve/replum boundary, the valve margins can be recognized as a constriction in the boundaries which only contains a few rows of narrow cells due to its slower expansion. During fruit maturation, the valve margins become the dehiscence zone which allows detachment of the valve from the replum through cell-cell separation, an important process in fruit development

for seed dispersal (reviewed in Robles and Pelaz, 2005; Seymour et al., 2008; Girin et al., 2009).

Shoot branching

Arabidopsis plants produce lateral shoots (branches) from axillary meristems (AMs) in the axils of rosette and cauline leaves. As mentioned above, AMs originate from the leaf primordia that form at flanks of the SAM. Whereas the abaxial domain of these primordia correlates with the formation of a leaf, the adaxial primordial domain is competent to form a meristem. AMs are, thus, secondary meristems, but they have the growth potential similar to the primary SAM. Branches initiate from the AMs and grow out into secondary lateral shoots, reiterating the development of the primary shoot axis. This growth potential confers plants great flexibility in development, and enables them to adapt their morphology to the environment as required. Because of the great variation in the degree and the pattern of branching, shoot axillary meristems are major determinants of plant architecture. Studies on shoot branching mutants have revealed a complex network of genes that are involved in the control of the formation of the AMs and outgrowth of the axillary buds (reviewed in McSteen and Leyser, 2005; Schmitz and Theres, 2005; Busov et al., 2008; Ongaro and Leyser, 2008; Leyser, 2009).

Initiation and subsequent development of the axillary meristem varies greatly between species. AMs may remain dormant as an axillary bud, mostly due to an effect called apical dorminance, or they might resume development at a later time after being triggered by a permissible signal (Bennett and Leyser, 2006; Leyser, 2009). In Arabidopsis, the AMs do not produce branches from the axil of rosette leaves until after the floral transition, whereas the cauline leaf AMs develop simultaneously with the leaf itself to produce secondary inflorescences (also referred to as paraclades: lateral branches that end in an inflorescence that repeats the main inflorescence). However, the primary shoot remains dominant to other lateral shoots, being the tallest and the most robust axis of growth.

Organ shape

Like shoot branching, also the final shape and size of the lateral organs produced by the SAM (e.g. leaves, flowers, and fruits) contributes to a large extend to the final plant form.

Next to proper meristems, including SAM, AMs and FMs, plants also contain additional regions with meristematic activity in their lateral organs. It is has recently been proposed that these regions are named quasi-meristems (Girin et al., 2009). According to these authors a quasi-meristem corresponds to a zone of active cell proliferation within an organ, giving birth to specialized and determi-

nate tissues. Although partially differentiated, the cells of these meristems still have meristematic attributes, allowing prolonged proliferation (Girin et al., 2009). Contrary to proper meristems, all the cells of quasi-meristems will eventually differentiate.

The presence of such meristem activities outside the proper meristem regions provides plants with an extra level to elaborate its body plan. It is currently thought that plants may sculpt plant form by modulating the degree (i.e. duration) of meristematic properties of the quasi-meristems (Girin et al., 2009). In this view, a prolonged quasi-meristematic state allows for an organ to modulate growth and shape during this time.

Well-known examples of quasi-meristems are the marginal and plate meristems in dicotyledonous leaves involved in, respectively, initiation and outgrowth of the leaf blade (Donnelly et al., 1999) and the determinate meristematic zones at the leaf base in monocots that determine the final size of the leaf blade. More recently, it was shown that the formation of dissected (compound) leaves also relies on the partial meristematic activity of quasi-meristems (Champagne and Sinha, 2004; Hay and Tsiantis, 2006; Barkoulas et al., 2008).

Recently, the replum tissue in the Arabidopsis fruit has been identified as a quasi-meristem and it was shown that its determinate meristematic activity is important for development of the central tissues of the fruit (Alonso-Cantabrana et al., 2007). In addition, initiation and development of ovule primordia have been shown to arise from a meristematic zone with determinate activity (Brambilla et al., 2007). Interestingly, to some extent the rib meristem (RM), which drives stem growth, has also been considered as a quasi-meristem (Girin et al., 2009). Although the RM is often considered as part of the SAM, it was initially defined as an independent structure within the stem (Ruonala et al., 2008). Cells within the RM differentiate rapidly and only due to the continuous provision of new cells from the SAM the RM appears to have indeterminate activity.

Phyllotaxis

Organogenesis does not randomly occur on the flanks of the SAM; instead, new primordia are organized in highly stereotypic pattern at the meristem periphery, showing remarkable precision of their position. For example, leave primordia are initiated in characteristic patterns on the shoot, a biological phenomenon referred to as phyllotaxis. The phyllotactic pattern exhibited by the primordia can be reflected by vegetative AMs, as AMs arise from the axils of the leaves. Since not only the leaves, but also flowers and floral organs of a plant or a flower are arranged in the defined divergence angles, the term phyllotaxis is frequently used to refer to the regular arrangement of all these organs. Both physiological and genetic studies have provided evidence that the plant hormone auxin plays

an essential role in both primordium initiation and in phyllotaxis (Alabadi et al., 2008). Regular phyllotaxis is found in nearly all higher plants, form mosses, ferns to gymnosperms and angiosperms. Phyllotactic patterns can be distichous, spiral, decussate or whorled (reviewed in Steeves and Sussex, 1989; Reinhardt and Kuhlemeier, 2001). Spiral phyllotaxis is the most common and best-studied pattern, and is also found in Arabidopsis. In spiral phyllotaxis, two successive primordia are positioned with a constant divergence angle of approximately 137°. Such separation of leaf primordia results in an ontogenetic spiral running up the shoot with a single leaf at each node.

Molecular basis of SAM initiation and maintenance

SAM maintenance

As described above, continuous development of the shoot depends on continuity of SAM activity. In Arabidopsis the WUSCHEL (WUS)-CLAVATA (CLV) feedback pathway confers stem cell pool homeostasis in the SAM (reviewed in Williams and Fletcher, 2005). *WUS* belongs to the plant specific *WOX*-family (*WUS*-like homeobox) of homeobox transcription factor genes. *WUS* is expressed in a cluster of cells at the basis of the CZ that is defined as organizing center (OC). Here it functions in maintaining the integrity of the overlying stem cell pool (Laux et al., 1996; Mayer et al., 1998). Cells expressing *WUS* produce a yet unknown non-cell-autonomous signal that acts positively on the expression of the *CLAVATA3* (*CLV3*) stem cell marker gene (Mayer et al., 1998; Schoof et al., 2000; Brand et al., 2002; Lenhard and Laux, 2003). *CLV3* encodes a ligand peptide that, although expressed in the tunica layers of the CZ, diffuses to the L3 layer of the CZ where it binds and activates the putative CLV1/CLV2 transmembrane receptor kinase complex, which in turn restricts the *WUS* expression domain to the OC (Clark et al., 1997; Fletcher et al., 1999; Trotochaud et al., 2000). This establishes a negative-feedback loop that forms a self-correcting mechanism to stabilize the stem cell pool size of the SAM (reviewed in Williams and Fletcher, 2005; Reddy, 2008) (Figure 1.2). Concordantly, mutations in *WUS* result in premature SAM termination due to the loss of stem cell identity in the CZ (Laux et al., 1996). On the contrary, loss-of-function mutations in any of the *CLV* genes lead to an enlarged SAM accumulating excess cell in the CZ (Clark et al., 1993, 1995; Clark et al., 1996; Fletcher et al., 1999). In *clv* mutants, the SAM continues to enlarge throughout shoot development, resulting in distorted patterns at organ formation, e.g. the formation of supernumerous floral organs (Clark et al., 1993; Fletcher et al., 1999). Confocal microscopy showed that the increase in meristem size of *clv3* is due to the reduced rate of recruitment of cells from the CZ to the PZ, indicating that CLV proteins actually promote cell differentiation (Laufs et al., 1998). Accordingly,

in *clv1* and *clv3* mutants, the enlargement of the CZ is accompanied by a great expansion of the *WUS* expression domain. Therefore, WUS and CLV essentially function to promote and restrict stem cell fate in the CZ, respectively. WUS expression is first initiated at early globular embryo stage in a subset of cells of the apical embryo and is required for *CLV3* expression in embryonic shoot meristem formation, while proper spatial *WUS* expression in turn requires CLV3 activity from the heart stage on, implying that this feedback loop is already functioning at this stage to regulate stem cell homeostasis (Mayer et al., 1998; Schoof et al., 2000).

In parallel to the WUS-CLV feedback loop, proper SAM function in Arabidopsis also requires the activity of the *SHOOTMERISTEMLESS* (*STM*) gene (Barton and Poethig, 1993; Long et al., 1996). Plants homozygous for strong loss-of-function *stm* mutations fail to establish and maintain a functional SAM, often do not undergo any postembryonic development, and show a severe fusion of the cotyledonary petioles (Barton and Poethig, 1993; Endrizzi et al., 1996; Long et al., 1996). Less severe mutants, however, do form a SAM that initiates primordia shortly after germination. Nevertheless, these meristems eventually terminate in ectopic central organs as a result of the complete incorporation of meristem cells in differentiating primordia. This implies that *STM* is not only essential for initial shoot meristem formation, but also for the subsequent maintenance of the SAM (Clark et al., 1996; Endrizzi et al., 1996). In addition, lack of *STM* results in fusions of cotyledons and other tissues, suggesting that *STM* expression in the PZ helps to define organ boundaries (Barton and Poethig, 1993).

STM encodes a transcription factor of the KNOTTED1-like homeobox (KNOX) class. Expression of *STM* is first activated at the late globular stage of embryogenesis in the presumptive SAM (Long and Barton, 1998). Post-embryonically, *STM* is expressed throughout meristems, including the CZ and PZ in the vegetative SAM, the IM and FMs. Notably, *STM* expression is excluded from the founder cells of the incipient organ primordia (Long et al., 1996; Long and Barton, 1998; Long and Barton, 2000). *STM* plays a significant role in the formation and maintenance of the SAM by negatively regulating organ formation genes in the shoot apex, thus suppressing cell differentiation (Long et al., 1996; Byrne et al., 2000).

Both *WUS* and *STM* are needed for indeterminate SAM activity. But what is the relationship between *WUS/CLV* pathway and *STM* in SAM development? Both *stm* and *wus* mutants lack meristematic potential in the SAM, leading to development arrest. However, their loss-of-stem-cell phenotypes are quite different. In *stm* mutants, shoot termination is caused by differentiation of meristematic cells into developing organs accompanied with the failure of stem cell maintenance. As a result, the cells of the SAM become vacuolated and cease to divide (Endrizzi et al., 1996; Byrne et al., 2000). The *wus* mutants, on the other hand, lack

stem cell identity altogether, yet do retain some disorganised, histologically distinct cells at the presumptive SAM position (Laux et al., 1996). Despite both *wus* and *stm* mutants lacking a functional SAM, both are capable of activating some kind of escape mechanisms characterized by the stunted vegetative and partial generative plant development (Barton and Poethig, 1993; Clark et al., 1996; Laux et al., 1996). The *wus* mutants reiteratively develop adventitious shoots from the presumptive SAM location, giving an abort-retry pattern of growth, whereas the *stm* phenotype arises from the formation of short-lived, transient meristems from or near petioles of cotyledons and leaves (Barton and Poethig, 1993; Clark et al., 1996; Laux et al., 1996).

These phenotypic characteristics along with genetic data resulted in a parallel model for *STM* and *WUS* in meristem function (Lenhard et al., 2002). While their onset during embryogenesis appears unlinked, *STM* and *WUS* control each others expression in a complex non-linear fashion throughout post-embryonic development (Clark et al., 1996; Endrizzi et al., 1996; Long and Barton, 1998; Mayer et al., 1998; Brand et al., 2002; Lenhard et al., 2002). In addition, *STM* expression appears to encompass the enlarged SAM of *clv* mutants, indicating that cross-talk occurs between the *WUS/CLV* loop and *STM*. However, WUS and STM also operate in parallel as they control different subsets of target genes. The main target of *WUS* is *CLV3*, which accomplishes stem cell identity in the CZ, while *STM* induces local expression of two *STM* homologs to suppress differentiation throughout the meristem and the mitotic cyclin *CycB1;1* to induce cell proliferation of the transit amplifying stem-cell daughters in the PZ (Lenhard et al., 2002).

SAM initiation

The earliest indication of SAM formation during embryogenesis is the onset of WUS expression in the four subepidermal cells of the apical domain of the 16-cell embryo (Schoof et al., 2000). Subsequently, this expression domain is confined to the presumptive OC position in the shoot meristem primordium through asymmetric cell divisions. At later stages, WUS function is required for CLV3 expression (Brand et al., 2002). This suggests that the first event in embryonic shoot meristem formation is the generation of a cell lineage that will give rise to the inductive niche cells, which in turn induce the overlying cells as stem cells. The partitioning of the embryo apex starts during the globular stage when the embryo is divided into the peripheral cotyledonary primordia and the central shoot meristem primordium. This process requires the successive activation of a set of putative NAC-domain transcription factor genes, CUP-SHAPED COTYLEDON1 (CUC1), CUC2, and CUC3 involved in repressing the outgrowth of the meristem region (Aida et al., 1997; Aida et al., 1999; Vroemen et al., 2003). In Arabidopsis, cuc1 cuc2 double mutants germinate with fused cotyledons, giving rise to a cup-

shape, and without any SAM at all (Aida et al., 1997). CUC1 and CUC2 expression is initially switched on in a few apical cells of the globular embryo only, but soon spreads in a stripe separating the two incipient cotyledon primordia (Aida et al., 1999; Takada et al., 2001). The spatial information for the expression of the CUC genes appears to be provided by the distribution of the growth regulator auxin, since CUC expression is affected in mutants where directional auxin transport is disrupted (Aida et al., 1999; Takada et al., 2001). CUC1 and CUC2 in turn activate STM expression, leading to the repression of outgrowth in the region between the two cotyledon primordia (Aida et al., 1999; Takada et al., 2001). On the other hand, as embryogenesis proceeds, STM in turn promotes CUC1 activity and is further required for defining the CUC2 expression domain (Aida et al., 1999; Aida et al., 2002). CUC2 and STM expression eventually resolve into complementary patterns at heart-stage, with STM expression being restricted to the centre of the incipient SAM and CUC2 to the boundaries between the cotyledons and the SAM (Aida et al., 1999). By the late globular stage the stem-cell niche is established and the embryo apex partitioned. During the following stages of embryogenesis, this information is translated into morphological structures: the cotyledonary primordia grow out and the shoot meristem structure becomes evident.

Initiation of lateral organs and axillary meristems

As mentioned above, organ formation takes place in the PZ of the SAM where a group of 15-30 cells derived from all three meristem layers becomes assigned to an incipient organ primordium (Irish and Sussex, 1990; Furner et al., 1996). One of the first signs of organ initiation is the down-regulation of *STM* expression in the organ founder cells, while *STM* stays expressed in the rest of the SAM (Long et al., 1996). This presumably allows the onset of expression in these cells of the MYB-like transcription factor gene *ASYMMETRIC LEAVES1* (*AS1*) and the AP2-like transcription factor gene *AINTEGUMENTA* (*ANT*), both of which are required for the establishment of the organ primordium and its outgrowth (Elliott et al., 1996; Byrne et al., 2000; Long and Barton, 2000). *AS1* in its turn negatively regulates the expression of two *STM* homologues, *BREVIPEDICELLUS* (*BP* a.k.a. *KNAT1* for *KNOTTED1-LIKE ARABIDOPSIS THALIANA1*) and *KNAT2* (Byrne et al., 2000). *AS2*, a member of the plant-specific *LATERAL ORGAN BOUNDARIES* domain (*LBD*) family, can physically and genetically interact with *AS1* and has been shown to function in the same pathway to promote leaf outgrowth via direct repression of *KNOX* gene expression (Byrne et al., 2002; Lin et al., 2003; Xu et al., 2003; Guo et al., 2008). The antagonistic role between *STM* and *AS1* provides a mode to balance SAM activity and to separate SAM from organ primordium development (Figure 1.3). Notably, down-regulation of

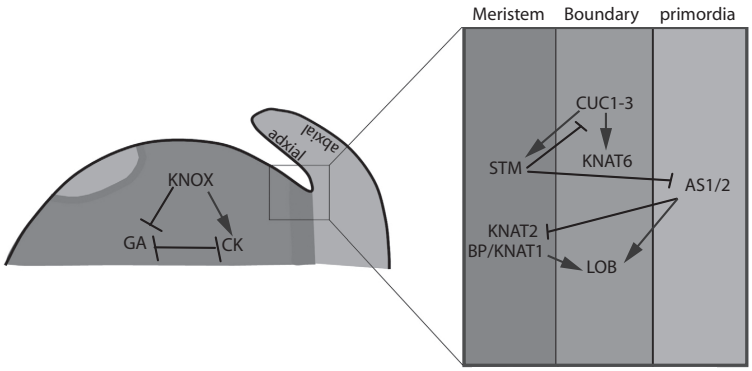

Figure 1.3 The role of *KNOX* genes in maintaining the SAM.
Summary of the genetic interactions between *KNOX* and *AS1/2* are shown in the inset (right panel) which is the region indicated in the pane (left panel). *STM* expression in the meristem (red), results in the inhibition of *AS1* expression, thereby allowing the expression of *BP/KNAT1* and *KNAT2*. On the other hand, *AS* expression at the leaf initiation sites (green) downregulates *STM* expression, to promote the formation of the leaf primordia and probably, to help the separation SAM from leaf development. During embryogenesis, CUC activity is required for the activation of *STM* in the SAM, whereas STM, in turn, restrict *CUC* expression in the boundaries between the SAM and the cotyledons. CUC also positively regulate *KNAT6* expression which is expressed at the SAM boundaries. *LOB* is expressed in the boundary region (purple) and can be induced by AS1/2 complex and BP/KNAT1 (for a detailed description see the text). Arrows indicate positive regulation and line ending in a bar indicates negative regulation. (adapted from Rast and Simon, 2008).
KNOX proteins functions to control hormonal balance in the SAM (left panel). KNOX proteins repress GA biosynthesis (GA 20-oxidase) and induce cytokinin (CK) production in the corpus of the SAM. GA and CK show antagonistic role in the maintenance of SAM activity (for a detailed description see the text). (adapted from Rast and Simon, 2008).

STM in primordia seems essential for lateral organ formation throughout plant development, since even in SAM-derived floral meristems *STM* expression is temporarily lost during the initiation process (Long and Barton, 2000).

Both primordia formed during the vegetative and generative growth phase initially execute a common developmental program (reviewed in Grbic, 2005). First, after established, the primordium grows outward, but remains contained within the SAM. Next, upward growth creates a cleft between the primordium and the SAM, and expression of genes that specify adaxial versus abaxial primordia (Figure 1.3) polarity becomes restricted to their corresponding primordia domains, thus dividing the primordia into two domains with distinct fates. The adaxial domain is competent to give rise to an axillary meristem, while the abaxial domain correlates with organ primordium formation. During the vegetative stage the organ primordium develops as a leaf, whereas in the adaxial domain, at the base of the developing leaf, a group of cells regain *STM* expression (Grbic and

Bleecker, 2000; Long and Barton, 2000). This group of *STM*-expressing cells then enlarges to become a morphologically visible AM in the axil of the leaf (Grbic and Bleecker, 2000; Long and Barton, 2000). After floral transition, the reproductive primordia develop as floral meristems. The primordium is also partitioned into a *STM*-expressing adaxial domain and a *STM*-negative abaxial domain. This lack of *STM* expression in the latter is reminiscent of leaf development, however, further leaf development is suppressed by, among others, the floral promoter LEAFY (LFY) (reviewed in Grbic, 2005).

Plant architecture: A TALE of two families

The KNOX-subfamily of plant TALE Homeodomain Proteins

As is clear, the KNOX homeodomain (HD) transcription factor protein STM plays essential roles in the regulation of key aspects of plant architecture.

The Arabidopsis genome contains in total eight *KNOX* genes, including *STM*. Based on phylogeny and expression patterns, *STM*, together with *BP/KNAT1, KNAT2* and *KNAT6*, belongs to the class I of *KNOX* genes, whereas *KNAT3*, *KNAT4*, *KNAT5* and *KNAT7* make up class II (Kerstetter et al., 1994; Reiser et al., 2000). Both classes have also been found in other dicots and monocots, as well as in gymnosperms, ferns and bryophytes. In algae there seems to be only one class of *KNOX* genes, displaying features of both class I and class II *KNOX* genes from higher plants (reviewed in Hake et al., 2004; Scofield and Murray, 2006).

Class I *KNOX* genes are typically expressed in the SAM, as well as in AMs and FMs, but excluded from initiating leaf primordia (reviewed in Hake et al., 2004; Scofield and Murray, 2006). In striking contrast, class II genes are more broadly expressed in plants (Serikawa et al., 1997; Truernit et al., 2006; Zhong et al., 2008). However, despite a few indicative roles of some class II *KNOX* genes in root and xylem development, their function remains enigmatic since their single mutants and overexpressors show wild-type phenotypes (Serikawa and Zambryski, 1997; Brown et al., 2005; Truernit et al., 2006).

A unique member of the KNOX family, lacking the HD, KNATM, has been recently identified. Strictly speaking, it is not a KNOX HD protein and it is thus characterized as an M subclass KNOX protein. The M subclass is conserved in dicotyledons (Kimura et al., 2008; Magnani and Hake, 2008).

KNOX proteins belong to the highly conserved class of three-amino-acid-loop-extension (TALE) homeodomain (HD) transcription factor proteins (Burglin, 1997). TALE HD proteins are characterized by the presence of three addi-

tional amino acid residues (PYP) in the loop between the first two helices of the helix-loop-helix-turn-helix structure that makes up the DNA-binding HD, when compared to the so-called typical HD proteins (Bertolino et al., 1995; Burglin, 1997).

KNOX genes are ancient (Burglin, 1998; Champagne and Ashton, 2001). Apart from the TALE HD domain, KNOX proteins contain different series of conserved domains (Figure 1.4). The ELK domain is the closest motif upstream of the HD. Next to it, towards the N-terminus of the protein, is the GSE domain which has been implicated in the regulation of protein stability (Nagasaki et al., 2001). A fourth conserved domain, the highly conserved MEINOX domain which consists of the KNOX1 and KNOX2 subdomains, separated by a flexible spacer (Burglin, 1997), is shared with the metazoan myeloid ecotropic integration site (MEIS) class of TALE HD proteins, where it is called MEIS domain (Bürglin, 1995; Burglin, 1997). MEIS class proteins include Drosophila Homothorax (Hth), and vertebrate Meis and Prep HD proteins. High sequence similarity between the MEIS and KNOX domains suggests that they have evolved from a common ancestral MEINOX domain (Burglin, 1997). In MEIS proteins, the MEIS domain has been shown to mediate interactions with a second group of TALE HD proteins, the pre-B cell (PBC) class proteins, that comprises Drosophila Extradenticle (Exd) and vertebrate Pbx proteins (Kamps et al., 1990; Nourse et al., 1990; Peifer and Wieschaus, 1990; Rauskolb et al., 1993). PBC class proteins also share an extra conserved domain in addition to the TALE HD, referred to as the bipartite PBC-A/B domain (Burglin and Ruvkun, 1992; Rauskolb et al., 1993; Burglin, 1997, 1998). PBC-MEIS interactions are mediated via their respective lineage-specific domains, the PBC-A and MEIS domains (Chang et al., 1997; Knoepfler et al., 1997; Rieckhof et al., 1997; Berthelsen et al., 1998). In most cases, PBC-MEIS heterodimerization is mandatory for nuclear translocation, functionality and stability of these proteins (Rieckhof et al., 1997; Abu-Shaar et al., 1999; Berthelsen et al., 1999; Mercader et al., 1999; Jaw et al., 2000; Longobardi and Blasi, 2003; Stevens and Mann, 2007).

Consistently, solitary expressed PBC and MEIS proteins reside in the cytoplasm and show reduced stability. In case of MEIS proteins this can be explained by the lack of a nuclear localization signal (NLS) (Abu-Shaar and Mann, 1998; Kurant et al., 1998; Haller et al., 2004). PBC monomers are actively excluded from the nucleus despite the presence of a conserved NLS in their HD. This exclusion mechanism involves the nuclear export receptor CRM1/exportin-1 (Fornerod et al., 1997; Fukuda et al., 1997; Kudo et al., 1997; Rieckhof et al., 1997; Kurant et al., 1998). In the MEIS-interacting PBC-A domain a set of conserved short leucine-rich stretches is recognized as nuclear export signals (NES) by the Importin β-family transport receptor CRM1 (Berthelsen et al., 1999; Kilstrup-Nielsen et al., 2003). Since both MEIS and CRM1 are able to interact with the PBC-A domain, it is postulated that by dimerization with MEIS proteins, PBC NES sequences are masked,

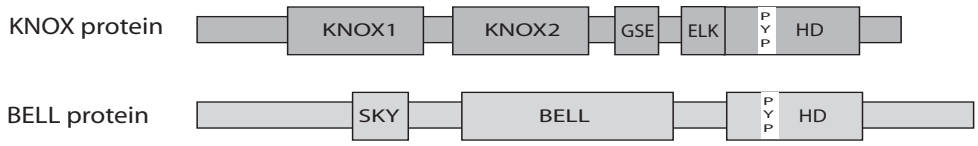

Figure 1.4 Plant TALE homeodomain proteins
KNOX and BELL TALE homeodomain proteins both contain a conserved homeodomain (HD) characterized by three additional amino acid residues proline (P)-tyrosine (Y)-proline (P) (shown in the narrow strip) in the loop between the first two helices of the three alpha–helices. KNOX members contain a few other conserved domains, the MEINOX domain (KNOX1 and KNOX2), an ELK and a GSE domain. BELL proteins harbor additional BELL and SKY domains.

thereby precluding recognition by CRM1 (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Kilstrup-Nielsen et al., 2003). As a consequence, PBC-MEIS heterodimers migrate to the nucleus, driven by the NLS present in the PBC-moiety.

The BELL -subfamily of plant TALE Homeodomain Proteins

Plants also contain a second group of TALE HD proteins named the BEL1-like (BELL) class (Reiser et al., 1995; Burglin, 1997). In addition to the TALE HD, BELL proteins share two adjacent N-terminal domains, designated SKY and BELL domains (Bellaoui et al., 2001) (Figure 1.4). Previously, it has been suggested that the animal PBCA/B domain finds its counterpart in the plant BELL domain (Bellaoui et al., 2001; Cole et al., 2006). However, unambiguous evidence for evolutionary conservation of PBC and BELL proteins is lacking (Becker et al., 2002).

The Arabidopsis genome contains thirteen BELL family members. Apart from *ARABIDOPSIS THALIANA HOMEOBOX1* (*ATH1*) and *BELL1* (*BEL1*), founding member and nomenclator of the family, respectively, BELL-family members have originally been systematically designated *BEL1-LIKE HOMEODOMAIN1* (*BLH1*) to *BLH11* (Modrusan et al., 1994; Quaedvlieg et al., 1995; Roeder et al., 2003; Smith et al., 2004). Later, *BLH2* and *BLH4* have been renamed *SAWTOOTH1* (*SAW1*) and *SAW2*, respectively, based on the *saw1 saw2* double mutant loss-of-function leaf phenotype (Kumar et al., 2007). *BLH8* is better known as *POUND-FOOLISH* (*PNF*), whereas its close paralog *BLH9*, due to the pleiotropic character of its loss-of-function phenotype, has been given various different names, including *PENNYWISE* (*PNY*), *BELLRINGER* (*BLR*), *REPLUMLESS* (*RPL*), *LARSON* (*LSN*) and *VAAMANA* (*VAA*) (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bao et al., 2004; Bhatt et al., 2004; Smith et al., 2004; Kanrar et al., 2008). For the sake of clarity, this gene will be referred to as *PNY* in the remainder of this document.

Like class II *KNOX* genes, *BELL* genes are more broadly expressed in plants, both in meristems and in more differentiated tissues (Reiser et al., 1995; Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Cole et al., 2006; Kumar et al., 2007; Proveniers, 2007; Yu et al., 2009).

Regulation of BELL and KNOX function by protein heterodimerization

Like PBC and MEIS proteins, BELL proteins have been shown to heterodimerize both *in vitro* and *in vivo* with KNOX proteins in a DNA-independent manner in both Arabidopsis and other plants, such as potato (*Solanum tuberosum*) and barley (*Hordeum vulgare*) (Bellaoui et al., 2001; Muller et al., 2001; Byrne et al., 2003; Chen et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Chen et al., 2004; Hackbusch et al., 2005; Cole et al., 2006). Although the combined SKY and BELL domains have been referred to as one MEINOX-interacting domain, single BELL domains were shown to be sufficient for heterodimerization with KNOX proteins (Bellaoui et al., 2001; Muller et al., 2001; Cole et al., 2006). Moreover, several BELL class members have recently been shown to translocate the KNOX protein STM, that lacks an efficient NLS, to the nucleus upon formation of a BELL-STM heterodimer (Cole et al., 2006). These authors further demonstrated that the conserved BELL domain is sufficient for interaction with STM, but not for nuclear translocation of STM protein (Cole et al., 2006), analogous to what has been reported for PBC-A domain–MEIS interactions (Kilstrup-Nielsen et al., 2003). Since BELL-KNOX heterodimerization has also been shown to be required for site-specific DNA binding (Smith et al., 2002), these interactions are probably required for their biological functions. This is further supported by the recent observation that ectopic activity of *BLH1* can be suppressed by additional introduction of a mutation in the gene encoding the BLH1-interacting KNOX protein KNAT3 (Pagnussat et al., 2007).

There is accumulating evidence showing that BELL-KNOX interactions are selective, meaning that each BELL protein mediates a subset of KNOX proteins and vice versa. In an *in vitro* DNA-binding assay, the maize BELL protein, KNOTTED1 INTERACTING PROTEIN (KIP) was found to bind KN1 with higher affinity than to other KNOX proteins (Smith et al., 2002). In Arabidopsis, BEL1 may also selectively interact with only few KNOX proteins (Bellaoui et al., 2001). Such selective interaction pairs may contribute to distinct plant developmental pathways or contribute to fine-tuning of the same processes. Due to the differential expression of TALE HD proteins in space and time, selectivity apparently can also be achieved at another level, reliant on overlapping expression patterns of the two interacting moieties. PNY, for example, can physically interact with both STM and BP/KNAT1 *in vitro* (Cole et al., 2006). In the vegetative SAM and the

IM, as well as in FMs the *PNY* expression domain overlaps with the *STM* expression domain. *BP/KNAT1* and *PNY* expression, on the other hand, only overlap in the SAM and IM. This suggests that both PNY-STM dimers and PNY-BP/KNAT1 dimers can be formed in these tissues, whereas in FMs only PNY-STM heterodimers might be present (Byrne et al., 2003; Smith and Hake, 2003).

Whereas KNOX-BELL interaction leads to nuclear localization of the transcription factor heterodimeric complex, interaction of BELL and KNOX proteins with members of the recently discovered plant-specific OVATE protein family (At OFP) can result in the relocalization of KNOX-BELL heterodimers from the nucleus to the cytoplasm (Hackbusch et al., 2005). In this way, OVATE proteins might be part of a regulatory mechanism that controls BELL-KNOX activity during plant development.

TALE homeodomain functions in the SAM

Studies on the biological functions of plant TALE HD proteins indicate that these proteins play essential roles in the regulation of various aspects of plant architecture, particularly through regulation of meristem activity. As discussed above, the KNOX class protein STM is essential for both SAM initiation and maintenance (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996; Long et al., 1996). A primary function of STM in meristem maintenance is regulation of cell division rates in the PZ and suppression of cell differentiation in the CZ as well as in the PZ (Long et al., 1996; Long and Barton, 2000; Byrne et al., 2002; Lenhard et al., 2002). Suppression of cell differentiation is mainly established by preventing the expression of *AS1* in the meristem and involves complex interactions within a network of the other class I KNOX homologues (Byrne et al., 2002). Two of these, BP/KNAT1 and KNAT6 have been shown to act redundantly with STM in meristem maintenance (Byrne et al., 2002; Belles-Boix et al., 2006). However, mutations in these homologues do not normally affect shoot meristem development or function, suggesting that STM provides the critical KNOX function for SAM development (Byrne et al., 2002; Douglas et al., 2002; Venglat et al., 2002; Dean et al., 2004; Belles-Boix et al., 2006).

Although all four class I *KNOX* genes are expressed in the SAM, their expression patterns are not identical. Whereas STM is expressed throughout the SAM in both CZ and PZ, the *BP/KNAT1* transcript is localized primarily in the PZ (Lincoln et al., 1994; Long et al., 1996; Long and Barton, 1998; Long and Barton, 2000; Ori et al., 2000; Douglas et al., 2002). *KNAT2* expression is confined to the L3 layer and the RZ of the SAM, while expression of its closest relative, *KNAT6*, is even more restricted to the boundaries between the SAM and developing primordia (Dockx et al., 1995; Pautot et al., 2001; Byrne et al., 2002; Hamant et al., 2002; Belles-Boix et al., 2006). As discussed above, biochemical and genetics studies

have shown that KNOX proteins associate with BELL proteins to form functional complexes regulating plant development.

Loss-of-function mutations in the *BELL* gene *PNY* have been shown to enhance SAM maintenance defects in weak *stm* alleles (Byrne et al., 2003; Bhatt et al., 2004). *pny* single mutants result in phyllotaxy defects that include both an increase in the number of lateral organs and displacement of organs along the stem, indicating that PNY might normally function to delay stem cell differentiation in the CZ and/or to control the specification of PZ cells as organ founder cells. In both cases, the presumed function of PNY is consistent with the PNY expression pattern in the SAM, that is very similar to that of *STM* (Byrne et al., 2003). As STM lacks a functional NLS and PNY has the capacity to target STM to the nucleus after formation of a PNY-STM heterodimer (Cole et al., 2006). PNY may be required for STM activity in the process of SAM maintenance. If true, this requirement for PNY seems to be only partial, since there is no noticeable difference in the size or organization of *pny* and wild-type SAMs (Byrne et al., 2003). Genetic redundancy between *BELL* genes might be a possible explanation. Indeed, mutants lacking both PNY and its close paralog PNF, one of several other BELL proteins capable of binding to STM, frequently display SAM maintenance defects during vegetative development, whereas no apparent aberrant phenotype was observed in *pnf* single mutants (Smith et al., 2004). Moreover, two additional BELL proteins, ATH1 and BLH3, both of which are expressed in the SAM (Cole et al., 2006), also possess the capacity to both interact with and to translocate STM to the nucleus (Cole et al., 2006).

In addition to STM, PNY can also physically interact with BP/KNAT1 (Smith and Hake, 2003; Bhatt et al., 2004; Hackbusch et al., 2005; Kanrar et al., 2006). Interestingly, PNY, like BP/KNAT1 is required for maintenance of stem cell fate in the absence of both STM and AS1 (Byrne et al., 2003).

Taken together, this suggests that several different BELL-class I KNOX heterodimer combinations might be active in the SAM. As can be inferred from over-expression experiments in transgenic Arabidopsis plants (Cole et al., 2006), these different BELL-KNOX complexes most likely contribute to different functions within the SAM.

TALEs in Leaf development

Intriguingly, the development of compound leaves shares molecular components with shoot development. Whereas in simple-leafed plants such as Arabidopsis, class I *KNOX* genes are expressed only in the SAM and become excluded from leaf primordia, in many compound leaf species they are expressed in both SAM and leaf primordia. In the latter species, after initial down-regulation in the incipient primordia, class I *KNOX* gene expression is reestablished in developing

primordia later on. Moreover, in compound leaf species a high correlation exists between leaf shape and class I *KNOX* expression patterns in leaf primordia (Bharathan et al., 2002). Since leaflets of compound leaves arise in a manner resembling the initiation of leaves from the SAM, this has led to the proposal that compound leaves represent a reinitiation of the ancestral shoot identity of leaves (reviewed in Uchida et al., 2009). In full support of this idea is the more recent finding that in the compound leaf species *Cardamine hirsuta* class I KNOX proteins promote compound leaf formation by prolonging the duration of cell division in specific regions of the leaf primordia, thereby delaying cellular differentiation (Hay and Tsiantis, 2006). Furthermore, in various simple-leafed species, class I *KNOX* genes, when ectopically expressed, can also cause dramatic changes in leaf morphology that are reminiscent of compound leaf development (reviewed in Bharathan et al., 2002; Hake et al., 2004), indicating that higher plants employ class I *KNOX* genes not only for essential functions for growth such as SAM formation and maintenance, but also for generating morphological changes in leaves. From this perspective it is worthwhile mentioning that plants have evolved several mechanisms that enable them to control class I KNOX function exclusively for modifying leaf shape without affecting essential SAM function, one of them involving KNATM-type proteins (Kimura et al., 2008). In tomato, a *KNATM* ortholog, the *PETROSELINUM* (*PTS*) gene, was found to be responsible for the natural variation in leaf shape between the Galapagean tomatoes *S. cheesmaniae* and *S. galapagense* (Kimura et al., 2008). *S. cheesmaniae* has compound leaves resembling cultivated tomato, whereas *S. galapagense* displays the so-called *pts* phenotype that resembles parsley leaf morphology and is characterized by increased primary and secondary leaflet production, and elevated leaf marginal serration. This natural variation arises from a single-nucleotide deletion in the PTS promoter, resulting in up-regulation of the gene product in leaves, but not in the SAM. PTS was further found to interact with the tomato BELL protein BIPINNATA (BIP), thereby repressing the interaction between LeT6, the tomato STM ortholog, and BIP, as well as subsequent nuclear localization of the LeT6-BIP transcriptional complex. Loss-of-function mutations in *BIP* cause highly compound leaves which recapitulate the *pts* phenotype. Thus, in the case of the natural variation in leaf shape between Galapagean tomatoes, leaf complexity is affected by the dosage of PTS within a protein-protein interaction network that involves at least PTS, LeT6 and BIP. Via leaf-specific regulation of the PTS dosage this modulation of the interaction network by PTS is achieved only in leaves without affecting the expression level of PTS in the SAM (Kimura et al., 2008).

In Arabidopsis, KNATM might similarly control TALE HD function by titrating the dose of BELL-KNOX heterodimers. Plants overexpressing *KNATM* display leaves with increased leaf serrations (Magnani and Hake, 2008). Similar phenotypes had previously been observed in plants lacking the two Arabidopsis

BIP orthologs BLH2/SAW1 and BLH4/SAW2 (Kumar et al., 2007). Whereas none of the single mutants displays a visible phenotype in development, *saw1 saw2* double mutants produce enhanced leaf serrations. Plants overexpressing both *BLH2/SAW1* and *KNATM* fully rescue both single overexpression phenotypes, implying that KNATM and BLH2/SAW1 have antagonistic functions in leaf margin development (Magnani and Hake, 2008). Since KNATM can also physically interact with BLH2/SAW1 and BLH4/SAW2, and both BLH2/SAW1 and BLH4/SAW2 have been shown to interact with a subset of KNOX proteins (Hackbusch et al., 2005; Kumar et al., 2007; Magnani and Hake, 2008), one might thus hypothesize that, in analogy to PTS, KNATM acts to control SAW-KNOX dosage, hence leaf lamina shape, by titrating SAW proteins into an inactive KNATM-SAW dimer. Similar to the tomato situation, this might disrupt functional SAW-KNOX complex(es). Alternatively, titration of BLH2/SAW1 and/or BLH4/SAW2 might allow for the formation of other functional BELL-KNOX interaction pairs. Indications for the latter come from the observation that there is a synergistic interaction between KNATM and BP/KNAT1 (Magnani and Hake, 2008), whereas BLH2/SAW1 and BLH4/SAW2 have an antagonistic relationship with BP/KNAT1. The latter is based on the finding that the SAW proteins establish leaf shape by repressing growth in specific subdomains of the leaf at least in part by repressing *BP/KNAT1* expression (Kumar et al., 2007). Accordingly, BLH2/SAW1 and BLH4/SAW2 on the one hand and BP/KNAT1 on the other hand are mostly expressed in mutually exclusive domains. However, both BLH2/SAW1 and BLH4/SAW2 were also found to heterodimerize with BP/KNAT1 (Kumar et al., 2007). As KNOX proteins, including BP/KNAT1, have been shown to be able to move over both short distances across cell layers, as well as to be transported over longer ranges via the phloem (Lucas et al., 1995; Kim et al., 2002; 2003; Ruiz-Medrano et al., 2004; Zambryski, 2004; Gallagher and Benfey, 2005; Kim et al., 2005), binding of SAW proteins to BP/KNAT1 could serve as an extra level of control to prevent the formation of additional, functional BP/KNAT1-BELL heterodimers within the *BLH2/SAW1* and *BLH4/SAW2* expression domains. Disruption of such SAW- BP/KNAT1 interactions by KNATM would account for the synergistic effects of KNATM and BP/KNAT1 that are observed in plants overexpressing these two proteins (Magnani and Hake, 2008).

