Control of Plant Architecture by the TALE Homeobox Genes ATH1 and PENNYWISE

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Plant architectuur staat onder controle van de TALE homeobox genen ATH1 en PENNYWISE

(met een samenvatting in het Nederlands)

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"Consider your seed; you were not made to live like brutes,
but to follow virtue and knowledge."

Dante Alighieri

The Divine Comedy, The Inferno, Canto 26 line 118-120

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Chapter 1

General Introduction

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Animal Development: From Homeosis to Homeobox Genes

A key step in our understanding of the molecular mechanisms underlying multicellular development was the identification of so-called homeotic selector genes in *Drosophila* (McGinnis *et al.*, 1984a, 1984b; Scott and Weiner, 1984). Impaired expression of homeotic selector genes results in transformations of one body part into another, a process referred to as homeotic transformation or homeosis (Bateson, 1894). In animals, homeosis is most strikingly reflected by the dominant *Antennapedia* (*Antp*) mutation in *Drosophila*, which results in replacement of head antennae with legs (Figure 1.1a) (Gehring *et al.*, 1967, 1987; Abbot and Kaufman, 1986; Schneuwly *et al.*, 1987). *Antp* and many other homeotic selector genes act as transcriptional regulators of discrete developmental programs and share a 180 bp consensus DNA sequence, called the homeobox (McGinnis *et al.*, 1984b). The translated homeobox motif folds into a characteristic DNA-binding structure, composed of three α-helices separated by a loop and a turn, named the homeodomain (HD) (McGinnis *et al.*, 1984a). Accordingly, the HD contributes to the sequence-specific recognition of HD transcription factor targets.

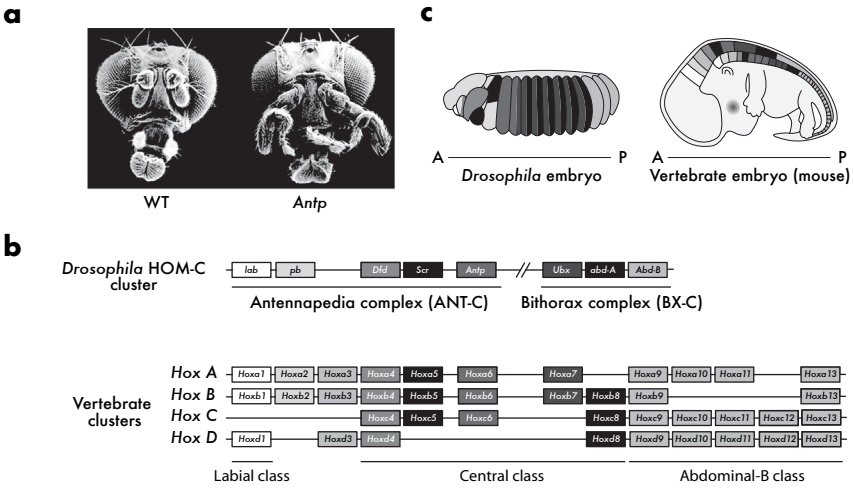


Figure 1.1 *Hox* Genes and Animal Development
(a) Heads of a wild type (left) and dominant *Antennapedia* (*Antp*) mutant (right). In *Antp* mutants, antennae are transformed into legs due to ectopic expression of the leg determining homeotic *Antp* gene in the head segment (Photograph of F.R. Turner, Indiana University, USA).
(b) A schematic of the *Hox* gene clusters (not to scale) in the genomes of *Drosophila* and a vertebrate (mouse). Gene colors are used to differentiate between *Hox* family members, and genes that are orthologous between clusters and species are labeled in the same color. Note that *Hox* expression domains along the anteroposterior (A-P) axis of the embryo's (Figure 1c) corresponds to the position of the respective *Hox* gene in its homeotic gene cluster. Gene abbreviations: *lab*, *labial*; *pb*, *proboscipedia*; *Dfd*, *Deformed*; *Scr*, *Sex combs reduced*; *Antp*, *Antennapedia*; *Ubx*, *Ultrabithorax*; *abd-A*, *abdominal-A*; *Abd-B*, *Abdominal-B*.
(c) A *Drosophila* and mouse embryo showing the approximate domains of *Hox* gene expression along their A-P body axis. Each segment will gain a specific body segment identity due to activation of the required developmental programs by the corresponding *Hox* genes
(adapted from Pearson et al., 2005).

The 'TALE' of Hox Cofactors

In metazoan genomes, including those of mice and humans, most homeobox genes that function as homeotic selector genes are positioned in highly conserved genomic clusters and are referred to as *Hox* genes (reviewed in Lemons and McGinnis, 2006). Examples of such clusters are the homeotic cluster (HOM-C) in *Drosophila*, consisting of the Antennapedia and Bithorax complexes (ANT-C and BX-C) and the four *Hox* gene clusters (*A-D*) of vertebrates (Figure 1.1b). Expression of *Hox* genes is restricted to discrete segmental zones along the anteroposterior (A-P) axis, reflecting the relative position of these genes in the homeotic clusters (Figure 1.1c). Subsequently, body segment identity is established by activation of the required segment-specific developmental programs.

Given the high degree of Hox target specificity *in vivo*, structural congruency of the DNA-binding HD, along with low DNA binding specificity *in vitro* raised the compelling question of how Hox proteins acquire *in vivo* target selectivity (Hoey and Levine, 1988). The characterization of cell-context specific Hox cofactors eventually helped to explain *in vivo* Hox target selectivity. Two important *Drosophila*

Hox cofactor encoding genes, *extradenticle* (*exd*) and *homothorax* (*hth*), were originally identified causing *Hox*$^{-/-}$-like homeotic transformations when mutated, without affecting *Hox* gene expression patterns (Peifer and Wieschaus, 1990; Rauskolb *et al.*, 1993; Rieckhof *et al.*, 1997; Pai *et al.*, 1998). Exd was shown to bind a subset of Hox proteins and subsequently enhance their target specificity both *in vitro* and *in vivo* (Chan *et al.*, 1994, 1997; Knoepfler and Kamps, 1995; Neuteboom *et al.*, 1995; Phelan *et al.*, 1995; Mann and Affolter, 1998; Passner *et al.*, 1999; Piper *et al.*, 1999; Ryoo and Mann, 1999; Ryoo *et al.*, 1999; LaRonde-LeBlanc and Wolberger, 2003). In addition, some Hox proteins interact with Hth *in vitro*, and Hth regularly binds *Hox* target loci in close proximity of Exd-Hox binding sites. This corresponds with *in vitro* data on the formation of Exd-Hox-Hth trimeric complexes and likely represents another way of establishing Hox target specificity (Chang *et al.*, 1997; Berthelsen *et al.*, 1998a; Mann and Affolter, 1998; Jacobs *et al.*, 1999; Ryoo *et al.*, 1999; Ferretti *et al.*, 2000; Ebner *et al.*, 2005; Williams *et al.*, 2005). Intriguingly, Exd and Hth belong to a divergent superclass of HD proteins that carry a three-amino-acid-loop-extension (TALE) between helices 1 and 2 of their HD (Bertolino *et al.*, 1995; Bürglin, 1995, 1997).

As for flies; worm, fish and vertebrate development greatly depends on TALE homeodomain proteins, and orthologues of both Exd and Hth are represented in all animal model organisms (Figure 1.2a) (reviewed in Moens and Selleri, 2006). Four vertebrate Exd orthologues, Pbx1-4, have been identified in fish, human and mouse genomes (Figure 1.2a) (Monica *et al.*, 1991; Popperl *et al.*, 2000; Selleri *et al.*, 2001, 2004; Wagner *et al.*, 2001; Waskiewicz *et al.*, 2002). They all share two conserved domains N-terminal of their TALE HD, referred to as PBC-A and PBC-B domains (Figure 1.2b) (Bürglin and Ruvkun, 1992; Rauskolb *et al.*, 1993; Bürglin *et al.*, 1997, 1998). Together, Exd and Pbx proteins form the PBC subclass of TALE homeodomain proteins (Figure 1.2a) (Bürglin and Ruvkun, 1992; Bürglin *et al.*, 1997). Hth and its multiple vertebrate orthologues, termed Meis proteins, constitute the MEIS subclass and share a highly conserved bipartite N-terminal MEIS domain (Figure 1.2a,b) (Bürglin, 1995, 1997; Kurant *et al.*, 1998). In addition, the MEIS subclass contains a closely related group of vertebrate Meis-like proteins called Prep (Figure 1.2a) (Bürglin, 1995; Bürglin *et al.*, 1997, 1998; Berthelsen *et al.*, 1998b; Fognani *et al.*, 2002; Haller *et al.*, 2002). Although MEIS and PBC domains substantially differ in sequence and function, traces of ancient conservation have been found and suggest divergence from a common ancestral domain (Bürglin, 1998).

Like Exd and Hth, vertebrate Pbx, Meis and Prep proteins can function as Hox cofactors and mutations have been implicated in homeotic transformations, disturbed organogenesis, cellular differentiation, multiple forms of cancer and other diseases involving disturbed cell proliferation (reviewed in Moens and Selleri, 2006).

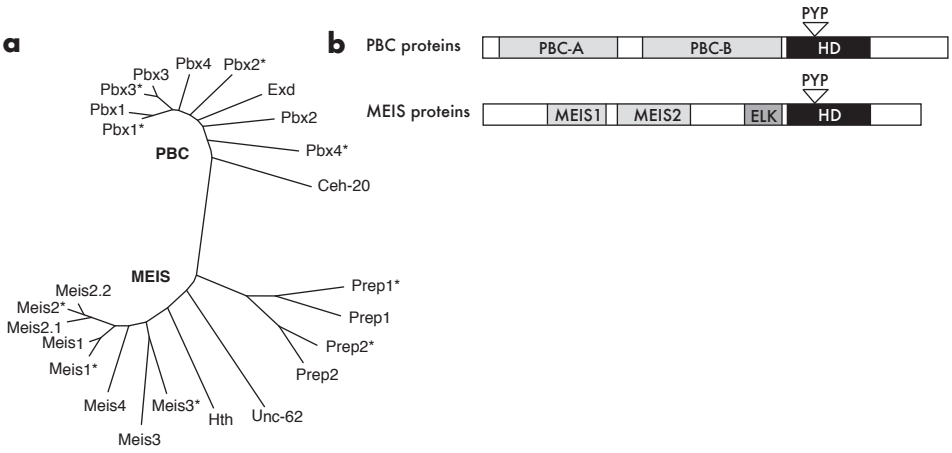


Figure 1.2 Animal TALE Homeodomain Protein Cladogram and Structure of Animal TALE Homeodomain Proteins

(a) Animal TALE homeodomain proteins can be divided into two subfamilies: the PBC family, including the vertebrate Pbx proteins, fly Extradenticle (Exd) and worm Ceh-20, and the MEIS family, including vertebrate Meis and Prep, fly Homothorax (Hth) and worm Unc-62 proteins. (*) indicate mouse proteins, unmarked proteins with the same name are their zebrafish orthologues.