As *KNATM* is not only expressed in leaves and KNATM protein has also been shown to interact with BELL proteins other than BLH2/SAW1 and BLH4/SAW2 (Magnani and Hake, 2008), KNATM might have a broader role in plant development by sequestering additional BELL and/or KNOX proteins.

Inflorescence development

In Arabidopsis, inflorescence growth is marked by internode development, flower specification and axillary branch development. The inflorescence stem constitutes the major part of the shoot and thus contributes significantly to the overall shoot architecture. Studies of the genetic control of inflorescence stem development have mainly focused on internode elongation and have resulted in the identification of various genes, including BP/KNAT1. The *bp* mutant is a classical mutant of Arabidopsis and presents a unique architectural phenotype (Koorneef et al., 1983). Mutations in *BP/KNAT1* result in short plants with compact internodes, bends at the nodes, and short, downward-pointing pedicels. Due to these defects the *bp* inflorescence is transformed to a distinctive structure bearing clusters of flowers which point downwards (Douglas et al., 2002; Venglat et al., 2002). The semi-dwarfed *bp* phenotype is caused by decreased cell division and cell expansion. The pedicel growth and angle defects are due to aberrant differentiation, growth and elongation of epidermal and cortical cells specifically in the abaxial side of pedicels (Douglas et al., 2002; Venglat et al., 2002). Corresponding to its role in inflorescence patterning, during floral transition *BP/KNAT1* expression is down-regulated in the SAM and becomes restricted to the cortex of the inflorescence stem and pedicels (Lincoln et al., 1994; Douglas et al., 2002; Smith and Hake, 2003). One of the processes regulated by BP/KNAT1 to achieve proper cell differentiation and that might, at least to a certain level, account for the drastic effects of the *bp* mutation on inflorescence development, is the modification of cell walls, in particular the regulation of lignin deposition, in the inflorescence stem (Mele et al., 2003).

It has been proposed that BP/KNAT1 functions to establish early internode patterning events operating in functional complexes together with PNY and PNF, respectively (Kanrar et al., 2006). Both proteins can interact with BP/KNAT1 (see Table 1.1) and, in the case of PNY, their expression patterns in the inflorescence and FMs overlap (Smith and Hake, 2003). Furthermore, coexpression of PNY with BP/KNAT1 in tobacco leaf cells results in nuclear co-localization of the two proteins (Bhatt et al., 2004). Similarly, a role for STM together with PNY and PNF in inflorescence growth has been suggested as inflorescence development is sensitive to the dosage levels of PNY, PNF and STM (Kanrar et al., 2006). However, these defects may be secondary effects resulting from primary SAM initiation and maintenance defects in these plants.

Apart from the earlier mentioned phyllotaxy defects, *pny* mutants, like *bp*, are semi-dwarfed due to randomly shorter internodes. As a result, *pny* inflorescence stems have clusters of cauline leaves and flowers/siliques. In addition, they display a slight increase in the number of axillary branches, indicating a partial loss of apical dominance (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake,

Table 1.1 BELL-KNOX protein-protein interactions

	STM	BP/ KNAT1	KNAT2	KNAT6	KNAT3	KNAT4	KNAT5	KNAT7	KNATM
ATH1	+*	+*	+	+		+		+	
BEL1	+	+	+	+			+		+
BLH1	+		+	+	+		+		+
BLH2/SAW1	+	+	+	+	+		+		+
BLH3	+*	*		+	+		+		
BLH4/SAW2	+	+	+	+	+		+		+
BLH5			+	+	+		+	+	
BLH6	+	+	+	+	+	+	+		
BLH7				+	+		+	+	
BLH8/PNF	+	+	+						
BLH9/PNY	+*	+*	+	+	+		+		*
BLH10				+			+		

KNOX proteins are indicated by different shades of colour: dark and light green cells indicate class I and class II KNOX proteins, respectively. BELL proteins are highlighted in blue. + and * indicate interactions found in yeast and in planta, respectively. Blank cells indicate no interactions have been found between the specific KNOX and BELL proteins (data were obtained from Bellaoui et al., 2001; Bhatt et al., 2004; Hackbusch et al., 2005; Cole et al., 2006; Kanrar et al., 2006; Viola and Gonzalez, 2006; Kumar et al., 2007; Magnani and Hake, 2008; Rutjens et al., 2009)

2003; Bao et al., 2004; Bhatt et al., 2004).

No apparent aberrant phenotype can be observed in *pnf* mutants. However, *pny* mutants lacking one functional allele of *PNF* (*PNF/pnf pny*; *pny*-hemi) show more severe defects in inflorescence internode and pedicel development than *pny* single mutants. In *pny*-hemi plants, pedicels are often fused to the main stem or to each other, indicating that the boundaries between the meristem and initiating flowers are not maintained. Moreover, both the main inflorescence and the branches, terminate prematurely with the fusion of several pedicels and flowers (Smith et al., 2004).

As might be expected, *pny bp* double mutants show a synergistic phenotype with extremely short internodes and increased branching (Smith and Hake, 2003; Bhatt et al., 2004).

Interestingly, both *pny* and *bp* phenotypes, including the stem elongation and internode patterning defects, can be fully, respectively partially, suppressed

by additional inactivation *KNAT6* (Ragni et al., 2008). Also loss of KNAT2 activity can partially rescue *bp* defects, but only in combined absence with KNAT6 or PNY. As in *bp* and *pny* mutants *KNAT6* is misexpressed in inflorescence stem internodes and pedicels, and *KNAT2* in pedicels, PNY and BP/KNAT1 most likely promote correct inflorescence growth by restricting *KNAT6* and, to a lesser extent, also *KNAT2* expression at this stage of development (Ragni et al., 2008).

Recently, a third *BELL* gene, *ATH1* has been found to be involved in the regulation of stem elongation (Cole et al., 2006; Gomez-Mena and Sablowski, 2008). In plants overexpressing *ATH1* vegetative growth is relatively unaffected, whereas growth of the inflorescence stem and pedicels is dramatically reduced, mostly due to limited cell proliferation (Cole et al., 2006; Gomez-Mena and Sablowski, 2008). As a result these transgenic plants maintain a rosette-like appearance throughout their lifecycle, resembling *pny bp* mutants. This then raises the question whether ATH1 and PNY-BP/KNAT1 might function as antagonists in the control of stem elongation and, if so, whether this antagonism involves interaction of ATH1 with KNAT2 and/or KNAT6.

Flowering time Control in Arabidopsis

Prior to the elaboration of inflorescence structures, plants must make the transition from the vegetative to the reproductive phase. The regulation of flowering time has been extensively studied in Arabidopsis (reviewed in Boss et al., 2004; Henderson and Dean, 2004; Schmitz and Amasino, 2007; Turck et al., 2008). Antagonistic action of promoting and repressing pathways prevents floral transition until the plant has reached a certain age or size and growth conditions are favorable for sexual reproduction and seed maturation. At a certain moment, the balance of promoting and repressing factors is such that the by then competent vegetative SAM is 'evoked' in a florally determined state by the activation of a set of so-called floral pathway integrators (FPI), including FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)/AGAMOUS-LIKE 20 (AGL20) (reviewed in Bernier, 1988; Mouradov et al., 2002; Boss et al., 2004). Mutational analyses have revealed four major genetic pathways controlling flowering in Arabidopsis. The photoperiod pathway promotes flowering in response to an increase in day length (reviewed in Bernier and Perilleux, 2005; Giakountis and Coupland, 2008; Turck et al., 2008; Zeevaart, 2008). Long day-dependent accumulation of the zinc finger TF protein CONSTANS (CO) results in the transcriptional activation of *FT*. FT encodes at least part of a systemic signal that induces flowering at the SAM (reviewed in Turck et al., 2008). Photoperiodic floral induction is antagonized by members of the floral repression pathway, which is considered to control the competence of the SAM to floral induction signals (Boss et al., 2004). The most prominent floral repressor is the MADS box

TF FLOWERING LOCUS C (FLC) (Michaels and Amasino, 2001; Simpson, 2004; Shindo et al., 2005). High levels of FLC make the plant incompetent to respond to flowering promoting factors. FLC delays flowering by directly repressing transcription of the FPI genes FT and SOC1 in leaves and the SAM. In addition, FLC prevents upregulation of the FT-interacting partner FD in the SAM (Lee et al., 2000; Hepworth et al., 2002; Michaels et al., 2005; Searle et al., 2006). The autonomous and vernalization flowering pathways converge at *FLC* to repress its expression (Simpson, 2004; Veley and Michaels, 2008). A fourth flowering pathway, the gibberellic acid (GA) hormonal promotion pathway, is important to induce flowering under non-inductive short day conditions via transcriptional activation of the FPI genes and the floral meristem identity (FMI) gene *LEAFY* (*LFY*) (reviewed in Parcy, 2005; Mutasa-Gottgens and Hedden, 2009).

Although no clear roles for KNOX proteins in the control of flowering time have been reported to date, there is accumulating evidence that BELL proteins play a key role during this crucial stage of development. Intriguingly, whereas *pny*-hemi plants show severe inflorescence patterning defects, complete loss of both *PNY* and *PNF* results in plants that lack any form of internode development and floral patterning. As a result *pny pnf* plants continue to produce leaves throughout their lifecycle (Smith et al., 2004). Although *pny pnf* plants respond to floral inductive signals by induction of FPI genes such as *SOC1*, *FT* and *FRUITFULL* (*FUL*) and by a gradual change in leaf identity from rosette leaves to more cauline leaf-like leaves, they fail to induce expression of the FMI genes *LFY*, *APETALA1* (*AP1*), and *CAULIFLOWER* (*CAL*) to complete the process of floral evocation (Smith et al., 2004). Because ectopic expression of *LFY* promotes flower formation in *pny pnf* plants, PNY and PNF are proposed to function as positive regulators of meristem competence in the process of floral evocation by promoting *LFY* expression during this developmental process (Kanrar et al., 2008).

In contrast, ATH1 has been identified as a positive regulation of *FLC* expression, thereby negatively controlling the competence of the SAM to respond to floral inductive signals (Proveniers et al., 2007). Accordingly, *ATH1* expression in the shoot apex is downregulated during the transition to reproductive development (Proveniers et al., 2007).

There is accumulating evidence that additional *BELL* genes are involved in the control of flowering time. Plants overexpressing *BLH6* show a delay in flowering time (Silva and Pelaz, 2007), whilst *BLH3* overexpressor lines flower slightly earlier in LD conditions (Cole et al., 2006; Silva and Pelaz, 2007).

Whether the activity of these five BELL proteins in the flowering process involves interaction with KNOX proteins or acts independent of them is yet to be tested.

Reproductive development

Floral patterning

During the final stages of reproductive development, the SAM co-initiates floral meristems (FMs) at its flanks. In Arabidopsis, FMs produce flowers with four types of lateral organs in a whorled pattern. Sepals are initiated first in the outermost whorl, followed by petals in the second whorl, and stamens in the third whorl. The FM is then consumed during the formation of the central carpels that will eventually form the gynoecium. The stem cell reservoir in FMs is therefore transient. Both termination of stem cell maintenance in the FM and subsequent development of carpels and stamens requires *AGAMOUS* (*AG*), which is activated in the centre of the FM by LFY and WUS (Mizukami and Ma, 1997; Busch et al., 1999; Lenhard et al., 2001; Lohmann et al., 2001). AG then represses *WUS* expression, causing stem cell maintenance to terminate and thus permitting the reproductive organs to develop.

Maintenance, but not initiation, of the floral stem cell population also requires STM. Analogous to the role of STM in maintaining the SAM, in weak alleles of *stm* as well as *STM* RNAi lines floral stem cells are not maintained and are instead consumed during the formation of petals and stamens (Clark et al., 1996; Endrizzi et al., 1996; Scofield et al., 2007). Apart from its role in FM stem cell maintenance there seems to be a specific requirement for STM in the process of carpel initiation and the formation of associated meristematic placental tissues, as progressive loss of *STM* causes floral phenotypes ranging from reduced formation of placental tissues and inhibited carpel fusion to complete loss of carpel development (Scofield et al., 2007).

Moreover, when ectopically expressed in flowers, *STM* causes formation of ectopic carpels and homeotic conversion of ovules to carpels within the gynoecium. Although KNAT2 is not essential for carpel development, similar phenotypes have been previously reported for plants overexpressing *KNAT2* (Pautot et al., 2001), suggesting that this role for STM is redundantly duplicated in KNAT2. This is also consistent with the almost identical expression patterns of *STM* and *KNAT2* in the FM, developing carpels, and associated placental tissues (Long et al., 1996; Long and Barton, 2000; Pautot et al., 2001).

Transcripts of two STM- and KNAT2-interacting BELL proteins, PNY and PNF, localize to all the four whorls of the developing flower (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bao et al., 2004; Smith et al., 2004; Yu et al., 2009). Single loss-of-function mutations in *pny* and *pnf* have no visible effect on flower development. However, two antimorphic *pny* alleles have been identified that, when grown at high temperatures, produce carpel-like structures in the first whorl of the flower during the late stages of inflorescence development, and

it was shown that PNY functions with a co-repressor complex to down-regulate *AG* in the outer whorls of the flower (Bao et al. 2004). A more recent study by Yu et al. (2009) that combined *pny* and *pnf* mutants with weak and strong alleles of *lfy* indicates that PNY and PNF also play a role in the specification of petals, stamens and carpels by positive regulation of *APETALA3* and *AG* expression in the inner whorls of the flower. At this time, it is unclear whether PNY and PNF interact with STM and/or KNAT2 to promote carpel formation in parallel with AG.

Fruit patterning

In Arabidopsis, the carpel is differentiated into stigma, style, and ovary tissues and occurs fused with a second carpel in a syncarpic pistil. After fertilization, the ovary develops into a fruit. The ovary is divided into three tissue zones: valve, replum and valve margin (reviewed in Roeder and Yanofsky, 2008). The valves correspond to the two carpel walls. They protect the seeds during their development and detach after maturation to promote seed dispersal process. The two valves are separated by a central ridge of replum tissue. Internal to each replum, a septum grows across the ovary and fuses postgenitally with septal tissue growing from the other side. At the valve/replum junction, a specialized stripe of tissue, termed the valve margin, will be formed that facilitates the detachment of the valves from the replum through the action of two different cell types. On the replum side of the valve margin, the separation layer forms, which permits the detachment of the valve from the replum through cell-cell separation (reviewed in Roeder and Yanofsky, 2008).

Girin et al. (2009) recently proposed that the replum can be regarded as a quasi-meristem on the basis of its function in fruit development and its trackable meristematic characteristics (Ferrandiz et al., 1999; Pautot et al., 2001; Balanza et al., 2006; Brambilla et al., 2007). Apart from the valves and valve margins, the development of all fruit tissues depends on this meristematic activity. As it is widely accepted that the carpels are modified leaves, the gynoecium can thus be seen as two modified cotyledons (the presumptive carpel valves) fused to two modified meristems (the presumptive repla), suggesting that the fruit and SAM share mechanistic similarities (Girin et al., 2009). In line with this, it has recently been suggested that the regulatory module involving AS1/AS2 and class I KNOX I proteins acts on the fruit mediolateral patterning as it does on the SAM/primordia patterning. Alonso-Cantabrana et al. (2007) showed that AS1 and BP/KNAT1 have antagonistic effects on replum development. Whereas BP/KNAT1 promotes replum development, AS1 represses it. *BP/KNAT1* is expressed in the replum and valve margins and is restricted to these tissues by the repressing action of AS1/AS2 in the valves. As a result, both plants that ectopically express *BP/KNAT1*, as well as *as1* and *as2* mutants show a similar enlarged replum and a reduction in

valve width (Alonso-Cantabrana et al., 2007). Despite a putative function of BP/KNAT1 in replum development, *bp* null mutants display no aberrant phenotype in this tissue. This might be explained by functional redundancy with STM that is also expressed in the replum (Long et al., 1996; Alonso-Cantabrana et al., 2007; Ragni et al., 2008).

BP/KNAT1 acts on replum development by interacting with PNY. In the fruit *PNY* expression is confined to the replum and mutations in *PNY* result in a reduced replum width and septum defects, and strong alleles lose the outer replum (Roeder et al., 2003). In *bp pny* double mutants replum defects are further enhanced (Alonso-Cantabrana et al., 2007). In addition, ectopically expressed *BP/KNAT1* is able to activate *PNY* in the valves and valve margins (Alonso-Cantabrana et al., 2007). However, the expression of *PNY* is not affected in a *bp* mutant background (Smith and Hake, 2003). Like BP/KNAT1, PNY is not indispensable for replum development (Roeder et al., 2003; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007). Instead, PNY enables replum development by repressing valve and valve margin development through negative regulation of the activity of the valve promoting genes *JAGGED* (*JAG*), *FILAMENTOUS FLOWER* (*FIL*), and *YABBY3* (*YAB3*), and by indirect repression of the activities of the *SHATTERPROOF1* (*SHP1*), *SHP2*, *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) valve margin identity genes (Roeder et al., 2003; Dinneny and Yanofsky, 2005; Alonso-Cantabrana et al., 2007; Girin et al., 2009).

Like in internode patterning, mutations in *knat6* completely rescue the replum and the septum defects seen in *pny* and *bp pny* plants (Ragni et al., 2008). In the fruit, *KNAT6* expression is restricted to the boundaries between the replum and valves. Therefore, *KNAT6* is likely to suppress replum formation by promoting the establishment of valve margins, reminiscent of the role of KNAT6 in the establishment of the boundaries between the SAM and the cotyledons (Belles-Boix et al., 2006). Despite this, in *knat6* mutants, no defects in replum, septum or valve margin can be observed (Ragni et al., 2008). Although *KNAT2* expression overlaps with that of KNAT6 in the carpel, this cannot easily be explained by functional redundancy with KNAT2. Unlike *knat6*, inactivation of KNAT2 does not rescue the *pny* or *bp pny* replum or septum defects (Ragni et al., 2008). In addition, *knat2 knat6* double mutants display no obvious fruit patterning defects (Ragni et al., 2008). Taken together, in the process of fruit patterning TALE HD proteins represent two opposing activities with PNY, BP/KNAT1, and STM as replum factors and KNAT2 and KNAT6 to specify valve margins.

Ovule development

In Arabidopsis, ovules arise from carpel tissue as new meristematic formations (Brambilla et al., 2007). Correct ovule development is dependent on a

proper balance between the activity of proteins controlling carpel identity and proteins controlling ovule integument identity. Carpel identity activity is mainly established by AG, whereas ovule integument activity is mainly supplied by three additional MADS box family members SEEDSTICK (STK), SHP1, and SHP2 (Brambilla et al., 2007; and references therein). Accordingly, in *stk shp1 shp2* triple mutants, ovule integuments are transformed into carpelloid structures (Pinyopich et al., 2003).

Conversions of integuments into carpelloid structures have also been observed in *bel1* mutants (Robinson-Beers et al., 1992; Modrusan et al., 1994; Reiser et al., 1995). Eventually, *bel1* mutants form abnormal ovules and, hence, mutant plants are female sterile (Robinson-Beers et al., 1992; Modrusan et al., 1994). When combined with the *stk shp1 shp2* triple mutant, the *bel1* phenotype is significantly enhanced (Brambilla et al., 2007). Consistent with its mutant phenotype, *BEL1* is expressed in developing ovules (Reiser et al., 1995; Bellaoui et al., 2001; Kumar et al., 2007). It has been proposed that BEL1 antagonizes the activity of AG in the ovule (Modrusan et al., 1994; Ray et al., 1994), most likely at a post-transcriptional level (Reiser et al., 1995). In addition, BEL1 and AG have also been suggested to redundantly interact in the determination of ovule identity (Western and Haughn, 1999). A model for BEL1 function in ovule development by Brambilla et al. (2007), partially based on their finding that BEL1 interacts with an AG-SEPALLATA3 (SEP3) dimer, takes both these observations into account. In this model AG-SEP dimers activate the carpel identity pathway, whereas trimeric BEL1-AG-SEP3 complexes antagonize this activity by controlling outer integument growth, probably by activating *INNER NO OUTER* (*INO*) expression and restricting *WUS* expression to the nucellus (Balasubramanian and Schneitz, 2002; Gross-Hardt et al., 2002; Brambilla et al., 2007). In addition, the BEL1-AG-SEP3 complex is thought to be stabilized by additional STK-SHP-SEP complexes.

Although BEL1 has been found to interact with several KNOX proteins, to date, no KNOX-derived functions have been attributed to the mediation of BEL1-associated ovule morphogenesis. Interestingly, overexpression of *KNAT2*, but not BP/*KNAT1*, induces carpeloid features in ovules accompanied by ectopic expression of AG in carpels and ovules (Pautot et al., 2001). However, in contrast to *bel1* ovules, the carpeloid structures induced by KNAT2 are derived from the nucellus instead of the integuments, indicating that BEL1 and KNAT2 act via different pathways.

Ovules generate the female gametophyte within sporophytic integuments. Recently, a novel female gametophyte mutant showing altered cell fates in the mature embryo sac, *eostre-1*, has been found. The *eostre-1* phenotype is caused by misexpression of *BLH1* in the emryo sac (Pagnussat et al., 2007). However, *BLH1* is normally not expressed in ovules and both *blh1* mutants and a double gene knockout of *BLH1* and its closest homolog *BLH5* have normal and func-

tional gametophytes, indicating that BLH1 is not essential for gametophyte development or function. Interestingly, inactivation of *KNAT3* rescued the *eostre-1* embryo sac defects, suggesting that *KNAT3* must be expressed and functional during megagametogenesis (Pagnussat et al., 2007).

TALE HD targets

Despite intensive research on TALE HD proteins, little is known about their targets. Regarding their interaction with DNA, binding-site selection and mutagenesis studies have indicated that, like their animal counterparts, plant TALE HD proteins bind sequences containing a TGAC core via their characteristic DNA-binding HD structure (Krusell et al., 1997; Smith et al., 2002; Chen et al., 2004; Tioni et al., 2005; Viola and Gonzalez, 2006; Bolduc and Hake, 2009; Viola and Gonzalez, 2009).

As can be inferred from the above, members of the Arabidopsis TALE HD gene family have important roles in plant meristems by regulating cell division and differentiation. But how these TF proteins actually regulate the behavior of meristem cells is still largely unknown. Previous studies have suggested that the plant growth regulator GA is incompatible with meristem activities and that TALE HD proteins promote meristem function partly through the regulation of GA metabolism (Hay et al., 2002; Jasinski et al., 2005). This idea is supported by the fact that class I KNOX proteins in tobacco (NTH15) and potato (POTH1) directly bind to and repress a gene encoding a GA 20-oxidase, a rate-limiting enzyme in GA biosynthesis (Tanaka-Ueguchi et al., 1998; Sakamoto et al., 2001; Chen et al., 2004). Furthermore, in the case of POTH1, the repression of GA 20-oxidase requires interaction with the potato BELL protein StBEL5 (Chen et al., 2004). Similarly, the rice class I *KNOX* gene *OSH1*, when ectopically expressed in tobacco, affects bioactive GA levels by suppressing GA 20-oxidase gene expression (Kusaba et al., 1998a; Kusaba et al., 1998b). In Arabidopsis, *AtGA20ox1* mRNA levels are reduced in leaves overexpressing the KNOX proteins STM or BP/KNAT1 (Hay et al., 2002), but whether this regulation is direct awaits confirmation. These authors further showed that constitutive signaling through the GA pathway is detrimental to meristem maintenance, suggesting that one of the functions of these KNOX proteins is to exclude transcription of this GA biosynthesis gene from the SAM (Hay et al., 2002).

TALE HD proteins have further been found to promote the expression of GA 2-oxidase genes that are expressed at the base of the SAM and developing leaf primordia (Jasinski et al., 2005; Gomez-Mena and Sablowski, 2008; Bolduc and Hake, 2009). GA 2-oxidases encode key components in the GA catabolism

pathway (Thomas et al., 1999). Since GA is a diffusible molecule the regulation of GA catabolism is thought to prevent a GA influx from developing leaf primordia and the stem into the stem cell zone of the SAM (Jasinski et al., 2005; Bolduc and Hake, 2009). In maize, KN1 was recently found to negatively modulate the accumulation of GA through direct control of *ga2ox1* expression (Bolduc and Hake, 2009). In Arabidopsis, ectopic expression of *STM* activates the expression of both *AtGA2ox2* and *AtGA2ox4*, but this regulation may be indirect (see below) (Jasinski et al., 2005).

Although it is clear that TALE HD proteins target GA activity, these studies also show that this connection cannot fully account for TALE HD action. Both early classic work and recent molecular genetic analyses suggest that more indeterminate programs of growth, including meristems depend on relatively high levels of cytokinin, a known growth regulator that promotes cell division (Veit, 2009) (Figure 1.3). In addition to limiting GA activity in the central tissues of the SAM, TALE HD activity is further suggested to promote an increase in cytokinin levels in this tissue (Kusaba et al., 1998b; Ori et al., 1999). KNOX proteins appear to have a major effect on cytokinin levels by directly activating *ISOPENTENYL TRANSFERASE* (*IPT*) genes that catalyse rate-limiting steps in cytokinin biosynthesis (Hewelt et al., 2000; Jasinski et al., 2005; Yanai et al., 2005). This interaction seems to be bidirectional as plants overproducing cytokinins express higher levels of *STM* and *BP/KNAT1* (Rupp et al., 1999; Frank et al., 2000). Moreover, direct application of cytokinin to the shoot or expression of *IPT7* in the STM expression domain can partially rescue *stm* SAM phenotypes (Yanai et al., 2005). Conversely, the instability of weak *stm* mutants is exacerbated by mutants that attenuate cytokinin signaling (Jasinski et al., 2005). These authors further reported that cytokinin promotes GA deactivation by activating *AtGA2ox2* and *AtGA2ox4* gene expression. Thus, possibly in response to TALE HD activity, cytokinin may promote the deactivation of GA at the boundary between leaves and the SAM, thereby reinforcing the low-GA regime established by KNOX proteins in the SAM. As Jasinski et al. (2005) further provided evidence that a combination of constitutive GA signaling and reduced CK levels is detrimental to SAM function, it has been proposed that TALE HD proteins may act as general orchestrators of growth-regulator homeostasis at the shoot apex of Arabidopsis by simultaneously activating cytokinin and repressing GA biosynthesis (Figure 1.3), thus promoting meristem activity.

Apart from establishing a hormonal regime favorable for meristem function by regulating GA and cytokinin activities, TALE HD proteins further regulate meristem development by allowing developmental patterning of the shoot apex via negative regulatory interactions with, among others, the AS1-AS2 complex to promote organ development. The latter activity of TALE HD proteins also seems to be mediated via plant hormone activities, especially those of auxin and

also ethylene.

The site of lateral organ initiation in the SAM is defined by both a local PINFORMED1 (PIN1)-mediated accumulation auxin (reviewed in Reinhardt et al., 2000) and down-regulation of KNOX expression by AS1-AS2 activity. A detailed expression analysis by live imaging of Arabidopsis meristem showed that *STM* is down-regulated at locations where *PIN1* is upregulated (Heisler et al., 2005). *PIN1* expression is low in the central part of the SAM and marks incipient primordia. Once the primordia position is determined, *STM* is specifically upregulated in the boundaries, while *PIN1* is excluded from the boundary domain (Heisler et al., 2005). Interestingly, several reports illustrated that misexpression of *KNOX* genes impedes polar auxin transport, suggesting that auxin transport or signaling and KNOX genes affect each other (Tsiantis et al., 1999; Scanlon et al., 2002; Scanlon, 2003; Hay et al., 2004, 2006). In case of *BP/KNAT1* it was shown that auxin converges with AS1 activity to repress *BP/KNAT1* expression and thus to promote leaf fate (Hay et al., 2006).

An indication that ethylene is involved in the regulation of meristem activity comes from the analysis of plants ectopically expressing KNAT2 (Hamant et al., 2002). These investigations indicate that *KNAT2* acts antagonistically to ethylene when overexpressed. This interaction may be relevant to SAM function as increased ethylene levels or a constitutive ethylene response reducse the number of cells in the L3 layer of the SAM, a defect that can be rectified by induction of KNAT2 activity (Hamant et al., 2002). Furthermore it was shown that ethylene restricts *pKNAT2-GUS* reporter expression in the SAM, whereas cytokinin treatment induces it, suggesting that ethylene and cytokinins act antagonistically in the SAM via KNAT2 to regulate the meristem activity (Hamant et al., 2002).

In conclusion, connections between TALE HD proteins and plant hormones provide a means of integrating developmental patterning signals with cell division and differentiation. However, although TALE HD-hormone interactions have begun to be mapped out their precise relationships are still unclear.

Outline of this Thesis

Chapter 1 reviews aspects of plant shoot architecture that are regulated by TALE HD proteins. Plants are the products of the meristem and the shoot apical meristem function is largely dependent on class I *KNOX TALE* HD genes that maintain the meristematic cells in an undifferentiated state by preventing the expression of differentiating factors. KNOX proteins interact with members of a second group of TALE HD proteins, the BEL1-like proteins. These interactions are known to be required for site-specific DNA binding and for nuclear localiza-

tion of the BELL-KNOX heterodimeric complex. Here, a number of developmental processes have been discussed that involves BELL and KNOX functions.

In **Chapter 2** we report that three BELL class TALE HD proteins ATH1, PNY and PNF are redundantly required for the initiation and maintenance of the Arabidopsis SAM throughout the lifecycle. All three proteins interact with KNOX TALE HD protein STM, and combined lesions in the three genes result in a full phenocopy of *stm* loss-of-function mutants. We propose that the meristem defects result from loss of combinatorial BELL-STM control. The process of nuclear translocation of BELL-KNOX dimers involves an ancient nuclear exclusion mechanism that also regulates the function of animal TALE HD counterparts.

In **Chapter 3** we investigate the functional relevance of additional ATH1-KNOX interactions in relation to boundary establishment. Based on their expression domain in the SAM, and the findings that lesion in *KNAT6* and *KNAT2* results in similar organ fusions that are characterized in *ath1* mutants, and their roles in stem growth, we suggest that ATH1 acts in conjunction with KNAT6 and KNAT2 at the interface between both the SAM and lateral organ primordia and between the SAM and the stem to establish proper meristem-leaf boundaries and to control stem elongation. Moreover, we provide data that ATH1 also affects KNOX activity by controlling KNOX expression.

Chapter 4 describes the overlapping and opposite function of ATH1, PNY and PNF at different developmental stage in Arabidopsis. Since partial rescuing of *pny* phenotype and *pny pnf* plants by removal of ATH1 activity was noticed before (Chapter 2), further studies continue on revealing their relationship. We found that depending on the developmental phase, ATH1 overlaps and opposes PNY/PNF function. In the vegetative stage, ATH1 and PNY are redundantly required for meristem initiation and maintenance. During the generative stage, ATH1 and PNY have opposite role in inflorescence patterning and fruit development. ATH1 also opposes both PNY and PNF in the process of floral evocation and floral meristem specification.

Chapter 2

Shoot apical meristem function in Arabidopsis requires the combined activities of three BEL1-like homeodomain proteins

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Summary

In plants, most of the above-ground body is formed post-embryonically by continuous organogenic potential of the shoot apical meristem (SAM). Proper SAM function requires maintenance of a delicate balance between the depletion of stem cell daughters into developing primordia and proliferation of the central stem cell population.

Here we show that Arabidopsis SAM initiation and maintenance, including that of floral meristems, requires the combinatorial action of three members of the BELL-family of TALE homeodomain proteins, ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1), PENNYWISE (PNY), and POUND-FOOLISH (PNF). All three proteins interact with the KNOX TALE homeodomain protein STM, and combined lesions in ATH1, PNY and PNF result in a phenocopy of *stm* mutations. Therefore, we propose that *ath1 pny pnf* meristem defects result from loss of combinatorial BELL-STM control. Further, we demonstrate that heterodimerization-controlled cellular localization of BELL and KNOX proteins involves a CRM1/exportin-1-mediated nuclear exclusion mechanism that is probably generic to control the activity of BELL and KNOX combinations.

We conclude that in animals and plants corresponding mechanisms regulate TALE homeodomain protein activity through controlled nuclear-cytosolic distribution of these proteins.

Introduction

In plants, proper function of the shoot apical meristem (SAM) requires maintenance of a delicate balance between the depletion of stem cell daughters into developing primordia and proliferation of the central stem cell population (Fletcher, 2002). In Arabidopsis, the *KNOTTED1*-like homeobox (KNOX) transcription factor gene *SHOOTMERISTEMLESS* (*STM*) plays an important role in this process (Barton and Poethig, 1993; Long *et al.*, 1996). Plants homozygous for strong loss-of-function *stm* mutations fail to establish a functional SAM (Barton and Poethig, 1993; Endrizzi *et al.*, 1996; Long *et al.*, 1996). Weaker mutants, however, do form a SAM that initiates primordia shortly after germination. Nevertheless, these meristems eventually terminate as a result of the complete incorporation of meristem cells in differentiating primordia. This implies that STM is not only essential for initial shoot meristem formation, but also for the subsequent SAM maintenance (Clark *et al.*, 1996; Endrizzi *et al.*, 1996).

A primary function of *STM* in meristem maintenance is suppression of cell differentiation in the stem cell area as well as in the peripheral zone (Byrne *et al.*, 2000; Lenhard *et al.*, 2002), and involves complex interactions within a network of redundant KNOX homologues (Byrne *et al.*, 2002). Two of these, *BREVIPEDICELLUS/KNOTTED1-LIKE ARABIDOPSIS THALIANA1* (*BP/KNAT1/KNAT1*) and *KNAT6* were shown to act redundantly with *STM* in meristem maintenance (Belles-Boix *et al.*, 2006; Byrne *et al.*, 2002). However, STM provides the critical KNOX function for SAM development (Byrne *et al.*, 2002; Dean *et al.*, 2004; Douglas *et al.*, 2002; Venglat *et al.*, 2002).

Together with the BEL1-like (BELL) proteins (Burglin, 1997; Reiser *et al.*, 1995) KNOX proteins make up the plant family of three-amino-acid-loop-extension (TALE) homeodomain (HD) transcription factors (Burglin, 1997). KNOX and BELL proteins can heterodimerize both *in vitro* and *in vivo* in a DNA-independent manner (Bellaoui *et al.*, 2001; Bhatt *et al.*, 2004; Byrne *et al.*, 2003; Chen *et al.*, 2004; Chen *et al.*, 2003; Cole *et al.*, 2006; Hackbusch *et al.*, 2005; Muller *et al.*, 2001; Smith and Hake, 2003). Moreover, several BELL proteins have recently been shown to drive (orthologues of) STM to the nucleus upon heterodimer formation (Cole *et al.*, 2006; Kimura *et al.*, 2008). Currently, the biological relevance of this mechanism is not clear.

In Arabidopsis, the BELL family consists of thirteen members. So far, for the majority of *BELL* genes their function remains unknown. One of the few exceptions is formed by *PENNYWISE* (*PNY* a.k.a. *BELLRINGER*, *REPLUMLESS*, *LARSON* and *VAAMANA*) (Bao *et al.*, 2004; Bhatt *et al.*, 2004; Byrne *et al.*, 2003; Ragni *et al.*, 2008; Roeder *et al.*, 2003; Smith and Hake, 2003). *pny* mutants have been identified in a variety of screens, and phenotypes include replum loss, inflorescence patterning defects, and, importantly, enhancement of *stm* phenotypes

(Bhatt *et al.*, 2004; Byrne *et al.*, 2003; Kanrar *et al.*, 2006; Ragni *et al.*, 2008; Roeder *et al.*, 2003; Smith and Hake, 2003). The latter is in line with the capacity of PNY to direct STM nuclear localization (Cole *et al.*, 2006). The need for PNY as a requirement for STM activity seems to be only partial (Byrne *et al.*, 2003). An obvious candidate to share this function with PNY is its paralogue POUND-FOOLISH (PNF) (Smith *et al.*, 2004), given its concerted action with STM and PNY in inflorescence development (Kanrar *et al.*, 2006; Smith *et al.*, 2004). However, *pnf* mutations do not affect weak *stm* phenotypes in any respect (Kanrar *et al.*, 2006).

Here, we show that SAM function in Arabidopsis, including embryonic initiation, post-embryonic maintenance, and floral meristem function, requires both PNY and PNF, together with a third related BELL protein, ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1) (Gomez-Mena and Sablowski, 2008; Proveniers *et al.*, 2007; Quaedvlieg *et al.*, 1995). All three BELL proteins dimerize with class I KNOX proteins, including STM, and combined lesions in ATH1, PNY and PNF result in a full phenocopy of *stm* loss-of-function mutants. We propose that the observed meristem defects result from loss of combinatorial BELL-STM control. The process of nuclear translocation/retention of BELL-KNOX complexes most likely underlies this combinatorial control, and involves a nuclear exclusion mechanism that is probably generally used to control the combined activity of BELL and KNOX proteins.

Results

ATH1 is required for proper vegetative SAM development

Recently, ATH1 has been reported essential for proper development of basal and lateral organ boundaries (Gomez-Mena and Sablowski, 2008) (Supplemental Figure 1). Meristem-organ boundaries likely function to prevent differentiation from depleting the central stem cell population. Here, we investigated whether ATH1 is required for proper SAM maintenance.

At 4 days post germination (dpg), wild-type and *ath1-1* display significant differences in SAM organization. In *ath1-1* the number of densely cytoplasmic, darker stained, meristematic cells is reduced when compared to wild-type (Wt) plants (Figure 2.1a,b), resulting in a decrease in SAM diameter (71.30±2.87µm in *ath1-1* vs. 104.37±4.01µm in Wt). In contrast, the *ath1-1* subapical region is more elongated (88.21±12.85µm vs. 48.28±2.94µm in Wt). At the same time, the number of rib zone cells that make up this region, recognizable by a highly vacuolated and larger appearance, is reduced in *ath1-1*. As a result, in *ath1-1* the rib zone cells are more longitudinally elongated than in Wt (Figure 2.1a,b). To examine

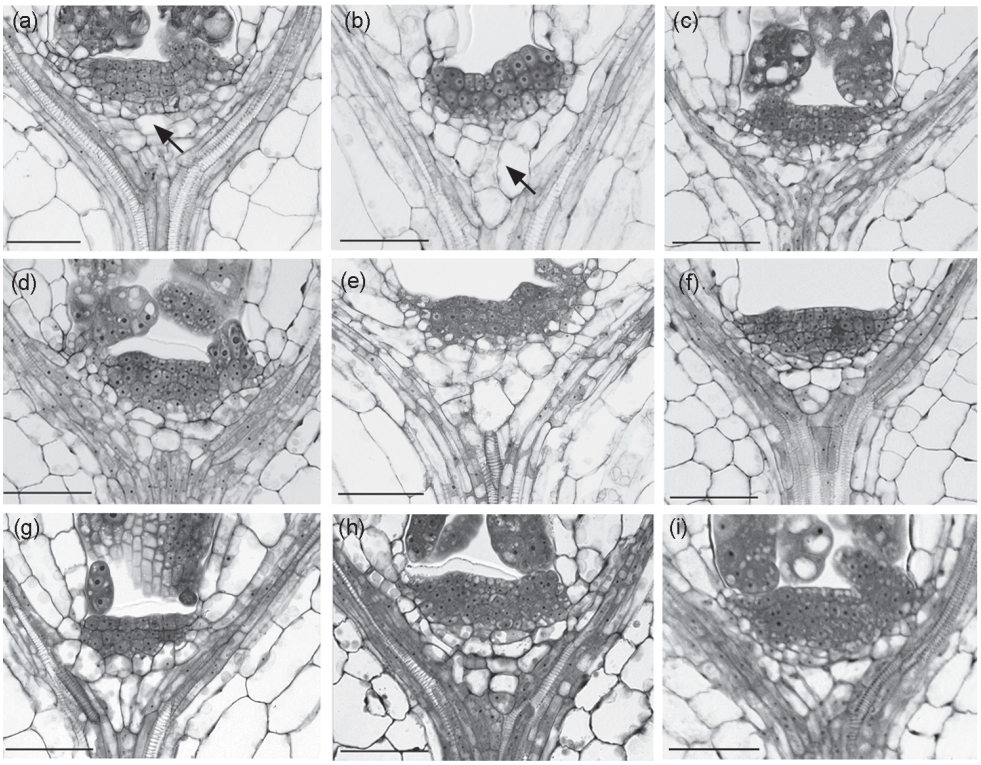


Figure 2.1 Loss of ATH1 affects vegetative SAM size and organization.
(a-i) Longitudinal median sections through apices of 4-day-old wild-type (a, d), *ath1-1* (b, e), *35Spro:ATH1-HBD ath1-1* (g-i), and *35Spro:ATH1-HBD* (c, f) plants. (a-c, g) Ethanol-treated control plants. (d-f, h, i) Dex-treated plants (1μM in (h), 10μM in (d-f, i)). Bar = 50μm. Arrows indicate rib zone cells.

whether the SAM defects in *ath1-1* plants can be completely attributed to loss of *ATH1*, we generated a fusion protein construct of ATH1 with the rat glucocorticoid receptor hormone binding domain (HBD) under the control of the CaMV 35S promoter (*35Spro:ATH1-HBD*). As can be seen in Figure 2.1 (a,g-i) both aspects of the altered SAM organization in *ath1-1* could be reverted back to a wild-type phenotype by nuclear expression of ATH1. In plants grown on as low as 1μM Dex, SAM diameter was restored from 68.98±2.83μm in control-treated plants to 98.18±2.57μm. Moreover, also in a wild-type background *35Spro:ATH1-HBD* induction resulted in an extra increase in meristematic cells (Figure 2.1a,c,d,f), whereas SAM diameter was not affected. Dex treatment itself had no visible effect on SAM size or organization in Wt or *ath1-1* (Figure 2.1a,b,d,e)

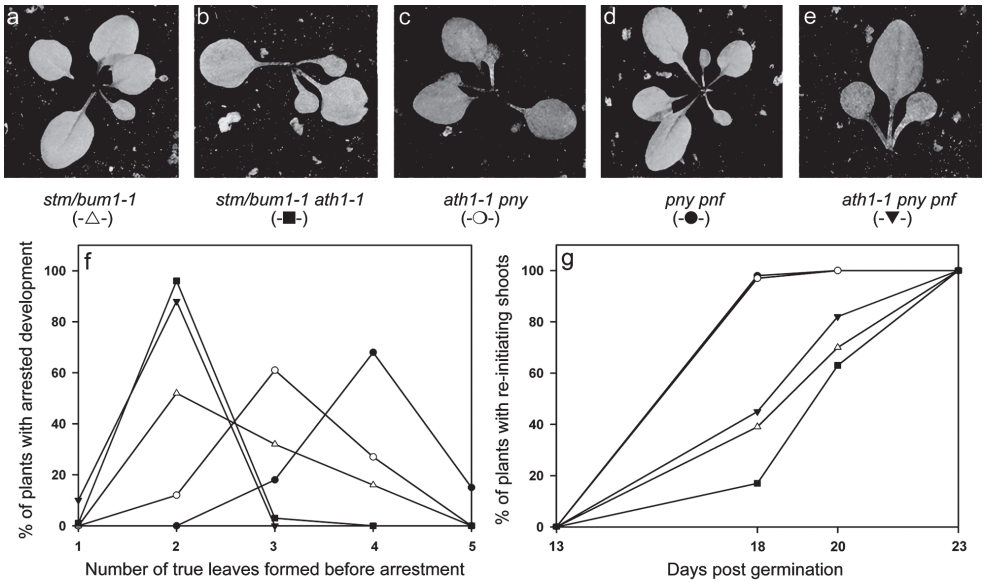


Figure 2.2 Maintenance of vegetative SAM function in Arabidopsis requires combinatorial activity of ATH1, PNY and PNF.
(a-e) SAM termination phenotypes in 8 to 13-day-old *stm-bum1-1* (a), *ath1-1 stm-bum1-1* (b), *ath1-1 pny40126* (c), *pny-40126 pnf-96116* (d), and *ath1-1 pny-40126 pnf-96116* (e) mutant plants. (f) Distribution per genotype of the leaf number at the moment of meristem termination. (g) Shoot re-initiation rate in arrested mutants.
Symbols in the graphs (f,g) correspond to the ones below the pictures (a-e).

ATH1 contributes with STM to SAM maintenance

ATH1 interacts with STM, BP/KNAT1, and KNAT6, all of which have been implicated in SAM initiation and/or maintenance (Supplemental Figure 2) (Cole *et al.*, 2006; Hackbusch *et al.*, 2005). To address the functional relevance of these interactions, we analyzed vegetative development of the respective double mutants.

Except for mild organ separation defects, *ath1* plants display a growth pattern comparable to wild-type plants when grown under standard conditions (Supplemental Figure 3) (Gomez-Mena and Sablowski, 2008; Proveniers *et al.*, 2007). Introduction of *bp-1*, *knat6-1*, or *knat6-2* in the *ath1-1* background did not significantly affect this growth pattern (not shown). In contrast, combination of *ath1-1* with the weak *stm* allele *bumbershoot1-1* (*stm-bum1-1*) severely enhanced the vegetative *stm-bum1-1* phenotype (Figure 2.2a,b,f,g), corroborating a role for ATH1 in vegetative SAM maintenance. The percentage of plants with terminated shoot growth increased from 28% in *stm-bum1-1* to 80% in double mutants. In

96% of the cases this occurred after the development of just two leaves, compared to two (52%), three (32%) or four (16%) leaves in *stm-bum1-1* (Figure 2.2a,b,f). Following developmental arrest, *ath1-1 stm-bum1-1* plants were capable of shoot reinitiation, although at a slower rate than *stm-bum1-1* plants (Figure 2.2g).

Post-germination SAM function requires the presence of ATH1, PNY and PNF

Intriguingly, *ath1-1* mutants seem to be able to recover from initial meristem defects (Figure 2.1b; 3a) as the size and structure of *ath1-1* inflorescence meristems (IM) did not fundamentally differ from those of control plants (Figure 2.4a,b).

An evolutionary tree based on full-length BELL protein sequences positions ATH1 in one sub-clade with PNY and PNF (Roeder *et al.*, 2003). Moreover, these three proteins share the same set of KNOX proteins as interaction partners (Supplemental Figure 2). To investigate whether they have overlapping functions during SAM maintenance and/or initiation, double and triple mutant combinations of *ath1-1*, *pny-40126*, and *pnf-96116* were analyzed for SAM defects.

As reported before, *pny* and *pnf* single mutant development progressed without obvious SAM termination defects (Supplemental Figure 3) (Bhatt *et al.*, 2004; Byrne *et al.*, 2003; Smith *et al.*, 2004; Smith and Hake, 2003). Accordingly, in both cases SAM size and organization was not affected throughout development (Figures 3a,c,d; 4a,c,d).

Whereas *ath1 pnf* double mutants were indistinguishable from *ath1* mutants (Figures 3g, 4f) (Supplemental Figure 3), combinations of both *ath1 pny* and *pny pnf* mutant resulted in SAM defects (Figure 2.2c,d,f). In 43% of *ath1 pny* seedlings and 34% of *pny pnf* seedlings, SAMs terminated between 8-13 dpg after the formation of two to four or five normal rosette leaves, respectively (Figure 2.2f). In agreement with the observed defects, in vegetative *ath1 pny* SAMs a further reduction in the number of meristematic cells was observed when compared to *ath1* plants (Figure 2.3a,e). Similarly, and in line with previous observations (Smith *et al.*, 2004), also *pny pnf* SAMs showed a reduction in the number of meristematic cells (Figure 2.3f).

After a 5-12 day developmental arrest, in both *ath1 pny* and *pny pnf* mutants new leaves were initiated (Figure 2g). In *pny pnf* these originated from the axils of pre-existing leaves, while in *ath1 pny* leaves also developed from petiole tissue. In addition, *ath1 pny* leaves were often aberrant in shape and included trumpet-shaped leaves and several types of leaf-leaf fusions (Supplemental Figure 4). While *pny pnf* double mutants fail to complete floral evocation (Smith *et al.*, 2004) (Figure 2.5d), all *ath1 pny* plants eventually flowered. Inflorescence internode length showed more variation than in wild-type plants (not shown), but

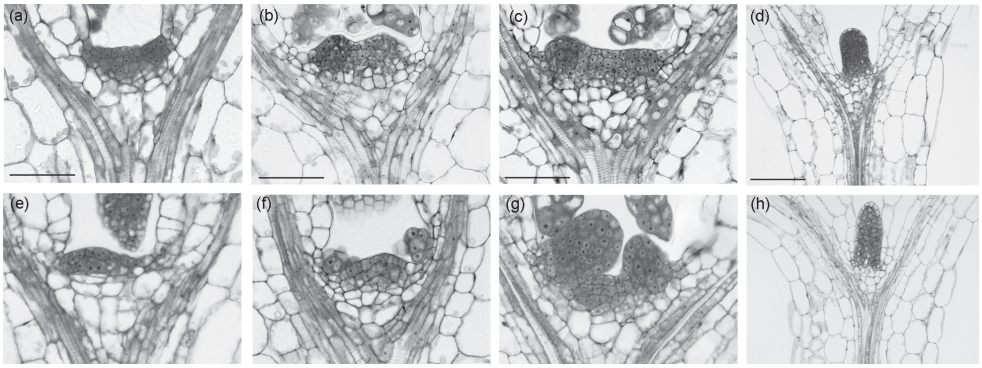


Figure 2.3 ATH1, PNY and PNF are redundantly required for vegetative SAM maintenance. (a-h) Longitudinal median sections through vegetative apices of 4-day-old *ath1-1* (a), *pny-40126* (b), *pnf-96116* (c), *stm-bum1-1* (d), *ath1-1 pny40126* (e), *pny-40126 pnf-96116* (f), *ath1-1 pnf-96116* (g), and *ath1-1 pny-40126 pnf-96116* (h) plants. Bar = 50µm (a-c,e-g)/100µm (d,h).

clusters of siliques or cauline leaves, typical for *pny*, or reiterative shoot formation, as in *stm-bum1-1*, were never observed (Figure 2.5a,b,g). Flowers produced a full complement of floral organs, but fertility was often significantly reduced due to incomplete stamen elongation (Figure 2.5j). Like both single mutants, the *ath1 pny* IM displayed a wild type-like organization (Figure 2.4a,e).

Severe enhancement of SAM phenotypes was observed in *ath1 pny pnf* triple mutants. Over a period of 5-13 dpg, all plants terminated after the formation of maximum two leaves (Figure 2.2e,f). In about 11% of the triple mutants the angle between the cotyledons was changed (Figure 2.2e), a phenotype we occasionally (<1%) also observed in *stm-bum1-1* and *ath1 pny* plants. As can be seen in Figure 2.3h, in *ath1 pny pnf* plants SAM termination resulted from complete consumption of meristematic cells by a single, central leaf primordium, similar as in *stm-bum1-1* (Figure 2.3d). Nevertheless, after a 10-18 day arrest triple mutants were able to reinitiate leaves from ectopic positions (Figures 2g), often aberrant in shape. Remarkably, removing *ATH1* from *pny pnf* plants restored the capability to undergo inflorescence development. All *ath1 pny pnf* plants formed several inflorescences from reinitiated shoots, although later than in wild-type, single mutant, or *ath1 pny* plants,. Similar to *stm-bum1-1* and *pny* (Bhatt *et al.*, 2004; Byrne *et al.*, 2003; Smith and Hake, 2003), no spiral phyllotaxy was evident and cauline leaves often clustered together (Figure 2.5a,f,g,h). Flowers were never observed. Instead at apical positions clusters of leaf-like structures were formed in triple mutant plants (Figure 2.5f,h,m). Often these displayed ectopic carpelloid features (Figure 2.5n,o). Eventually, all inflorescences terminated prematurely, often with central sepal-like organs, but always new inflorescences were initiated (Figure 2.5h). In accordance, we failed to detect proper IMs in *ath1 pny pnf* inflorescences, whereas the apical leaf-like structures could be clearly recognized (Figure 2.4g).

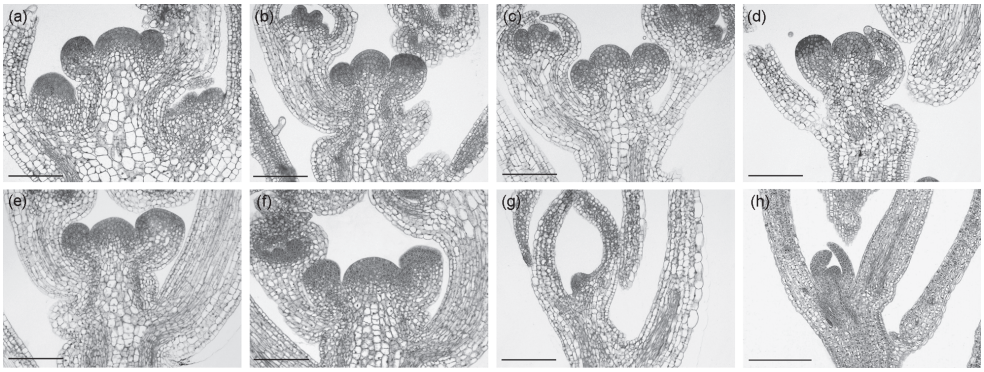


Figure 2.4 ATH1, PNY and PNF are redundantly required for floral meristem establishment.
(a-h) Longitudinal cross-sections through inflorescence apices of 22 to 35-day-old wild-type (a), *ath1-1* (b), *pny-40126* (c), *pnf-96116* (d), *ath1-1 pny40126* (e), *ath1-1 pnf-96116* (f), *ath1-1 pny-40126 pnf-96116* (g), and *stm-bum1-1* (h) plants. Bar = 100µm
Inflorescences were harvested just after bolting when they measured 1-1.5 cm.

Similar structures were observed in sections of *stm-bum1-1* inflorescences where they enclose floral buds (not shown) or organized meristem-like tissue flanked by leaf primordia (Figure 2.4h). In triple mutants in such positions only ectopic meristem tissue/primordia lacking clear organization could be distinguished (Figure 2.4g). Floral primordia or buds were never observed.

ath1 pny pnf mutants are clearly more severely affected with respect to

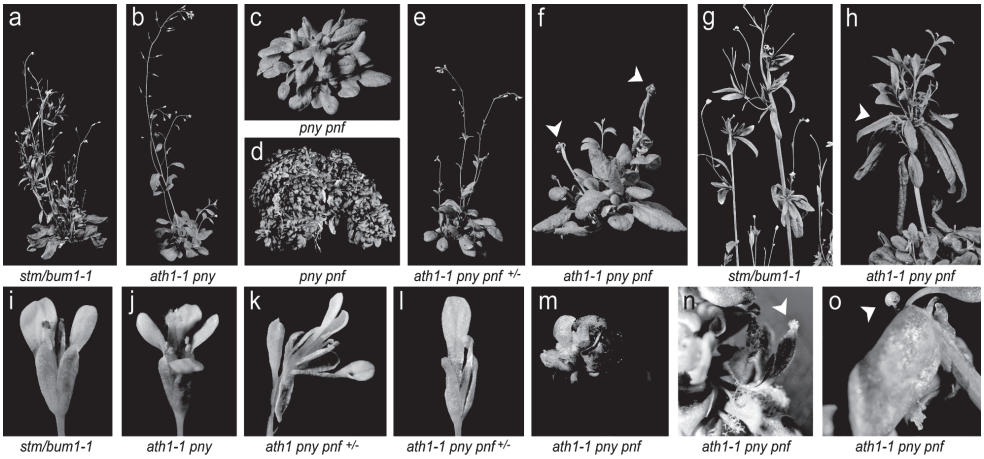


Figure 2.5 Reproductive development of plants lacking combinations of ATH1, PNY and PNF.
(a) *stm-bum1-1*.
(b) *ath1-1 pny-40126*.
(c) Non-flowering *pny40126- pnf-96116* plant, same age as (B).

(d) >1-year-old *pny-40126 pnf-96116* plant, lacking inflorescence development.
(e) *ath1-1 pny-40126 PNF/pnf-96116*. Note the reduction in flower number when compared to (B).
(f) *ath1-1 pny-40126 pnf-96116* same age as (B) and (C), showing inflorescence development. Arrows indicate apical terminating clusters of leaf-like structures.
(g) Close-up of *stm-bum1-1* inflorescence.
(h) Close-up of *ath1-1 pny-40126 pnf-96116* inflorescence, showing clustered cauline leaves (arrow) and reinitiated inflorescences terminated with a central sepal-like organ.
(i) Typical *stm-bum1*-1 flower lacking a central gynoecium. Also sepal and petal numbers are reduced.
(j) *ath1-1 pny* flower. Due to impaired elongation of stamens only 4 out of 6 stamens are visible.
(k,l) *ath1-1 pny-40126 PNF/pnf-96116* flowers lacking a central gynoecium, showing mild (k) to severe (l) additional floral phenotypes. The central stamen in (l) shows basal petaloid characteristics.
(m) Close-up of *ath1-1 pny-40126 pnf-96116* inflorescence apex from (f), showing a knot of apical leaf-like tissue.
(n,o) *ath1-1 pny-40126 pnf-96116* inflorescences bearing carpelloid structures (arrows) such as ovule tissue (n) and stigmatic tissue (o).

floral development than *stm-bum1-1* plants. However, floral phenotypes typically found in *stm* mutants, such as the lack of a central gynoecium, (Clark *et al.*, 1996; Endrizzi *et al.*, 1996; Scofield *et al.*, 2007), were observed in *ath1 pny PNF/pnf* plants (Figure 2.5i,k,l). This points to a dosage requirement for PNF in floral development. The severity of floral defects in *ath1 pny PNF/pnf* plants increased acropetally, and ranged from flowers with the gynoecium replaced by a central filament (not shown) to flowers lacking a fourth whorl altogether (Figure 2.5k). In the latter, in the outer three whorls a large variation in organ number was seen, and often chimaeric organs were present (Figure 2.5l).

Embryonic SAM initiation relies on ATH1, PNY and PNF

All studied double and triple mutant BELL combinations displaying SAM termination phenotypes are capable of forming at least one leaf at an apical position after germination. Therefore, it is unclear whether the observed defects are exclusively caused by progressive post-embryonic consumption of the initial SAM by the developing leaf primordia or by combination of embryonic SAM initiation defects and post-embryonic SAM maintenance defects.

Both *pnf* (not shown) and *ath1-1 pnf* embryos were able to initiate a dome-shaped SAM of similar dimensions as in wild type (Figure 2.6a,d). However, embryonic SAM phenotypes were observed in *ath1*, *pny*, and *ath1 pny* embryos, and in the offspring of *pny/PNY pnf* (~25%), *pny PNF/pnf* (~25%), and *ath1 PNY/pny pnf* (~25%) plants (Figure 2.6b,c,e,f,h). In all cases the number of cells in the embryonic meristem was reduced. In addition, in all combinations containing the *ath1-1* mutation and in the presumable *stm-bum1-1* embryos the meristem appeared

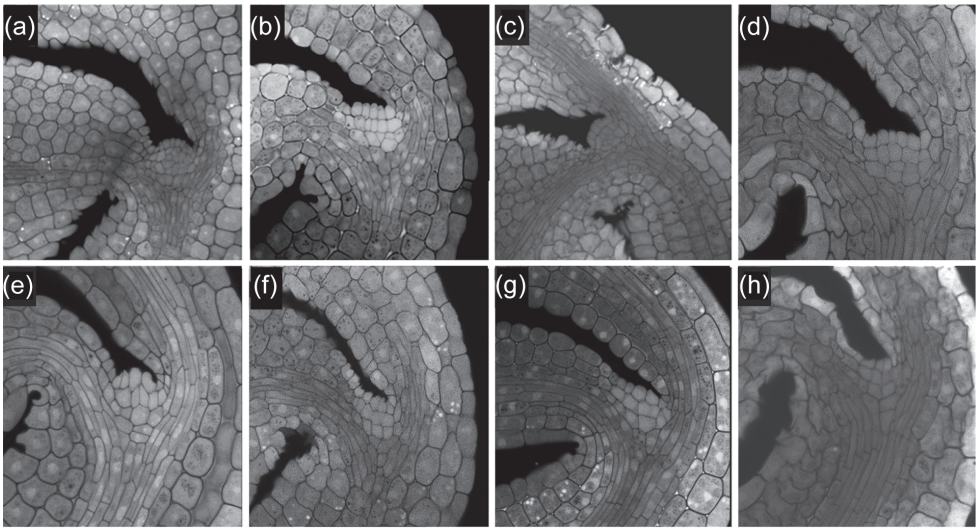


Figure 2.6 ATH1, PNY and PNF are redundantly required for embryonic SAM establishment.
(a-h) Confocal Laser Scanning Microscopy Images of Arabidopsis Embryos showing the SAM region of wild-type (a), *ath1-1* (b), *pny-40126* (c), *ath1-1 pnf-96116* (d), *pny-40126 pnf-96116* (e), *ath1-1 pny40126* (f), *stm-bum1-1* (g), and *ath1-1 pny40126 pnf-96116* (h) embryos.

more flattened and consisted of larger cells than a wild-type embryonic meristem (Figure 2.6b,d,f,g,h). In about one quarter of the *pny PNF/pnf* offspring, cells in the shoot meristem area were also enlarged, but they still composed a dome-shaped SAM. The more severely affected embryos were among the *ath1 pny* and *ath1 PNY/pny pnf* offspring, consistent with displayed post-germination phenotypes. In these embryos SAM phenotypes were highly comparable to those observed in the presumptive *stm-bum1-1* embryos (Figure 2.6f,g,h). The observed partial loss of an embryonic shoot meristem in mutants that lack different combinations of ATH1, PNY, and PNF, shows that these three BELL proteins also contribute to embryonic SAM initiation. Based on the differences in severity of the observed defects in *ath1 pnf, pny pnf*, and *ath1 pny* single mutants, ATH1 and PNY most likely contribute to this process to a larger extend than PNF.

Taken together, our findings suggest that proper shoot meristem function requires the combined activities of the BELL proteins ATH1, PNY, and PNF throughout Arabidopsis development.

BELL domains harbor conserved putative leucine-rich NES sequences and interact with the Arabidopsis nuclear export receptor AtCRM1

Cooperative interactions of BELL-KNOX complexes, including ATH1-STM and PNY-STM, are known to mediate nucleo-cytoplasmic partitioning of the respective heterodimers (Bhatt *et al.*, 2004; Cole *et al.*, 2006; Kimura *et al.*, 2008; Lee *et al.*, 2008). Since STM is devoid of a functional NLS, the BELL moiety likely forms the driving force behind targeting of BELL-STM dimers to the nucleus (Cole *et al.*, 2006). Therefore, depletion of functional nuclear-localized BELL-STM complexes in the SAM is a plausible explanation for the observed *stm*-like defects in SAM initiation and maintenance in *ath1 pny pnf* plants.

Nucleo-cytoplasmic partitioning of proteins is often determined by the relative accessibility of nuclear export signals (NES) and/or nuclear localization signals (NLS) (Merkle, 2004). NES sequences are short motifs that were originally characterized as a series of leucines arranged in a characteristic spacing pattern: L-x2/3-L-x2/3-L-x-L (where x is any residue). Many NESs have been identified, including those in Arabidopsis proteins (Haasen *et al.*, 1999; Kotak *et al.*, 2004; Subramanian *et al.*, 2004; Zeidler *et al.*, 2004) and it is now clear that bulky hydrophobic amino acids, including isoleucine, valine, methionine, and phenylalanine can be present instead of leucine at key positions (la Cour *et al.*, 2004). This type of NES sequences is specifically recognized by the nuclear export receptor CRM1/exportin-1 (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Kudo *et al.*, 1997; Kurant *et al.*, 1998; Ossareh-Nazari *et al.*, 1997; Rieckhof *et al.*, 1997; Stade *et al.*, 1997). In the nucleus, CRM1/exportin-1 binds to leucine-rich NES containing proteins, and in a multi-step process the targeted proteins are translocated to the cytoplasmic side of the nuclear pore complex, and then released into the cytoplasm (Merkle, 2004).

Using the split-YFP system, we were able to confirm that ATH1 and STM efficiently drive each other into the nucleus in plant cells (Figure 2.7a-e). Our finding that solitary eGFP:ATH1 does not end up solely in the nucleus (Figure 2.7b,f), and similar findings for other solitary expressed BELL proteins (Cole *et al.*, 2006), indicate the presence of an active export mechanism that relocates nuclear localized BELL proteins back into the cytoplasm.

Members of the BELL family show very weak homology outside the conserved SKY-, BELL- and homeodomains (Becker *et al.*, 2002; Bellaoui *et al.*, 2001). Hence, if present, a conserved, leucine-rich NES is expected to be located in one of these three domains. From a mechanistic point of view, such a conserved NES sequence is expected to coincide with the KNOX interacting domain. Although combined SKY and BELL domains have been referred to as MEINOX-interacting domain, single BELL domains are sufficient for heterodimerization with KNOX

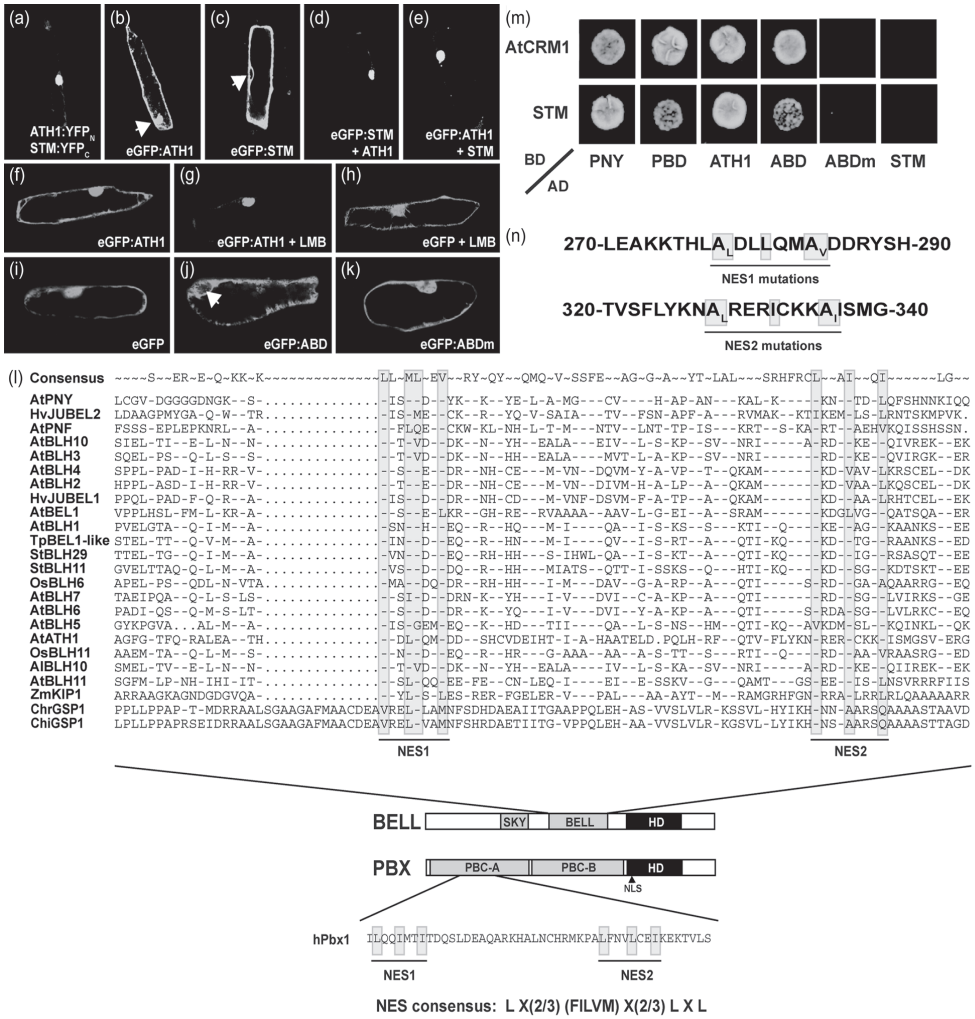


Figure 2.7 A conserved CRM1-dependent nuclear export mechanism is involved in controlling plant TALE homeodomain protein sub-cellular localization.

(a-k) Confocal Z-stacks of onion epidermal cells expressing fluorescent tag-fused proteins.

(a) Reconstitution of YFP as a result of ATH1 and STM heterodimerization. Strong fluorescence in the nucleus indicates the prevailing presence of STM-ATH1 dimers in the nucleus.

(b) eGFP:ATH1 localizes to the nucleus (arrow) and the cytoplasm.

(c) eGFP:STM localizes to the cytoplasm. Note the eGFP-depleted nucleus (arrow).

(d) Nuclear localization of eGFP:STM when co-expressed with non-tagged ATH1.

(e) Nuclear localization of eGFP:ATH1 when co-expressed with non-tagged STM.

(f) as in (b)

(g) Complete nuclear localization of eGFP:ATH1 in the presence of 100 nM leptomycin B (LMB).

(h) Sub-cellular distribution of non-fused eGFP is not affected by 100 nM LMB. Compare to (i)

(i) Sub-cellular distribution of non-fused eGFP. Due to its small size eGFP can diffuse freely across the nuclear membranes.

(j) Sub-cellular distribution of the eGFP-fused ATH1 BELL domain (eGFP:ABD). Although the fusion protein is small enough to diffuse across the nuclear membranes (compare to (k)), eGFP:ABD resides in the cytoplasm. The arrow indicates the eGFP:ABD depleted nucleus.
(k) Sub-cellular distribution of an eGFP-fused ABD with mutations in the NES motifs (eGFP:ABDm). Mutations are as depicted in (n).
(l) Amino acid sequence alignment of BELL domains from multiple BELL proteins from both monocot and dicot species. Grey boxes indicate the conserved SKY and BELL domains, a black box indicates the homeodomain (HD). Conserved amino acids that meet the NES consensus are shaded in gray. For comparison, the NES-containing PBC-A domain sequence of human PBX1 (hPBX1), and the NES consensus are shown. (~) marks positions within the BELL consensus with amino acid variation. Amino acids corresponding to the consensus are marked with (-).
(m) Yeast two-hybrid interactions of full-length PNY and ATH1, the PNY and ATH1 BELL domains (PBD and ABD, respectively), and the ABD with mutated NES sequences (ABDm) with AtCRM1/AtXPO1a (CRM1) and STM.
(n) Partial protein sequence of the mutated ABD indicating the amino acid changes that were introduced to impair NES function of the conserved motifs (gray boxes) in the ABD. Numbers are corresponding to the first and last amino acid of the fragments, AX represents a modified amino acid, were X shows the original amino acid replaced by alanine (A).

proteins (Cole *et al.*, 2006; Muller *et al.*, 2001). Intriguingly, the majority of BELL domain sequences, of both mono- and dicot origin, harbor two stretches of highly conserved leucines, isoleucines or one of the other large hydrophobic amino acids, with a spacing characteristic of a leucine-rich NES (Figure 2.7l). In the BELL-related proteins from the green alga *Chlamydomonas* that were previously shown to translocate as heterodimers with the KNOX protein GSM1 from the cytosol to the nucleus (Lee *et al.*, 2008), the first of these two NES sequences can be found (Figure 2.7l).

CRM1/Exportin-1 activity is specifically inhibited by leptomycin B (LMB) (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Kudo *et al.*, 1999; Kudo *et al.*, 1998; Wolff *et al.*, 1997). Arabidopsis contains two genes encoding highly similar CRM1/Exportin-1 orthologues, designated AtCRM1/AtXPO1a (At5g17020) and AtXPO1b (At3g03110) (Haasen *et al.*, 1999; Merkle, 2001; Merkle, 2004), the first of which has been shown to function as a LMB-sensitive nuclear export receptor in Arabidopsis (Haasen *et al.*, 1999).

Interestingly, LMB causes complete nuclear translocation of ATH1-fusion protein in cells expressing eGFP:ATH1 (Figure 2.7g), similar to as when co-expressed with STM (Figure 2.7a,e). Solitary eGFP, on the other hand, was not affected by LMB treatment (Figure 2.7h). This suggests that BELL proteins are targeted by a CRM1-dependent nuclear export mechanism.

This hypothesis was further explored by testing the capacity of ATH1 and PNY to interact with CRM1. As shown in Figure 2.7m, both PNY and ATH1 were able to interact with AtCRM1/AtXPO1a in a yeast system. Since the putative NES sequences are located in the BELL domain, we further tested for interactions between AtCRM1/AtXPO1a and ATH1 and PNY BELL domains. Importantly,

both BELL domains interacted with AtCRM1 (Figure 2.7m). In addition, these domains were sufficient for interaction with STM, meaning that they harbor both the AtCRM1/AtXPO1a interaction site and the STM binding site (Figure 2.7m).

ATH1 BELL domain NES sequences are functional in plant cells

To determine whether the two conserved BELL NES sequences in the ATH1 BELL domain (ABD) are functional, an ABD:eGFP fusion protein was transiently expressed in onion epidermal cells. The size exclusion limit for bidirectional diffusion through nuclear pore complexes is estimated to be 40–60 kDa (Gorlich and Mattaj, 1996; Subramanian *et al.*, 2004). Therefore, both eGFP (29 kDa) and ABD:eGFP (42 kDa) can potentially diffuse over the nuclear membrane. As expected, single eGFP protein was evenly distributed over the cytoplasm and the nucleus (Figure 2.7i). However, ABD:eGFP was excluded from the nucleus (Figure 2.7j). Point-mutations disrupting the NES consensus sequences (eGFP:ABDm) resulted in redistribution of the fluorescence pattern over both cytoplasm and the nucleus (Figure 2.7k), meaning that fusion of ABD to eGFP did not impair the nuclear localization of eGFP due to size exclusion and that the ABD NESs are functional.

Next, we tested whether these NES mutations also affected the interaction between the ATH1 BELL domain with AtCRM1. As can be seen in Figure 2.7m, the ATH1 BELL domain with both NESs mutated was not able to interact with AtCRM1. Moreover, these mutations also impaired the ability to interact with STM (Figure 2.7m), indicating that the interaction domains of STM and AtCRM1 in the ATH1 BELL domain physically overlap.

Taken together, these findings imply a competition model in which BELL-STM heterodimerization interferes with AtCRM1 recognition and subsequent nuclear export of BELL proteins. As a result, BELL-STM complexes accumulate in the nucleus.

Discussion

SAM maintenance requires a tightly controlled balance between renewal of the central positioned stem cell pool and the division rate of stem cell daughters that provide organ founder cells. In Arabidopsis, the WUSHEL-CLAVATA feedback loop continuously adjusts the size of the shoot stem cell pool (reviewed in Williams and Fletcher (2005), whereas the KNOX TALE HD protein STM acts as a suppressor of differentiation throughout the meristem and regulates cell division

rate in the transit amplifying stem cell daughter zone (Byrne *et al.*, 2000; Lenhard *et al.*, 2002; Long and Barton, 2000; Long *et al.*, 1996). STM is not only essential for SAM maintenance, but also for SAM initiation during embryogenesis (Barton and Poethig, 1993; Clark *et al.*, 1996; Endrizzi *et al.*, 1996; Long *et al.*, 1996).

Here we show that, throughout the life cycle, Arabidopsis SAM function, including initiation of floral meristems, requires the combinatorial action of three closely-related BELL-family TALE HD proteins: ATH1, PNY, and PNF. Since these proteins are capable of forming heterodimers with STM, this suggests that STM recruits different BELL proteins to ensure proper meristem function during above-ground plant development.

BELL-KNOX combinatorial control of SAM function

In animals, heterodimerization of MEIS- and PBC-class TALE HD proteins is usually mandatory for stability, nuclear translocation, and, hence, functionality of these proteins (Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999; Jaw *et al.*, 2000; Longobardi and Blasi, 2003; Mercader *et al.*, 1999; Rieckhof *et al.*, 1997; Stevens and Mann, 2007; Waskiewicz *et al.*, 2001). As reported by Cole and co-workers (2006), STM lacks an efficient NLS and observations made by both these authors and us (Figure 2.7) suggest that STM resides in the cytoplasm by default. In contrast, ATH1 and PNY can be found in cytosol and nucleus (Figure 2.7) (Cole *et al.*, 2006), likely as result of the nuclear exclusion mechanism that operates on them. Upon heterodimerization, these BELL and KNOX proteins become completely nuclear (Figure 2.7) (Bhatt *et al.*, 2004; Cole *et al.*, 2006). As such, STM, on the one hand, and ATH1, PNY and, most likely, also PNF on the other hand, are interdependent for stable nuclear localization, and, as a consequence, for functionality. Therefore, the *stm*-like phenotypes in *ath1 pny pnf* triple mutants are most likely directly due to the lack of nuclear-localized STM-complexes in the SAM, although we cannot exclude that reduced function of the STM-relatives BP/KNAT1 and KNAT6 also contributed to these meristem defects. Actually, the situation might be even more complex, since there is accumulating evidence suggesting that BELL proteins, including ATH1 and PNY, not only affect KNOX activity through heterodimer formation with KNOX proteins, but also by spatially restricting KNOX gene expression (Gomez-Mena and Sablowski, 2008; Kumar *et al.*, 2007; Ragni *et al.*, 2008).

As suggested by the recent observation that ectopic activity of BLH1 can be suppressed by introduction of a *knat3* mutation (Pagnussat *et al.*, 2007), the formation of BELL-KNOX heterodimers to compose functional complexes is a common theme in plant development. The findings of these authors further indicate that BELL proteins are not merely chaperones to guide KNOX proteins to the nucleus, in agreement with what is known for animal PBC-MEIS complexes (Ste-

vens and Mann, 2007). Therefore, the BELL proteins studied here most likely operate as functional units together with STM, where different combinations have both unique and overlapping targets. This is corroborated by over-expression experiments in transgenic Arabidopsis plants, that imply that different STM-BELL heterodimers contribute different functions (Cole *et al.*, 2006). Clear phenotypic differences between individual single mutants and between the various double mutant combinations also point to overlapping and specific functions of ATH1, PNY and PNF in the SAM. Thus, whereas STM seems responsible for differences between meristematic and primordial cell identity (Byrne *et al.*, 2000; Byrne *et al.*, 2002; Cole *et al.*, 2006), different BELL partners in the SAM most likely account for, currently poorly characterized, zone-specific functions of STM in this process. In this light, it will be challenging to determine the exact localization of specific BELL-STM dimers in the SAM and to identify their specific and overlapping targets. Such information is essential to understand the function of the various TALE HD heterodimers in shoot meristem function in general.