(b) Both PBC and MEIS TALE homeodomain proteins contain a divergent homeodomain (HD) containing a three amino acid loop extension (TALE) between the first and second of three α-helices. The TALE motif virtually always consists of a proline (P)–tyrosine (Y)–proline (P) (Bürglin, 1997; 1998). PBC proteins contain the additional lineage-specific PBC-A and PBC-B domains. Conversely, the bipartite MEIS domain (Bürglin, 1997; Mann and Affolter, 1998) is specific for and conserved among MEIS and PREP proteins (Ryoo et al., 1999). Black rectangles represent the homeodomains (HD) and light gray boxes represent conserved lineage specific domains within PBC and MEIS proteins. The dark grey ELK-box represents a third conserved domain among MEIS proteins. The precise role of this domain has not been determined yet (adapted from Moens and Selleri, 2006).

Interestingly, TALE cofactor binding to Hox proteins does not necessarily result in target gene activation. Recent evidence indicates that TALE-Hox homeodomain complexes can act as either repressors or activators of transcription depending on additional factors, mainly chromatin modifiers, that are recruited (Pinsonneault *et al.*, 1997; Saleh *et al.*, 2000b; Kobayashi *et al.*, 2003; Gebelein *et al.*, 2004).

Hox-Independent Functions for TALE Homeodomain Proteins

Unlike *Hox* genes, *PBC* and *MEIS* TALE homeobox genes are not clustered in genomes and representatives are, unlike *Hox* genes, conserved outside the animal kingdom, implying a broader role for these genes besides their function as *Hox* cofactors (Bürglin, 1997, 1998). Indeed, animal TALE homeobox genes have already been implicated in several developmental processes that involve cooperating with non-Hox homeodomain proteins and even non-homeodomain transcription factors (reviewed in Moens and Selleri, 2006). Processes include the specification of borders for eye development, wing blade outgrowth and patterning of the embryonic peripheral nervous system in *Drosophila*.

One interesting *Hox*-independent process that involves TALE homeobox genes in vertebrates is the establishment of myogenic potential (Berkes *et al.*, 2004;

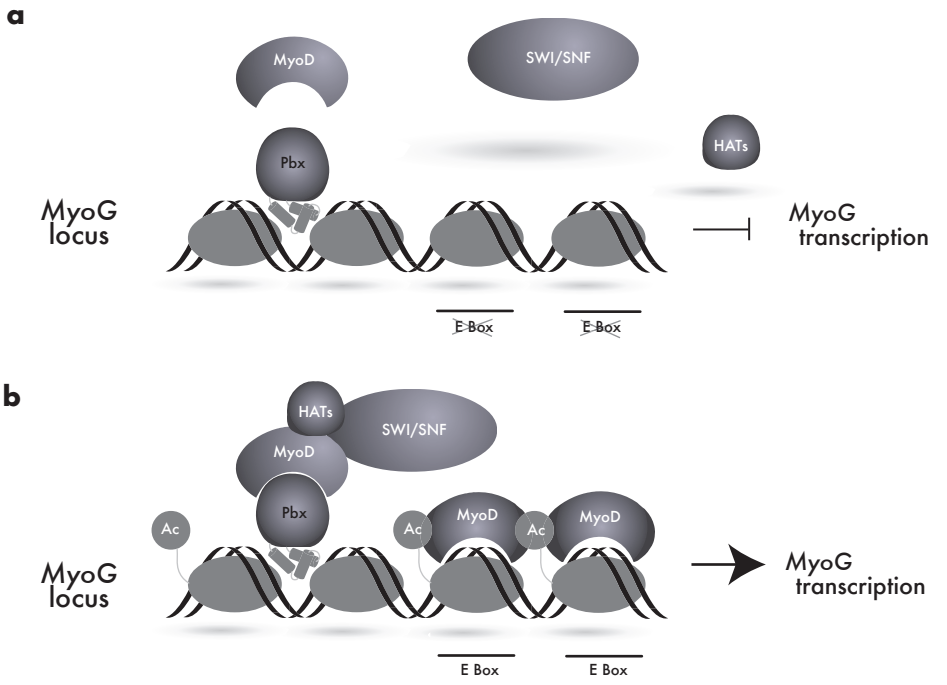


Figure 1.3 'Molecular Beacon' Model for TALE Homeodomain Protein Function
(a) During muscle differentiation, *Myogenin* (*Myog*) can be activated by the bHLH transcription factor MyoD. However, MyoD binding sites (E-boxes) are unavailable due to a repressed chromatin state of the *MyoG* locus. For activation of *MyoG*, MyoD is first recruited to a constitutively *Myog*-bound Pbx-Meis TALE HD complex (for convenience only Pbx is shown).
(b) Subsequently, MyoD recruits chromatin remodeling proteins (HATs and SWI/SNF complexes) that physically modify the *Myog* locus (for example histone acetylation (Ac)), thereby establishing MyoD binding potential at the E-boxes, which allows *Myog* transcription (adapted from de la Serna et al., 2005).

de la Serna *et al.*, 2005). Specification and differentiation of muscle progenitor cells depends on the proper timing of target gene activation by the basic helix-loop-helix (bHLH) transcription factor MyoD (Braun *et al.*, 1989; Weintraub *et al.*, 1989). Initial binding of MyoD to one of its target loci, *Myogenin* (*Myog*), does not involve direct contact with DNA, but rather acts via binding to a *Myog* bound Pbx-Meis complex (Figure 1.3a) (Berkes *et al.*, 2004; de la Serna *et al.*, 2005). Upon binding to Pbx, MyoD targets chromatin remodeling factors to the *Myog* locus, which establish MyoD binding potential by physically modifying the repressed chromatin status of *Myog* (Figure 1.3b) (de la Serna *et al.*, 2005). Surprisingly, Pbx and Meis were found to be constitutively bound to *Myog* DNA even when the gene was repressed (Berkes *et al.*, 2004). This resulted in a model in which Pbx-Meis complexes act as 'molecular beacons', allowing spatiotemporal controlled cofactors to dock on target genes and determine their transcriptional status (Berkes *et al.*, 2004). Further support for such a 'molecular beacon' function for TALE homeodomain proteins recently came from Shim and colleagues (2006), who showed that mouse Meis2 and

Pbx2 are constitutively bound to the *eph8A* locus during midbrain development and exert both *eph8A* repression and activation depending on cell context specific cofactors (Shim *et al.*, 2006).

Nuclear Exclusion as a Conserved Mechanism to Control Animal TALE Homeodomain Protein Activity

PBC proteins are widely present in animal embryos, but, surprisingly for transcription factors, mainly reside in the cytoplasm where they are unstable (Mann and Abu-Shaar, 1996; Aspland and White, 1997; Rieckhof *et al.*, 1997; Kurant *et al.*, 1998; Pai *et al.*, 1998; Jaw *et al.*, 2000; Saleh *et al.*, 2000a; Waskiewicz *et al.*, 2001; Longobardi and Blasi, 2003). Nuclear PBC proteins can be transiently found in specific regions throughout animal embryogenesis (Mann and Abu-Shaar, 1996; González-Crespo *et al.*, 1998; Kurant *et al.*, 1998). Interestingly, nuclear localized PBC protein normally coincides with expression of *MEIS* class genes (Rieckhof *et al.*, 1997; Mercader *et al.*, 1999). For example, during early stages of embryogenesis the *Drosophila* Hox protein Sex combs reduced (Scr) controls leg-specifying target genes in conjunction with nuclear Exd and Hth. However, later in development Scr acts as a suppressor of *hth* expression, resulting in a cytoplasmic translocation of Exd protein and a shift in target gene selectivity of Scr (Ryoo and Mann, 1999).

Surprisingly, MEIS class proteins lack a nuclear localization signal (NLS) and are localized cytoplasmically in the absence of PBC proteins, where they show strongly reduced stability (Abu-Shaar and Mann, 1998; Kurant *et al.*, 1998; Haller *et al.*, 2004). Thus, both PBC and MEIS proteins depend on each others presence for nuclear translocation and stability. Moreover, PBC and MEIS proteins directly interact both *in vitro* and *in vivo* via their conserved N-terminal PBC-A/B and MEIS domains, respectively (Figure 1.2b) (Chang *et al.*, 1997; Knoepfler *et al.*, 1997; Rieckhof *et al.*, 1997; Berthelsen *et al.*, 1998a). Deletion constructs of PBC proteins revealed a conserved NLS in the N-terminus of the HD, which explains the nuclear localization of MEIS proteins after forming PBC-MEIS heterodimers (Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999; Saleh *et al.*, 2000a). Thus, PBC proteins are the driving force behind nuclear PBC-MEIS complexes. However, cytoplasmic localization of PBC proteins in the absence of MEIS partners implies the presence of a mechanism which actively retains PBC proteins out of the nucleus (Rieckhof *et al.*, 1997; Kurant *et al.*, 1998). Intriguingly, two putative nuclear export signals (NES) were identified in the MEIS-interacting PBC-A domain (Figure 1.4a) (Berthelsen *et al.*, 1999; Kilstrup-Nielsen *et al.*, 2003). NES sequences are short leucine-rich stretches recognized by the nuclear export receptor CRM1/exportin-1 of the Importin β-family of transport receptors (Fornerod *et al.*, 1997; Kudo *et al.*, 1997; Fukuda *et al.*, 1997). A conserved cysteine residue in the CRM1 NES recognition domain is sensitive to binding of leptomycin B (LMB), a potent cytotoxin, which blocks CRM1 function (Wolff *et al.*, 1997; Kudo *et al.*, 1999). Incubation of PBC