Mechanistic conservation of a NES-based nuclear exclusion mechanism

Despite lack of unambiguous evidence for evolutionary conservation of PBC and BELL proteins (Becker *et al.*, 2002), it has been suggested that the animal PBCA/B domain finds its counterpart in the plant BELL domain (Bellaoui *et al.*, 2001; Cole *et al.*, 2006). This is based on the mechanistic conservation of protein-protein interactions of MEIS/KNOX with PBC/BELL and the interdependence of both these animal and plant TALE HD proteins for heterodimerization to become nuclear. Here we show that this mechanistic conservation can be extended, since the BELL domain contains two functional NESs that harbor conserved sequences essential for both recognition by AtCRM1/AtXPO1a and for interaction with STM. Intriguingly, animal PBC proteins are capable of interacting with both MEIS proteins and CRM1 via a single conserved contact surface in the PBC-A domain (Berthelsen *et al.*, 1999; Berthelsen *et al.*, 1998; Kilstrup-Nielsen *et al.*, 2003). The latter results in nuclear exclusion of PBC proteins, whereas PBC-MEIS interactions cause nuclear accumulation of resulting dimers by masking of NESs in the PBC A/B domain from CRM1 (Kilstrup-Nielsen *et al.*, 2003). Analogously, in plants BELL-KNOX interaction likely precludes AtCRM1/AtXPO1a-recognition of and interaction with BELL proteins. Driven by a yet unidentified NLS present in the BELL-moiety, BELL-KNOX heterodimers then accumulate in the nucleus according to a similar principle.

Recently, also in green algae the mechanism of nuclear import upon heterodimerization of otherwise cytoplasmic BELL-related and KNOX proteins has been described (Lee *et al.*, 2008). Of the two conserved NES sequences present in

the BELL domains of higher plant BELL proteins, the first one seems to be also conserved in the *Chlamydomonas* BELL-related proteins (Figure 2.7) and is located in the KNOX-interaction domain (Lee *et al.*, 2008). Database searches revealed that the *Chlamydomonas reinhardtii* genome contains at least one CRM1-homologue (GenBank: XM_001695542). As such, also the NES-based nuclear exclusion mechanism involving CRM1 might be mechanistically conserved.

In conclusion, a similar mechanism is active in plant, algae and animal cells to control the cellular localization of BELL(-related) and PBC proteins, respectively, implying a functional convergence of BELL(-related) and PBC proteins in controlling TALE HD protein activity.

ATH1 and PNY/PNF have opposite roles in determination of SAM phase identity

In addition to their established role in meristem initiation and maintenance, ATH1, PNY and PNF have also been proposed to function as competency regulators of the SAM in the process of floral evocation. By controlling expression of the floral pathway integrator (FPI) gene *FT* and the floral meristem identity (FMI) genes *LFY*, *AP1* and *CAL*, PNY and PNF act as positive regulators of meristem competence. *pny pnf* plants respond normally to floral inductive signals, but the *pny pnf* SAM fails to complete floral evocation, as internode and floral patterning events do not take place (Kanrar *et al.*, 2008; Smith *et al.*, 2004). During vegetative development FPI and, consequently, FMI gene levels are kept low by the activity of floral repressors, such as FLC. We recently identified ATH1 as a negative regulator of flowering time through positive regulation of *FLC* expression (Proveniers *et al.*, 2007). As such, ATH1 and PNY/PNF are antagonists in flowering time control. Interestingly, antagonistic and synergistic interactions have also been reported for the KNOX proteins BP/KNAT1 and KNAT6 (Belles-Boix *et al.*, 2006; Byrne *et al.*, 2002; Ragni *et al.*, 2008). The observed restoration of inflorescence development in *pny pnf* plants after introduction of an *ath1* mutation is in line with the reported roles for ATH1, PNY and PNF in floral competency control. However, vernalization treatments known to stably repress *FLC* expression have no effect on floral transition in *pny pnf* plants (our observations) (Smith *et al.*, 2004). Therefore, the exact nature of the antagonistic relationship between ATH1 and PNY/PNF in this process is currently not clear.

Taken together, BELL proteins are not only indispensable for initiation and maintenance of the SAM but also for determination of SAM phase identity. Whether the latter also involves combinatorial control by BELL-KNOX dimers, remains to be examined.

ATH1, PNY and PNF have both overlapping and opposite roles in inflorescence development

In *pny pnf* plants internode development is severely impaired (Smith *et al.*, 2004). Even after floral stimulation, these plants lack cell expansion in the rib zone, the area from which internode tissue originates. In *ath1* mutants rib zone cells are more elongated than in the wild-type (Figure 2.2.1) and ATH1 has recently been proposed to act as a growth modulator at the interface between stem and SAM (Gomez-Mena and Sablowski, 2008). Most importantly, internode elongation is, at least partially, restored in *ath1 pny pnf* plants. This suggests that also in the process of internode patterning ATH1 and PNY/PNF act as antagonists. Such an antagonism is further supported by *pny*-like inflorescence phenotypes in mild *ATH1* overexpressor plants (Supplemental Figure 5).

Even though in *ath1 pny pnf* mutants the ability to develop an inflorescence with elongated internodes is restored, floral patterning events do not take place. Previously, PNY and PNF have been shown to regulate aspects of floral specification (Bao *et al.*, 2004; Kanrar *et al.*, 2006; Smith *et al.*, 2004). *ATH1* is expressed at late stages of developing stamens and carpels (Gomez-Mena and Sablowski, 2008), but it is not clear whether ATH1 is actually involved in the development of reproductive organs. The enhancement of floral defects in *ath1 pny PNF/pnf* plants (Figure 2.5) when compared to those in *pny PNF/pnf* plants (our observations) (Kanrar *et al.*, 2006; Smith *et al.*, 2004), indicates that ATH1 is functional at later stages of flower development as well.

Experimental procedures

Plant Materials and Growth Conditions

Mutant alleles used were: *ath1-1* and *ath1-3* (Proveniers *et al.*, 2007), *pny-40126* (Smith and Hake, 2003), *pnf-96116* (Smith *et al.*, 2004), *stm-bum1-1* (Jasinski *et al.*, 2005), *knat6-1*, *knat6-2* (Belles-Boix *et al.*, 2006), and *bp-1* (Venglat *et al.*, 2002). Columbia-8 (Col-8) was used as wild type. Mutations present in other backgrounds were backcrossed three times to Col-8. *ath1 pny pnf* mutants were propagated from *ath1 PNY/pny pnf* parents. Plants were grown in long days (16 hrs light/8 hrs darkness) on soil as before (Proveniers *et al.*, 2007), or in petridishes (1x MS supplemented with 1% plant agar). For dexamethasone (Dex; Sigma) treatments, an appropriate amount of Dex (1mM in 95% EtOH) was added to the growth medium to arrive at the desired final concentration, or 1000x diluted in water with 0.2% (w/v) Silwet surfactant and applied by spraying.

Plasmid construction and yeast two-hybrid analysis

ATH1, PNY, PNF, STM, BP/KNAT1, KNAT2-7 and AtCRM1 open reading frames, as well as the ATH1 and PNY BELL domains, and the mutated ATH1 BELL domain were inserted in pDONR201 (Invitrogen) by Gateway™ recombination. ATH1 NES mutations were introduced using site-directed mutagenesis by the University of Texas Medical Branch (UTMB, Sealy Center for Molecular Science, Galveston, TX, USA). For yeast two-hybrid analysis the ProQuest™ Two-Hybrid System with Gateway™ Technology (Invitrogen) was used in combination with the yeast strain PJ694A (James *et al.*, 1996).

For the *35Spro:ATH1-HBD* construct, the hormone binding domain (HBD) coding sequence of the rat glucocorticoid receptor was PCR-amplified from pG795 (Schena *et al.*, 1991), adding 5′ SacI and 3′ MunI restriction sites. After SacI/MunI digestion, the fragment was ligated in the SacI/EcoRI-sites of the 35S-CaMV cassette (www.pgreen.ac.uk). Subsequently, a Gateway™ Reading frame A cassette (Invitrogen) was cloned upstream of the HBD sequence using the SmaI restriction site. The *ATH1* CDS was then inserted by Gateway™ recombination. Finally, the resulting 35S:ATH1-HBD cassette was isolated by EcoRV restriction digestion and ligated into the SmaI-site of pCAMBIA1300 (www.cambia.org). *35Spro:ATH1-HBD* lines were selected for displaying characteristic *ATH1* overexpression phenotypes (Cole *et al.*, 2006; Gomez-Mena and Sablowski, 2008) upon Dex treatment. Selected lines were also crossed to *ath1-1*.

Confocal microscopy and sectioning

For confocal microscopy mature embryos were stained with aniline blue and treated as in Bougourd et al. (2000).

For sectioning, plant material was fixed overnight in a solution containing 1% glutaraldehyde and 4% formaldehyde in 50mM sodium phosphate buffer, pH 7.2. Subsequently the plant material was dehydrated in gradual steps: 10%, 30%, 50%, 70%, 90% and 2x 100% ethanol. Infiltration and embedding in Technovite 7100 (Kulzer, Hereaus) was performed as instructed by the manufacturer. Longitudinal 4 µm sections were made on a Reichert-Jung 1140 rotary carrying a disposable Adamas steel knife. Sections were stained with 0.1% Ruthenium red (Sigma) in distilled water for 2-5 minutes at room temperature. SAM sizes (6-12 individual plants/genotype) were determined from median longitudinal sections using a VIDAS RT image analysis system (Zeiss/Kontron). SAM diameter is defined as the distance in a straight line between the cotyledonary boundaries. For the size of the subapical region the distance between the lower border of the population of more intensely stained, meristematic cells and the base where the hypocotyl vascular strands converge was determined.

Transient expression in onion epidermal cells

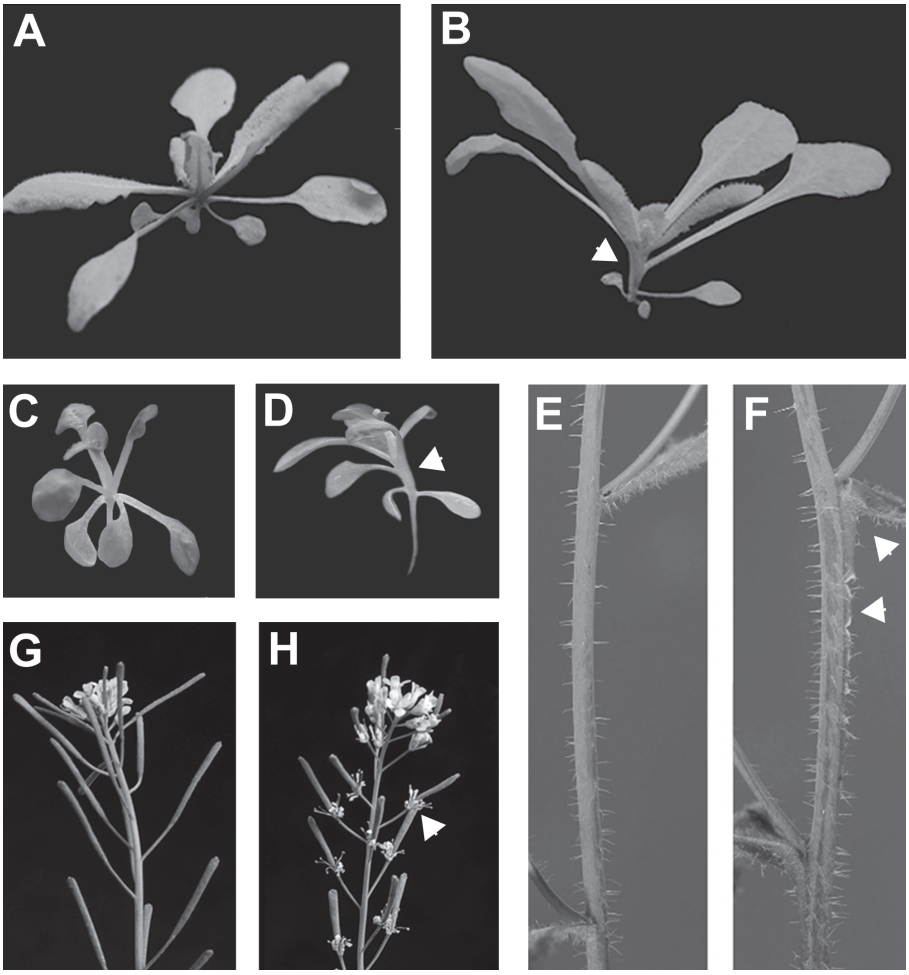
For N- and C-terminal GFP-fusions, pK7WGF2 and pK7FWG2 (Karimi *et al.*, 2002), respectively, were used. pARC233 (N-terminal YFP-halve) and pARC234 (C-terminal YFP-halve) were used for split-YFP fusions. Neither single ATH1-, nor single STM:eYFP$_{N/C}$ fusions resulted in detectable signal when transiently expressed. Also combinations with complementing non-chimeric eYFP-halves or non-interacting eYFP$_{N/C}$-linked proteins did not result in any detectable signal.

For transient expression, gold particles (1.0µm diameter; BioRad) were coated with DNA and delivered into onion epidermal cells using a helium-driven particle accelerator (PDS-1000/He; Bio-Rad) at 1100 psi rupture disc bursting pressure. Bombarded tissue was kept in Petri dishes on damp filter paper for 12–16 h in darkness. Epidermal peels were transferred to PBS-buffer containing microscope glass slides. For Leptomycin B (LMB) (1mM in ethanol, LC Laboratories) experiments, PBS-buffer was supplemented with 100nM LMB and tissue was incubated in darkness for 2 more hours.

Using a Zeiss Confocal Laser Scanning Microscope, median Z-stack projections were made through nuclei of fluorescing cells.

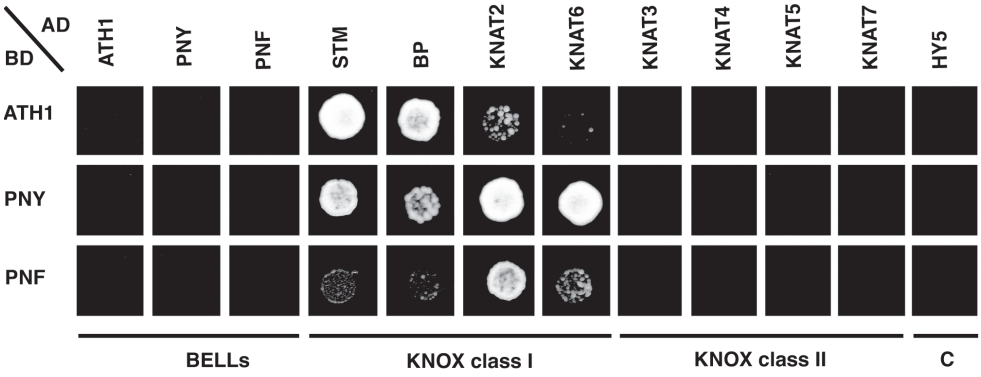
Acknowledgements

We thank Enric Belles-Boix and Véronique Pautot (INRA Versailles, France) for Arabidopsis KNOX and HY5 yeast two-hybrid clones and for *knat6-1* and *knat6-2* seeds; Richard Immink (Wageningen UR & Plant Research International, The Netherlands) for pARC233 and pARC234; Steven Spoel for pG795, the NASC and ABRC Arabidopsis Stock Centers for providing mutant seeds, and Frits Kindt and Ronald Leito for photography and artwork. We are grateful to The Netherlands Organisation for Scientific Research (STW-NWO) for funding this research (Project UPB.5562).

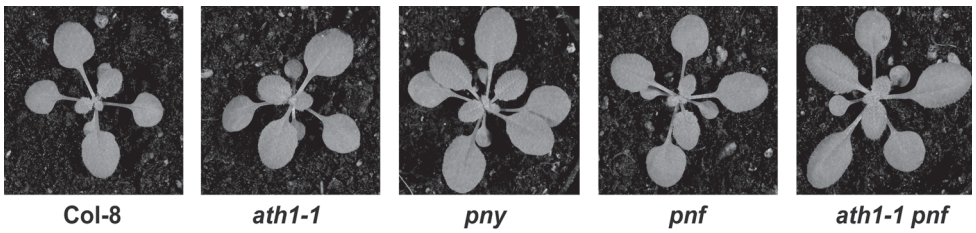


Supplemental Figure 1 Organ fusion phenotypes characteristic for *ath1* mutants.
(a) Rosette of Col-8 plant. Cotyledon and leaf petioles emerge from a central point and are clearly separated from each other and from central tissue surrounding the SAM.
(b) *ath1-1* mutant plant of same age as (a), showing extended fusions of leaf base/petiole tissue to each other and/or to central tissue, giving the impression of internode elongation during rosette growth.
(c) Plate-grown Col-8 plant with centrally emerging cotyledon and leaf petioles.
(d) Plate-grown *ath1-1* mutant with severe leaf petiole fusions. The arrow marks the point where the petiole of the first true leave separates from the fused tissue.
(e) Part of Col-8 inflorescence stem showing the bases of the first two cauline leaves.
(f) Part of *ath1-1* inflorescence as in (e) with cauline leaf-stem fusions. Upper arrow marks the margin of the cauline leaf that is fused at the base down along the stem. Lower arrow marks fused cauline leaf margin tissue torn in several places during subsequent stem elongation.

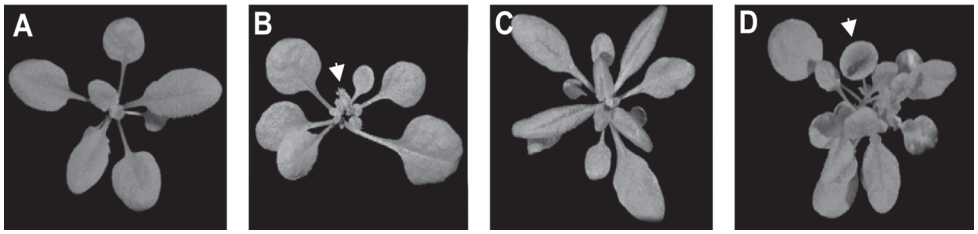
(g) Wild-type (Ler) inflorescence were sepals, petals and stamens normally separate from the developing fruit after pollination.
(h) *ath1-1* (Ler) inflorescence. In contrast to wild type (g), floral organ shedding does not occur after pollination as can be seen by the sepals, petals and stamen that remain attached to the developing fruit (arrow).



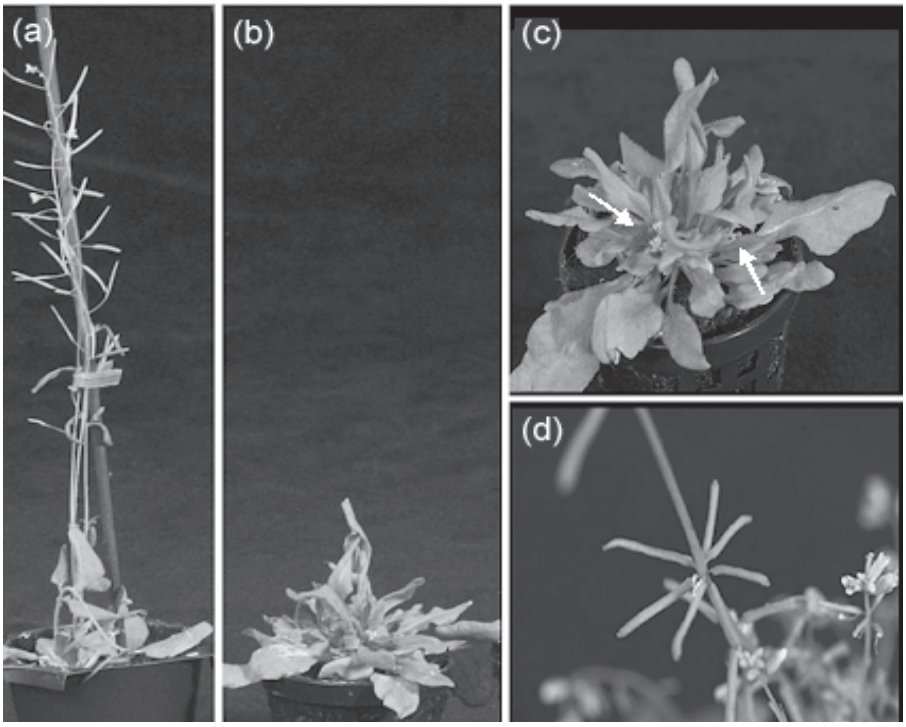
Supplemental Figure 2 BELL proteins ATH1, PNY and PNF interact with class I KNOX proteins.
ATH1, PNY and PNF were tested in a yeast two-hybrid system for mutual interactions and interactions with class I and II KNOX proteins. Full-length proteins were fused to the GAL4 DNA-binding domain (BD) and to the GAL4 transcriptional activation domain (AD) for pair wise yeast two-hybrid interaction analysis. As a negative control (C) the bZIP TF protein HY5 was used.



Supplemental Figure 3 Vegetative development of *ath1-1*, *pny-40126*, *pnf-96116*, **and** *ath1-1 pnf-96116* **mutants.**
From left to right: 15-day-old, long-day grown wild-type, *ath1-1*, *pny-40126*, *pnf-96116* and *ath1-1 pnf-96116* rosette plants. N.B.: The higher number of rosette leaves in *pny*, when compared to the other genotypes, produced in this growing period indicate a higher leaf initiation rate in this background.



Supplemental Figure 4 Vegetative development of *ath1-1 pny-40126* **double mutants.**
(a) Wild-type plant with leaves formed in a regular pattern from the central positioned SAM.
(b) After arresting, *ath1-1 pny-40126* plants start forming escape shoots from the presumptive SAM area in a disorganized way (arrow). Plant is of same age as (a).
(c) Wild-type plant at the start of bolting.
(d) *ath1-1 pny-40126* plant of same age as (c). Aberrant-shape leaves, including trumpet-shaped leaves (arrow), originate from both the presumptive SAM area and from petioles of existing leaves, causing a bushy appearance.



Supplemental Figure 5 Induced *ATH1* **expression affects whole-plant morphology.**
(a) Ethanol control-treated mature *35Spro:ATH1-HBD* plant. (b) 10μM Dex-treated *35Spro:ATH1-HBD* plant of same age as in (a) that lacks inflorescence internode elongation. (c) Same plant as in (b), arrows indicate non-elongating inflorescences. (d) Inflorescence of less severely-affected 10μM Dex-treated *35Spro:ATH1-HBD* plant with clusters of siliques.

Chapter 3

ATH1 interacts with *KNAT2* and *KNAT6* in basal organ boundary formation and control of stem growth

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Summary

Plant organogenesis results from the activity of the meristems. Lateral shoot organ primordia are initiated from the flanks of the shoot apical meristem (SAM). Patterning and elaboration of tissues is primarily achieved through coordination of differential growth in distinct domains, which are separated by morphological boundaries. Previously, it was found that the BEL1-like ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) TALE HD protein is important for SAM initiation and maintenance, the proper establishment of meristem-organ boundaries and stem elongation. In yeast two-hybrid systems, ATH1 associates with the class I KNOX TALE HD proteins KNAT2 and KNAT6. Here, we investigated the biological relevance of ATH1-KNAT2/6 interactions in relation to boundary establishment. We show that loss-of-function mutations in these *KNOX* genes result in defects at the cauline leaf-stem boundary, similar to those observed in *ath1* plants. This phenotype is greatly enhanced by combined loss of ATH1 and KNAT2/6. In addition, ectopic A*TH1* expression dramatically restricts the growth of the subapical region below the SAM. This effect is dependent on the presence of *KNAT6*, and to a lesser extent, *KNAT2*. As we further found that ATH1 is required for proper expression of these two *KNOX* genes, these results suggest that ATH1 acts in conjunction with *KNAT2* and *KNAT6* at multiple levels at the interface between both the SAM and lateral organ primordia, and between the SAM and the stem to establish proper basal organ boundaries and to control stem elongation.

Introduction

Plant shoot architecture is primarily established in the shoot apical meristem (SAM). Stem cells in the central zone (CZ) of the SAM generate multi-potent daughters that are partitioned to the peripheral region where they make up the organ founder cells that eventually become incorporated into lateral organ primordia (Fletcher, 2002a). Below the CZ, the rib meristem (RM) sustains stem growth. The continuous production of leaf-stem units during indeterminate growth is a coordinated effort of both SAM and RM. The RM is composed of small densely cytoplasmic cells that produce the ribs of vacuolated and elongated cells that make up the rib zone (RZ). Together, the RM and the RZ move the SAM upwards during plant development (Bernier et al., 1981; Harkess and Lyons, 1993).

Patterning and elaboration of tissues is primarily achieved through coordination of differential growth in distinct domains during organogenesis. These domains are separated by morphological boundary regions, which isolate cell groups with different identities (Aida and Tasaka, 2006). Organ-to-organ boundaries keep apart distinct constituent founder cells with determinate fate between adjacent developing organs. Meristem-to-organ boundaries, on the other hand, not just separate cells with indeterminate fate from determinate organ founder cell, but they also mediate communication between the meristem and lateral organs, which is crucial for both meristem maintenance and organ patterning (Aida and Tasaka, 2006).

Meristem-organ boundaries are further important determinants of plant architecture, since axillary meristems are initiated from leaf axils, which in turn develop from the boundary of leaf primordia and the shoot meristem (reviewed in Schmitz and Theres, 2005). Despite the importance of boundaries, a clear understanding of the molecular mechanisms that control their identity and function is lacking. Studies on boundary regions have focused mainly on their establishment at the interfaces between lateral organ primordia and at the interfaces between the meristem and initiating lateral organs (Tooke and Battey, 2003; Rast and Simon, 2008), while the interface between the SAM and the stem has received little attention (Sablowski, 2007).

Molecular genetic studies have identified a number of regulatory genes that act largely redundantly to control boundary formation during embryogenesis and post-embryonic development (reviewed in Rast and Simon, 2008). A number of these, including *BLADE ON PETIOLE1 (BOP1)/BOP2*, *LATERAL ORGAN BOUNDARIES (LOB), LATERAL SUPPRESSOR (LAS)*, *REGULATORS OF AXILLARY MERISTEMS (RAX),* the recently identified *LATERAL ORGAN FUSION 1* (*LOF1*)/*LOF2* genes, and the *CUP-SHAPED COTYLEDON11-3 (CUC1-3)* genes, encode transcription factors that are specifically expressed in the meristem-organ boundary area (Hepworth et al., 2005; Norberg et al., 2005; Hibara

et al., 2006; Ha et al., 2007; McKim et al., 2008; Lee et al., 2009). The CUC genes are probably the best studied boundary genes. They act redundantly as general boundary factors for both organ-organ and meristem-organ separation (Aida and Tasaka, 2006). During embryogenesis, the CUC genes redundantly control organ separation, such that single mutations in *CUC1*, *CUC2* or *CUC3* cause only subtle defects, whereas any combination of two *cuc* mutations results in fusion along the cotyledon margins and a dramatic cup-shaped cotyledon phenotype (Aida et al., 1997; Aida et al., 1999; Ishida et al., 2000; Takada et al., 2001; Vroemen et al., 2003). CUC2 and CUC3 also function to specify boundaries during post-embryonic development (Hibara et al., 2006; Raman et al., 2008). The *CUC* genes prevent fusion by locally repressing growth at the boundaries between individual organs and between the SAM and organ primordia (Takada et al., 2001).

Meristem-organ boundaries are proposed to function in preventing differentiation from depleting the central stem cell population. As a result, maintenance of these boundaries is an important aspect in SAM maintenance. In Arabidopsis, the class I Knotted1-like homeobox (*KNOX)* genes *SHOOTMERISTEMLESS* (*STM*), *BREVIPEDICELLUS/ KNOTTED1-LIKE ARABIDOPSIS THALIANA1* (*BP/ KNAT1*) and *KNAT6* have been identified as central regulators of SAM maintenance (Byrne et al., 2000; Byrne et al., 2002; Lenhard et al., 2002; Belles-Boix et al., 2006). Next to this, *STM* and *KNAT6* have been implicated to contribute to meristem-organ boundary formation, providing further evidence of a tight connection between organ separation and SAM maintenance. Whereas initial activation of *STM* and subsequent initiation of a SAM during early embryogenesis depends on the redundant activities of CUC1 and CUC2 (Aida et al., 1999; Takada et al., 2001), later during embryogenesis STM is required for proper spatial expression of *CUC2* to separate cotyledons (Aida et al., 2002). As a result, in *stm* mutants the petioles of the cotyledons are (partially) fused to each other (Clark et al., 1996; Long et al., 1996). Although no such defects can be observed in *knat6* mutants, in *stm knat6* double mutants petiole fusions are extended to the cotyledon lamina, indicating that KNAT6 contributes to cotyledon boundary establishment in embryos in the absence of STM (Belles-Boix et al., 2006). Consistent with this role, the expression domain of *KNAT6* in the SAM marks the boundaries between the SAM and cotyledons (Belles-Boix et al., 2006).

KNOX proteins interact with members of the BEL1-like (BELL) homeodomain transcription factor class in both Arabidopsis and other species (Bellaoui et al., 2001; Muller et al., 2001; Chen et al., 2003; Bhatt et al., 2004; Hackbusch et al., 2005; Cole et al., 2006; Kumar et al., 2007; Lee et al., 2008; Rutjens et al., 2009). Such BELL-KNOX interactions are required for site-specific DNA binding (Smith et al., 2002; Chen et al., 2004) and for nuclear localization of the transcription factor heterodimeric complex (Bhatt et al., 2004; Cole et al., 2006; Kimura et al., 2008; Rutjens et al., 2009). We recently demonstrated that heterodimerization-control-

led cellular localization of BELL and KNOX proteins involves an evolutionary conserved nuclear exclusion mechanism that probably acts generic to control the activity of BELL-KNOX heterodimers (Rutjens et al., 2009). Taken together, this strongly indicates that BELL-KNOX interactions are evolutionarily conserved and that their interaction is required for biological function.

Several studies have shown that STM interacts with two paralogous BELL proteins, PENNYWISE (PNY; also known as BELLRINGER, REPLUMLESS, VAAMANA) and POUND-FOOLISH (PNF) (Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Hackbusch et al., 2005; Cole et al., 2006; Kanrar et al., 2006; Rutjens et al., 2009). In *pny PNF/pnf* plants pedicels are often fused to the main stem (Smith et al., 2004). This boundary defect was found to be sensitive to gene dosage levels of STM, and it was proposed that PNY-STM and PNF-STM heterodimers regulate maintenance of the boundary between initiating floral primordia and the inflorescence meristem (Kanrar et al., 2006).

More recently, a third *BELL* gene, *ARABIDOPSIS THALIANA HOMEOBOX1* (*ATH1*), has been reported to modulate growth at the interface between the stem, meristem, and organ primordia (Gomez-Mena and Sablowski, 2008). Mutations in *ATH1* result in boundary defects throughout post-embryonic shoot development, including partially fused rosette leaf petioles to central stem tissue, cauline leaf-stem fusions, partial fusions at the base of stamens and sepals and floral organ abscission defects (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). In addition, in *ath1* seedlings the subapical region is longitudinally enlarged due to reduced inhibition of growth of cells in the rib zone (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). Although ATH1 functions in the SAM in association with STM (Rutjens et al., 2009) and STM is involved in the establishment of several types of SAM-organ boundaries, the boundary and rib zone defects observed in *ath1* mutants cannot be simply explained by reduced activity of STM. In addition to STM, in a yeast two-hybrid assay ATH1 also binds to the three other class I KNOX proteins, BP/KNAT1, KNAT2 and KNAT6 (Rutjens et al., 2009). Moreover, there is a significant overlap in the expression patterns of *ATH1* on the one hand, and *BP/KNAT1*, *KNAT2*, and *KNAT6* on the other hand (Ori et al., 2000; Lin et al., 2003; Belles-Boix et al., 2006; Proveniers et al., 2007; Gomez-Mena and Sablowski, 2008; Ragni et al., 2008; Rutjens et al., 2009). Therefore, we investigated the biological relevance of these additional ATH1-KNOX interactions in relation to boundary establishment. We show that ATH1 acts in conjunction with KNAT2 and KNAT6 at the interface between both the SAM and lateral organ primordia and between the SAM and the stem to establish proper meristem-leaf boundaries and to control stem elongation.

Results

ATH1 interacts with *KNAT2* and *KNAT6* to control proper establishment of cauline leaf-stem boundaries

Perhaps the most striking boundary defects that can be observed in *ath1* mutants are the typical cauline leaf-stem fusions (Rutjens et al., 2009). These are visible as strands of leaf blade tissue attached to the inflorescence stem, subtending and continuous with either one or both margins of a cauline leaf (Figure 3.1b,c). Cauline leaf-stem fusions were present in all *ath1-1* mutants tested, but these were never observed in Col-8 wild-type control plants (Figure 3.1a). The severity of this boundary defect decreases acropetally, meaning that it is most prominent in the oldest cauline leaves (Table 3.1). In *ath1-1* fusions could be found up till the third cauline leaf, but in most cases they were restricted to the lower two cauline leaves (Table 3.1). To test whether ATH1 requires any of the class I KNOX proteins that were shown to interact with ATH1 (Rutjens et al., 2009) in establishing proper meristem-cauline leaf boundaries, *stm*, *bp*, *knat2*, and *knat6* single mutants were examined for cauline leaf separation defects in the primary inflorescence. Defects of this kind were never observed in the *stm* and *bp* alleles analyzed and in both cases, when combined with *ath1-1*, they did not further enhance this particular defect in this background (data not shown), suggesting that STM and BP/KNAT1 are not involved in the initiation and/or maintenance of these specific meristem-organ boundaries.

Previously it was reported that *knat2*, *knat6*, and *knat2 knat6* mutants display a wild-type phenotype (Belles-Boix et al., 2006; Ragni et al., 2008). However, detailed analysis of *knat2-5*, *knat6-1*, *knat6-2*, *knat2-5 knat6-1*, and *knat2-5 knat6-2* mutants revealed that all these mutants to some extent display cauline leaf-stem boundary defects (Figure 3.1f-h, insets). In 24%, 57% and 43% of the analyzed *knat2-5*, *knat6-1* and *knat6-2* plants, respectively, leaf-stem fusions were observed. Mostly, fusions were restricted to the lowest node of the inflorescence stem and only one of the two leaf margins was fused. Surprisingly, combined loss of *KNAT2* and *KNAT6* did not significantly enhance these defects (Table 3.1).

In contrast, loss of either *KNAT2* or *KNAT6* in the *ath1-1* mutant background resulted in a marked increase of fusion events involving both cauline leaf margins when compared to *ath1-1* single mutants. In addition, boundary defects more often extended to the upper leaf positions in the inflorescence (Table 3.1). In *ath1-1 knat2-5 knat6-2* triple mutants these defects were stronger and occasionally fusions up to the fifth cauline leaf position were observed (Figure 3.1l).

These results indicate that KNAT2 and KNAT6 redundantly act with ATH1 in the establishment of proper meristem-cauline leaf boundaries.

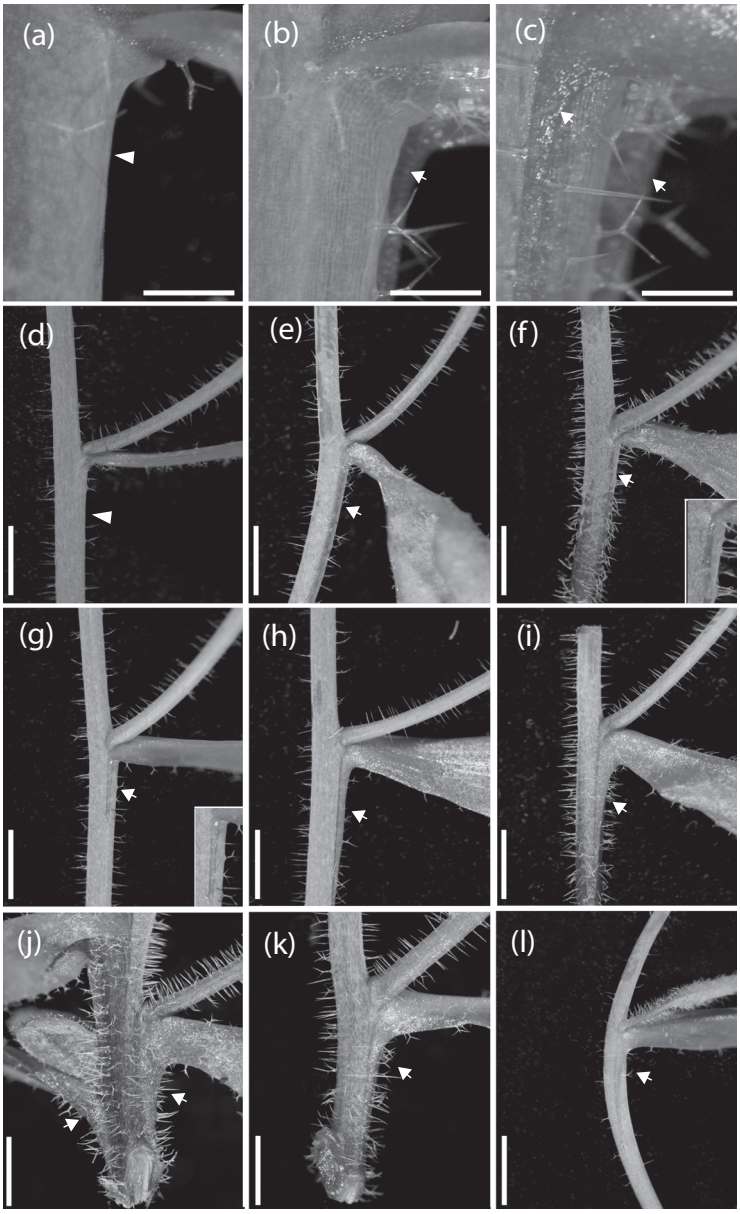

Figure 3.1 *ATH1* interacts with *KNAT2* and *KNAT6* to control cauline leaf-stem boundary development.
(a) Wild-type (Col-8) cauline leaf-stem boundary without leaf lamina fusions (arrowhead).
(b) Example of a cauline leaf-stem fusion where only one of the two leaf lamina are fused to the stem (arrow).
(c) Example of a cauline leaf-stem fusion where both leaf margins are fused to the stem (arrows).
(d) - (l) Cauline leaf-stem boundaries in wild-type (d), *ath1-1* (e), *knat2-5* (f), *knat6-2* (g), *knat2-5 knat6-2* (h), *ath1-1 knat2-5* (i), *ath1-1 knat6-2* (j), and *ath1-1 knat2-5 knat6-2* (k, l) plants. In (d)-(k)

the lowest cauline leaf positions are shown; (l) shows the highest cauline leaf (5th position). Arrowheads indicate the absence of fused tissue, arrows indicate the presence of fused tissue. The insets show the close-ups of the arrow marked area in (f) and (g). Bar=1mm (a-c) or 1cm (d-l).