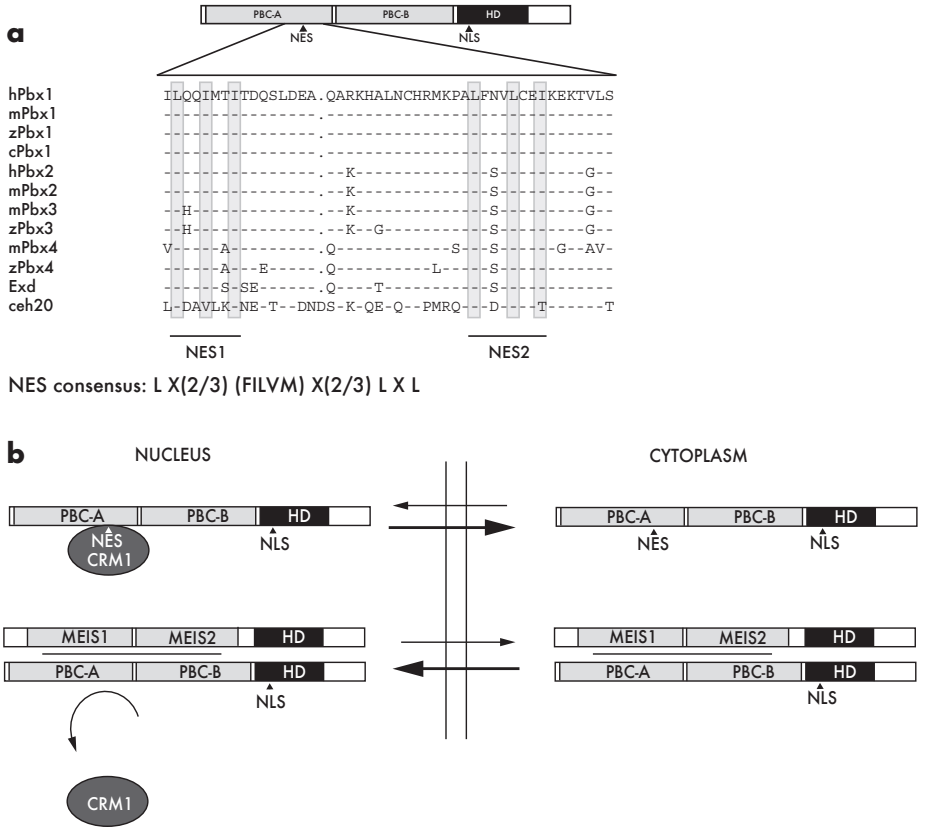


Figure 1.4 Subcellular Localization of Animal TALE Homeobox Proteins Depends on Counteracting Nuclear Export and Nuclear Localization Signals in PBC Proteins

(a) Schematic representation of a PBC protein. The PBC-A domain harbors two closely positioned and highly conserved nuclear export signals (NES), whereas the homeodomain (HD) contains a functional nuclear localization signal (NLS). The NES containing region is magnified and depicted as an alignment of a fragment of the human (h) Pbx1 PBC-A domain with the corresponding regions from mouse (m), zebrafish (z), chicken (c), *Drosophila* (Exd) and *Caenorhabditis elegans* (ceh20) PBC proteins. Dashes indicate conserved amino acids and dots indicate absent ones. Boxed amino acids share homology to the NES consensus (L)-(2/3X)-(FILVM)-(2/3X)-(L)-(X)-(L) (Henderson and Eleftheriou, 2000).

(b) A model for the regulation of subcellular localization of PBC and MEIS proteins. PBC subcellular localization is a dynamic equilibrium between cytoplasm and nucleus established by the counterbalancing activities of its NES and NLS sequences. In animal embryos PBC proteins that, driven by their NLS, end up in the nucleus are actively exported. This process requires the NES sequences located within the PBC-A domain. Upon recognition of these NES sequences by the nuclear export receptor CRM1 (dark grey oval), PBC is relocated to the cytoplasm. As a result, most monomeric PBC protein resides in the cytoplasm. However, PBC proteins can form stable dimers with MEIS proteins through interaction of their respective PBC-A/B and MEIS domains. Since the PBC MEIS- and CRM1-interaction domains coincide, it is postulated that upon dimerization with MEIS proteins PBC NES sequences become masked, thereby precluding recognition by CRM1. As a consequence, PBC-MEIS heterodimers become nuclear, driven by the nuclear localization signal (NLS) of PBC. Black rectangles represent the homeodomains (HD). Light gray boxes represent conserved lineage-specific domains in PBC and MEIS proteins. A black horizontal line indicates protein–protein contacts (adapted from Kilstrup-Nielsen et al, 2003).

expressing cells with LMB results in translocation of Exd to the nucleus, implying that CRM1-dependent nuclear export is part of the mechanism that retains PBC cytoplasmic in the absence of MEIS partners (Abu-Shaar *et al.*, 1999; Berthelsen

et al., 1999; Kilstrup-Nielsen *et al.*, 2003). Moreover, the PBC-A domain directly interacts with the CRM-1 nuclear export receptor *in vitro* and a diffusible GFP: PBC-A domain fusion localizes to the cytoplasm, while addition of LMB results in re-localization to both the cytoplasm and nucleus (Kilstrup-Nielsen *et al.*, 2003). Interestingly, CRM1 and MEIS binding sites were found to overlap in the PBC-A domain (Berthelsen *et al.*, 1999; Kilstrup-Nielsen *et al.*, 2003). Therefore, it was postulated that PBC nuclear export can be overcome by blocking recognition by the CRM1 receptor due to dimerization with MEIS proteins (Figure 1.4b) (Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999; Kilstrup-Nielsen *et al.*, 2003).

Nuclear localization of PBC proteins is not in all cases dependent on presence of MEIS proteins. In such cases, nuclear translocation is enabled by a distinct cell context specific mechanism (Berthelsen *et al.*, 1999; Kilstrup-Nielsen *et al.*, 2003). For example, in *Drosophila* and vertebrate embryos, as well as in undifferentiated *Drosophila* SL2 cells, a strong cytoplasmic retention of PBC proteins is observed. However, some more differentiated vertebrate cells types allow nuclear accumulation of PBC proteins in the absence of MEIS partners (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. The high conservation of the PBC-B phosphorylation sites, as well as similar behavior of vertebrate and fly PBC proteins in these experiments, strongly suggests a biological relevance for MEIS independent nuclear activity of PBC proteins, but this needs to be confirmed (Kilstrup-Nielsen *et al.*, 2003).

In summary, TALE HD proteins form a tightly controlled group of key regulators in animal development and function by potentiating binding and/or increasing binding specificity of several classes of homeodomain and non-homeodomain transcription factors. One way of doing so is by acting as constitutively target-bound molecular 'beacons' that need cofactors to define the transcriptional outcome of the recruited protein complexes.

Plant Development: from Flowers to Flowering Time

The MADS World of Plant Homeosis

Although multicellular life has evolved independently in the plant and animal lineages, fundamental principles of their development show striking parallels. Nevertheless, extensive comparison of plant and animal development at the molecular level revealed that the causal factors involved seldom show homology

(reviewed in Meyerowitz, 2002; Willemsen and Scheres, 2004). For instance, the mechanism of patterning by the combinatorial action of Hox transcription factors, as described earlier for animal body segment identity, shows striking parallels with the establishment of radial flower organ identity. The Arabidopsis flower consists of four concentric whorls which all contain a single type of flower organ (Figure 1.5a,b). Whorl organ identity is established by the combinatorial action of five floral homeotic genes (FHG), *APETALA1* (*AP1*), *AP2*, *AP3*, *PISTILATA* (*PI*) and *AGAMOUS* (*AG*), which are expressed differentially over three domains (A,B and C) along the radial axis of the developing flower (Figure 1.5c,d) (Yanofsky *et al.*, 1990; Jack *et al.*, 1992; Goto and Meyerowitz, 1994; Gustafson-Brown *et al.*, 1994; Jofuku *et al.*, 1994). The ABC model explains how three partially overlapping expression domains of FHG result in four different organ identities (Figure 1.5a-d) (Bowman *et al.*, 1991; Coen and Meyerowitz, 1991; Meyerowitz *et al.*, 1991). Misexpression of FHG results in homeotic transformations of whorl identity and combined loss of A, B and C gene function causes replacement of all floral organs with leaf-like structures, suggesting that leaf identity is the ground state of lateral shoot organ development (Figure 1.5e-g) (Bowman *et al.*, 1991). Moreover, impaired FHG functioning does not result in loss of whorl or organ number, suggesting that FHG, like animal *Hox* genes are required for segment organ identity, but have no role in establishing initial segmentation patterns. In conclusion, the mode of action of FHG strongly resembles that of animal *Hox* genes. The five FHG are not, however, homologous to *Hox* genes, nor are they positioned in genomic clusters. Four of the five described FHG, *AP1*, *AP3*, *PI* and *AG*, encode transcription factors of the MADS-box class. In addition, a second group of MADS-box proteins, SEPALLATA1 (SEP1) to SEP3, act as FHG cofactors in whorl 2 to 4 (Pelaz *et al.*, 2000, 2001). Hence, of eight homeotic selector genes involved in setting up Arabidopsis floral organ identity, seven encode MADS-box transcription factors. Strikingly, similarities between *Hox* and MADS-box genes extend beyond their involvement in establishing segment identity. Divergence of appendage morphology in animals is largely directed by *Hox* gene evolution and a similar mechanism of gene duplication and diversification appears to be used by MADS-box genes to catalyze differences in flower morphology between plant species (reviewed in Irish and Litt, 2005). In addition, memorizing initiated gene expression patterns of both animal *Hox* and plant MADS-box genes over many cell divisions is mediated by two highly conserved groups of chromatin remodeling proteins, the *Polycomb* group (PcG) and *trithorax* group (trxG) proteins (reviewed in Goodrich and Tweedie, 2002; Li, 2002; Alvarez-Venegas *et al.*, 2003).

In plants, the MADS-box family is strongly over-represented, and additional roles for MADS-box genes in plant development have been elucidated (reviewed in Riechmann and Meyerowitz, 1997; de Bodt *et al.*, 2003; Messenguy and Dubois, 2003). Taken together, MADS-box proteins function in a plethora of developmental

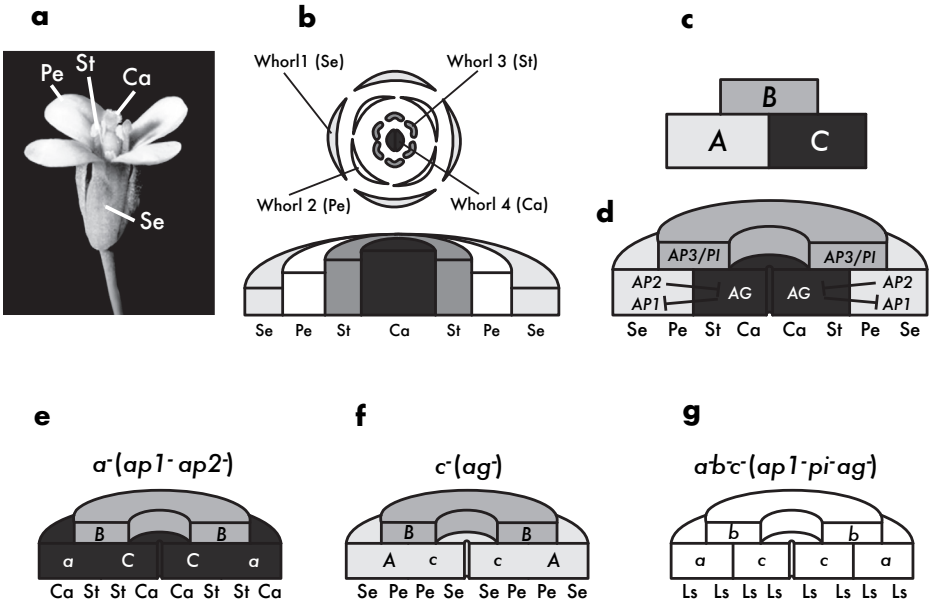


Figure 1.5 The ABC of Floral Homeosis
(a-b) The Arabidopsis flower consists of four organ types which are positioned in a similar number of concentric whorls. Four sepals (Se) are the outermost whorl 1 organs, whereas four petals (Pe), six stamen (St) and 2 carpels (Ca) form the organs of whorls 2,3 and 4, respectively.
(c) Three partial overlapping gene expression domains, *A*, *B* and *C* form the basis of the four whorl identities.
(d) Combinatorial expression of class *A* (*APETALA1* (*AP1*) and *AP2*), *B* (*AP3* and *PISTILLATA* (*PI*)) and *C* (*AGAMOUS* (*AG*)) genes specifies four domains (*A*/*AB*/*BC*/*C*) and concomitantly, results in formation of four organ types (Se/Pe/St/Ca). Expression of *AG* (*C* class gene) in the *A* domain is prevented by *AP2* (*A* class gene), whereas *AG* on its turn suppresses *AP1* (*A* class gene) expression in the *C* domain (for a detailed description see the text).
(e) Mutations in *A* class genes ($ap1^{-}$ $ap2^{-}$) result in spread of *C* class gene (*AG*) expression into the A class expression domain and conversion of whorl one and two organ identity into that of whorl four and three, respectively.
(f) Reciprocally plants disturbed in *C* (ag^{-}) gene class function show homeotic transformations of the inner two whorls.
(g) Mutations in all three gene classes ($a^{-}b^{-}c^{-}$) result in a complete loss of floral organ identity and in formation of leaf like structures (Ls). These homeotic transformations reflect the ground-state leaf identity of floral primordia. The picture was adapted from Weigel World (http://www.weigelworld.org).

processes, including floral transition, floral and fruit patterning, shattering of floral organs and lateral root development in both dicot and monocot species.

Evolutionary Divergence at the Molecular Level: The Shoot Apical Meristem

Despite the lack of solid data on the role of plant homeobox genes as homeotic factors, homeobox genes are widely involved in plant development (reviewed in Chan *et al.*, 1998; Ito *et al.*, 2002; Tsiantis and Hay, 2003; Byrne, 2006). A striking example is the initiation and maintenance of two apical stem cell pools throughout the life span of plants. In contrast to animal embryogenesis, during which a complete stereotype body pattern elaborates and most pluripotent stem cells are lost, plant embryogenesis is characterized by the formation of an incomplete body plan,

equipped with two indefinite apical pools of pluripotent stem cells. These stem cell pools are situated in the shoot apical meristem (SAM) and root meristem (RAM) and are established during early embryogenesis, but remain quiescent during the remaining embryonic phase. Upon germination, activated meristems provide the developing plant with cells to realize the post-embryonic initiation of lateral organs, allowing the high developmental plasticity needed to adapt to prevalent environmental conditions.

The embryonic initiation and proper maintenance of the Arabidopsis SAM during vegetative and generative development requires the *WUSCHEL* (*WUS*) and *SHOOT MERISTEMLESS* (*STM*) genes, which belong to the plant specific WOX-family (*WUS*-like homeobox) of homeobox genes and to the TALE superclass of homeobox genes, respectively (Barton and Poethig, 1993; Laux *et al.*, 1996; Long *et al.*, 1996; Bürglin, 1997; Mayer *et al.*, 1998; Haecker *et al.*, 2004). *WUS* is expressed in the central sub-apical organizing center (OC) of the SAM from where it functions in the maintenance of the overlying stem cell pool in the central zone (CZ) of the SAM (Figure 1.6a) (Laux *et al.*, 1996). Mutations in *WUS* result in a loss-of-SAM phenotype, due to lack of stem cell identity of CZ cells (Laux *et al.*, 1996). Contrary to the *wus* phenotype, mutations in the *CLAVATA* (*CLV*) genes result in a dramatic enlargement of the CZ and underlying *WUS* expression domain (Clark *et al.*, 1993, 1996; Kayes and Clark, 1998; Laufs *et al.*, 1998; Fletcher *et al.*, 1999). The small, secreted CLV3 ligand peptide is a key player in maintaining the correct size of the OC and consequently the CZ (Clarck *et al.*, 1996; Fletcher *et al.*, 1999). *CLV3* expression is restricted to CZ stem cells and their primary daughters, but its gene product diffuses towards the underlying OC where it activates the putative CLV1/CLV2 transmembrane receptor kinase complex, which in turn restricts the *WUS* expression domain (Figure 1.6b) (Jeong *et al.*, 1999; Trotochaud *et al.*, 1999; Lenhard and Laux, 2003). *WUS*, on the other hand, positively acts on CLV3 expression in the CZ via an unknown non-cell-autonomous signal (Mayer *et al.*, 1998; Schoof *et al.*, 2000; Brand *et al.*, 2002). This creates the WUS/CLV negative-feedback loop that delicately stabilizes the stem cell pool size of the SAM (Figure 1.6b)

Besides the WUS/CLV feedback-loop that controls stem cell pool size, *STM* is necessary for establishment and maintenance of the Arabidopsis SAM (Barton and Poethig, 1993; Long *et al.*, 1996). *STM* is widely expressed in the meristem including the stem-cell containing CZ and the lateral peripheral zone (PZ) (Figure 1.6a), but is excluded from incipient primordia cells by yet unknown factors (Figure 1.6a and c) (Long *et al.*, 1996). A primary function of *STM* in meristem maintenance is suppression of differentiation. This is partially established by preventing expression of the differentiation factor *AS1* in the meristem (Figure 1.6c), since loss of *AS1* function rescues weak *stm* mutants (Byrne *et al.*, 2000). Notably, down regulation of *STM* in primordia seems essential for lateral organ formation, since even in SAM-

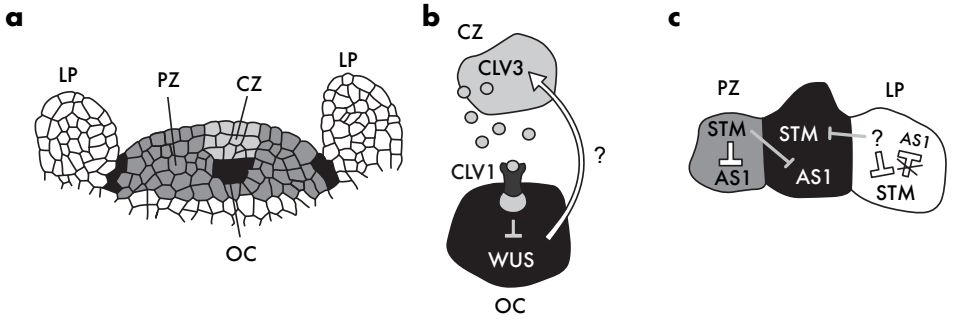


Figure 1.6 Shoot Apical Meristem Maintenance by the Concerted Actions of WUS and STM
(a) Schematic representation of the Arabidopsis shoot apical meristem (SAM). The central zone (CZ) contains the shoot stem-cell pool. The organizing center (OC) functions non-cell autonomously to maintain the overlying stem-cell pool. Lateral cells in the peripheral zone (PZ) show a high division rate, but remain undifferentiated until they become incorporated into lateral organs (here depicted as leaf primordia (LP)).
(b) The WUS/CLV loop. CZ stem cells secrete the signal peptide CLAVATA 3 (CLV3), which migrates to the underlying organizing center (OC). Here, the CLV1 receptor complex is activated by CLV3, resulting in restriction of the *WUSCHEL* (*WUS*) expression domain and, correspondingly, the OC. WUS on its turn activates CLV3 expression in the overlying CZ via an unknown non-cell autonomous signal (depicted as a question-mark) (for a detailed description see the text).
(c) The role of STM in maintaining the SAM. *STM* is expressed throughout the meristem (including the CZ, OC and PZ), but is downregulated at sites of leaf initiation and in leaf primordia (LP) by yet to define factors (depicted as a question-mark). STM acts as a suppressor of differentiation throughout the meristem dome, at least partially by preventing spread of *ASYMMETRIC LEAVES1* (*AS1*) expression into meristem cells (here depicted as PZ cells). However, *AS1* is highly expressed in differentiating leaf primordia (LP) due to the down-regulation of STM in these cells, resulting in a tightly controlled border of meristem identity versus organ differentiation (for a detailed description see the text) (adapted from Sablowski, 2004; Scheres, 2007).