Table 3.1 *ATH1* interacts with *KNAT2* and *KNAT6* to control proper establishment of cauline leaf-stem boundaries

Plant genotype	no fusions	1st[a] single[b]	1st double[c]	2nd single	2nd double	3rd single	3rd double	4th single	4th double	5th single
Col-8	**21**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
ath1-1	**0**	**8**	**13**	**5**	**6**	**3**	**0**	**0**	**0**	**0**
knat2-5	**16**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**
knat6-1	**9**	**11**	**1**	**2**	**0**	**0**	**0**	**0**	**0**	**0**
knat6-2	**12**	**9**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
knat2-5 knat6-1	**13**	**8**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
knat2-5 knat6-2	**7**	**14**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**0**
ath1-1 knat2-5	**0**	**1**	**20**	**9**	**11**	**4**	**3**	**1**	**0**	**0**
ath1-1 knat6-1	**0**	**1**	**20**	**4**	**15**	**4**	**4**	**0**	**1**	**0**
ath1-1 knat6-2	**0**	**2**	**19**	**1**	**16**	**7**	**3**	**3**	**0**	**0**
ath1-1 knat2-5 knat6-2	**0**	**0**	**21**	**0**	**18**	**2**	**12**	**2**	**9**	**3**

Cauline leaf-stem fusions at different inflorescence stem positions.
In total, 21 plants were scored for each genotype.
[a]The number indicates the position of the cauline leaf on the inflorescence stem. 1st indicates the oldest cauline leaf, 2nd the following leaf etc.
[b]Single refers to cauline leaves fused to the stem at only one of the two leaf margins (see Figure 3.1 b).
[c]Double indicates cauline leaves fused to the stem at both leaf margins (see Figure 3.1 c).

KNAT2 and KNAT6 function in floral organ abscission

Another readily visible defect of *ath1* plants is that floral organs do not abscise normally after fertilization and remain attached to the flower base (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). This non-shedding phenotype

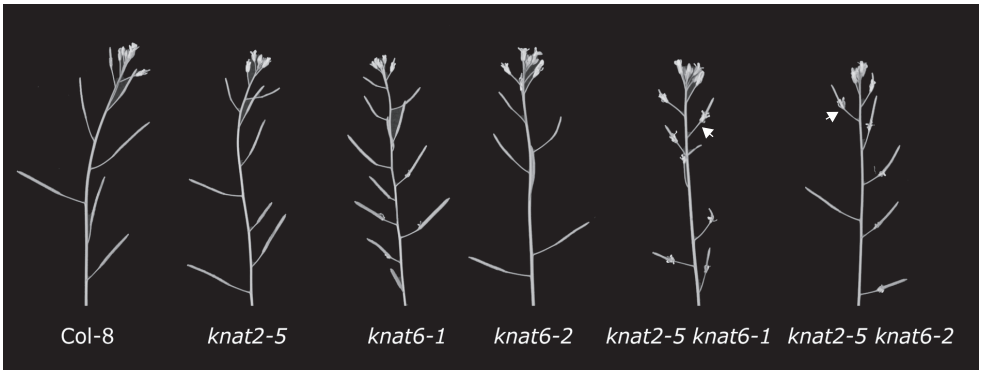

Figure 3.2 KNAT2 and KNAT6 function in floral organ abscission.
From left to right: Inflorescences of Col-8, *knat 2-5*, *knat6-1*, *knat6-2*, *knat2-5 knat6-1* and *knat2-5 knat6-2* plants. Arrows indicate the delayed abscission of floral organs.

represents a general defect in the basal region of flowers that results in a developmental delay in the formation of a functional sepal abscission zone and complete absence of a functional stamen abscission zone (Gomez-Mena and Sablowski, 2008). Since both KNAT2 and KNAT6 are expressed in basal parts of flowers (Ragni et al., 2008) (Figure 3.2), we also examined *knat2-5*, *knat6-1*, *knat6-2*, *knat2-5 knat6-1*, and *knat2-5 knat6-2* mutants for floral organ shedding phenotypes. A weak floral organ abscission defect was noticed in *knat2 knat6* double mutants, whereas the single mutant flowers displayed wild-type morphology. If left undisturbed after fertilization, *knat2 knat6* floral organs were retained longer at the base of the flower than in wild-type control plants (Figure 3.2). However, these defects were too weak to be quantified using a petal breakstrength meter (Melinka Butenko, personal communication). Flowers of plants combining above-mentioned *knat* mutations with the *ath1-1* mutation were morphologically indistinguishable from *ath1-1* flowers (data not shown).

Despite lack of quantitative data, the observed *knat2 knat6* floral phenotype provides an indication that ATH1 might also act together with KNAT2 and KNAT6 to establish basal boundaries in the flower.

ATH1 depends on KNAT6 to inhibit stem and pedicel elongation

ATH1 modulates growth at the interface between the stem or pedicel and organ primordia. ATH1 also prevents stem elongation during the vegetative phase by restricting growth of the rib zone (Gomez-Mena and Sablowski, 2008). As a result, in *ath1* seedlings the subapical region is longitudinally enlarged (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009) (Figure 3.3). At the same

time, during vegetative growth in *ath1-1* the SAM diameter is decreased due to a reduction in the number of meristematic cells in the L1, L2 and L3 layers of the SAM. Both SAM and rib zone defects are already clearly visible in 4-day-old seedlings (Rutjens et al., 2009). In line with its proposed function in restricting growth of the rib zone, constitutive *ATH1* expression severely inhibits growth of the inflorescence stem and pedicels, resulting in plants that maintain a rosette-like appearance even when flowering (Cole et al., 2006; Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009).

Both *KNAT2* and *KNAT6* are expressed in the SAM domain that is affected by *ath1* mutations (Dockx et al., 1995; Belles-Boix et al., 2006). Moreover, manipulation of the *KNAT2* expression domain in the SAM has been shown to affect the number of cells in the L3 layer under certain conditions (Hamant et al., 2002). The possibility that ATH1 depends on KNAT2 and/or KNAT6 to maintain a proper organization of the SAM and underlying rib zone was intestigated by examining the SAM of 4-day-old *knat2* and *knat6* plants for *ath1*-like defects. In line with previous observations, no obvious aberrations in SAM organization were observed in these mutants (Hamant et al., 2002; Belles-Boix et al., 2006) (data not shown).

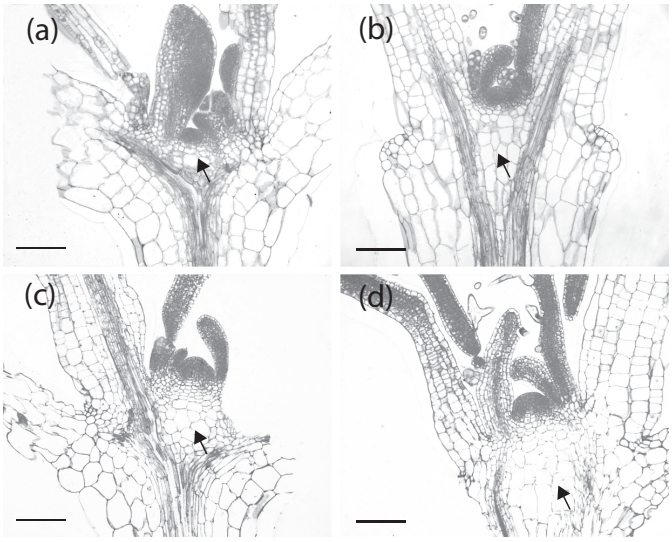

Figure 3.3 ATH1 restricts the growth of the subapical region.
(a-d) Longitudinal median sections through the shoot apices of 8-day-old (a,b) and 15-day-old (c,d) seedlings of Col-8 (a,c) and *ath1-1* (b,d) plants. Bar=100 µm. Arrows indicate the RZ in the SAM.

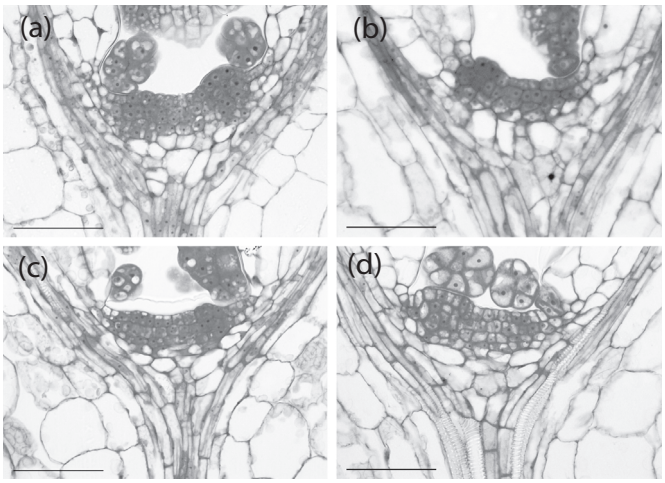

Figure 3.4 SAMs are not affected in *knat2 knat6* double mutants.
(a-d) Longitudinal median sections through vegetative apices of 4-dpg-old Col-8 (a), *ath1-1* (b), *knat2-5 knat6-1* (c) and *knat2-5 knat6-2* (d) plants. Bar=50 µm.

Considering a potential redundant role for both KNAT proteins in SAM organization, also sections were made of *knat2-5 knat6-1* and *knat2-5 knat6-2* double mutant SAMs. Such plants displayed a SAM and rib zone organization that was essentially similar to that of control plants (Figure 3.4). This clearly shows that combined loss of *KNAT2* and *KNAT6* cannot account for the morphological defects seen in the SAM of *ath1-1* plants.

Both the stem-cauline leaf boundary defects and the floral organ abscission phenotype in *knat2 knat6* mutants were quite subtle and the possibility remains that SAM defects could be too subtle to be observed. Therefore, we also checked whether absence of KNAT2 and/or KNAT6 has any consequences in plants that constitutively express *ATH1*. For this purpose wild-type plants and *knat2*, *knat6*, and *knat2 knat6* mutants were transformed with a HA-tagged version of ATH1 under control of the CaMV 35S promoter (Pro35S:*HA-ATH1*) and T1 plants were analyzed for stem and pedicel elongation defects (Table 3.2). In all backgrounds, a number of transformants (ranging from 4-12%) displayed clear *ath1* loss-of-function phenotypes, most likely due to co-suppression (Table 3.2). The remaining plants were categorized into four groups, based on the severity of the inhibition of stem and pedicel elongation: wild-type looking plants (not shown), and severe, mild, or weak *ATH1* overexpression phenotype plants (Figure 3.5, Table 3.2). Plants with a severe *ATH1* overexpression phenotype had a very compact stature with inflorescences ranging from 0 to 5 cm in height (Figure 3.5a,d). Mild

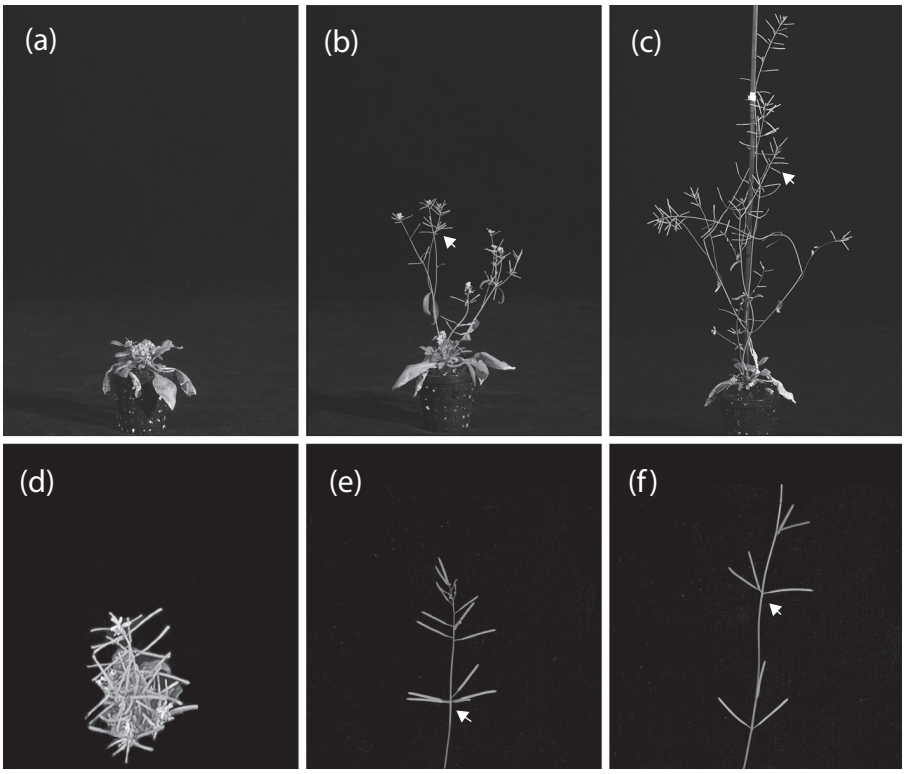

Figure 3.5 Ectopic Expression of *ATH1* inhibits stem and pedicel elongation.
(a-c) Overall plant stature of *Pro35S:HA-ATH1* plants and (d-f) close-ups of the inflorescence stem and pedicels of the plants comparable to those shown in (a-c), displaying the different effects of ectopic expression of *ATH1* in these plants. Strong phenotype *Pro35S:HA-ATH1* plants are displayed in (a) and (d); mild phenotype *Pro35S:HA-ATH1* plants in (b) and (e), and weak phenotype *Pro35S:HA-ATH1* plants in (c) and (e). Arrows indicate clusters of siliques.

ATH1 overexpressor plants were semi-dwarfed with inflorescence heights ranging from 5 to 15 cm in length. In addition, in both strong and mild phenotype plants pedicels were shortened (≤0.3 cm in length vs. approximately 0.8 cm on average in wild-type plants) and siliques were often clustered (Figure 3.5b,e). Weak phenotype plants carried relatively normal inflorescences with lengths ranging from 15 cm to wild-type length (40 cm on average). However these plants could be easily distinguished from wild-type looking plants because of the presence of clustered siliques and/or a significant number of flowers with shortened pedicels in the main inflorescence (≤0.3 cm in length) (Figure 3.5c,f).

Despite the high percentage wild-type looking plants in all backgrounds (Table 3.2), clear differences were observed between some of the backgrounds.

Table 3.2 *knat6* mutation suppresses *ATH1*-induced inhibition of stem elongation

Plant background	Total transformants	Phenotypes of 35S:HA-ATH1[a]					
		Co-suppression	Wild type	strong	mild	weak	%[b]
Col	**162**	**17**	**134**	**3**	**4**	**4**	**6.8**
knat2-5	**189**	**22**	**153**	**0**	**7**	**7**	**7.5**
knat6-1	**138**	**6**	**132**	**0**	**0**	**0**	**0**
knat6-2	**136**	**12**	**124**	**0**	**0**	**0**	**0**
knat2-5 knat6-1	**167**	**17**	**150**	**0**	**0**	**0**	**0**
knat2-5 knat6-2	**187**	**20**	**20**	**0**	**0**	**0**	**0**

Phenotype classes of *35S:HA-ATH1* plants in wild type and *knat2* and *knat6* mutant (combination) backgrounds.
[a]*ATH1* over-expression phenotypes were categorized into four groups based on distinct severity (see Figure 3.3).
[b]Percentage shown was calculated based on the number of plants that found displaying characteristics of *ATH1* over-expressor phenotype described (strong, mild, weak, see Figure 3.3).

Whereas 7% of both wild-type (Col-8) and *knat2-5* Pro35S:*HA-ATH1* T1 plants (respectively 11/162 and 14/189 transformants) displayed a typical ATH1 overexpression phenotype, we never observed any morphological indications for *ATH1* overexpression in any of the *knat6* Pro35S:*HA-ATH1* T1 plants (274 plants in total) or *knat2 knat6* Pro35S:*HA-ATH1* T1 plants (354 plants in total) (Table 3.2). This strongly suggests that ATH1 loses the ability to inhibit stem and pedicel growth in the absence of *KNAT6* and indicates that, although no SAM defects were observed in *knat6* mutants, ATH1 and KNAT6 might interact with each other to control subapical growth in Arabidopsis. Given the absence of severe phenotype Pro35S:*HA-ATH1* T1 plants in the *knat2-5* background (vs. 3/11 in the wild-type background), KNAT2 may also be involved in this process, though to a much lesser extent.

ATH1 is required for proper *KNAT2* expression

There is accumulating evidence that BELL proteins, including ATH1, function to affect KNOX activity not only by forming heterodimers with KNOX proteins, but also through spatially restricting *KNOX* gene expression (Kumar et al., 2007; Gomez-Mena and Sablowski, 2008; Ragni et al., 2008). Our findings that KNAT2 and KNAT6 are partially overlapping in function with ATH1 fit with both a model in which KNAT2 and/or KNAT6 act as protein partners of ATH1

and with a model in which ATH1 acts upstream of both KNAT genes to control their expression. Therefore, *KNAT2* and *KNAT6* expression patterns in *ath1-1* mutants were compared to those in wild-type plants.

The expression pattern of *KNAT2* was determined using a translational *pKNAT2:GUS* (for β-glucuronidase) fusion (Dockx et al., 1995). In agreement with previous studies (Dockx et al., 1995), during vegetative development *KNAT2* was highly expressed in the shoot apex of wild-type plants. In addition, a strong GUS staining was also detected in the root (Figure 3.6a). Close examination of the SAM of these plants further confirmed that *KNAT2* SAM expression was confined to the cells of the L3 layer and rib zone (Figure 3.6e). Surprisingly, no GUS activity was detectable in the SAM of both 4-day-old (Figure 3.6b, f) and 10-day-old (Figure 3.6r) *pKNAT2:GUS ath1-1* seedlings, while *GUS* was still highly expressed in the roots (Figures 3.6r).

Previously, using a glucocorticoid inducible system (*35Spro:ATH1-HBD*), we have shown that the *ath1-1* SAM defects can be restored to a wild-type situation after induction of nuclear expression of ATH1 (Rutjens et al., 2009). To test whether induction of ATH1 also leads to recovery of *KNAT2* expression, *pKNAT2-GUS ath1-1* plants were crossed with *35Spro: ATH1-HBD ath1-1* plants and F1-offspring plants were tested for *GUS* expression. Indeed, plants grown on 1 μM dexamethasone (Dex), showed a reversion of *ath1* SAM defects (Rutjens et al., 2009) and a strong KNAT2-GUS signal reappeared in the L3 layer and rib zone of the SAM (Figure 3.6k,o). In ethanol control-treated *35Spro:ATH1-HBD pKNAT2-GUS ath1-1* plants, a clear blue staining was visible in roots, similar to *pKNAT2-GUS ath1-1* plants, but no SAM GUS activity could be detected (Figure 3.6c,g), showing that there was no significant leakage of the glucocorticoid system. In wild-type plants containing both *35Spro:ATH1-HBD* and *pKNAT2-GUS,* induction of ATH1 resulted in broadening of the *KNAT2* expression domain in the SAM and ectopic expression in the hypocotyl and cotyledonary petiole regions closest to the SAM (Figure 3.6l,p). As Dex treatment itself had no effect on GUS activity in the shoot apex of *pKNAT2-GUS* plants, this enlargement of the *KNAT2* expression domain can be attributed to ectopic expression of *ATH1* (Figure 3.6i,m).

After floral transition, in control plants high level of *KNAT2* promoter activity wase observed in the inflorescence stem at early bolting stages (Figure 3.6s). At later stages, *GUS* expression in the stem became restricted to a stripe of cells along the side of the stem. These stripes of blue staining initiated directly above a cauline leaf and, while gradually decreasing in intensity, extended till the next cauline leaf node (Figure 3.6u, inset). Interestingly, these stripes of GUS expressing tissue co-localize with the area were stem-cauline leaf fusions can be observed in *knat2* seedlings. As reported before (Dockx et al., 1995; Pautot et al., 2001), strong *GUS* activity was further detected at paraclade junctions and in the

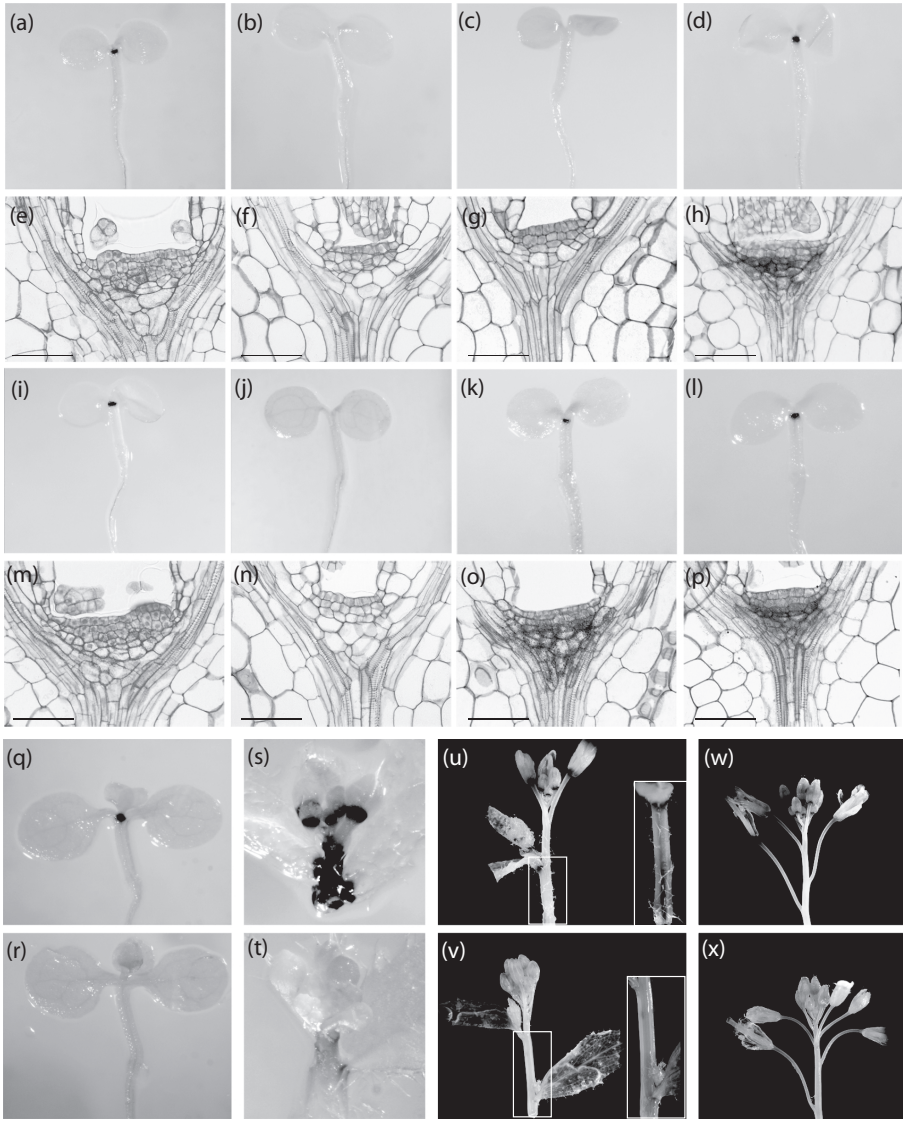

Figure 3.6 *ATH1* is required for proper *KNAT2* expression.
(a-d) Whole-mount GUS staining of 4-day-old wild-type (a), *ath1-1* (b), *35Spro:ATH1-HBD ath1-1* (c) and *35Spro:ATH1-HBD* (d) seedlings harbouring a *pKNAT2-GUS* construct. GUS activity can be observed in the shoot apex (a,d). Note the presence of KNAT2-GUS activity in the roots of (a-d). (c, d) Ethanol-treated control plants.
(e-h) Longitudinal median sections through the shoot apex of seedlings shown in (a-d).
(i-l) Whole-mount GUS staining of 4-day-old wild-type (i), *ath1-1* (j), *35Spro:ATH1-HBD ath1-1* (k), and *35Spro:ATH1-HBD* (l) seedlings harbouring a *pKNAT2-GUS* construct. (i-l) Dexamethasone-treated plants (1 μM).
(m-p) Longitudinal median sections through the shoot apex of seedlings shown in (i-l).
(q) Whole-mount GUS staining of a 10-day-old wild-type (Col-8) seedling harbouring a *pKNAT2-GUS* construct, showing high KNAT2-GUS activity in the shoot apex.
(r) Whole-mount GUS staining of a 10-day-old *ath1-1* seedling harbouring a *pKNAT2-GUS* con-

struct. No GUS activity was detected in the shoot apex.
(s-x) Whole-mount GUS staining of inflorescences showing GUS activity in the stem and flowers in Col-8 (s, u, w) and in *ath1-1* (t, v, x) plants harbouring a *pKNAT2-GUS* construct at an early bolting stage (the oldest flower is at about stage 9) (s and t), in elongating inflorescences (u and v) and in elongated inflorescence (w and x). Rectangle insets in (u) and (v) show close-ups of the indicated stem region. Bar=50 µm.

receptacle of flowers (Figure 3.6u,w). Thus, overall the expression of *KNAT2* is consistent with the *knat2* mutant phenotypes described above.

Unlike in the vegetative *ath1-1* SAM, in mature *ath1-1* plants the KNAT2-GUS signal was not completely absent. Instead, in all inflorescence tissues mentioned above GUS activity was dramatically decreased with activity levels near detection limits in the receptacle of older flowers and at paraclade junctions (Figure 3.6t,v,x).

Thus, ATH1 is also essential for proper induction of *KNAT2* expression in addition to acting as a protein partner of KNAT2. However, ATH1 is not indispensable for *KNAT2* expression, as can be concluded from the presence of GUS activity in the roots and inflorescences of *ath1* loss-of-function plants. Moreover, since *ath1* mutations significantly affect tissue morphology in the *KNAT2* expression domain (Rutjens et al., 2009), it is possible that the observed effects of ATH1 on *KNAT2* promoter activity, at least in the SAM, are indirect as a result of changes in cell identity. In case of a direct effect, ATH1 most likely depends on a localized co-factor, since ectopic expression of ATH1 can induce *KNAT2* promoter activity in the SAM domain of *ath1-1* mutants and adjoining parts of connected tissues, but not outside of this domain.

ATH1 is required for proper *KNAT6* expression

A transcriptional *pKNAT6*:*GUS* fusion (Belles-Boix et al., 2006) was used to determine the expression pattern of *KNAT6* in *ath1-1* mutants. The KNAT6 expression pattern is more restricted than that of KNAT2. Belles-Boix et al. (2006) observed that p*KNAT6-GUS* is first expressed during the early torpedo stage of embryogenesis when GUS activity is present at the boundaries between the presumptive SAM and the cotyledons. This pattern is maintained in mature embryos. In line with these findings, we detected high GUS activity in the SAM region of mature wild-type *pKNAT6-GUS* embryos (Figure 3.7a). In addition, GUS expression was also detected at the very tip of the cotyledons where the hydathodes originate (Figure 3.7a). In *ath1-1* embryos, *pKNAT6-GUS* expression in the SAM region was completely absent; whereas expression at hydathodes was still present (Figure 3.7b). *KNAT6* promoter activity remained absent from the SAM of *ath1-1* mutants during post-embryonic development (Figure 3.7d,h), whereas

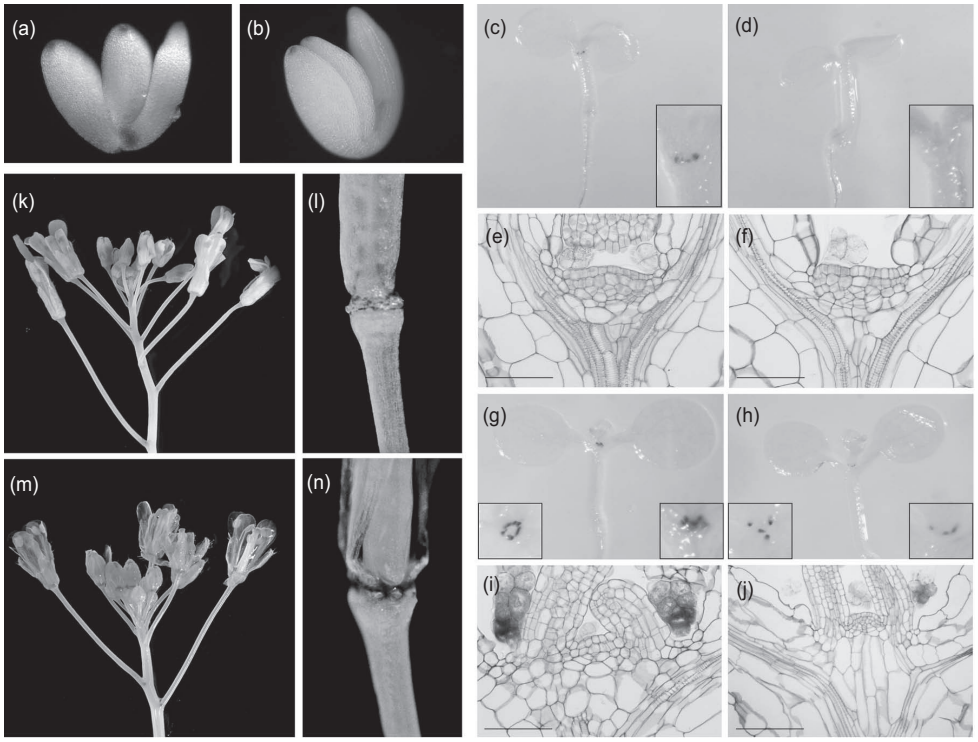

Figure 3.7 *ATH1* is required for proper *KNAT6* expression.
(a) Whole-mount GUS staining showing the activity of the *KNAT6*-promoter in the embryonic SAM and at the top of the cotyledons in wild-type plant harbouring a *pKNAT6-GUS* construct.
(b) Absence of GUS activity in the embryonic SAM region and presence of GUS activity at the tips of the cotyledons in *ath1-1* plants harbouring a *pKNAT6-GUS* construct.
(c) Expression of *KNAT6-GUS* in the shoot apex of 4-day-old seedlings in wild-type plants.
(d) Absence of *KNAT6-GUS* expression in the shoot apex of 4-day-old *ath1-1* seedlings.
(e) Longitudinal sections through the shoot apices of wild-type plants as shown in (c), showing GUS activity in the SAM and stipules.
(f) Longitudinal sections through the shoot apices of *ath1-1* plants as shown in (d), showing the absence of GUS activity in the SAM, but presence in the stipules.
(g) Expression of *KNAT6-GUS* in the shoot apex of 10-day-old wild-type seedling. Note the inset on the left showing the top view of the *GUS* expression pattern in the shoot apex of (g). The inset on the right shows a close-up of the side view of the shoot apex of (g).
(h) Expression of *KNAT6-GUS* in the shoot apex of 10-day-old *ath1-1* seedlings. The inset on the left shows the top view of the *GUS* expression pattern in the shoot apex of (h). Inset on the right show a close-up of the side view of the shoot apex of (h).
(i) Longitudinal sections through the shoot apex of a wild-type plant as shown in (g), showing GUS activity in the SAM and stipules.
(j) Longitudinal sections through the shoot apex of *ath1-1* plants as shown in (h), showing the presence of GUS activity in the stipules, whereas it is absent in the SAM.
(k-n) Whole-mount GUS staining of inflorescences (k, m) and siliques (stage 17) (l, n) of wild-type (k, l) and *ath1-1* (m, n) plants harbouring a *pKNAT6-GUS* construct.
Bar=50 µm in (e, f, i) or 100 µm (j).

in control plants high *GUS* expression could be detected in this region both at 4 days post germination (dpg) and at 10 dpg (Figure 3.7c,g). Sections of this region show that *KNAT6-GUS* expression in the SAM is restricted to a small group of cells that mark the boundaries between the SAM and the emerging primordia (Figure 3.7e,i). In wild-type seedlings *GUS* was further expressed in roots and stipules (Figure 3.7c,e,g,i). Expression in these tissues was not affected by the *ath1-1* mutation (Figure 3.7d,f,h,j). In a top view, in control plants the GUS activity in the stipules in combination with GUS activity in the boundaries between the SAM and leaf primordia is nicely visible as blue ring around the SAM (Figure 3.7g, inset). In *ath1-1* mutants, only isolated spots that mark the stipules can be seen in these plants, instead of a ring shape as in wild type (Figure 3.7h, inset).

During generative development in wild-type plants, *KNAT6-GUS* activity was detected in axils of the floral pedicels and at the base of the floral organs where the floral organ abscission zones are located (Figure 3.7k,l) as described by Ragni et al. (2008). A highly similar pattern of *GUS* expression was seen in *ath1-1* mutants (Figure 3.7m,n). However, in contrast to what was observed for *KNAT2-GUS* expression, in *ath1* inflorescences the expression level of *KNAT6-GUS* in these tissues was noticeably higher than in wild-type plants (Figure 3.7k-n). This suggests that during this developmental stage loss of *ATH1* triggers upregulation of *KNAT6* expression. ATH1 and KNAT6 might operate together in a functional complex and one can speculate that KNAT6 upregulation is might be the result of a feedback mechanism to compensate for the loss of *ATH1* expression.

Our findings show that, ATH1 is required for proper expression of *KNAT6* as observed for *KNAT2*. It is currently unclear how ATH1 regulates *KNAT* gene expression.

Expression of *CUC* and *LOB* boundary genes is controlled by ATH1

Activation of *KNAT6* is CUC dependent (Belles-Boix et al., 2006). This and the various boundary defects in *ath1* mutants raise the possibility that ATH1 might function in the organ boundary pathway controlled by *CUC* genes (Aida and Tasaka, 2006), we also tested whether ATH1 might be required for normal *CUC* gene expression. In contrast to what has been reported by Gomez-Mena and Sablowski (2008), quantitative RT-PCR analyses (qPCR) on 10-day-old-seedling material revealed that expression of *CUC1* and *CUC3* was significantly affected by changes in *ATH1* levels (Figure 3.8a-d). In two different *ath1* alleles tested, an 85% reduction in *CUC1* expression was observed (Figure 3.8a). Conversely, in *35S:ATH1-HBD* seedlings Dex-induced nuclear localization of ATH1 resulted in a 25-fold increase in *CUC1* transcript levels (Figure 3.8b,d). At the same time,

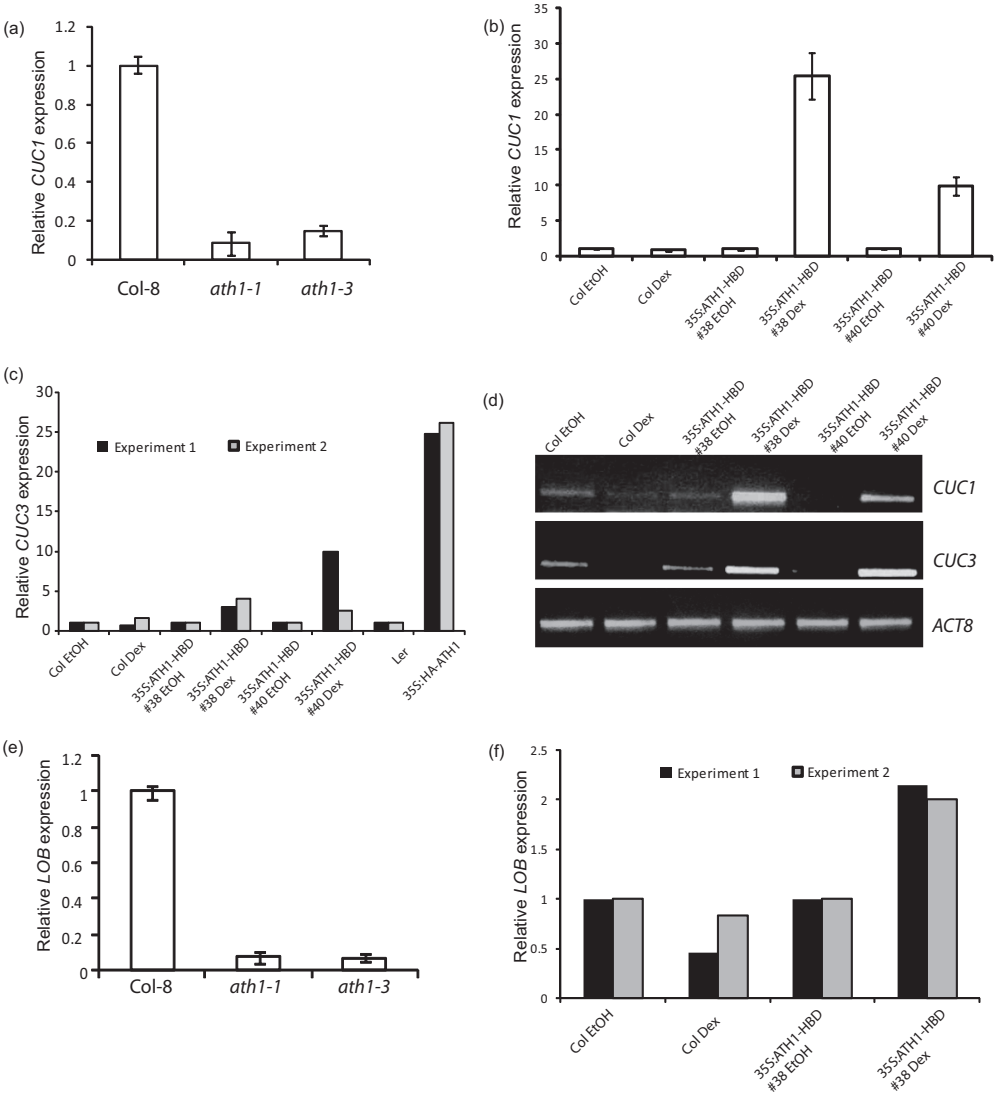

Figure 3.8 *ATH1* affects the expression of boundary-specific genes.
(a) Real-time qRT-PCR levels of *CUC1* expression in 10-day-old wild type, *ath1-1*, and *ath1-3* mutants. For each cDNA sample, the *CUC1* expression level was normalized to the *ACTIN-2* (*ACT2*) control.
(b) Real-time qRT-PCR results for transcript levels of *CUC1* in 15-day-old wild type, *35Spro:ATH1-HBD* line # 38, and *35Spro:ATH1-HBD* line #40 plants treated with either EtOH (control) or Dexamethasone (Dex) (10 µM Dex, 8hrs). For each cDNA sample, the *CUC1* expression level was normalized to the *ACT2* control.
(c) Real-time qRT-PCR results for transcript levels of *CUC3* in 15-day-old wild type, *35Spro:ATH1-HBD* line #38 and *35Spro:ATH1-HBD* line #40 plants plants treated with either EtOH (control) or Dex (10 µM Dex, 24 hrs). For each cDNA sample, the *CUC3* expression level was normalized to the *ACT2* control.

(d) RT-PCR results showing that ATH1 affects both *CUC1* and *CUC3* transcript level of 15-day-old wild type, *35Spro:ATH1-HBD#38* and *35Spro:ATH1-HBD#40* plants. Dex indicates plants that were treated with 10 µM Dex throughout their development. EtOH indicates ethanol control treated plants. Experiments were repeated three times and resulted in the same pattern shown here. *ACT8* was used as internal control.
(e) Real-time RT-PCR results for *LOB* (*At 5g63090*) expression in 10-day-old wild type, *ath1-1*, *ath1-3* mutants. For each cDNA sample, the *LOB* expression level was normalized to the *ACT2* control.
(f) Real-time RT-PCR results for transcript levels of *LOB* in 10-day-old wild type, *35Spro:ATH1-HBD#38* plants under control condition (EtOH supplemented) and under inductive (Dex) condition (10 µM Dex, 8hrs). For each cDNA sample, the *LOB* expression level was normalized to the *ACT2* control.
Each reaction was made in duplicate, and experiments were repeated three times for (a), (b), and (e). Error bars indicate SD. For (c) and (f), experiments were repeated twice and each one was indicated in the figures. Note that the level of each corresponding line under control condition was set at a value of 1.

expression of *CUC3* was 4-fold upregulated (Figure 3.8c,d), whereas constitutive expression of *ATH1* in Landsberg *erecta* (Ler) 35S:HA-ATH1 seedlings resulted in a 25-fold upregulation of *CUC3* expression (Figure 3.8c). No significant differences were found for *CUC2* expression in any of these experiments (data not shown). These results indicate that *ATH1* acts upstream of *CUC1/3* in the process of boundary establishment.