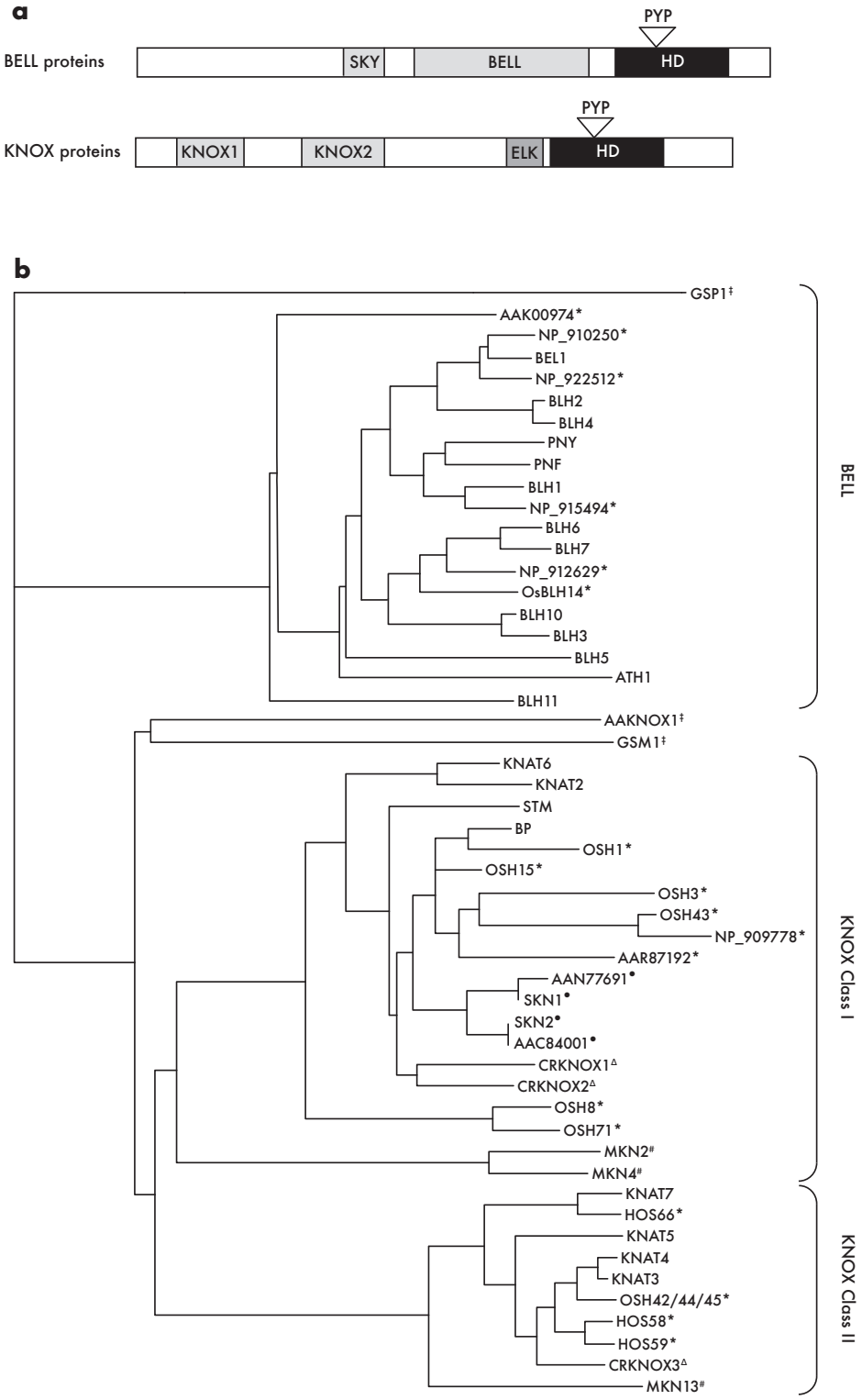
derived floral meristems, *STM* expression is temporarily lost during the initiation process (Long and Barton, 2000). Like *wus* mutants, *stm* plants lack a functional SAM and show early arrests of development (Barton and Poethig, 1993; Long *et al.*, 1996). In addition, lack of *STM* results in fusions of cotyledons and other tissues, suggesting that *STM* expression in the PZ helps to define organ boundaries (Barton and Poethig, 1993). Both *stm* and *wus* mutants lack stem cells, but their loss-of-stem-cell phenotypes are different. In *stm* plants, differentiation of stem cells into developing organs appears to be the main reason for SAM-loss (Byrne *et al.*, 2000). *wus* plants, on the other hand, lack stem cell identity altogether, but do contain morphologically distinct cells at the presumptive SAM location (Laux *et al.*, 1996). Interestingly, even though both *wus* and *stm* mutants lack a functional SAM, both are capable of activating, so far unknown, escape mechanisms which allow stunted vegetative and partial reproductive development (Barton and Poethig, 1993; Clark *et al.*, 1996; Laux *et al.*, 1996). *wus* mutants reiteratively form transient shoots from the presumptive SAM area, whereas *stm* plants reinitiate growth from escape meristems formed on 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

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). However, WUS and STM also function in parallel since they control different sets of genes, suggesting that the combinatorial outcome of their targets is compulsory for SAM function. The main target of *WUS* is the stem cell marker *CLV3*, while *STM* induces local expression of two *STM* homologues 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).

TALEs From the Plant Kingdom

As mentioned above, *STM* belongs to the highly conserved TALE superclass of homeobox genes (Bürglin *et al.*, 1997). *STM* is the Arabidopsis orthologue of the first homeobox gene cloned from plants, *KNOTTED1* (*KN1*) from maize (Vollbrecht *et al.*, 1991; Long *et al.*, 1996). Like *STM*, *KN1* is necessary for SAM initiation and maintenance (Kerstetter *et al.*, 1997; Vollbrecht *et al.*, 2000). STM and KN1 are also referred to as KNOX proteins, and in addition to their conserved TALE HD, share the N-terminal MEIS domain with the metazoan MEIS class of TALE HD proteins (Figure 1.7a) (Bürglin, 1995, 1997). The high sequence similarity between the animal MEIS and plant KNOX domains suggests that they have evolved from a common ancestral MEINOX domain (Bürglin, 1997). Next to *STM*, Arabidopsis contains 7 additional *KNOX* genes (Figure 1.7b), of which *BREVIPEDICELLUS* (*BP* a.k.a. *KNAT1* for *KNOTTED1-LIKE ARABIDOPSIS THALIANA1*) and *KNAT6* were recently shown to act redundantly with *STM* in meristem function (Byrne *et al.*, 2002; Belles-Boix *et al.*, 2006). According to phylogeny and expression patterns, *STM*, *BP*, *KNAT2* and *KNAT6* form the class I *KNOX* genes, whereas class II is formed by *KNAT3*, *KNAT4*, *KNAT5* and *KNAT7* (Figure 1.7b) (Kerstetter *et al.*, 1994; Reiser *et al.*, 2000). *KNOX* genes have been identified in other dicots and monocots, as well as in gymnosperms, ferns and even bryophytes, whereas algae seem to contain a single *KNOX* gene type which shows characteristics of both class I and class II *KNOX* genes from higher plants (Figure 1.7b) (reviewed in Hake *et al.*, 2004). The Arabidopsis *KNOX* genes have been extensively characterized and specific expression patterns have been described. However, single mutant phenotypes have only been observed in *stm* and *bp* loss-of function plants and up till now no functions have been attributed to *KNAT2*, nor to any of the class II *KNOX* genes (Dockx *et al.*, 1995; Serikawa *et al.*, 1997; Serikawa and Zambryski, 1997; Bryne *et al.*, 2002; Belles-Boix *et al.*, 2006; Truernit *et al.*, 2006).

Besides the KNOX class, plants contain a second group of TALE HD proteins referred to as the BELL (BEL1-like) class (Figure 1.7a-b) (Reiser *et al.*, 1995; Bürglin, 1997). Phylogenetic analysis of the HD placed BELL proteins close to the KNOX and MEIS classes (Bürglin *et al.*, 1997). BELL proteins share their



conserved HD, and in addition, two closely positioned N-terminal domains, called SKY and BELL domain, of which the BELL domain has been repeatedly implicated in heterodimerization with KNOX proteins (Figure 1.7a) (Bellaoui *et al.*, 2001; Müller *et al.*, 2001; Cole *et al.*, 2006). Interaction of the KNOX and BELL proteins might reflect a common evolutionary origin of the BELL and KNOX domains, as was shown for the interacting PBC and MEIS domains (Bürglin, 1998). Moreover, it is tempting to speculate on functional resemblance of the PBC and BELL proteins in controlling sub-cellular localization of PBC-MEIS and BELL-KNOX heterodimers, respectively. Secondary structure analysis predicted that BELL domains consist of two α-helices, whereas the KNOX, MEIS and PBC domains all contain a single α-helix in the C-terminal part (Becker *et al.*, 2002). Furthermore, attempts to align the BELL domain to the KNOX, MEIS and PBC domains failed to unambiguously show that the BELL domain evolved from a common ancestral MEINOX domain (Becker *et al.* 2002), while this was shown before for the PBC domain (Bürglin, 1998). However, despite the absence of structural resemblance between the PBC and BELL proteins, several BELL class members have recently been shown to translocate the KNOX protein STM to the nucleus upon formation of BELL-KNOX heterodimers (Cole *et al.*, 2006). This implies a functional convergence of BELL and PBC proteins in controlling TALE HD protein localization and activity (Cole *et al.*, 2006).

In Arabidopsis, the *BELL* family consists of thirteen members (Figure 1.7b). The majority of BELL genes have unknown functions as is the case for most of the *KNOX* genes. Moreover, loss-of-function of most single *BELL* genes doesn't result in clearly visible defects (B.R. and M.P., unpublished data). For *BEL1* a function in the production of lateral primordia within developing ovules was proposed (Reiser *et al.*, 1995). Loss-of-function *bel1* mutants show a defect in ovule development, primarily in integument morphogenesis and as a consequence mutant plants are female sterile (Robinson-Beers *et al.*, 1992; Modrusan *et al.*, 1994).