Another gene that is typically expressed at the boundaries of lateral organs throughout plant development, and whose expression pattern is highly similar to that of *KNAT6*, is the *LATERAL ORGAN BOUNDARY* (*LOB*) gene (Shuai et al., 2002; Husbands et al., 2007). Similar to *CUC1*, in both *ath1-1* and *ath1-3* seedlings *LOB* transcript levels were severely reduced (7% of wild-type plants levels, Figure 3.8e). Conversely, in Dex-treated *35S:ATH1-HBD* plants, LOB expression showed a 3-fold increase (Figure 3.8f). This indicates that *ATH1* also acts upstream of *LOB* to control its transcript accumulation. During vegetative development, ATH1 seems a major player in boundary establishment by controlling the expression of boundary specific genes like *CUC1*, *CUC3* and *LOB*.

Discussion

In plants, organ formation and the growth of stem tissue require a continuous supply of new cells. Shoot meristems can provide these owing to the activity of stem cells that reside in the central zone of the SAM. In plant shoot meristems the stem cells are separated from determinate organ founder cells by morphological boundaries, recognizable as regions with reduced cell growth activity. These

boundaries are crucial for normal plant development, as by separating meristem and organ domains they contribute to a large extent to maintenance of these same domains. Morphological boundaries largely overlap with gene expression boundaries. These gene expression boundaries are set and maintained by a whole set of transcription factors that interact with and regulate the expression of meristematic and organ-specific genes. However, it is still largely unknown what the downstream factors are and how downstream cascades lead to the normal patterns of morphogenetic parameters such as cell division and expansion, or expression patterns of organ identity genes (reviewed in Aida and Tasaka, 2006; Rast and Simon, 2008).

Previously, the BELL transcription factor protein ATH1 has been shown to be important for both SAM initiation and maintenance and the proper establishment of meristem-organ boundaries (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). As a result, mutations in *ATH1* give rise to boundary defects throughout post-embryonic shoot development. Here, we started to elucidate the molecular mechanism(s) through which ATH1 functions in this particular process. Down-regulation of the boundary specific genes *CUC1*, *CUC3* and *LOB* in *ath1* seedlings and, conversely, an increase in the expression of these genes after induction of ATH1, suggests that ATH1 acts through these three genes in meristem-organ boundary establishment, at least during vegetative growth. This fits with earlier findings of Hibara et al. (2006) that CUC1 and CUC3, together with CUC2, fulfil redundant but partially distinct functions in shoot organ boundary formation throughout the Arabidopsis life cycle. It was concluded that ATH1 converges on the regulation of basal organ boundaries downstream of *CUC* expression (Gomez-Mena and Sablowski, 2008). However, this conclusion was based on *CUC1* expression levels only and measured in a single tissue, developing flowers, whereas our experiments were done in seedlings. During vegetative growth, ATH1 contributes to the establishment of the boundary at the base of rosette leaf petioles with stem tissue (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). Thus, in the process of meristem-organ boundary development ATH1 might rely on different downstream acting factors during different developmental stages.

We further discovered that the closely related KNOX transcription factor proteins KNAT2 and KNAT6 function in conjunction with ATH1 at the interface between the SAM and initiating cauline leaves and at the SAM-stem and floral meristem-pedicel interfaces to contribute to the establishment of meristem-organ boundaries and to modulate stem/pedicel elongation. Until now the role of KNAT6 and, especially KNAT2, remained unclear. Although misexpression of *KNAT6* and, to a lesser extent, *KNAT2* can have dramatic effects on inflorescence architecture (Ragni et al., 2008), and KNAT6 has been shown to contribute redundantly with STM to SAM function and boundary maintenance during embryogenesis (Belles-Boix et al., 2006). Here we report phenotypical alterations for

knat2, *knat6*, and *knat2 knat6* plants and show that both in *knat2* and *knat6* mutants cauline leaves were occasionally fused to the inflorescence stem, and that *knat2 knat6* double mutants display subtle floral organ abscission defect. Similar defects were previously described for *ath1* mutants. The cauline leaf boundary defect was markedly enhanced by the combined absence of ATH1, KNAT2 and KNAT6. This suggests that KNAT2 and KNAT6 redundantly contribute with ATH1 to proper establishment of post-embryonic shoot meristem-organ boundaries. The observed KNAT2 and KNAT6 expression patterns are consistent with such a function.

Does ATH1 operate together with KNAT2 and KNAT6 in heterodimeric BELL-KNOX complexes?

KNOX proteins have repeatedly been shown to interact with BELL proteins. BELL-KNOX interactions are required for site-specific DNA binding (Smith et al., 2002) and for nuclear localization of the transcription factor heterodimeric complex (Bhatt et al., 2004; Cole et al., 2006; Kimura et al., 2008; Rutjens et al., 2009), strongly suggesting that these interactions are a prerequisite for biological function. The latter is further supported by our recent observation that plants that combine mutations in three STM-interacting BELL proteins, ATH1, PNY and PNF, phenocopy *stm* loss-of-function mutants (Rutjens et al., 2009). Further, it has been suggested that different combinations of BELL-KNOX transcription factors may regulate different downstream genes (Cole et al., 2006). In a yeast two hybrid system, ATH1 not only interacts with STM, but also with KNAT2 and KNAT6 (Rutjens et al., 2009). The overlap in *knat2*, *knat6* or *knat2 knat6* mutant phenotypes, on the one hand and *ath1* mutant phenotypes, on the other hand, together with the observed requirement of ATH1 for KNAT6 to inhibit stem elongation fits with a model in which ATH1-KNAT2 and ATH1-KNAT6 heterodimers operate as functional complexes. However, these data also fit with a model in which ATH1 acts upstream of both KNAT2 and KNAT6 as their transcriptional activator. Our expression analyses indicate that the latter is a valid option as at several stages of plant development ATH1 affects both *KNAT2* and *KNAT6* expression. In the vegetative SAM *KNAT2* and *KNAT6* expression completely depends on ATH1 activity, whereas after floral transition, ATH1 is required to maintain wild-type levels of *KNAT2* in both stem and flowers and of *KNAT6* in pedicels and flowers. Interestingly, similar results were previously reported for the interaction between ATH1 and *BP/KNAT1* expression (Gomez-Mena and Sablowski, 2008).

It is currently not clear whether the effect of ATH1 on *KNAT2* and *KNAT6* expression is a direct result of ATH1 functioning as transcriptional activator or an indirect consequence of changed cell identities due to changes in *ATH1* levels. Moreover, it might be possible that ATH1 acts both as heterodimeric partners and

as upstream regulator of KNAT2 and KNAT6. In case ATH1 acts as an upstream regulator of *KNAT2* and *KNAT6*, our findings that the *ath1-1* boundary defects are more severe than those of *knat2 knat6* double mutants, suggest that KNAT2 and KNAT6 represent only part of the mechanism through which ATH1 acts in boundary development. Perhaps ATH1 can interact with additional KNOX protein(s) that functions in this process. The finding that the *ath1-1* cauline leaf defects were severely enhanced when combined with *knat2* and/or *knat6* mutations, also suggest the presence of an additional BELL protein(s) that functions in this process and interacts with KNAT2 and/or KNAT6.

A role for BELL proteins in controlling KNOX expression has been reported before. Kumar et al. (2007) found that the BELL proteins SAWTOOTH1 (SAW1) and SAW2 repress *BP/KNAT1* and *STM* expression in leaves. Similarly, Ragni et al. (2008) showed that during inflorescence and fruit development PNY acts to restrict *KNAT6* and *KNAT2* expression domains. As BP/KNAT1 was found to have a similar effect as PNY on the expression of these two genes in these organs, it has been suggested that BP/KNAT1 and PNY operate together in a heterodimeric complex. In all these cases it is unclear whether the BELL effects on KNOX expression are direct or indirect, but it was proposed that these examples of KNOX co-factors regulating *KNOX* expression might represent a general mechanism that creates a feedback loop that is critical for determining the phenotypic readouts of KNOX activities in diverse organs (Hay and Tsiantis, 2009). Although the ATH1-*KNAT2/6* transcriptional interaction might also be part of such a feedback loop, there is a striking difference with the previously reported cases. In the reported cases, BELL proteins operate as repressors of *KNOX* gene expression to set the boundaries of their expression domains, whereas ATH1 seems to function as a transcriptional activator that is required for wild-type levels of *KNOX* gene expression within their normal expression domains.

Taken together, it is clear that plant TALE HD proteins operate within a complex network of interactions and that for a better understanding of their functions it will be necessary both to determine the spatio-temporal distribution of BELL-KNOX heterodimers *in planta* and to identify their combinatorial targets.

A role for ATH1 at the interface between the RZ and the SAM

In the vegetative SAM *KNAT2* is specifically expressed in the cells of the L3 layer and the underlying rib meristem/rib zone (RM/RZ). The absence of *KNAT2*-promoter activity in the SAM of *ath1-1* mutants corroborates our previous observation that ATH1 specifically functions in proper establishment of these tissues in the SAM (Rutjens et al., 2009). Although the SAM and the RM were originally recognized as separate areas with distinct morphogenetic tasks, this

distinction gradually became unclear due to the tendency to include the RM in the SAM (Esau, 1977). Recently, Ruonala et al. (2008) provided support for the classic notion that the RM is a morphogenetic unit in its own right, indicating that the two meristematic regions of the apex, SAM and RM, might function as semi-independent but interacting units. Similar to initiating lateral organs, the RZ is continuously supplied with cells by the SAM. Cells within the RM differentiate rapidly and only due to this continuous provision of new cells from the SAM the RM appears to have indeterminate activity. Thus, one could envision that also a boundary exists between the SAM and the RM. The RM generates the pith of both the stem and petioles (Vaughan, 1955). During the vegetative to generative transition, cells in the RM/RZ become mitotically more active and give rise to the inflorescence stem (Kwiatkowska, 2008). In *ath1* seedlings the subapical region is longitudinally enlarged. This is partly due to reduced inhibition of growth of cells in the RM/RZ and partly because of incorporation of fused leaf petioles (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). In contrast, increased levels of *ATH1* inhibit cell proliferation in the inflorescence stem, thereby nearly reducing the inflorescence to a rosette of flowers and siliques (Gomez-Mena and Sablowski, 2008). In tobacco, a complete lack of cell division in the RM/RZ area had a similar effect: stem elongation was prevented and plants grew as a rosette (Rinne et al., 2005). In Arabidopsis the start of bolting coincides with downregulation of *ATH1* expression (Proveniers et al., 2007; Gomez-Mena and Sablowski, 2008). Taken together, this strongly suggests that ATH1 is involved in the establishment of a boundary at the SAM-RM interface.

In poplar, *CENTRORADIALIS-LIKE1* (*CENL1*), the ortholog of Arabidopsis *TERMINAL FLOWER1* (*TFL1*), is proposed to act as a modifier of RM activity (Ruonala et al., 2008). An acceleration of stem elongation coincides with upregulation of *CENL1* in the RM (Ruonala et al., 2008). Similarly, in Arabidopsis local upregulation of *TFL1* expression in the RM/RZ coincides with floral transition and, the onset of bolting (Bradley et al., 1997; Kardailsky et al., 1999; Ratcliffe et al., 1999; Conti and Bradley, 2007). Therefore, in addition to its established function in inflorescence meristem identity, CENL1/TFL1 has been proposed to function also in promoting stem elongation (Ruonala et al., 2008). Interestingly, in tomato it was recently found that local balances of the TFL1 ortholog SP and its close relative SFT regulate, among others, the growth of stems and the formation of abscission zones (Shalit et al., 2009). ATH1 is not only involved in these processes, but similar to TFL1, also in the control of floral transition (Proveniers et al., 2007). Possibly another function of ATH1 at the SAM-RM interface might be to control the activity of *TFL1*.

Experimental Procedures

Plant materials and growth conditions

Mutant alleles used were *ath1-1* (Proveniers et al., 2007), *stm-bum1-1* (Jasinski et al., 2005), *knat2-5*, *knat6-1*, *knat6-2*, *knat2-5 knat6-1*, and *knat2-5 knat6-2* mutants (Belles-Boix et al., 2006), and *bp-1* (Venglat et al., 2002). Columbia-8 (Col-8; N60000) was used as the wild type. The translational KNAT2-GUS (Dockx et al., 1995) and transcriptional KNAT6-GUS (Belles-Boix et al., 2006) fusions have been described previously. Transgenic line *Pro35S*:HA-ATH1 (Proveniers et al., 2007) and *Pro35S:ATH1-HBD* lines (Rutjens et al., 2009) were created before. For ectopic overexpression of ATH1 in *knat2* and *knat6* background, *Pro35S:HA-ATH1* construct (Proveniers et al., 2007) was introduced into these plants. Transgenic seeds were selected using a Leica MZFIII stereomicroscope equipped with GFP3 (470 nm/525±50 nm) and YFP (510 nm/640±50 nm) filter stes.

Mutations/transgenes present in other backgrounds were backcrossed three times to Col-8. Plants were grown in long days (16-h light/8-h darkness) on soil as before (Proveniers et al., 2007), or in Petri dishes (1x MS supplemented with 1% plant agar). For Dex (Sigma, http://www.sigmaaldrich.com/) treatments, an appropriate amount of Dex [1 mM in 95% ethanol (EtOH)] was added to the growth medium to arrive at the desired final concentration, or 1000x diluted in water with 0.2% (w/v) Silwet surfactant (Momentive performance materials, http://www.momentive.com/oi/) and applied in liquid media. Same amount of 95% ethanol (EtOH) were used for the control treatment. Plants for induction of gene expression analysis were grown either on Dex-supplemented (10 µM) plates, or without Dex but seedlings were then transferred to the liquid media containing Dex (10 µM) in 20 ml liquid media in continuous light in separate flasks and harvested after 8 hrs or 24 hrs treatment.

Histochemical analysis of beta-glucuronidase (GUS) activity, sectioning and microscopy

Embryos, seedlings, inflorescence were immersed in the staining solution (50mM sodium phosphate buffer PH 7.2, 0.2% TritonX-100, 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM X-glucoronide) and incubated 12 hrs (for plants containing *pKNAT2:GUS*) or 18 hrs (for plants containing *pKNAT6:GUS*) in the dark at 37ºC. After staining, plant samples were cleared and dehydrated several times in 70% ethanol and visualized the localization of the GUS activity using LEICA MZ16F microscope with LEICA DFC 300FX digital camera (Germany). For SAM sectioning, plants material (either GUS-stained or non-stained) were fixed, dehydrated and sections were made as described before

(Rutjens et al., 2009). Cauline leaf-stem fusions were examined under Nomarski optics on a Zeiss (Jena, Germany) Axioskop 2 light microscope.

Analysis of gene expression

RNeasy minkit columns (Qiagen, http://www.qiagen.com) were used to isolate RNA for quantitative (Q)-PCR and RT-PCR analysis. Total RNA was DNaseI treated (RNase-free; Fermentas GmbBH, Germany, http://www.fermentas.com) to remove genomic DNA. Absence of DNA was analyzed by performing a PCR reaction (40 cycles, similar to the real-time PCR program) on the DNaseI-treated RNA using Taq-DNA polymerase. cDNA was synthesized using MLV reverse transcriptase (Promega, Madison, WI, USA) according to the instructions of the manufacturer. Q-PCR was performed using ABIprim7700 Sequence detector and Cybergreen™ chemistry (ABI, Foster City, CA, USA). Expression levels were calculated relative to *ACTIN2* (At3g18780) levels using the Q-gene method that takes the relative efficiencies of the different primer pairs into account (Muller et al., 2002). Primers were designed according to the recommendations of the PCR master-mix manufacturer (ABI) or designed to be gene-specific by the CATMA consortium (http://www.CATMA.org). Primer sequences can be obtained from the authors.

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Chapter 4

Overlapping and Opposite roles of Three *BEL1*-like Homeobox Genes during different stages of Arabidopsis Development

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Summary

Plant BEL1-like (BELL) and Knotted1-like homeobox (KNOX) transcription factors (TF) play a central role in diverse morphogenetic programs. Genetic studies have shown extensive overlapping functions amongst the members of each subclass. Here we investigate the role of three previously characterized BELL proteins, ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1), PENNYWISE (PNY) and POUND-FOULISH (PNF) in generative development. We previously concluded that these proteins are redundantly required for proper shoot apical meristem initiation and maintenance in Arabidopsis. In this study, opposite functions of ATH1, PNY and PNF during development are described. We show that ATH1 opposes PNY function in stem growth, floral internode patterning, the maintenance of apical dominance, and septum formation in the fruit. Moreover, ATH1 opposes the joint function of PNY and PNF in the process of floral evocation and subsequent floral meristem specification. Our data suggest that these BELL proteins have overlapping roles during vegetative stage while they have opposite functions during generative development.

Introduction

In plants, the three amino acid loop extension (TALE) homeodomain (HD) super-family, comprises the Knotted1-like homeobox (KNOX) subfamily and the BEL1-like (BELL) transcription factor (TF) subfamily (Reiser et al., 1995; Burglin, 1997). These BELL and KNOX proteins are important morphogenetic regulators. TALE HD proteins are characterized by the presence of three additional amino acid residues (PYP) in the loop between the first two helices of the helix-loop-helix-turn-helix structure that makes up the DNA-binding HD, when compared to the other HD proteins (Bertolino et al., 1995; Burglin, 1997). In addition to the TALE HD, BELL-subclass proteins share two adjacent N-terminal domains, designated SKY and BELL domains (Burglin, 1997; Bellaoui et al., 2001), whereas KNOX-subclass proteins share the highly conserved MEINOX domain (Burglin, 1997). These lineage specific domains enable BELL and KNOX proteins to dimerize with members of the other subclass (Bellaoui et al., 2001; Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Hackbusch et al., 2005; Cole et al., 2006; Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). BELL-KNOX interactions are required for site-specific DNA binding (Smith et al., 2002) and for nuclear localization of the transcription factor heterodimeric complex (Bhatt et al., 2004; Cole et al., 2006; Rutjens et al., 2009). Heterodimerization-controlled cellular localization of BELL and KNOX proteins involves an evolutionary conserved nuclear exclusion mechanism that is probably generic to control the activity of BELL and KNOX combinations (Rutjens et al., 2009) BELL–KNOX interactions have been reported in Arabidopsis, potato, tomato, maize, rice, barley, and green algae (Bellaoui et al., 2001; Muller et al., 2001; Smith et al., 2002; Chen et al., 2003; Nagasaki et al., 2005; Kimura et al., 2008; Lee et al., 2008). Taken together, this strongly suggests that BELL-KNOX interactions are evolutionarily conserved and that their interaction is required for biological activity.

In Arabidopsis, the *KNOX* and *BELL* genes that have been functionally characterized to date often show overlapping roles with other members of their subclass, indicating that functional redundancy within each subfamily seems to be a common feature. For example, three of the four Arabidopsis class I KNOX proteins play key roles in shoot apical meristem (SAM) function. Whereas SHOOTMERISTEMLESS (STM) provides the critical KNOX function for initiation and maintenance of the SAM (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996), both BREVIPEDICELLUS/KNOTTED1-LIKE ARABIDOPSIS THALIANA1 (BP/KNAT1) and KNAT6 contribute redundantly with STM in this process (Byrne et al., 2002; Belles-Boix et al., 2006). In addition, KNAT6 has been reported to have a functionally redundant role with STM in meristem-organ boundary formation (Belles-Boix et al., 2006), whereas the two closest related class I KNOX proteins, KNAT2 and KNAT6, have an overlapping function in the

establishment of cauline leaf-stem boundaries (Chapter 3). Similarly, within the BELL protein family, the closely related BEL1-LIKE HOMEODOMAIN2/SAWTOOTH1 (BLH2/SAW1) and BLH4/SAW2 proteins act redundantly to limit leaf margin growth (Kumar et al., 2007), whereas the paralogs PENNYWISE (PNY) (also known as BELLRINGER, REPLUMLESS, LARSON and VAAMANA) and POUND-FOOLISH (PNF) act concertedly to control a wide number of developmental processes throughout the Arabidopsis lifecycle, including SAM initiation and maintenance, the transition from vegetative to reproductive development, inflorescence architecture and floral patterning (Smith et al., 2004; Kanrar et al., 2006; Kanrar et al., 2008; Rutjens et al., 2009; Yu et al., 2009).

Intruigingly, TALE HD proteins that have overlapping functions in one developmental process, can have opposite roles in a second process. For instance, in addition to their overlapping role in SAM function, BP/KNAT1 and KNAT6, are reported to have counteracting functions in inflorescence patterning and in replum and septum formation during fruit development (Alonso-Cantabrana et al., 2007; Ragni et al., 2008). Misexpression of *KNAT6* in the inflorescence of *bp* mutants suggests that this antagonism involves spatial limitation of the *KNAT6* expression domain by BP/KNAT1 (Ragni et al., 2008). PNY has similar effect as BP/KNAT1 on the *KNAT6* inflorescence expression (Ragni et al., 2008). PNY can interact with BP/KNAT1 (Hackbusch et al., 2005) and it has been suggested that both proteins operate together in a heterodimeric complex to restrict *KNAT6* expression (Hay and Tsiantis, 2009). Spatial restriction of gene expression by other members of the family might be a common theme in the regulation of Arabidopsis TALE HD function, since also SAW1 and SAW2 have been shown to set the expression boundaries of other TALE HD genes. As part of their role in leaf development, both proteins prevent *BP/KNAT1*, *STM*, and *KNAT6* expression in leaves (Kumar et al., 2007).

Interestingly, a combination of overlapping and antagonistic functions among members of the same TF family has also been described for the class III HD-leucine zipper proteins (Prigge et al., 2005) and it has been suggested that this type of interactions could provide flexibility in the regulation of distinct phases of development during plant growth.

One of the most prominent phase transitions in plant development is the switch from vegetative to generative growth. A major determinant in flowering time control is the competence of plants to respond to floral inducing signals (Bernier, 1988). We previously identified the BELL gene *ARABIDOPSIS THALIANA HOMEOBOX1* (*ATH1*) (Quaedvlieg et al., 1995) as a specific, positive regulator of the floral repressor *FLOWERING LOCUS C* (*FLC*), a major regulator of floral competence in Arabidopsis (Proveniers et al., 2007). During vegetative development *ATH1* is expressed in both the SAM and leaf primordia. Prior to floral transition ATH1 becomes gradually down-regulated in the SAM (Proveniers

et al., 2007; Gomez-Mena and Sablowski, 2008). Down-regulation of *ATH1* expression is accompanied by a similar decrease in *FLC* expression, indicating that ATH1 functions as a competency modulator of the vegetative SAM (Proveniers et al., 2007). In contrast, PNY and PNF have been proposed to function as positive regulators of meristem competence (Smith et al., 2004). Plants that carry lesions in both *PNY* and *PNF* lack any form of inflorescence development and floral patterning. As a result *pny pnf* plants continue to produce leaves throughout their lifecycle (Smith et al., 2004). Ectopic expression of *LEAFY* (*LFY*) in these plants restores flower formation, indicating that PNY and PNF function as positive regulators of meristem competence in the process of floral evocation by promoting *LFY* expression (Kanrar et al., 2008).

Recently, we identified overlapping roles for ATH1, PNY, and PNF in embryonic SAM initiation and SAM maintenance during vegetative growth (Rutjens et al., 2009). Taken together, this suggests that also ATH1, on the one hand, and PNY and PNF, on the other hand, have both overlapping and opposite functions. An ATH1-PNY antagonism is supported by our previous observations that clusters of siliques, typical for *pny* mutants, were absent in *ath1-1 pny-40126* inflorescences, whereas this phenotype could be induced by ectopic expression of *ATH1* (Rutjens et al., 2009). Furthermore, introduction of the *ath1-1* mutation in *pny pnf* plants partially restored internode elongation in this background. However, triple mutants lacked a proper inflorescence meristem and floral patterning events did not occur (Rutjens et al., 2009). As *ath1-1 pny pnf* plants display severe SAM defects already during early seedling development, the observed defects in the inflorescences of these plants might be an indirect consequence of the initial SAM defects. Previous preliminary analyses indicated a difference in allele strength between the two *ath1* alleles studied, *ath1-1* and *ath1-3*, the former having the strongest effect on flowering time (Proveniers et al., 2007). Here we demonstrate that while exhibiting all characteristic defects attributed to loss of *ATH1* function, *ath1-3* is indeed a weaker allele than *ath1-1*. Using this weaker *ath1* allele, we examined the opposite roles of ATH1 and PNY/PNF in more detail. We show that ATH1 totally opposes PNY function in inflorescence patterning and fruit development and that of PNY and PNF together in the control of floral transition and floral meristem specification.

Results

ath1-1 and *ath1-3* affect SAM morphology to a different extent

Based on the absence of full-length *ATH1* mRNA in *ath1-1* and *ath1-3* seedlings both alleles have previously been regarded as null alleles (Proveniers et al., 2007). In line with this, *ath1-1* phenotypes are highly comparable to those of *ath1-3* with respect to meristem-leaf boundary defects, floral organ abscission, and SAM displacement in continuous darkness (Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). However, when assayed for *FLC* activity *ath1-1* plants display a greater reduction in expression levels than *ath1-3* plants and this is reflected in a slightly earlier flowering of the former plants (MP and BR, unpublished results), indicating that there still might be a difference in allele strength between both alleles.

This potential difference in allele strength was examined in more detail by comparing the SAM morphology of *ath1-1* and *ath1-3* mutants. ATH1 is required for proper SAM organization and particularly affects the subapical region (Rutjens et al., 2009). In a previous paper we reported that in 4-day-old *ath1-1* mutants the subapical region is significantly elongated when compared to wild-type plants: 88.21±12.85 µm vs. 48.28±2.94 µm, respectively (Rutjens et al., 2009). In addition, the *ath1-1* mutation negatively affects SAM width: 71.30±2.87 µm in *ath1-1* vs.

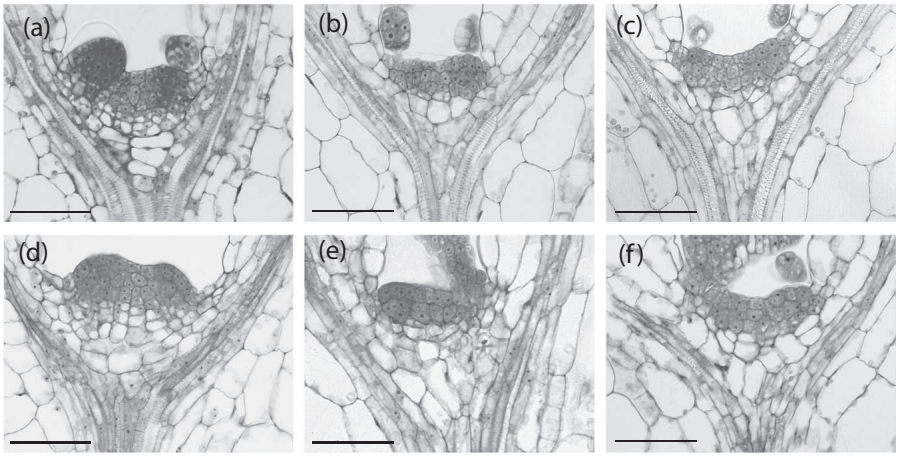

Figure 4.1 *ath1-1* and *ath1-3* affect SAM morphology to a different extent.
(a)-(f) Longitudinal median sections through vegetative apices of 4-day-old wild-type (a), *ath1-1* (b), *ath1-3* (c), *pny-40126* (d), *ath1-1 pny-40126* (e) and *ath1-3 pny-40126* (f) plants. Bar=50 µm.

104.37±4.01 µm in wild-type plants (Rutjens et al., 2009). In the same experiments we also measured these characteristics in *ath1-3* mutants, but we did not previously report on them. Both *ath1* alleles exhibit essentially the same aberrations in SAM organization. Highly comparable to *ath1-1, ath1-3* mutants also showed a longitudinal increase in the size of the subapical region (87.04±12.56µm) and a reduction in SAM diameter (77.04±3.25µm). In both cases this is accompanied with a reduction in the densely cytoplasmic, darker stained, meristematic cells when compared to the wild-type situation (Figure 4.1a,b,c). However, when compared to each other, the number of meristematic cells in *ath1-3* is not as severely reduced as in *ath1-1*, suggesting that the *ath1-3* allele has a weaker effect on SAM morphology than *ath1-1*. This is further corroborated by the differences in SAM composition of *ath1-1* and *ath1-3* plants when combined with a mutation in *PNY* (Figure 4.1d,e,f). As was previously found for *ath1-1 pny* mutants (Rutjens et al., 2009) and consistent with overlapping functions of ATH1 and PNY during vegetative development, introduction of the *pny-40126* mutation caused a further reduction in the number of meristematic cells in the SAM of *ath1-3* plants (Figure 4.1b,e,c,f). Nevertheless, in *ath1-3 pny* mutants still a considerable number of L3 cells can be identified, whereas in *ath1-1 pny* hardly any meristematic cells underlying the outer two cell layers of the SAM is left (Figure 4.1e,f). Importantly, we never observed growth cessation due to SAM termination in *ath1-3 pny* seedlings, whereas previously we found that over 40% of *ath1-1 pny* seedlings display a temporary developmental arrest due to SAM maintenance defects (Rutjens et al., 2009).

Based on these morphological data, it can be concluded that there is indeed a difference in allele strength between *ath1-1* and *ath1-3*, the latter being the weaker allele, at least with respect to SAM function.

The *ath1-3* mutation rescues the *pny* inflorescence patterning defects

To get a better idea of the antagonistic interaction between ATH1 and PNY/PNF, we combined the weaker *ath1-3* allele with *pny-40126*, *pnf-96116* and *pnf-33879* mutations, respectively.

Except for mild organ separation defects, *ath1-3* mutant morphology was highly comparable to that of wild-type plants when grown under standard conditions (Proveniers et al., 2007; Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). Only the average height of *ath1-3* plants was significantly higher than that of wild-type controls (Figure 4.3a), consistent with the proposed role of *ATH1* in restricting stem growth (Gomez-Mena and Sablowski, 2008; Chapter 3). As reported before (Smith et al., 2004), no apparently aberrant phenotype was observed in either *pnf-33879* or *pnf-96116* mutants, and, like *ath1-1 pnf-96116* (Rutjens et al., 2009), *ath1-3 pnf-96116* and *ath1-3 pnf-33879* double mutants were

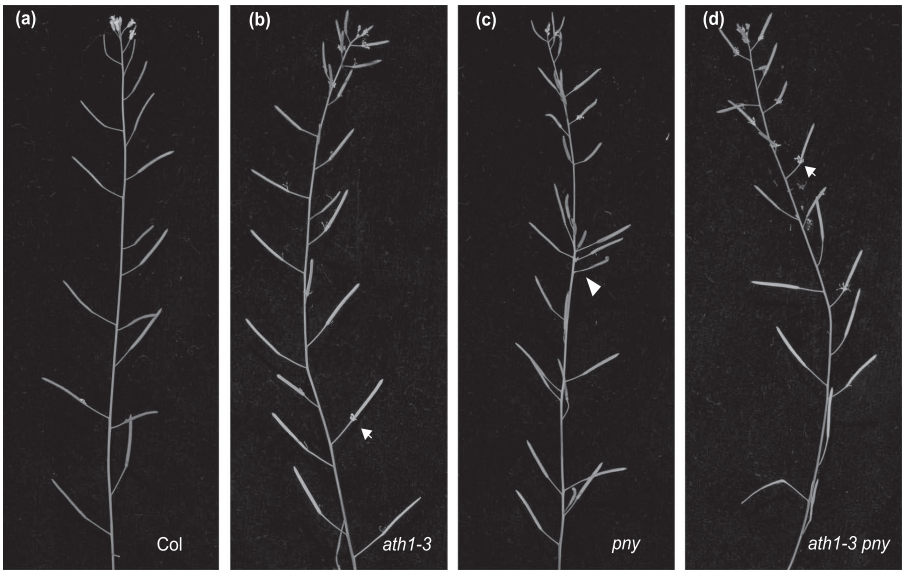

Figure 4.2 Inflorescence phenotypes of wild-type plants and *ath1-3*, *pny* and *ath1-3 pny* mutants.
(a) Wild-type inflorescence.
(b) *ath1-3* inflorescence.
(c) *pny* inflorescence with irregular internode length distribution.
(d) *ath1-3 pny* inflorescence.
Arrows indicate the floral abscission defects caused by the *ath1-3* mutation. The arrow head indicates a cluster of siliques typically observed in pny mutants.

indistinguishable from the *ath1* single mutant in SAM organization and overall plant morphology (data not shown). On the other hand, *pny* mutants, are characterized by a reduced stature and a bushy appearance due to partial loss of apical dominance. In addition, inflorescence internodes are often absent or severely reduced in length so that flowers occur at irregular intervals along the stem. In combination with a relative displacement of the flowers along the radial axis of the stem this gives rise to typical inflorescences with clusters of siliques (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Ragni et al., 2008) (Figure 4.2c). In *ath1-1 pny* mutants, the bushy appearance is maintained due to SAM termination and formation of transient meristematic tissues during vegetative development. After floral transition, inflorescence internode lengths shows more variation than in wild-type plants, but siliques are no longer clustered in groups in *ath1-1* plants (Rutjens et al., 2009).

Surprisingly, introduction of *ath1-3* in the *pny* background almost completely suppressed the *pny* inflorescence defects, whereas the *pny* mutation had no effect on the typical *ath1* organ boundary and floral organ abscission defects in the same background (Figure 4.2d). Whereas the *pny* mutants were semi-dwarfed,

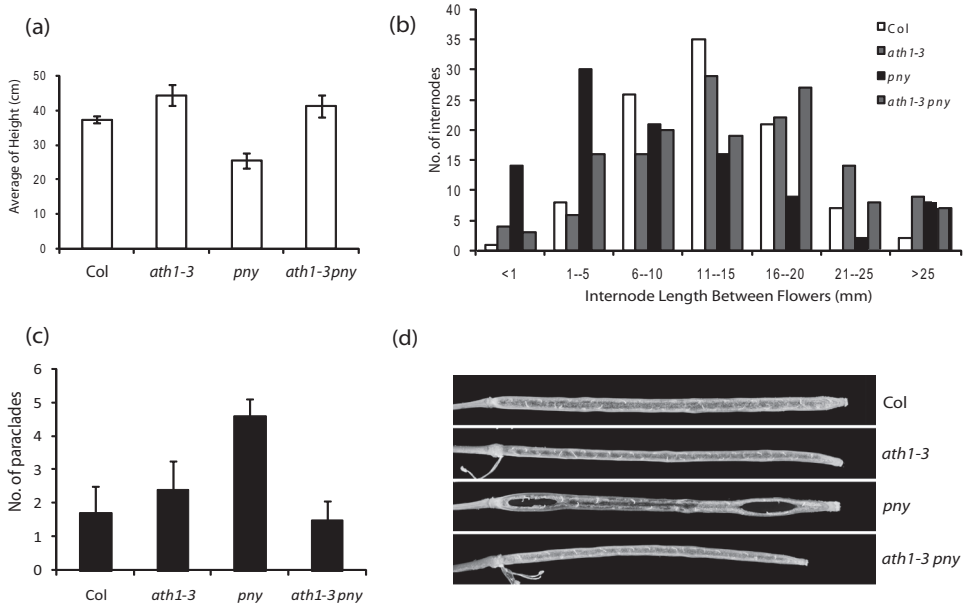

Figure 4.3 The *ath1-3* mutation rescues the *pny* inflorescence defects.
(a) Average height of wild-type, *ath1-3*, *pny*, and *ath1-3 pny* plants.
(b) Histogram representing the distribution of the length of the internode between two successive siliques. Internodes between the 1st and 11th silique (acropetally) were analysed.
(c) Average number of rosette paraclades of wild-type, *ath1-3*, *pny* and *ath1-3 pny* plants.
(d) Sseptum phenotypes of wild-type, *ath1-3*, *pny*, and *ath1-3 pny* fruits. Note the retained stamen at the base of the siliques in the *ath1-3* background.
Ten plants per genotype were used for the analyses of (a)-(c). Error bars indicate SD.

about 12 cm shorter on average than wild-type, the average height of the *ath1-3 pny* plants was comparable to that of wild-type plants (Figure 4.3a). The distribution of organs along the inflorescence in *ath1-3 pny* double mutants was more regular than in *pny* plants (Figure 4.3b). In *pny* plants the majority (65%) of the floral internodes was equal to or shorter than 10 mm, whereas only 39% of the *ath1-3 pny* floral internodes fell into these categories, compared to 35% in wild-type plants. In wild-type plants the majority of the internodes (56%) had a length in between 11 and 20 mm. In *pny* plants this level was reduced to 25%, while introduction of the *ath1-3* mutation almost recovered the wild-type situation with 46% of the floral internodes in the category between 11 and 20 mm (Figure 4.3b).

The partial loss of apical dominance seen in the pny mutants was also rescued in the *ath1-3 pny* double mutant (Figure 4.3c). Whereas *pny* plants produced four to six rosette paraclades, both wild-type plants and the *ath1-3 pny* double mutants produced one to three rosette paraclades (Figure 4.3c).

The *ath1-3* mutation reverses the *pny* septum defects during fruit development

In addition to the inflorescence patterning defects, *pny* mutants also exhibit defects in fruit development as the *pny* fruit shows a narrower replum and ovary septum fusion defects (Byrne et al., 2003; Roeder et al., 2003; Ragni et al., 2008). As *ATH1* is expressed at late stages of carpel development (Gomez-Mena and Sablowski, 2008), we examined whether ATH1 also antagonizes PNY function during this stage of development by comparing septum morphology in *pny*, *ath1-3*, and *ath1-3 pny* fruits (Figure 4.3d).

In line with previous observations (Ragni et al., 2008), sixty out of sixty *pny* fruits showed clear septum fusion defects (Figure 4.3d). The septa in *ath1-3* fruits had a wild-type morphology (Figure 4.3d). In contrast, in *ath1-3 pny* mutants this defect was almost completely restored, as fifty-seven out of sixty examined fruits had intact, non-fused spectra (Figure 4.3d), the other three showing only minor defects (not shown).