ARABIDOPSIS THALIANA HOMEOBOX 1 (*ATH1*) is the first Arabidopsis *BELL* gene described and was suggested to function in the signaling cascade that induces photomorphogenesis in plants. *ATH1* was originally identified in a screen for light-regulated transcription factors and is positively regulated by light in Arabidopsis seedlings. *ATH1* is derepressed in mutants that show constitutive

◄ **Figure 1.7 Plant TALE Homeodomain Proteins**

(a) Both BELL and KNOX TALE homeodomain proteins contain a divergent homeodomain (HD) characterized by a 3 amino acid loop extension (TALE) between the first and second of three α-helices. The TALE motif consists of a proline (P)–tyrosine (Y)–proline (P) (Bürglin, 1997; 1998). BELL proteins contain additional SKY and BELL domains. Conversely, KNOX proteins contain the bipartite KNOX domain and the small ELK domain, which show strong homology with the animal MEIS and ELK domains, respectively (Bürglin, 1997; Bellaoui et al., 1998). Black rectangles represent the homeodomains (HD) and light gray boxes represent conserved lineage-specific domains within BELL and KNOX proteins.

(b) Evolutionary relationship of plant TALE homeodomain proteins. Homeodomain sequences of all known *Arabidopsis* (unmarked), rice (*), gymnosperm (●), fern (Δ), bryophyte (#), and green algae (‡) BELL and KNOX proteins were aligned using the Muscle alignment program (Edgar, 2004). An unrooted neighbor-joining tree was generated. (adapted from Hake et al. 2004).

photomorphogenesis such as *cop1* and *det1*, hence its suggested function. However, evidence for a direct interaction was not provided (Quaedvlieg *et al.*, 1995).

The best studied member of the *BELL* family is *PENNYWISE* (*PNY* a.k.a. *BELLRINGER*, *REPLUMLESS*, *LARSON* and *VAAMANA*) (Byrne *et al.*, 2003; Roeder *et al.*, 2003; Smith and Hake, 2003; Bao *et al.*, 2004, Bhatt *et al.*, 2004). *pny* mutants were isolated in a number of screens and phenotypes include loss of the replum, patterning defects of the inflorescence and, as observed for *bp* and *knat6* mutants, enhancement of weak *stm* phenotypes (Byrne *et al.*, 2002; Roeder *et al.*, 2003; Smith and Hake, 2003; Bhatt *et al.*, 2004; Kanrar *et al.*, 2006; Belles-Boix *et al.*, 2006). Besides genetic interactions with *STM*, *PNY* also interacts with *BP*, since *pny* mutations synergistically enhance the *bp* inflorescence phenotype (Byrne *et al.*, 2002; Smith and Hake, 2003; Bhatt *et al.*, 2004). A likely explanation for the observed genetic interactions is the fact that PNY is able to dimerize with both STM and BP *in vitro* and in heterologous yeast-two-hybrid systems (Byrne *et al.*, 2003; Smith and Hake, 2003; Bhatt *et al.*, 2004). Moreover, in leek epidermal cells, PNY-STM dimerization results in the nuclear localization of STM (Cole *et al.*, 2006). These authors also showed that an isolated BELL domain can interact with STM, but it does not promote the nuclear translocation of STM protein (Cole *et al.*, 2006). Again, these data demonstrate the remarkable functional resemblance between the PBC and BELL proteins, since both PBC and BELL domains are sufficient to interact with MEIS and KNOX proteins, respectively, but do not promote the nuclear accumulation of the dimer (Kilstrup-Nielsen *et al.*, 2003; Cole *et al.*, 2006).

Flowering: a Switch of Shoot Apical Meristem Fate

A fascinating aspect of plant development is the switch from vegetative to reproductive development. During the process of floral transition the primary SAM changes from initiating leaf primordia into forming floral meristems (FM) at its flanks. Once the SAM has changed from vegetative to reproductive development it is referred to as inflorescence meristem (IM). Like the vegetative SAM, the IM stays indeterminate and gives rise to the flower bearing inflorescence (IF). During IF development secondary inflorescence shoots emerge from resting meristems in the axils of rosette and cauline leaves. These morphological changes are at least partially caused by reprogramming the expression of numerous genes in the SAM during the floral switch (Schmid *et al.*, 2003). The specification of FM at the IF flanks largely depends on the cooperative action of three floral meristem identity (FMI) genes, *LEAFY* (*LFY*) (Schultz and Haughn, 1991), *APETALA1* (*AP1*) (Irish and Sussex, 1990) and *CAULIFLOWER* (*CAL*) (Figure 1.8a) (Kempin *et al.*, 1995).

Unlike the indeterminate state of vegetative and inflorescence meristems, each FM terminates after forming a single flower. FM identity is initiated by LFY, since in *lfy* mutants early flowers are completely replaced by leaf-like structures and secondary inflorescences (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel *et al.*, 1992). The capacity of *LFY* to induce floral identity is further demonstrated by the formation of ectopic floral tissues on Pro_{35S}:*LFY* plants (Weigel and Nilsson, 1995). Interestingly, *LFY* transcript can be detected in all lateral organ primordia that arise from the SAM. In leaf primordia and older leaves, low constitutive expression of *LFY* can be detected, whereas floral induction results in a dramatic rise of *LFY* levels throughout FM primordia (Bradley at al, 1997; Blázquez *et al.*, 1997; Hempel *et al.*, 1997). A quantitative role for *LFY* in floral timing is further suggested by delayed flowering in heterozygous *LFY/lfy* plants and reduced flowering time in plants with increasing copy numbers of the *LFY* allele (Blázquez *et al.*, 1997). *LFY* encodes a plant specific nuclear localized protein and exerts its function as transcriptional regulator of at least *AP1* and *CAL* (Figure 1.8a) (Parcy *et al.*, 1998; Wagner *et al.*, 1999; William *et al.*, 2004). The lack of a fully penetrating *lfy* phenotype is caused by a redundant mechanism, which eventually activates *AP1* and *CAL* independent of *LFY* and results in delayed flower formation (Huala and Sussex, 1992; Bowman *et al.*, 1991). In turn, *AP1* and *CAL*, two highly redundant MADS-box transcription factors, are responsible for *LFY* up-regulation in developing flowers (Irish and Sussex, 1990; Mandel *et al.*, 1992; Bowman *et al.*, 1993; Kempin *et al.*, 1995; Mandel and Yanofsky, 1995b; Ferrándiz *et al.*, 2000a). Like *LFY*, ectopic expression of *AP1* or *CAL* causes precocious flowering. Both transcripts are uniformly expressed at high levels throughout young floral meristems and are absent from vegetative tissues (Mandel *et al.*, 1992; Kempin *et al.*, 1995; Mandel and Yanofsky, 1995b). During later stages of flower development, *AP1* and *CAL* expression becomes restricted to the two outer whorls, where at least *AP1* functions in the specification of petal and sepal identity (Figure 1.8a) (Irish and Sussex, 1990; Bowman *et al.*, 1991; Mandel and Yanofsky, 1995b).

The activity of the FMI genes is counteracted by the shoot-identity gene *TERMINAL FLOWER1* (*TFL1*), and vice versa (Figure 1.8a). By excluding FMI gene expression in the IM, TFL1 is a key player in maintaining the IM in an indeterminate state (Shannon and Meeks-Wagner, 1991; Bradley *et al.*, 1997; Ohshima *et al.*, 1997; Liljegren *et al.*, 1999). Expression of *TFL1* is restricted to the subapical domain of the vegetative SAM and IM, and increases during the floral transition (Figure 1.8a) (Bradley *et al.*, 1997). Accordingly, *TFL1* is excluded from FM and ectopic *TFL1* expression causes a dramatic delay in *LFY* and *AP1* up-regulation in FM (Ratcliffe *et al.*, 1998; Kobayashi *et al.*, 1999). Reciprocally, loss-of-function alleles of *tfl1* result in floral differentiation of the IM due to misexpression of *LFY* and *AP1* in the IM (Liljegren *et al.*, 1999). A second early marker for inflorescence identity is the MADS-box gene *FRUITFULL* (*FUL*),

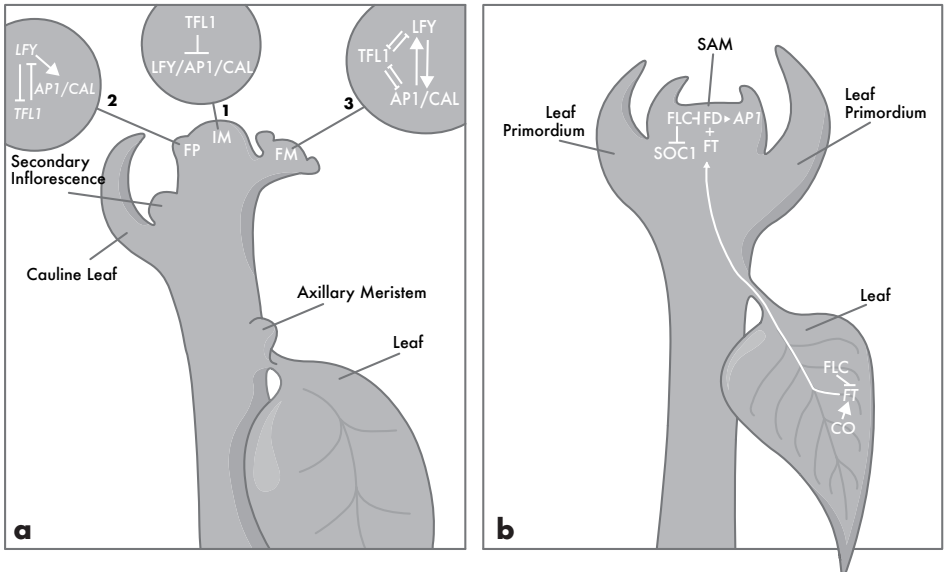


Figure 1.8 Floral Induction at the SAM

(a) Interactions between LFY, AP1/CAL and TFL1 during inflorescence development.

During inflorescence meristem (IM) development TFL1, a repressor of floral meristem identity, prevents the spread of the floral meristem identity (FMI) genes *LFY* and *AP1*/*CAL* into the IM (1). At the flanks of the IM small domains of cells, the flower primordia (FP), gain floral fate by the local induction of *LFY* (2). LFY on its turn prevents expression of *TFL1* in the flower primordium to allow further floral development. Moreover, LFY is involved in the up-regulation of two other FMI genes, *AP1* and *CAL* in the flower primordium. *LFY* and *AP1*/*CAL* are highly expressed in floral meristems (FM) (3), where they mutually promote each others expression and continue to inhibit *TFL1* expression (for a detailed description see the text) (adapted from Bauerle and Dean, 2006).

(b) The photoperiod pathway and FLC antagonize on the floral pathway integrators FT, FD and SOC1 in the control of flowering time.

In long-day photoperiods, CO protein is able to induce expression of *SOC1* in the shoot apical meristem (SAM) and of *FT* in leaf vasculature cells. The gene product of *FT* is capable of traveling to the SAM via an unknown mechanism. Upon arrival in the SAM, *FT* mRNA is translated and FT protein binds FD. Subsequently, the FT-FD dimer induces expression of the floral meristem identity gene *AP1*. Contrary to the floral inducing activity of CO, the floral repressor FLC directly represses transcription of the floral pathway integrator *FT* in leaves and that of *FD* and *SOC1* in the SAM to prevent induction of *AP1*. (adapted from Bäuerle and Dean, 2006).

which is closely related to the FMI genes *AP1* and *CAL* (Mandel and Yanofsky, 1995a; Ferrándiz *et al.*, 2000a). *FUL* expression is strongly up-regulated in the inflorescence upon floral induction and *ful* mutants show a slight delay in flowering time (Mandel and Yanofsky, 1995a; Ferrándiz *et al.*, 2000a). Like *TFL1*, *FUL* is excluded from early stage floral primordia. However, *FUL* mRNA reappears in the center of older flowers where it functions in carpel and fruit development (Gu *et al.*, 1998; Ferrándiz *et al.*, 2000a, 2000b). Consistent with its spatio-temporal expression pattern, *AP1*, and not *LFY,* is responsible for *FUL* repression during FM and flower development (Mandel and Yanofsky, 1995a).

Recently, two redundantly acting TALE-homeobox genes of the *BELL* subclass, *PNY* and *POUND-FOOLISH* (*PNF*) have been implicated in inflorescence development (Smith *et al.*, 2004; Kanrar *et al.*, 2006). *pny pnf* double mutants are characterized by a complete lack of inflorescence evocation and flower formation,

whereas *pny* single mutants show only a weak disturbance of inflorescence phyllotaxis and *pnf* mutants are indistinguishable from wild type (Smith *et al.*, 2004). Remarkably, vegetative development in *pny pnf* plants appears unaffected and morphological changes that accompany the floral switch of the SAM, as well as induction of genes involved in the transition, including *FUL,* appear normal (Smith *et al.*, 2004). Thus, the presence of either *PNY* or *PNF* is compulsory for IF and flower development, but not for primary steps in the floral switch. In *pny pnf* plants severely reduced levels of *LFY* and *AP1* transcripts can explain the lack of flower formation, but this cannot account for the dramatic non-bolting phenotype, since *lfy ap1* doubles show normal inflorescence development and only lack floral meristem identity (Huala and Sussex, 1992).

The Control of Flowering Time: Integrating External Cues and Endogenous Signals at the SAM

Upstream of *LFY* and *AP1* lies the genetic convergence point of several pathways that control the proper temporal initiation of flowering. Plants, in their natural environment, constantly monitor a comprehensive set of external cues to determine the proper season for reproduction. Of these, light quality, light quantity and temperature are most prominent. Environmentally derived information along with the intrinsically controlled competence of the plant to respond to flower inducing stimuli orchestrate the optimal timing for reproduction, which is a prerequisite for high offspring rates. Most signals involved in flowering time converge at the transcriptional control of the so-called floral pathway integrator (FPI) genes *FLOWERING LOCUST* (*FT*), *FD* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), which on their turn induce expression of, at least, *LFY* and *AP1* (Figure 1.9) (reviewed in Bernier, 1988; Mouradov *et al.*, 2002; Boss *et al.*, 2004).

Photoperiod and other Flower Promoting Signals

The diurnally perceived amount of light is a major parameter for plants to monitor seasonal changes. Increasing day length is often used by plants to trigger reproductive growth during favorable spring and summer conditions (reviewed in Bernier and Périlleux, 2005). Like many other species, Arabidopsis flowers late in short day light regimes (SD, 8h light and 16h darkness) when compared to growth in long days (LD, 16h light and 8h darkness). This facultative LD early flowering response is the result of specific activation of the photoperiod pathway under these conditions. Photoperiod perception takes place in the leaves, where LD conditions result in the accumulation and stabilization of the CCH zinc finger protein CONSTANS (CO)

in phloem companion cells (Figure 1.8b and 1.9) (Putterill *et al.*, 1995; Valverde *et al.*, 2004). CO in its turn cell-autonomously activates expression of the florigen component *FT* (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Samach *et al.*, 2000; Suarez-Lopez *et al.*, 2001; Abe *et al.*, 2005; Huang *et al.*, 2005; Wigge *et al.*, 2005). Transport of *FT* gene product via the phloem results in its accumulation in the SAM, where FT protein binds the SAM specific bZIP transcription factor FD to trigger flowering by inducing expression of the FPI MADS-box gene *SOC1* and the FMI genes *AP1* and *LFY* in the SAM (Figure 1.8b) (Abe *et al.*, 2005; Huang *et al.*, 2005; Wigge *et al.*, 2005). In addition to the photoperiod pathway, the hormonal response pathway that involves gibberellin (GA), also acts as a floral promotion pathway (Wilson *et al.*, 1992). Under non-inductive SD conditions GA promotes floral induction, whereas in LD, impaired GA biosynthesis or signaling hardly has an effect on the timing of flowering (Wilson *et al.*, 1992; Reeves and Coupland, 2001). The effect of GA on flowering in SD partially runs via transcriptional activation of *LFY* through a GA-responsive SD element in its promoter (Blázquez and Weigel, 1998, 2000). Also *SOC1* is upregulated by GA, independently of *LFY*, implying that the GA pathway acts at different hierarchical steps of the floral induction pathway (Borner *et al.*, 2000; Moon *et al.*, 2003).

Floral Repression:

FLOWERING LOCUS C and its Relatives

The photoperiod and GA pathways stimulate flowering, but the *FLOWERING LOCUS C* (*FLC*) gene plays a prominent role in floral repression (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). In contrast to CO, the MADS-box protein FLC suppresses *FT* and *SOC1* expression in leaf vasculature and prevents *SOC1* and *FD* upregulation in the SAM via direct binding to cis-regulatory elements located in the promotors or introns of these FPI loci (Figure 1.8b and 1.9) (Searle *et al.*, 2006). In accordance, during vegetative development *FLC* expression is restricted to the meristematic regions of both shoot and root, and to cotyledon and leaf vasculature (Michaels and Amasino, 2000; Sheldon *et al.*, 2002; Bastow *et al.*, 2004), although root specific expression of *FLC* seems unrelated to flowering time control (Searle *et al.*, 2006).

Next to *FLC*, the Arabidopsis genome contains five additional *FLC*-like genes (*FLM/MAF1* to *MAF5* for *FLOWERING LOCUS M/ MADS BOX AFFECTING FLOWERING1-5*) (Michaels and Amasino, 1999; Ratcliffe *et al.*, 2001, 2003; Scortecci *et al.*, 2001, 2003). For *FLM* and *MAF2* a role in floral repression has been established in parallel to *FLC*, but data on their targets is lacking (Ratcliffe *et al.*, 2001, 2003; Scortecci *et al.*, 2001, 2003). Functions of *MAF3* to

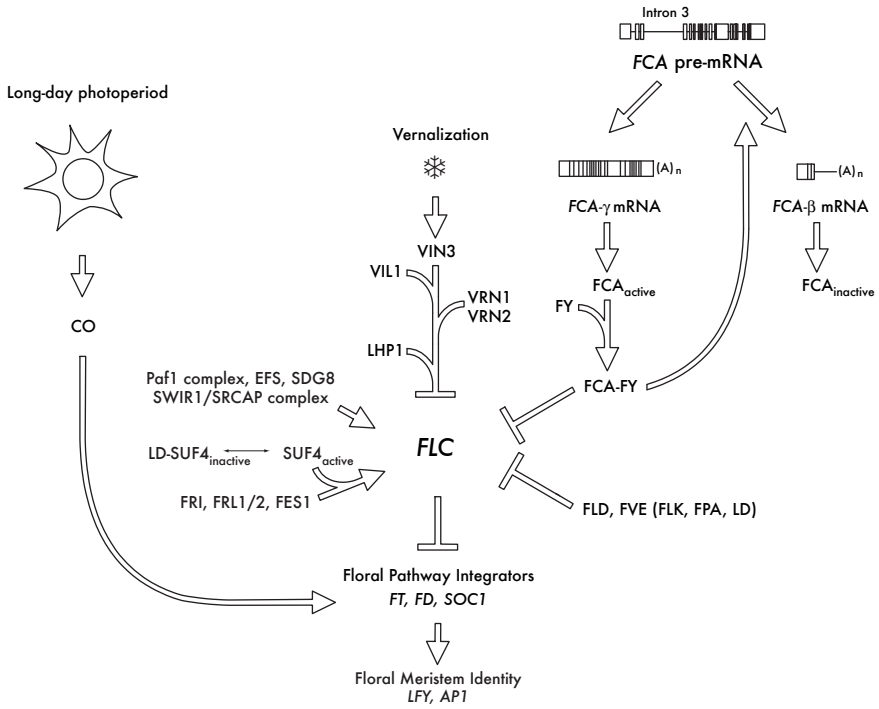


Figure 1.9 The Control of Flowering Time: an Integrated Diagram
For a detailed description see the text. Note: not all genes discussed are represented in the figure.

MAF5 in flowering time control, on the other hand, remain elusive (Ratcliffe *et al.*, 2003).