The *ath1-3* mutation restores internode elongation and floral meristem formation in *pny pnf* mutants

Perhaps the most dramatic phenotype involving *pny* is the non-flower-producing phenotype of *pny pnf* plants. These double mutants fail to complete floral evocation in response to floral inductive signals, producing a non-flowering phenotype (Smith et al., 2004). The *ath1-3* mutants are early flowering (Figure 4.4d) and introduction of the *ath1-1* mutation in *pny pnf* plants partially restores internode elongation in this background. However, these plants lack a proper

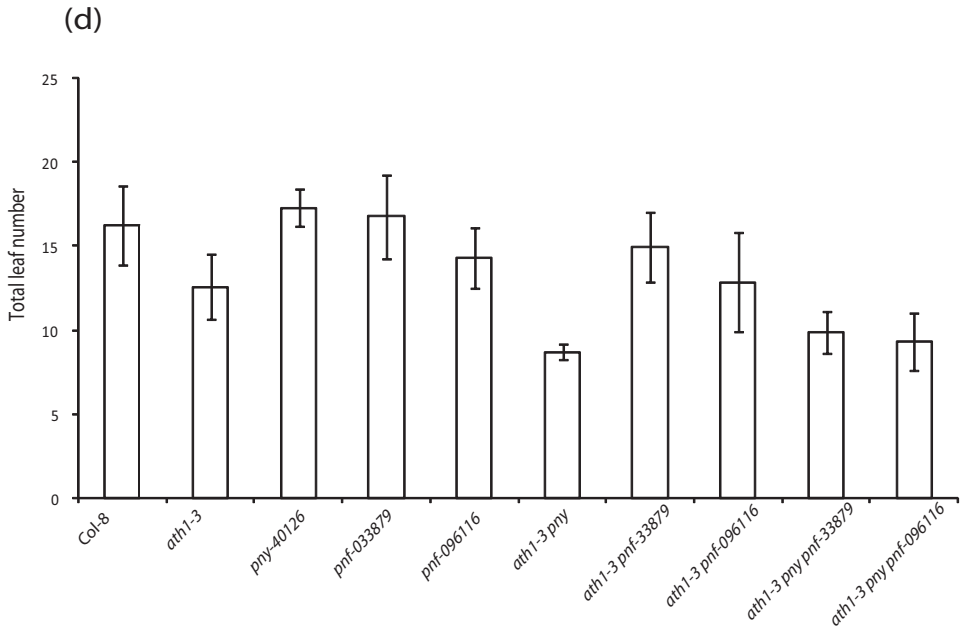

Figure 4.4 The *ath1-3* mutation restores inflorescence growth and floral meristem formation in pny pnf mutants.
(a) 4-month-old *pny pnf-096116* plant, lacking inflorescence development.
(b) 8-week-old *ath1-3 pny pnf-096116* plant with restored inflorescence development.
(c) 8-week-old *ath1-3 pny pnf-033897* plant with restored inflorescence development. The arrow indicates fertility defects: absence of siliques on the pedicel along most of the inflorescence stem.
(d) Histogram representing flowering time of *ath1-3*, *pny*, *pnf* and *ath1-3 pny*, *ath1-3 pnf* double and *ath1-3 pny pnf* triple mutants compared to wild-type control plants. Two pnf alleles (*pnf-096116* and *pnf-033879*) were used. Total leaf number formed at flowering was determined. Plants were grown under long day conditions. At least 14 plants per genotype were scored. Error bars indicate SD.

inflorescence meristem and floral patterning events never occur (Rutjens et al., 2009). To examine whether these remaining defects are an indirect consequence of the strong SAM defects already manifested during early *ath1-1 pny pnf* seedling growth or can be directly attributed to ATH1, we generated *ath1-3 pny-40126 pnf* triple mutants. Remarkably, no developmental arrest was observed in either *ath1-3 pny pnf-96116* or *ath1-3 pny pnf-33879* plants, suggesting that loss of *PNF* did not significantly deteriorate the *ath1-3 pny* SAM defects or that any such SAM defects could be overcome later in the triple mutants during plant development. As a result, during the vegetative growth stage these plants were indistinguishable from *ath1-3* plants (data not shown). It is also remarkable that the *ath1-3* mutation

restored the ability to specify flower meristems in both *pny pnf-96116* and *pny pnf-33879* mutants (Figure 4.4a-c). Stem elongation and internode patterning defects were also rescued, providing *ath1-3 pny pnf* plants with a wild-type stature (Figure 4.4). These findings strongly suggest that the presence of inflorescence and floral patterning defects in *ath1-1 pny pnf-96116* plants are an indirect consequence of severely impaired vegetative SAM function that cannot be overcome later in development.

It is further noteworthy that *ath1-3 pny* plants flowered earlier than *ath1-3* single mutants, whereas *pny* mutants behaved as wild-type (Figure 4.4d). This indicates that PNY has a functionally redundant role with ATH1 as a repressor of flowering. The flowering time of *ath1-3 pny pnf* triple mutants was not significantly different from that of *ath1-3 pny* plants (Figure 4.4d), suggesting that PNF does not contribute to the timing of floral transition.

Although normal flowers and siliques were produced in both triple mutant lines, the fertility of *ath1-3 pny pnf-33879*, but not *ath1-3 pny pnf-96116* plants, was severely affected as many flowers displayed floral patterning defects. In one experiment, only 4% (22/530) of the *ath1-3 pny pnf-33879* flowers were fertile, whereas in the same experiment 82% (137/168) of the *ath1-3 pny pnf-96116* flowers produced seed. Many *ath1-3 pny pnf-33879* flowers lacked pistils or contained non-fused carpels. In addition, often ectopic stigmatic tissue on petals was observed, as well as petal-stamen and stamen-stamen fusions (data not shown). In both triple mutant lines, in the fruits that developed, the *pny* septum fusion defects rescued by *ath1-3* were partially reversed by the *pnf* mutation. In 16% of the *ath1-3 pny pnf-96116* fruits and 85% of the *ath1-3 pny pnf-33879* fruits open septa were observed, indicating that both *pnf* mutations (partially) reversed the *ath1*-3-mediated restoration of the *pny* septum defects. This suggests that ATH1 and PNF have opposite functions in this part of fruit patterning. The observed differences in fertility and fruit development between both triple mutant lines further indicate that *pnf-33879* is a stronger allele than *pnf-96116*. A recent study by Yu et al. (2009) examined the role of PNY and PNF in floral patterning using *pny* PNF/*pnf-33879* and *pny lfy-10 PNF/pnf-33879* alleles. This study revealed that PNY and PNF play a role in the specification of petals, stamens and carpels during flower development. Since *ath1-3 pny pnf-33879* plants displayed severe flower defects, this indicates that the *ath1-3* mutation cannot rescue flower patterning defects caused by the combined absence of *PNY* and *PNF*.

Discussion

Plant BELL and KNOX proteins are members of the conserved TALE HD superfamily of transcription factors and, they have been found, in analogy with their animal counterparts, to form functional complexes with members of the other class to regulate various developmental processes (Byrne et al., 2003; Cole et al., 2006; Kanrar et al., 2006; Pagnussat et al., 2007; Kimura et al., 2008; Rutjens et al., 2009). So far, the function of the majority of Arabidopsis TALE HD genes remains unclear. This might be partially explained because of a high level of genetic redundancy between family members (Smith et al., 2004; Belles-Boix et al., 2006; Kumar et al., 2007; Ragni et al., 2008; Rutjens et al., 2009). Another aspect that hampers comprehensive analysis of TALE HD function in plants is the hugely pleiotropic effects that can be observed in some TALE HD loss-of-function mutants. This pleiotropy is also reflected by the fact that each BELL protein associates with a subset of KNOX proteins and vice versa (Hackbusch et al., 2005). Perhaps the best example of a TALE HD protein that is involved in a wide array of developmental processes is PNY. *pny* mutants have been identified in a variety of screens, and phenotypes include enhancement of *stm* phenotypes, aberrant organ initiation patterns, loss of apical dominance, phyllotaxy defects, reduced internode elongation, organ boundary defects, replum loss, and floral patterning defects (Byrne et al., 2003; Roeder et al., 2003; Smith and Hake, 2003; Bao et al., 2004; Bhatt et al., 2004; Smith et al., 2004; Kanrar et al., 2006; Yu et al., 2009). Next to this, in a number of cases mutations in (combinations of) TALE HD genes have a dramatic effect early during development, thereby interfering with a proper characterization of their function later in development. Strong *stm* alleles, for instance, are seedling lethal. However, in this case identification and characterization of weaker alleles have shown that STM is also involved in floral specification and internode development (Endrizzi et al., 1996; Kanrar et al., 2006; Scofield et al., 2007).

Previously, we demonstrated that the BELL proteins ATH1, PNY, and PNF are partners of STM and have a partially redundant function in embryonic SAM initiation and SAM maintenance during vegetative growth (Rutjens et al., 2009). At the same time, results from this study indicated that later in development ATH1 might have a function opposite to that of PNY and/or PNF. However, severe SAM defects already at the onset of post-embryonic development in the studied *ath1-1 pny pnf* plants interfered with proper interpretation of the phenotypes observed in these plants at later developmental stages.

Here we use the weaker-strength *ath1* allele *ath1-3* and show that ATH1 in all processes studied opposes PNY function in inflorescence development. In addition, the observed SAM defects in *ath1-3* and enhancement of these defects in *ath1-3 pny* plants corroborate our previous findings that ATH1 and PNY have

partial overlapping functions during the vegetative growth stage.

ATH1 and PNY have opposite roles in inflorescence development

The data presented here show that introduction of the *ath1-3* mutation in a *pny* mutant background almost fully suppresses all *pny* inflorescence defects investigated, including the reduced stem elongation, floral internode patterning defects and the partial loss of apical dominance. Moreover, the *pny* septum fusion defects were almost completely restored in *ath1-3 pny* double mutant plants (Figure 4.2 and 4.3). Taken together, this suggests that during the generative phase of development ATH1 and PNY have opposite functions. Although *ath1-3* inflorescences grow taller than those of wild-type plants, whereas *pny* plants are semi-dwarfed, this antagonistic function of ATH1 is not directly reflected by the *ath1* mutant phenotype. In contrast, in plants ectopically expressing ATH1, phenotypes typical for *pny*, such as reduced stem elongation and clustered siliques, can be observed (Rutjens et al., 2009). As regulation of TALE HD gene expression by other family members is not an uncommon feature in Arabidopsis (Kumar et al., 2007; Ragni et al., 2008), in wild-type plants (localization of) *ATH1* expression might be controlled by PNY. Loss of PNY function would then result in ectopic/increased activity of *ATH1*, with the typical *pny* defects as a result. However, we never observed any septum fusion defects in plants that ectopically express ATH1 (D.B. and M.P., unpublished observation), indicating that the situation might be more complex.

Interestingly, restoration of *pny* inflorescence patterning and fruit development defects has also been found by Ragni et al. (2008) in plants that combine a *pny* mutation with loss of KNAT6 and KNAT2 function. These authors further showed that the inflorescence and fruit patterning aberrations seen in *pny* can be attributed to misexpression of *KNAT6* and to a lesser extent of *KNAT2*. ATH1 is capable of interacting with both KNAT proteins (Rutjens et al., 2009) and the work presented in Chapter 3 of this thesis indicates that such interactions might have biological relevance. Thus, it is likely that ATH1-KNAT6 and ATH1-KNAT2 heterodimeric complexes antagonize PNY function in inflorescence and fruit development. The observed restoration of *pny* defects in *ath1-3 pny* plants and *knat2 knat6 pny* plants might have a similar basis: the abolishment of ectopic KNAT2 and KNAT6 activity. In *ath1-3 pny* plants the loss of protein function is due to absence of functional heterodimeric complexes. This scenario is supported by the recent finding that removal of a putative interacting KNOX partner from plants ectopically expressing a BELL gene can result in restoration of the wild-type phenotype (Pagnussat et al., 2007). ATH1 and PNY might normally function in mutually exclusive domains in the inflorescence stem, similar to the

situation proposed for KNAT2 and KNAT6, on the one hand, and PNY and BP/KNAT1, on the other hand, in the valve margins and the replum during fruit development, respectively (Alonso-Cantabrana et al., 2007; Ragni et al., 2008; Girin et al., 2009b). PNY might spatially control ATH1 activity by restricting the expression domain of its partners. ATH1, for its part, might keep PNY activity within physical boundaries by controlling the expression of one or more PNY-interacting KNOX partner(s). A likely candidate is *BP/KNAT1*, as BP/KNAT1 has been suggested to operate together with PNY in inflorescence patterning (Smith and Hake, 2003; Rast and Simon, 2008). Moreover, ATH1 has been proposed before to antagonize BP/KNAT1 function in the control of internode elongation (Gomez-Mena and Sablowski, 2008), although further experimental support for this proposal is still lacking. Supportive evidence for a putative antagonistic function of ATH1 and BP/KNAT1 comes from the observation by Xiao-Qun et al. (2006) that *bp* mutants exhibit an enlarged floral organ abscission zone, whereas *ath1* mutants are impaired in proper establishment of this region (Gomez-Mena and Sablowski, 2008).

ATH1 opposes PNY and PNF in the process of floral evocation

The *ath1-1* mutation partially rescues the non-bolting in *pny pnf* plants (Rutjens et al., 2009). Here we report that introduction of the weaker *ath1-3* mutation in the *pny pnf* background entirely restores the ability to complete floral evocation in these plants.

In Arabidopsis, floral transition is regulated by antagonistic action of promoting and repressing pathways. Floral transition occurs once the plant has reached a certain age or size and growth conditions are favourable for sexual reproduction and seed maturation. At floral transition, the balance of promoting and repressing factors is such that the by then competent vegetative SAM is 'evoked' in a florally determined state by the activation of a set of so-called floral pathway integrators (FPI). In the SAM, these FPI proteins then activate floral meristem identity (FMI) genes to finally specify the floral fate of meristems (reviewed in Bernier, 1988; Mouradov et al., 2002; Boss et al., 2004).

PNY and PNF have been proposed to function as positive regulators of meristem competence in the process of floral evocation by promoting LFY expression (Smith et al., 2004; Kanrar et al., 2008). Although *pny pnf* plants respond to floral inductive signals by induction of FPI gene expression such as *SUPPRESSOR OF CONSTANS1* (*SOC1*), *FT* and *FRUITFULL* (*FUL*), they fail to induce expression of the FMI genes *LFY*, *APETALA1* (*AP1*), and *CAULIFLOWER* (*CAL*) to complete the process of floral evocation.

In contrast, ATH1 has been identified in our lab as a negative regulator of

floral competency through positive regulation of *FLC* expression (Proveniers et al., 2007). The observed restoration of floral transition in *ath1-3 pny pnf* plants is, thus, in line with the reported roles for ATH1, PNY and PNF. However, the exact nature of the antagonistic relationship between ATH1 and PNY/PNF in this process is still not clear. Both vernalization treatments known to stably repress *FLC* expression and the introduction of a *flc* null mutation have no effect on floral evocation in *pny pnf* plants (B.R. and M.P., our unpublished data), indicating that regulation of *FLC* activity is not part of this antagonism.

During vegetative development *ATH1* is expressed in both the SAM and leaf primordia. In response to floral inductive signals *ATH1* becomes gradually down-regulated in the SAM (Proveniers et al., 2007; Gomez-Mena and Sablowski, 2008). Currently, we do not know whether this response is retained in *pny pnf* mutants. Prolonged *ATH1* expression in the SAM might explain the non-bolting phenotype of *pny pnf* plants, as plants that constitutively express ATH1 often fail to form an elongated inflorescence. However, we never observed absence of floral patterning in plants that overexpress *ATH1*.

The *pny pnf* mutants lack the ability to express the FMI genes *LFY*, *AP1* and *CAL* to normal levels upon floral inductive stimuli (Kanrar et al., 2006). In *ap1 cal lfy* triple mutants flowers are transformed into shoots. Under standard growth conditions these plants do not produce flower meristems. These plants do form an elongated shoot, but these shoots never make a complete transition to the reproductive phase as they fail to produce flower meristems (Weigel et al., 1992; Bowman et al., 1993; Schultz and Haughn, 1993). Overexpression of *LFY* can restore flower formation in *pny pnf* double mutants, but it does not suppress the internode elongation defect (Kanrar et al., 2006). Therefore, the *pny pnf* phenotype might be a combination of two independent, separate defects: the inability to form an elongated shoot plus loss of the capacity to develop floral meristems. ATH1 seems to be involved in both. In line with this, ATH1 has been shown to play a role in inhibiting stem growth and was proposed to contribute to the rosette habit of Arabidopsis by inhibiting internode elongation during vegetative development (Gomez-Mena and Sablowski, 2008). It will be interesting to test whether ATH1 is also, involved in the regulation of FMI gene expression. However, for a better understanding of the role of ATH1, PNY and PNF in these processes a more comprehensive analysis, including the identification of direct and more downstream targets, is needed.

Overlapping and opposite roles for ATH1 and PNY during plant development

Our results suggest a functional redundant role for ATH1 and PNY as floral repressors, in addition to their opposite roles in inflorescence and flower de-

velopment. This is in line with the previous identification of *pny* in a screen for early flowering mutants in non-inductive short days in our lab (Rutjens, 2007). During vegetative development ATH1 and PNY have overlapping roles, whereas in generative development their functions become opposite. A combination of overlapping and antagonistic functions among members of the same TF family has also been described for the class III HD-leucine zipper proteins and MADS-box factors and it has been suggested that this type of interactions could provide flexibility in the regulation of distinct phases of development during plant growth (Prigge et al., 2005; Gregis et al., 2008; Gregis et al., 2009).

At the same time our findings indicate that PNY can function both as a floral repressor and a floral activator. The basis for this dual function is presently unknown, but, in analogy to the MADS-box protein AP1 that initially prevents but at a later moment promotes floral organ identity (Gregis et al., 2008; Gregis et al., 2009), this might depend on the (TALE HD) protein partners with which PNY interacts during the different phases of the plant lifecycle. TALE HD interactions have been extensively mapped and studied using the yeast two-hybrid system, but *in planta* spatio-temporal information about these interactions is still lacking. Therefore, in addition to the above-suggested identification of TALE HD controlled regulatory gene networks, detailed information on where and when in the plant TALE HD complexes are present will be essential to come to a better understanding of the function of TALE HD proteins in plant development.

Experimental Procedures

Plant materials and growth conditions

Mutant alleles used were: *ath1-1* and *ath1-3* (Proveniers *et al.*, 2007), *pny-40126* (Smith and Hake, 2003), *pnf-96116*, *pnf-33879* (Smith *et al.*, 2004). Columbia-8 (Col-8) was used as wild type. Plants were grown in long days (16 hrs light/8 hrs darkness) on soil as before (Proveniers *et al.*, 2007), or in Petri dishes (1x MS supplemented with 1% plant agar). Flowering time was measured by counting the total leaf number.

Phenotypic Analysis, microscopy and sectioning

Quantitative analyses of the different genotype were performed on 8-week-old plants. Ten plants for each genotype were used for phenotypic analyses. Septum defects were examined under Nomarski optics on a Zeiss (Jena, Germany) Axioskop 2 light microscope with a Nikon (Tokyo, Japan) DXM1200 digital cam-

era. Sectioning followed the procedure as described before and SAM size (6-12 individual plants/ genotype) were determined (Rutjens et al., 2009).

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Chapter 5

Summarizing discussion

TALE HD combinatorial control of plant development

Regulatory gene networks are an important mechanism for organizing developmental processes according to genetic information. At the basis of these regulatory networks are transcription factor (TF) proteins that act as molecular switches by controlling the activity of downstream target genes. An important aspect of regulatory gene networks is combinatorial control: specific combinations of *cis*-elements are present in the promoter regions, and only specific combinations of TF proteins bind to these elements. As a result, a large number of genes can be differentially regulated by only a limited set of TF proteins. Therefore, TF protein-protein interactions are of key importance for proper functioning of regulatory gene networks.

In eukaryotes homeodomain protein-based combinatorial control of gene expression is a widely used mechanism to control basal developmental processes. Both animals and plants utilize TALE homeodomain proteins to subdivide their body plan. In animals MEIS/PBC TF heterodimer-mediated networks are involved in the specification of the anterior-posterior axis of animals, whereas in plants their KNOX/BELL orthologs play an important role in plant architecture in general and, more specifically, in shoot meristem functions (reviewed in Mann and Morata, 2000; Hake et al., 2004). To date up to sixty-two different possible BELL-KNOX combinations have been identified (Chapter 1 Table 1.1), mostly using a yeast two-hybrid system (Bellaoui et al., 2001; Hackbusch et al., 2005). BELL-KNOX interactions are selective, and *in vitro* experiments indicate that these interactions are most likely required for site-specific DNA binding (Smith

et al., 2002; Smith and Hake, 2003). In addition, there are indications that different BELL-KNOX transcription factor pairs may regulate different downstream target genes (Cole et al., 2006). However, whether all these plant TALE heterodimers have functional relevance *in planta* and whether they are employed for combinatorial control of developmental processes is currently not clear.

In this thesis we addressed (part of) this question by functional characterization of the BELL protein ATH1, and to a lesser extent also of two related BELL proteins PNY and PNF, in relation to their interacting partners, the class I KNOX proteins STM, BP/KNAT1, KNAT2 and KNAT6.

Our finding that combined mutations in three genes that encode STM-interacting BELL proteins, *ATH1*, *PNY*, and *PNF*, phenocopy *stm* mutants (Chapter 2), not only highlights the role of these BELL proteins in SAM function, but also provides a strong indication that in plants TALE HD proteins operate as heterodimeric complexes. Since ATH1 was further found to be required for proper establishment of meristem-organ boundaries and for control of stem growth in collaboration with a different set of KNOX proteins, KNAT2 and KNAT6 (Chapter 3), this indicates that combinatorial control of developmental processes by TALE HD proteins might also be a common feature in plants.

BELL proteins: more than KNOX co-factors

The complex network of interactions that BELL and KNOX proteins operate in is not limited to the formation of BELL-KNOX heterodimers. The work described in Chapter 3 of this thesis shows that ATH1 affects the expression of two genes encoding the proteins that are able to heterodimerize with, *KNAT2* and *KNAT6* (Chapter 3). During the vegetative growth stage, ATH1 is required for wild-type levels of *KNAT2* and *KNAT6* in the SAM (Chapter 3). In generative tissues, ATH1 seems to have an opposite role in controlling *KNAT6* expression, as *ath1* mutants display elevated levels of *KNAT6* expression (Chapter 3). As STM has also been found to be normally required for *KNAT2* and *KNAT6* expression (Belles-Boix et al., 2006; Scofield et al., 2007) and ATH1 functions together with STM in the vegetative SAM (Chapter 2), during this growth stage, it is quite likely that *KNAT2* and *KNAT6* expression is controlled by the ATH1-STM protein pair. However, how exactly ATH1 affects *KNAT2* and *KNAT6* expression after floral transition remains currently unclear. As discussed in Chapter 3, a role of BELL proteins in controlling *KNOX* expression has been reported before. Kumar et al. (2007) found that the SAW1 and SAW2 repress *BP/KNAT1* and *STM* expression in leaves. Similarly, Ragni et al. (2008) showed that during inflorescence and fruit development PNY acts to restrict *KNAT6* and *KNAT2* expression domains. It has been put forward that these examples of BELL proteins regulating *KNOX* expression might represent a general mechanism that creates a feedback loop that is

critical for determining the phenotypic read-outs of TALE HD heterodimer activities in diverse organs and/or different developmental stages (Hay and Tsiantis, 2009). It will be interesting to see whether the regulation of *KNAT2/6* expression by ATH1 after floral transition also forms part of such a fine-tuning mechanism, especially in the light of the observed ATH1-PNY antagonism during this developmental stage (Chapter 4).

Apart from forming functional heterodimers, BELL and KNOX proteins might also interact with each other to sequester members of the other family by titrating them in an inactive dimer in certain tissues and/or during certain developmental stages, thus preventing the formation of additional, functional, but locally unwanted BELL-KNOX heterodimers. Such sequestration mechanisms might especially be important to set and/or maintain boundaries of gene expression domains during development. The BP/KNAT1-SAW interaction has been proposed to be part of such a sequestration mechanism (Kumar et al., 2007). The SAW proteins establish leaf shape by repressing growth in specific subdomains of the leaf at least in part by repressing *BP/KNAT1* expression (Kumar et al., 2007). Accordingly, BLH2/SAW1 and BLH4/SAW2, on the one hand, and BP/KNAT1, on the other hand, are mostly expressed in mutually exclusive domains. However, both BLH2/SAW1 and BLH4/SAW2 interact with BP/KNAT1 (Kumar et al., 2007). As KNOX proteins, including BP/KNAT1, have been shown to be able to move over both short distances across cell layers, as well as to be transported over longer ranges via the phloem (Lucas et al., 1995; Kim et al., 2002; 2003; Ruiz-Medrano et al., 2004; Zambryski, 2004; Gallagher and Benfey, 2005; Kim et al., 2005), binding of SAW proteins to BP/KNAT1 is thought to serve as an extra level of control to prevent the formation of additional, functional BP/KNAT1-BELL heterodimers within the *BLH2/SAW1* and *BLH4/SAW2* expression domains.

We identified BP/KNAT1 as one of the interacting partners of ATH1 (Chapter2), but we could find no functional overlap for both proteins (Chapter3). In contrast, BP/KNAT1 and ATH1 seem to have antagonistic functions, especially in controlling stem growth and floral organ abscission (Douglas et al., 2002; Venglat et al., 2002; Xiao-Qun et al., 2006; Gomez-Mena and Sablowski, 2008; Rutjens et al., 2009). Therefore, the BP/KNAT1 interaction might also be part of sequestration mechanism. Given the antagonistic relationship of ATH1 and PNY in inflorescence development (Chapter 4) and the proposed cooperation of BP/KNAT1 with PNY in this process (Smith et al., 2004; Rast and Simon, 2008), titrating BP/KNAT1 in an inactive ATH1-BP/KNAT1 dimer might prevent formation of BP/KNAT1-PNY complexes in the *ATH1* expression domain. Taken together, BELL and KNOX interactions exceed the protein-protein interaction level, thus forming a complex network of interactions throughout development, that is most likely set up to continuously fine-tune TALE HD heterodimer activity.

BELL and KNOX: always a TALE of two?

The transcriptional context in which TALE HD proteins act is an important aspect of their function as it likely modulates not only target selection but also nuclear versus cytoplasmic localization (Bhatt et al., 2004; Hackbusch et al., 2005; Cole et al., 2006; Kimura et al., 2008; Lee et al., 2008; Rutjens et al., 2009). In Chapter 2 we have shown that heterodimerization-controlled cellular localization of ATH1 and STM involves an evolutionary conserved CRM1/exportin-1-mediated nuclear exclusion mechanism. Since all currently identified BELL proteins contain conserved NES sequences in their BELL domains (Chapter 2), this is probably a generic mechanism to control the activity of plant TALE HD proteins (Figure 5.1). The question is whether this is the only mechanism to control subcellular localization and, thus, functioning of these proteins.

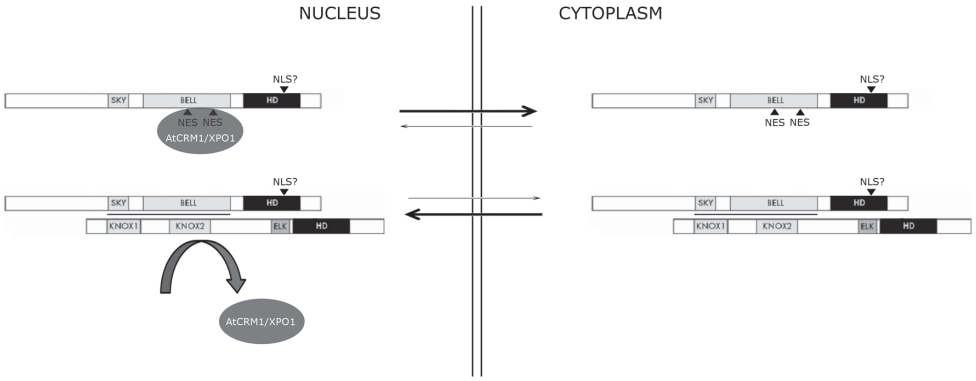

Figure 5.1 BELL-KNOX heterodimerization controls plant TALE homeobox nuclear localization through masking of nuclear export signals.
A proposed model for the regulation of sub-cellular localization of plant TALE HD proteins, based on the model for the regulation of subcellular localization of animal TALE HD proteins as proposed by Kilstrup-Nielsen et al. (2003). In plants BELL proteins are actively exported from the nucleus, a process requiring NES sequences located within their conserved BELL domain that are recognized by the nuclear export receptor AtCRM1 (dark grey oval). BELL proteins form stable dimers with KNOX proteins through interaction of the BELL and KNOX domains. The BELL-KNOX binding surface coincides with the region required for nuclear export, thereby shielding it from recognition by AtCRM1. The newly formed complex translocates into the nucleus owing to a yet unidentified NLS located within BELL proteins. Black rectangles represent the homeodomains (HD). Light gray boxes represent conserved amino-terminal regions within BELL and KNOX proteins. A black horizontal line indicates protein–protein contacts.

In animals, nuclear localization of the BELL-orthologous PBC proteins is not in all cases dependent on the presence of the KNOX-orthologous MEIS proteins. In these cases, nuclear translocation is enabled by a distinct cell context specific mechanism (Berthelsen et al., 1999; Kilstrup-Nielsen et al., 2003). In the absence of MEIS binding partners, nuclear localization of PBC proteins in differentiated cell types can be achieved by phosphorylation of the PBC-B domain (Kilstrup-Nielsen et al., 2003). Such phosphorylation possibly leads to conformational changes of PBC proteins that mask the NES and allow nuclear accumulation, even in the absence of MEIS proteins.

Previously we have identified ATH1 as a repressor of floral transition (Proveniers et al., 2007). The results described in Chapter 4 support these findings and, moreover, identify PNY as a functional redundant component in this process. Currently, no such function has been described for KNOX proteins. In addition, our ATH1 yeast two-hybrid analyses have only identified class I KNOX proteins as putative interacting partners within the plant family of TALE HD proteins (Chapter 2). *ATH1* is, among others, expressed in leaf primordia and mature leaves (Quaedvlieg et al., 1995; Proveniers et al., 2007; Gomez-Mena and Sablowski, 2008), whereas class I KNOX gene expression is excluded from these tissues. Moreover, also other BELL proteins have been identified in processes that can currently not be associated with KNOX protein activity, such as ovule development (BELL1) (Reiser et al., 1995; Brambilla et al., 2007), the phyA high irradiance response (BLH1) (Staneloni et al., 2009), nitrate assimilation in response to the fungus-*Piriformospora india* (BLH1) (Sherameti et al., 2005), and disease resistance and pathogen defense (rice *OsBIHD1*) (Luo et al., 2005). Next to this, PNY has been found to act as a direct repressor of the floral homeotic gene *AGAMOUS* (*AG*) (Bao et al., 2004). The DNA sequence motif that, in this case, is bound by PNY is significantly different from the consensus binding motif that has been described for KNOX proteins (Bao et al., 2004). Taken together, this suggests that, in analogy to animal PBC proteins, plant BELL proteins also can function independent of KNOX proteins.

Over the years, BELL proteins have been found to physically interact with several non-KNOX TF proteins, including MADS box proteins (Hackbusch et al., 2005; Brambilla et al., 2007; Ogura et al., 2008). It will be interesting to find out if these protein-protein interactions also result in stable nuclear translocation of BELL proteins or whether additional mechanisms, such as phosphorylation of specific residues in the BELL domain, are required for this.

Perspective

Despite accumulating knowledge on the function of individual BELL and KNOX proteins and the interaction network formed by them, we still have only

limited information on how this network operates during plant development. For a better understanding of the role of BELL and KNOX proteins the next step to take will be a comprehensive, systems biology approach. Such an approach includes the identification of direct and more downstream targets, both of identified BELL-KNOX combinations and of individual TALE HD proteins, in a genome-wide fashion. Further, to allow the construction of TALE HD controlled regulatory gene-networks that are involved in distinct developmental processes, it will also be necessary to get detailed information on which TALE HD complexes are present *in planta*, where and when during development.

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summary

In nature, the plant world impresses us with a large variety of forms and structures. But our knowledge of how plants are built is still limited. Using molecular genetics, scientists have begun to unravel the molecular mechanisms controlling plant architecture (or stature). As productivity of agricultural and horticultural crops is tightly associated with the plant body plan, understanding the regulation of plant architecture is of particular importance for crop yield improvement, horticultural manipulation, and biofuel production.

The model plant *Arabidopsis thaliana*, the first plant that has its entire genome sequenced, provides a good system for studying plant architecture. In Arabidopsis, it has been found that TALE homeodomain (HD) family transcription factors are involved in many aspects of plant development. One of the quintessential roles of TALE HD proteins is to maintain the shoot meristem function. The shoot apical meristem (SAM) is the source of all post-embryonic above-ground plant organs. During development, cells in the SAM keep the ability of self-renewing while allowing a continuous production of plant organs like leaves, stems and branches. It is thus of great importance to maintain proper meristem activity. A subclass of TALE HD proteins, the class I KNOX proteins, are crucial regulators in this process. For instance, mutations in the *KNOX* gene *SHOOTMERISTEMLESS (STM)* cause dramatic phenotypes as in these mutants an active SAM is lacking. As a result, these plants cease to grow.

Plants also contain a second group of TALE HD proteins, belonging to the BELL subclass. It has been shown that KNOX proteins can interact with BELL proteins. However, the biological relevance of these interactions is not well-understood. We focused our research on the functional characterization of the related BELL proteins ATH1, PNY and PNF in relation to their interacting partners, the class I KNOX proteins STM, KNAT1/BP, KNAT2 and KNAT6. Our finding that combined loss of the three genes that encodes STM-interacting BELL proteins, *ATH1*, *PNY* and *PNF*, phenocopy *stm* mutants (Chapter 2), not only highlights the role of these BELL proteins in SAM function, but also strongly indicates that TALE HD proteins act as heterodimeric complexes *in planta*. We also discovered that ATH1 is required for proper establishment of meristem-organ boundaries and for control of stem growth in collaboration with a different set of KNOX proteins, KNAT2 and KNAT6 (Chapter 3). These data suggest that KNOX-BELL mediated interactions might be a common mechanism used by plants to specify their body plan, just like the MEINOX/PBC proteins, the KNOX/BELL counterparts in the animal kingdom, shape the anterior-posterior axis.

Interestingly, a more complicated network seems to underlie the function of KNOX-BELL heterodimeric complexes as our data suggest that BELL proteins can interact at multiple levels with members of the KNOX class (Chapter 3). Ad-

ditional complexity is added by our results on the two BELL proteins, ATH1 and PNY, that have both overlapping and antagonistic functions depending on the developmental stage of the plant (Chapter 4).

In conclusion, the research presented in this thesis strongly indicates that BELL and KNOX proteins act as functional heterodimeric units to control different aspects of plant architecture. BELL-KNOX interactions extend to other molecular levels, most likely for fine-tuning TALE HD heterodimer activity.

Samenvatting in het Nederlands

De plantenwereld wordt gekenmerkt door een grote verscheidenheid in vormen en structuren. Onze kennis over de regulatoire processen die aan deze enorme variatie ten grondslag liggen is echter beperkt. Met behulp van moleculaire genetica is nu een begin gemaakt met het ophelderen van de moleculaire mechanismen die betrokken zijn bij de regulatie van plantenarchitectuur. Omdat er een sterk verband is tussen de mate van productiviteit van land- en tuinbouwgewassen en hun driedimensionale bouw (architectuur van de plant), is een beter begrip van deze mechanismen zeer wenselijk, met name met betrekking tot opbrengstverbetering voor landbouwgewassen, verbetering van tuinplanten en productie van biobrandstoffen.

Arabidopsis thaliana, de Zandraket, is een uitermate geschikt modelsysteem voor het bestuderen van aan plantarchitectuur gerelateerde processen. Vanuit eerdere Arabidopsis studies is het bekend dat een specifieke groep van regulatoire eiwitten, de zgn. TALE homeodomein eiwitten, betrokken is bij een groot aantal ontwikkelingsprocessen in de plant. In planten kan deze eiwitfamilie worden onderverdeeld in twee subgroepen: de zgn. KNOX-type TALE homeodomein eiwitten en de zgn. BELL-type TALE homeodomein eiwitten. Een van de voornaamste functies van leden van deze groep is het vormen en, later tijdens de ontwikkeling, in stand houden van het apicale scheutmeristeem. Dit meristeem bestaat uit een groep van een paar honderd stamcellen die ervoor zorgen dat steeds opnieuw weefsels kunnen worden aangemaakt. Het apicale scheutmeristeem staat daardoor aan de basis van alle bovengrondse plantendelen. Tijdens de ontwikkeling van de plant behouden de stamcellen in het apicale scheutmeristeem het vermogen zich continu te vernieuwen om een continue aanmaak van plantenorganen zoals bladeren, bloemen en stengels tijdens de ontwikkeling te garanderen. Er bestaat dus een dynamisch evenwicht tussen de aanmaak van nieuwe stamcellen en de vraag naar bouwstenen (cellen) vanuit de zich ontwikkelende organen. Voor een normale ontwikkeling van de plant is het van essentieel belang dat dit evenwicht niet verstoord wordt. Leden van de KNOX-klasse van TALE homeodomein eiwitten spelen hierbij een zeer belangrijke rol. In planten die een

mutatie dragen in het gen dat codeert voor het KNOX TALE homeodomain eiwit SHOOTMERISTEMLESS (STM), bijvoorbeeld, is de balans zo verstoord dat alle stamcellen worden opgebruikt voordat er voldoende nieuwe stamcellen worden aangemaakt. Als gevolg hiervan bezitten deze mutanten al vroeg tijdens de ontwikkeling geen apicaal scheutmeristeem meer, en stoppen ze met groeien.

Het is bekend dat KNOX TALE homeodomain eiwitten een interactie kunnen aangaan met leden van de BELL TALE homeodomain eiwitfamilie. In totaal zijn er zo'n tweeënzestig mogelijke, verschillende KNOX-BELL combinaties beschreven in de literatuur. Wat de biologische relevantie van al deze interacties is is echter nog verre van duidelijk. Het werk beschreven in dit proefschrift richt zich vooral op het beantwoorden van deze vraag. Centraal binnen dit onderzoek staan de BELL eiwitten ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1), PENNYWISE (PNY) en POUNDFOOLISH (PNF) en hun eiwitpartners, de KNOX eiwitten STM, KNOTTED1-like from ARABIDOPSIS THALIANA1/BREVIPEDICELLUS (KNAT1/BP), KNAT2 en KNAT6.

In hoofdstuk twee wordt beschreven dat zowel ATH1, PNY en PNF een complex kunnen vormen met STM en dat zulke KNOX-BELL interacties essentieel zijn voor de functionaliteit van homeodomein eiwitten van de TALE klasse. Dit wordt verder onderstreept door bevindingen die laten zien dat planten die zowel ATH1, PNY als PNF missen, fenotypisch niet zijn te onderscheiden van mutanten die STM missen. Dit betekent dat het STM eiwit de balans controleert tussen aanmaak van nieuwe stamcellen en de vraag naar stamcellen vanuit de ontwikkelende organen samen met de BELL eiwitten ATH1, PNY en PNF.

Dit is echter niet de enige functie van deze BELL eiwitten tijdens de ontwikkeling van de plant. In hoofdstuk drie zijn de bevindingen weergegeven die laten zien dat ATH1 verder benodigd is voor het afbakenen van de grenzen tussen het scheutmeristeem en gedifferentieerde weefsels, zoals bladeren en de stengel. De beschreven resultaten duiden er sterk op dat ATH1 deze eerste functie beoefent samen met twee andere KNOX eiwitten, namelijk KNAT2 en KNAT6.

Het algemene beeld dat hieruit ontstaat, in combinatie met de resultaten van andere onderzoeksgroepen, is dat in de plant verschillende KNOX-BELL interacties worden ingezet bij een verscheidenheid aan ontwikkelingsprocessen die uiteindelijk bepalend zijn voor de driedimensionale structuur van de plant.

Tegelijkertijd wordt uit de in hoofdstuk drie beschreven resultaten duidelijk dat het KNOX-BELL netwerk in planten complexer is dan voorheen gedacht. Het ATH1 eiwit gaat namelijk niet alleen een fysieke interactie aan met de KNAT2 en KNAT6 eiwitten, ATH1 is ook onmisbaar voor een normale expressie van de genen die coderen voor deze twee KNOX eiwitten. Waarschijnlijk laat dit extra niveau van KNOX-BELL interactie een nauwkeuriger afstemming ("fine-tuning") toe van KNOX-BELL gemedieerde ontwikkelingsprocessen.

In hoofdstuk vier staat de rol van de BELL eiwitten ATH1, PNY en PNF bij

bloei-inductie en de architectuur van de bloeiwijze (stengellengte, afstand tussen de bloemen, vorming van bloemmeristemen) centraal. Verrassend genoeg blijken ATH1 en PNY een overlappende rol te spelen in het eerste proces, beiden remmen de bloei, terwijl na de florale transitie beide eiwitten een tegenovergestelde functie hebben. Waar ATH1 stengelgroei remt, is PNY nodig voor een normale stengelgroei. Daarnaast hebben planten die zowel PNY als PNF missen de capaciteit om bloemen te vormen verloren en deze eigenschap kan weer worden hersteld door ook ATH1 in deze planten uit te schakelen. Of bij al deze BELL eiwit gemedieerde processen ook KNOX eiwitten betrokken zijn is op dit moment nog niet duidelijk.

Samenvatten kunnen we stellen dat de uiteindelijke driedimensionale structuur van een plant voor een groot deel bepaald wordt door de activiteiten van TALE homeodomein eiwitten gedurende alle fasen van de ontwikkeling. Het hier gepresenteerde onderzoek vormt daarbij een sterke aanwijzing dat BELL en KNOX eiwitten samen als functionele eenheid verschillende aspecten van plantenarchitectuur controleren.

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The first experience in the journey of doing a PhD is actually meeting great people. I have been indebted in this course to my professor and promotor Sjef Smeekens, for having me in your group, for your inspiring encouragement, for your wisdom and for all your kind support.

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Another significant part of learning how to do science is based on discussions with Johannes Hanson, my officemate. Both the depth and the width of your experience often amazed me. Special thanks to you, for the friendly and warm atmosphere you carry around in the group, for being so supportive and for your help in microarray experiments and all the other science stuff.

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Special thanks to Frits Kindt and Ronald Leito for unwavering assistance, I was always glad to find your door open, and all your photographs and skilful art work. I very much enjoyed working with such kind and cheerful people like the two of you.

I owe debt to my colleagues who are the basis of the entire research group. Maureen, we had an enjoyable time, making dumplings, visiting interesting restaurant and, of course, talking about science. Thanks for your friendship and all your supports. Micha, many thanks to you for helping me with the layout of this thesis. Jingkun, thanks for good company in a foreign world. Thanks to Jolanda,

for all the help that I received from you in these years. Thanks to other colleagues in the group for the nice chat, useful discussions and especially for sharing the experience in both the scientific and non-schientific fields.

It is a pleasure to thank some former colleagues too. Thanks to Bas Rutjens for the collaborative work on 'meristem function'; you'll find the obvious fingerprints of our collaboration at the second chapter of this thesis. Marco, thanks a lot for helping me starting the sectioning technique and being always so kind and patient! Bas Dekkers, thanks for all the help when I started in the group and for so much kindness and hospitality. Leónie, good luck with your current research in Wageningen! Sheng, I have missed your agreeable presence in the group for more than two years already!

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But I cannot deny that living in a different continent isn't always easy. Fortunately there are friends who share that experience and who carry around them the light of my home country wherever they linger. Yuan Gao, my special friend, I am very glad that you followed me from Leiden to Utrecht! Many thanks to your mother too, for taking good care of me. Ying Sun, Zhi Ding, Lei Sun, Kai Ye and Qilian Li, Dan Ye, Qing Gu, Hongtao Zhang, Jian Xu, I'll never forget all the nice hours we spent and all the help you gave.

I am deeply indebted to Jay Achterberg, my special friend for all your understanding, encouragement, and support. It's my luck to have you at my side at this very time. You are an energetic source which keeps producing new ideas! I am sincerely thankful for your numerous help, insightful comments and your company.

I am grateful to Dick Ladage of Optima Grafische Communicatie for his kind help and for showing and offering me the most excellent thesis offset printing I've seen to date.

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Dongping

Curriculum vitae

Dongping Bao was born on the 18th of January 1973 in Zhangjiagang, Jiangsu province, China. In 1996, she started her university education at Yangzhou University, China, studying agronomy. She moved to Nanjing University and obtained MSc in plant science in 2003. She came to the Netherlands in 2004 for a post-master traineeship in Institute Biology Leiden (IBL). Starting from 21 March 2005, she has worked as a PhD student at Utrecht University in Prof. Sjef Smeekens' laboratory for Molecular Plant Physiology under the supervision of Dr. Marcel Proveniers. Her research has been mainly focused on the functional characterization of BELL-KNOX interactions. In September 2009 she continued her postdoctoral research at Utrecht University with Prof. Ben Scheres and Renze Heidstra at the Department of Molecular Genetics, on a CBSG (Centre for BioSystems Genomics) 2012 project to reveal the function of the downstream targets of PLETHORA (PLTs) in embryogenesis and root development.

Publications

Bas Rutjens, **Dongping Bao**, Evelien van Eck-Stouten, Marco Brand, Sjef Smeekens and Marcel Proveniers. (2009) Shoot apical meristem function in Arabidopsis requires the combined activities of three BEL1-like homeodomain proteins. *Plant J* 58,641-654.

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Song Ping, Xia Kai, Wu Chuanwan, **BAO Dongping** et al (2001). Differential Response of Floret Opening in Male-Sterile and Male-Fertile Rices to Methyl Jasmonate. *Acta Botanica Sinica* 2001, 43(5) 480-485.

Song Ping, Xia Kai, Chen Lili, **Bao Dongping** et al (2000). Response of glume opening of different indica rice to methyl jasmonate. *Acta Bot. Boreal. -Occident. Sin.* 20 (6):1155-1161.

Colour Supplement

Figure 1.1 Developmental stages of Arabidopsis.

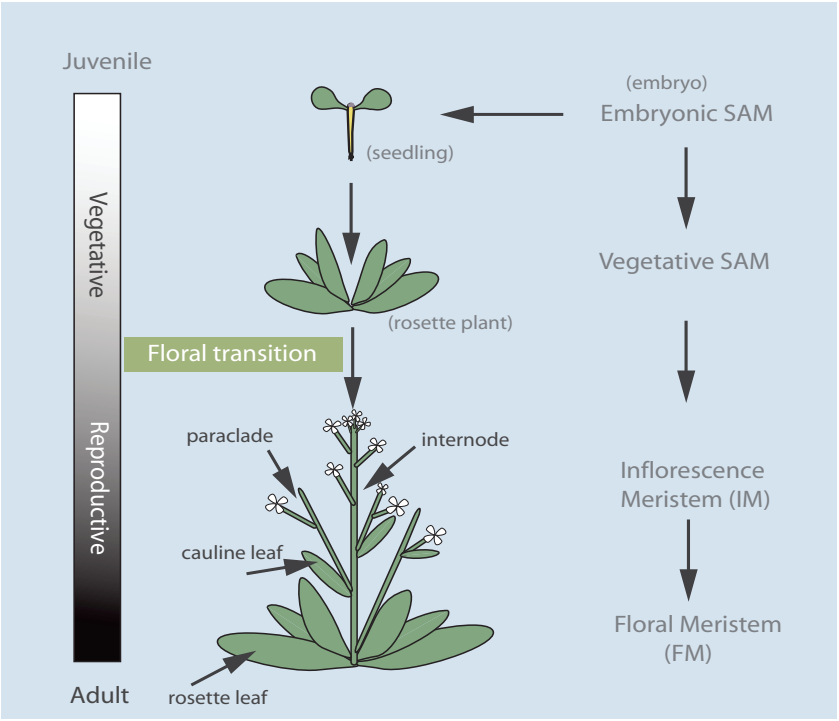


Figure 1.2 Structure of the shoot apical meristem (SAM) and the WUS-CLV loop.

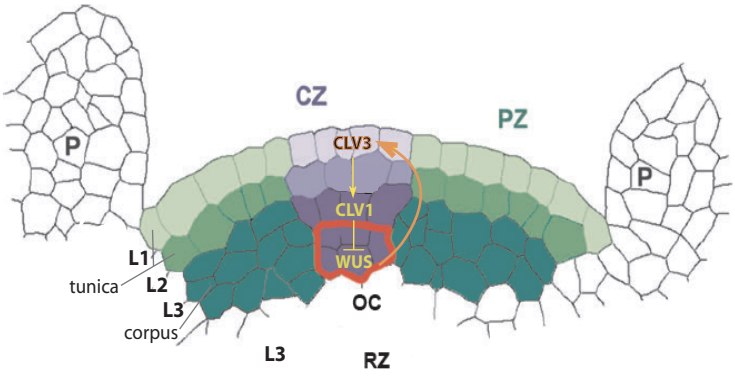


Figure 1.3 The role of *KNOX* genes in maintaining the SAM.

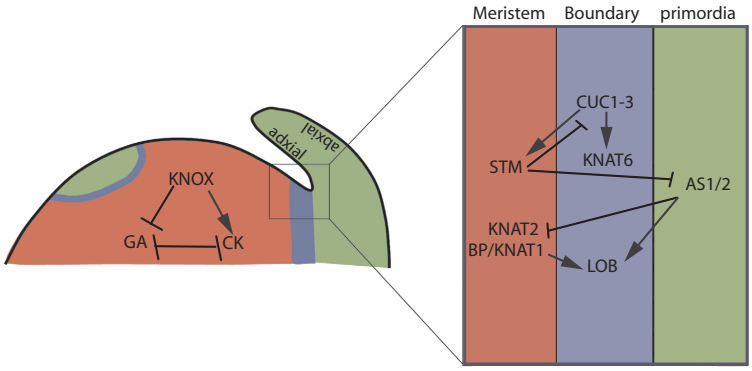


Figure 1.4 Plant TALE homeodomain proteins

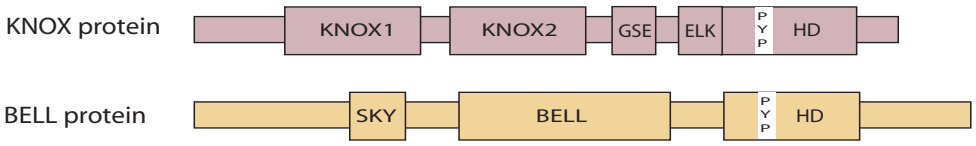
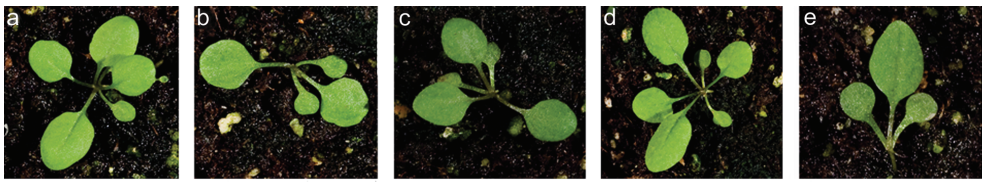


Figure 2.2 Maintenance of vegetative SAM function in Arabidopsis requires combinatorial activity of ATH1, PNY and PNF.

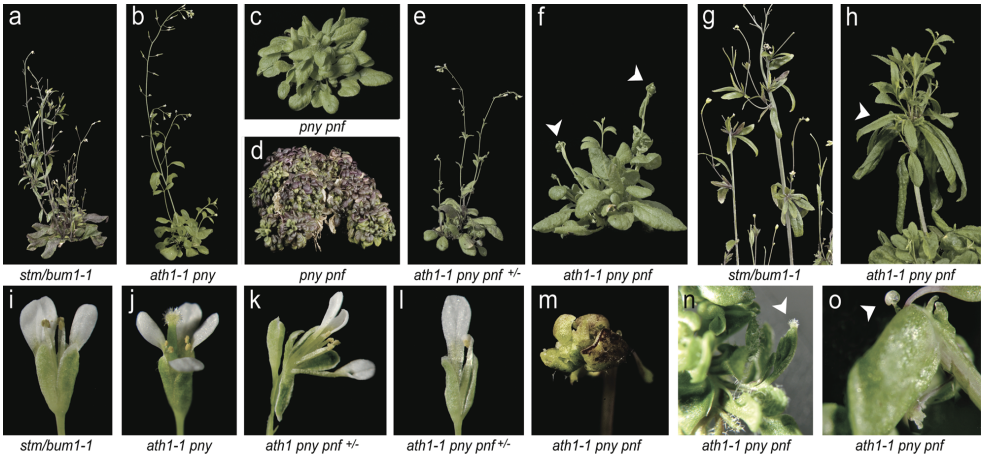


stm/bum1-1 (-△-) | *stm/bum1-1 ath1-1* (-■-) | *ath1-1 pny* (-○-) | *pny pnf* (-●-) | *ath1-1 pny pnf* (-▼-)

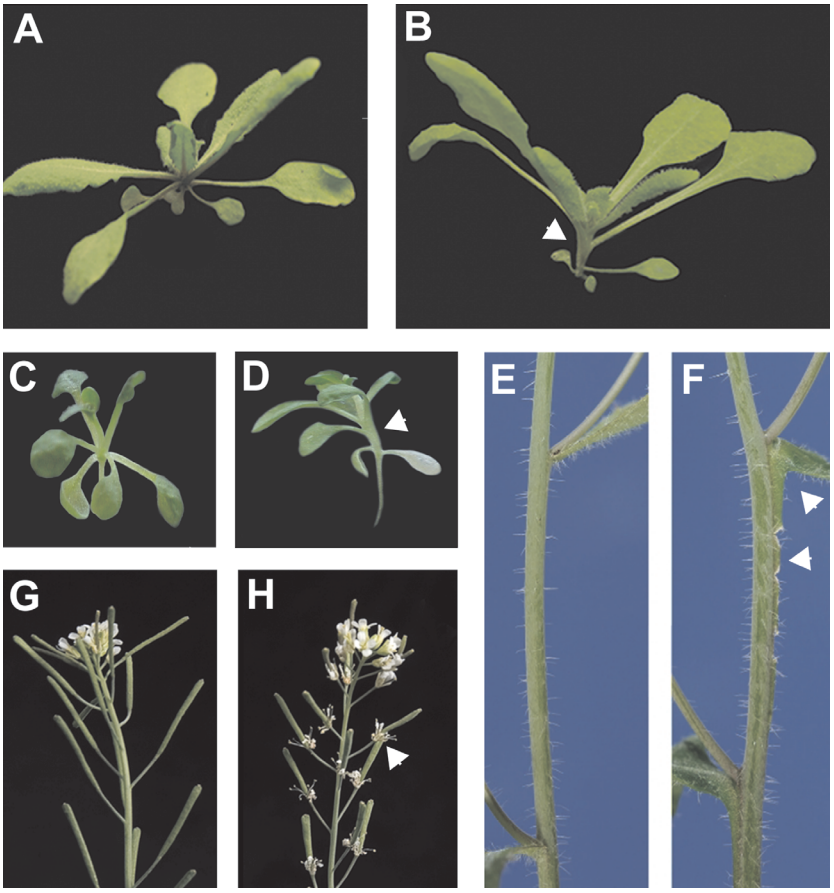
Supplemental Figure 3 Vegetative development of *ath1-1, pny-40126, pnf-96116,* and *ath1-1 pnf-96116* mutants.

Col-8 | ***ath1-1*** | ***pny*** | ***pnf*** | ***ath1-1 pnf***

Figure 2.5 Reproductive development of plants lacking combinations of ATH1, PNY and PNF.



Supplemental Figure 1 Organ fusion phenotypes characteristic for *ath1* mutants.



Supplemental Figure 4 Vegetative development of *ath1-1 pny-40126* double mutants.

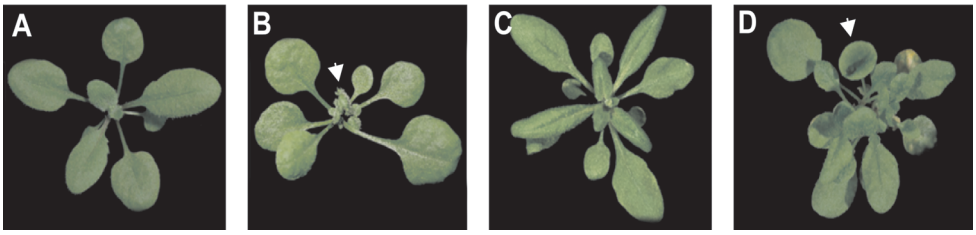


Figure 3.3 ATH1 restricts the growth of the subapical region.

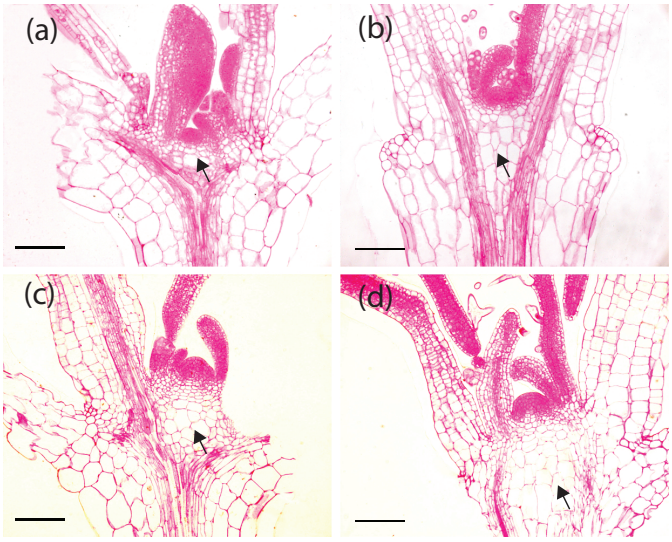


Figure 3.4 SAMs are not affected in *knat2 knat6* double mutants.

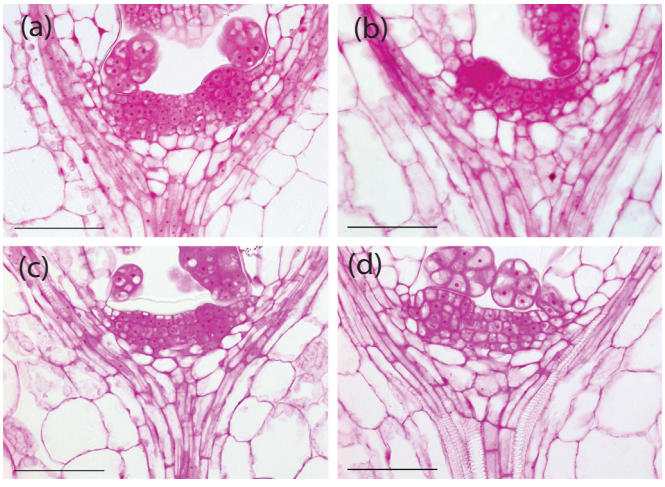


Figure 3.1 *ATH1* interacts with *KNAT2* and *KNAT6* to control cauline leaf-stem boundary development.

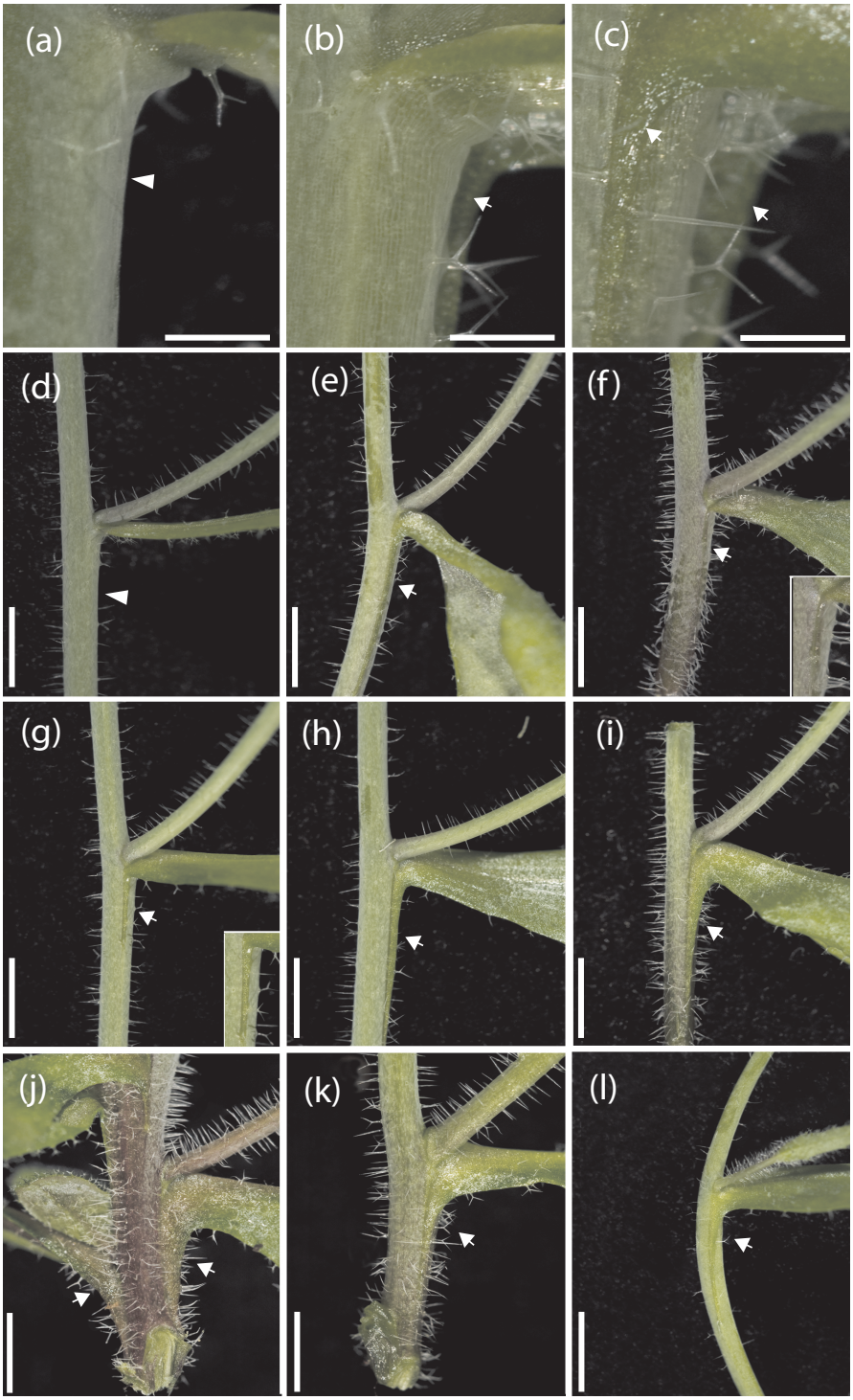


Figure 3.6 *ATH1* is required for proper *KNAT2* expression.

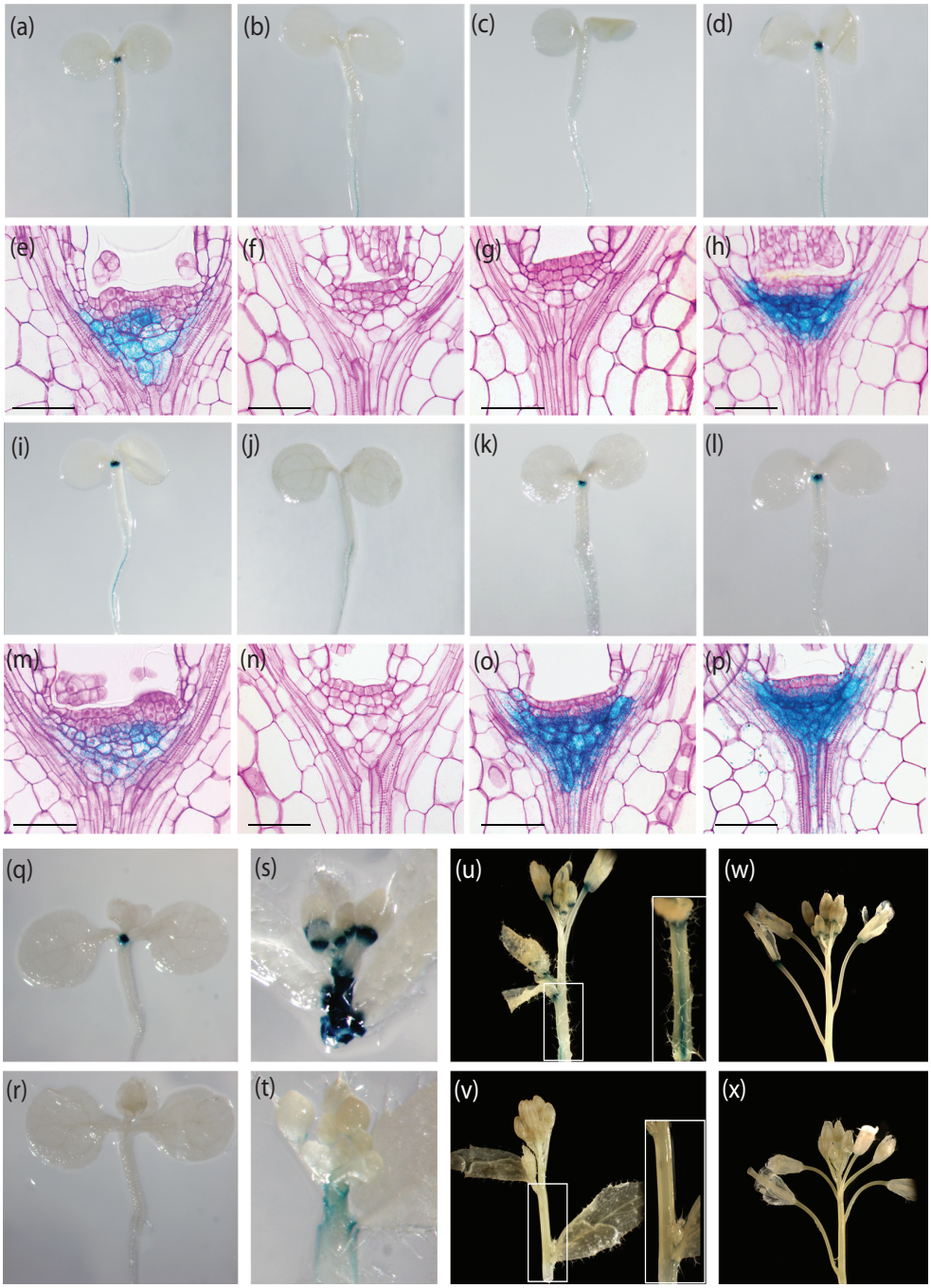


Figure 3.7 *ATH1* is required for proper *KNAT6* expression.

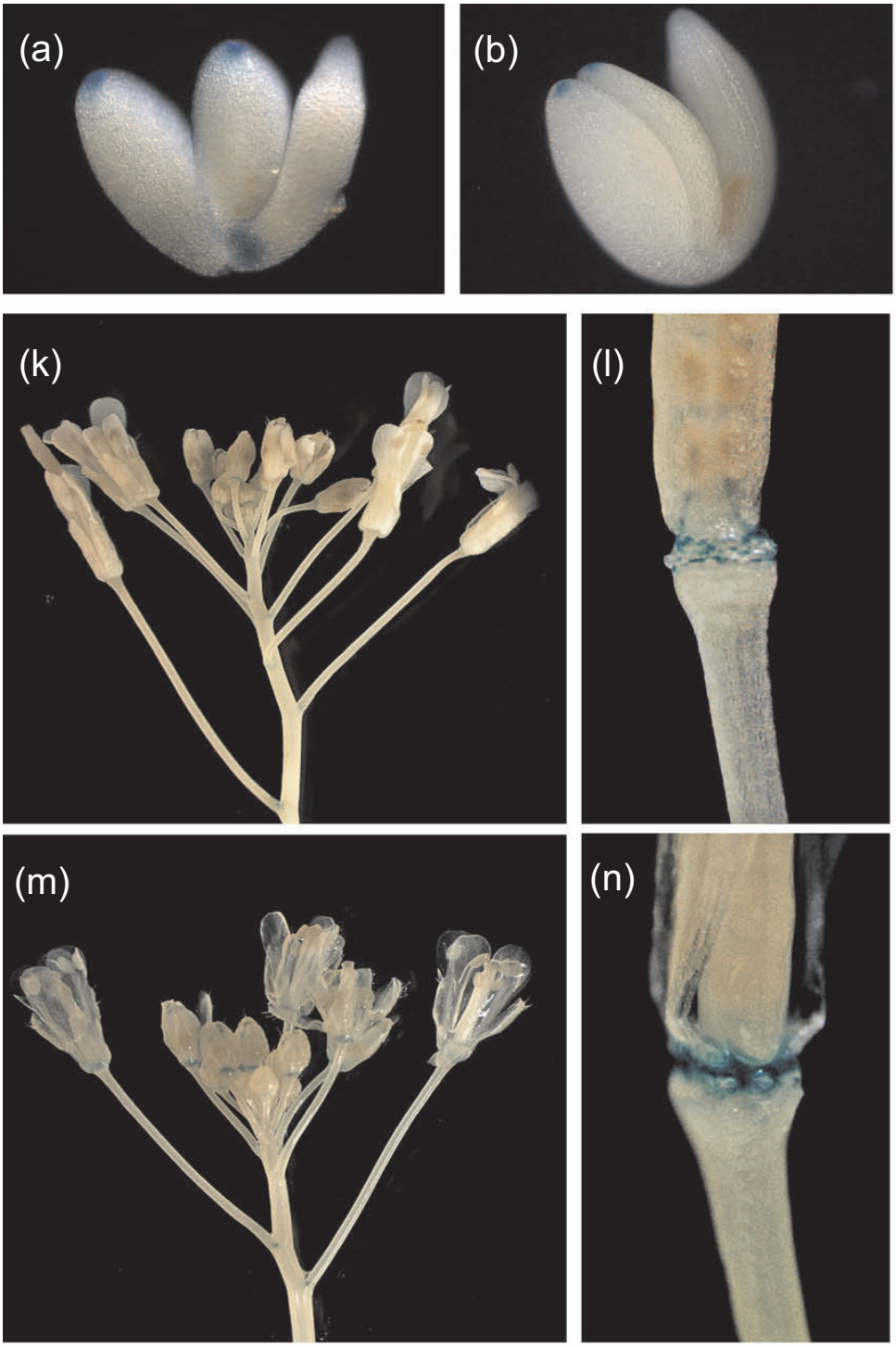


Figure 3.7 *ATH1* is required for proper *KNAT6* expression.

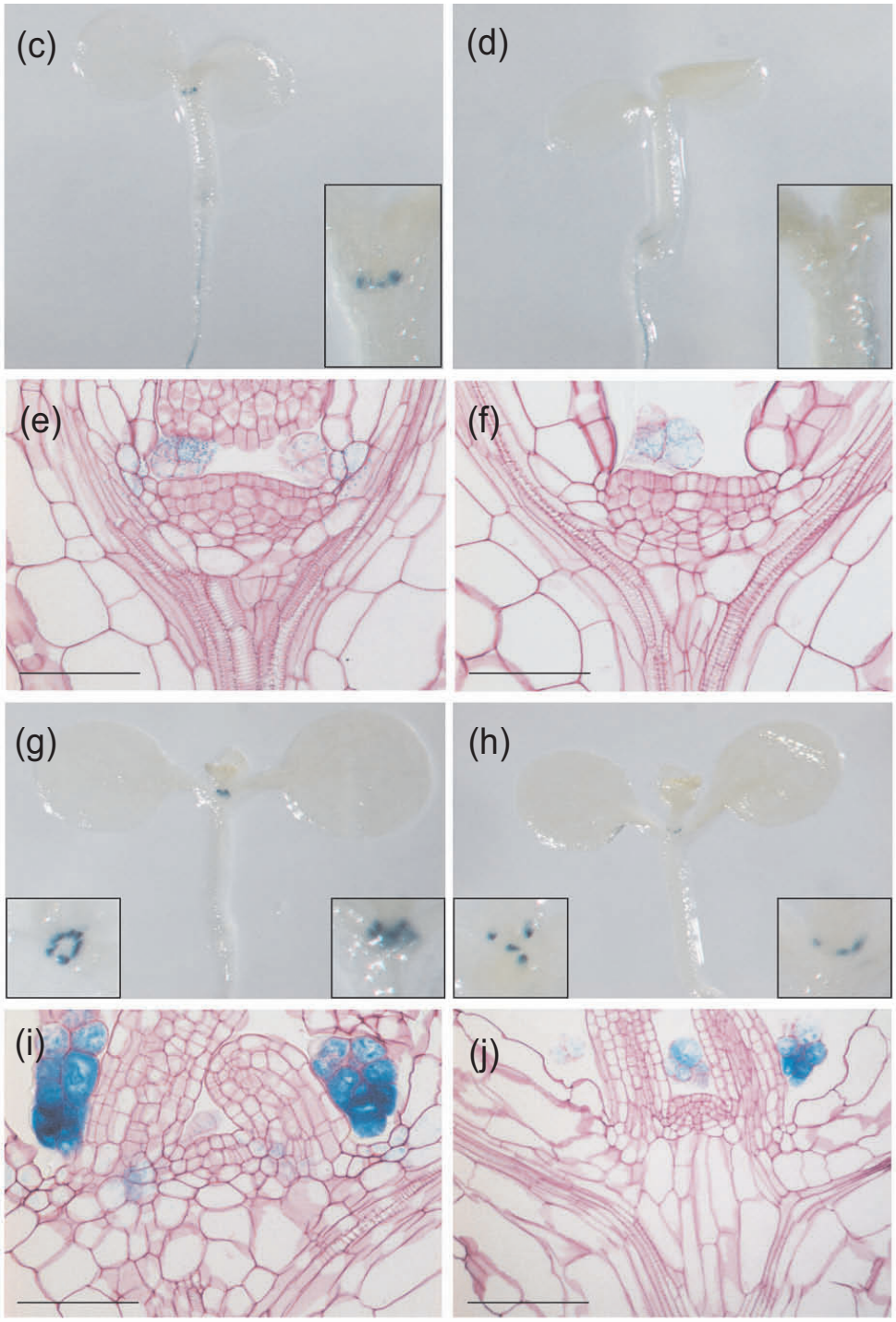


Figure 4.1 *ath1-1* and *ath1-3* affect SAM morphology to a different extent.

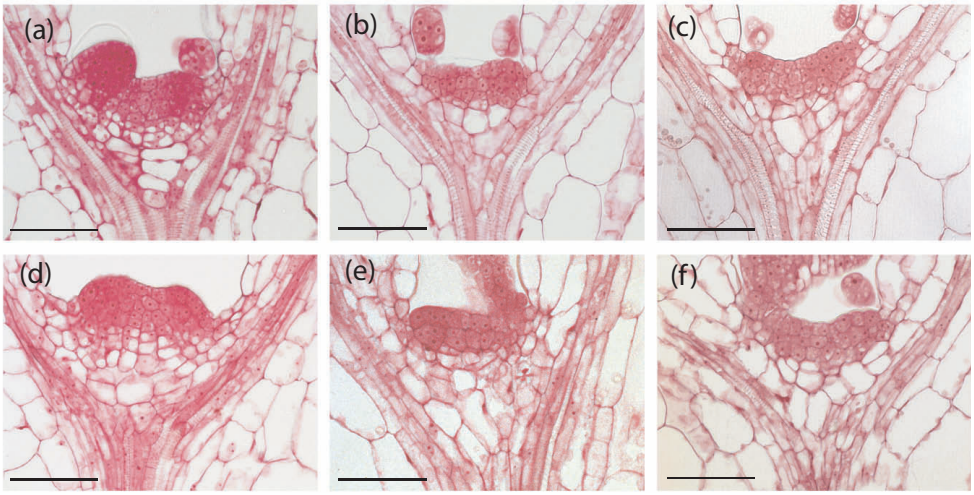


Figure 4.3 The *ath1-3* mutation rescues the *pny* inflorescence defects.

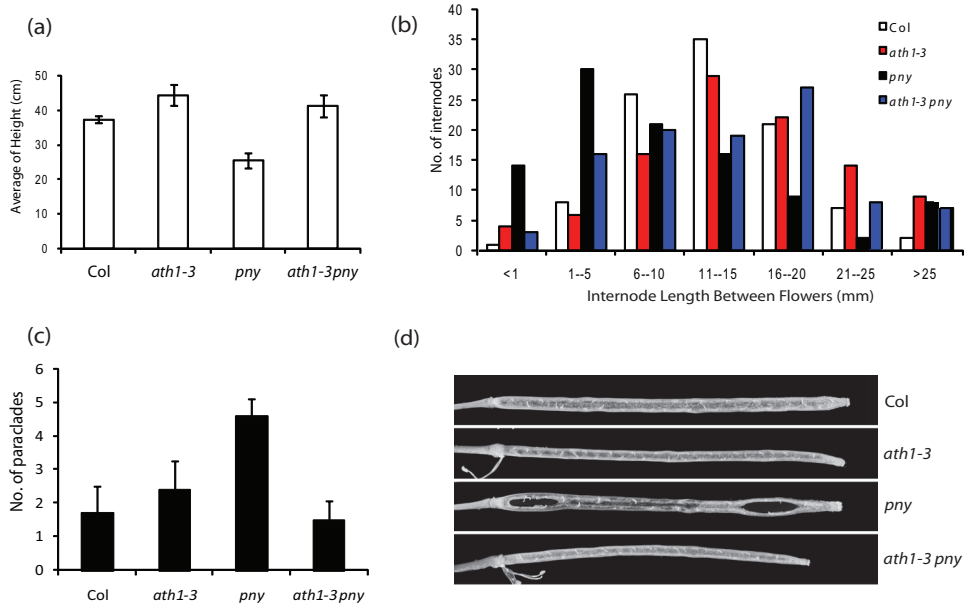
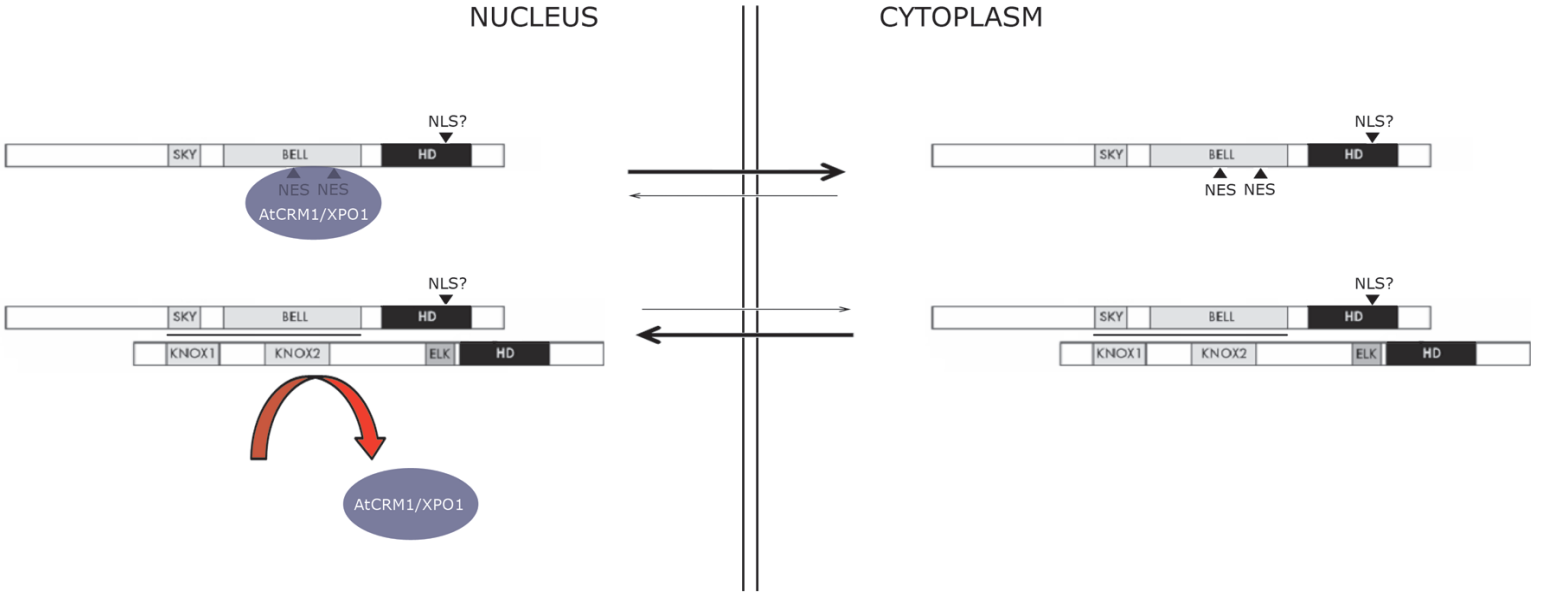


Figure 5.1 BELL-KNOX heterodimerization controls plant TALE homeobox nuclear localization through masking of nuclear export signals.



PENGUIN CLASSICS

Ho Meo Box
Moonlight on the Fourth Floor

a TALE of considerable effort

translation by Jeansin Terexion