Activation of *FLC* Expression

In winter-annual accessions of Arabidopsis, strongly enhanced FLC levels suppress flowering during the primary growth season. In contrast, rapid-cycling accessions complete their life cycle within one growth season due to lack of floral inhibiting levels of *FLC*, which permits flowering. High levels of *FLC* transcription are mostly attributable to the presence of a single dominant locus called *FRIGIDA* (*FRI*) (Figure 1.9) (Johanson *et al.*, 2000; Le Corre *et al.*, 2002; Gazzani *et al.*, 2003; Shindo *et al.*, 2005). Interestingly, lesions in *FLC* fully suppress FRI-mediated late flowering (Michaels and Amasino, 2001), suggesting that none of the *MAF* genes are regulated by *FRI*. The importance of *FRI* in contributing to flowering time variation amongst natural occurring Arabidopsis accessions was recently demonstrated by analyzing the flowering behavior and genotype of a comprehensive set of 192 accessions (Shindo *et al.*, 2005). From this study it was concluded that 70% of the flowering time variation between winter-annual and rapid-cycling accessions is contributable to allelic variation at the *FRI* locus. Moreover, *FRI*

appears an evolutionary hot spot in flowering time since 20 independent natural loss-of-function alleles of *FRI* have been identified to date (Shindo *et al.*, 2005).

In screens for mutants that suppress FRI-mediated late flowering, two *FRI* homologues, *FRIGIDA-LIKE1* (*FRL1*) and *FRL2* have been identified that act in parallel with *FRI* in *FLC* induction (Figure 1.9) (Michaels *et al.*, 2004; Schläppi, 2006). The deduced protein sequence of *FRI* and its relatives does not reveal the molecular mechanism by which these plant specific proteins regulate *FLC* expression levels (Johanson *et al.*, 2000; Michaels *et al.*, 2004). However, recent progress in the characterization of additional factors essential for FRI-mediated *FLC* induction has begun to shed light on this enigmatic relationship. Two of them, *FRIGIDA ESSENTIAL1* (*FES1*) (Schmitz *et al.*, 2005) and *SUPPRESSOR OF FRIGIDA4* (*SUF4*) (Kim *et al.*, 2006a; Kim *et al.*, 2006b), encode zinc-finger proteins. For SUF4, binding to *FLC* chromatin has been demonstrated in a 5' region known to harbor important elements for *FLC* up-regulation (Sheldon *et al.*, 2002; Kim *et al.*, 2006b). Moreover, SUF4 interacts with both FRI and FRL1 *in vitro* and in heterologous yeast-two-hybrid experiments (Kim *et al.*, 2006b), implying the presence of a regulatory FRI-SUF4-FRL1 complex at the *FLC* promoter. The FES1 CCCH zinc-finger domain is regularly found in proteins that control messenger processing by direct interactions with the 3' untranslated region of mRNA (Carballo *et al.*, 1998; Cheng *et al.*, 2003). However, whether FES1 directly binds *FLC* transcript remains to be determined (Figure 1.9) (Schmitz *et al.*, 2005)

FRI mediated induction of *FLC* coincides with increased levels of histone-3 lysine-4 tri-methylation (H3K4me3) at the *FLC* locus (He *et al.*, 2004). This type of modification at the 5' end of genes is an epigenetic mark to maintain an active chromatin state, and, in case of *FLC*, requires the Arabidopsis RNA polymerase II (Pol II) associated factor1 (Paf1) complex. The Paf1 complex, originally identified in yeast, is involved in transcriptional initiation/elongation and chromatin regulation of a subset of yeast genes (Squazzo *et al.*, 2002; Ng *et al.*, 2003). It exerts its function by interacting with Pol II and recruitment of Set1, a H3 methyltransferase involved in H3K4me3 at the transcriptional start site of target genes (Roguev *et al.*, 2001; Santos-Rosa *et al.*, 2002). Upon transcriptional activation the Paf1-complex progressively parts from Set1 and associates with Set2, a second H3 methyltransferase involved in transcriptional elongation and suppression of spurious intragenic transcription by progressive H3K36 methylation towards the 3' end of transcribed regions (Carrozza *et al.*, 2005; Kizer *et al.*, 2005; Morris *et al.*, 2005). Orthologues of the yeast Paf1 complex, as well as of SET1 and SET2 have been identified in Arabidopsis and cause early flowering when mutated due to attenuated expression of members of the *FLC* clade (Figure 1.9). EARLY FLOWERING7 (ELF7) (He *et al.*, 2004), VERNALIZATION INDEPENDENCE 4 (VIP4) (Zhang and Nocker, 2002), VIP5 (Oh *et al.*, 2004) and VIP6/ELF8 (He *et al.*, 2004; Oh *et al.*, 2004) are orthologues of the Paf1 complex members Paf1, Leo1, Rft1 and Ctr9 respectively,

whereas EARLY FLOWERING IN SHORT DAYS (EFS) and SET DOMAIN GROUP8 (SDG8) encode Set1 and Set2 orthologues (Soppe *et al.*, 1999; Kim *et al.*, 2005; Zhao *et al.*, 2005). Interestingly, transcript levels of other members of the *FLC* clade are also affected in plants disturbed in Paf1 complex and Set functioning, implying that these genes fulfill a broader role in transcriptional control.

A second group of proteins implicated in controlling expression of the *FLC* clade, ACTIN-RELATED PROTEIN6 (ARP6, a.k.a. SUPPRESSOR OF FRIGIDA3 (SUF3)/ EARLY IN SHORT DAYS1 (ESD1)) (Choi *et al.*, 2005; Deal *et al.*, 2005; Martin-Trillo *et al.*, 2006), PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (PIE1) (Noh and Amasino, 2003) and SERRATED LEAVES AND EARLY FLOWERING (SEF) (March-Díaz *et al.*, 2007), are all orthologues of SWIR1/SRCAP-complex components (Figure 1.9) (Deal *et al.*, 2007; March-Díaz *et al.*, 2007). In yeast and animals, ATP-dependent SWIR1/SRCAP-complexes are recruited by H3K4me3 loci and adjust their histone composition by substituting histone H2A with the active loci associated variant H2A.Z (Krogan *et al.*, 2003; Kobor *et al.*, 2004; Mizuguchi *et al.*, 2004; Ruhl *et al.*, 2006). Mutations in the corresponding Arabidopsis genes result in reduced expression of *FLC* and other *FLC* clade members, which correlates with an extremely early flowering phenotype in non-inductive SD (Choi *et al.*, 2005; Deal *et al.*, 2005; Martin-Trillo *et al.*, 2006; March-Díaz *et al.*, 2007). In addition, ARP6 and PIE1 physically interact with SEF (March-Díaz *et al.*, 2007), and both *arp6* and *pie1* mutants show alterations in H2A.Z accumulation in the *FLC*, *MAF4* and *MAF5* loci, while other H2 variants remain unchanged (Deal *et al.*, 2007).

Other factors required for mRNA accumulation of *FLC* and its relatives may act post-transcriptionally, such as ABA HYPERSENSITIVE 1 (ABH1), which encodes the large subunit of the eukaryotic nuclear mRNA cap-binding complex, the zinc-finger protein SERRATE that likely acts in a miRNA gene-silencing pathway, and the HUA2 protein that is required for the efficient processing of *AG* pre-mRNA (Bezerra *et al.*, 2004; Doyle *et al.*, 2005; Grigg *et al.*, 2005).

Floral Induction by Repression of *FLC*:

The Autonomous Floral Promoting Pathway

FLC acts as a quantitative floral repressor or floral rheostat, meaning that increasing *FLC* levels coincide with a corresponding delay in flowering time (Michaels and Amasino, 2001; Simpson, 2004). Floral inhibiting *FLC* levels are readily reached in the presence of dominant *FRI* alleles or mutations in the autonomous floral promoting pathway. Screens for mutants that flower late in both LD and SD conditions, while remaining susceptible to environmental signals such as photoperiod and vernalization resulted in the identification of the so-called autonomous floral

promoting pathway (Koornneef *et al.*, 1991). To date, the autonomous pathway comprises a group of seven proteins, FCA, FY, FVE, FPA, FLOWERING LOCUS D (FLD), LUMINIDEPENDENS (LD) and FLOWERING LATE KH MOTIF (FLK) that share *FLC* as a common target (Koornneef *et al.*, 1991, 1998; Sanda and Amasino, 1995; Lim *et al.*, 2004). Moreover, like FRI, the autonomous pathway seems to act specifically via *FLC*, since *flc* null alleles fully suppress late flowering of autonomous pathway mutants (Michaels and Amasino, 2001). However, despite its floral promoting capacities, activity of the autonomous pathway is easily overruled by dominant *FRI*.

The autonomous pathway is not a single linear pathway (Figure 1.9) (Koornneef *et al.*, 1998). Extensive genetic characterization of double and triple mutants resulted in the reorganization of the autonomous pathway into several semi-redundant genetic branches that converge at the *FLC* locus (Koornneef *et al.*, 1998). Moreover, linearity of the pathway is implausible because none of the pathway members seems to influence expression levels of any other established pathway component (Macknight *et al.*, 1997, 2002; Simpson *et al.*, 2003; Ausin *et al.*, 2004; Lim *et al.*, 2004). Surprisingly, although natural variation is found amongst *FRI* alleles, autonomous pathway mutants have so far not been isolated from screens for natural variation. This suggests that autonomous pathway genes fulfill broader roles in development. In case of *FCA* and *FY*, this is corroborated by the wide set of genes they control, including genes not involved in the flowering process (Marquardt *et al.*, 2006). Moreover, *fy* null mutants appear lethal and the combination of a hypomorphic *fy* allele with loss-of-function *fpa* alleles also result in lethality (Koornneef *et al.*, 1998; Simpson *et al.*, 2003). In addition, *FPA* has been found to regulate the GA biosynthesis pathway and *FVE* may function as a negative regulator of cold stress signaling (Meier *et al.*, 2001; Kim *et al.*, 2004).

Recently, information on the biochemical activity of several autonomous pathway members became available. *FCA*, for instance, encodes a plant-specific RNA binding protein, whereas FY forms a conserved RNA 3' end-processing/polyadenylation factor (Figure 1.9) (Macknight *et al.*, 1997; Simpson *et al.*, 2003). FCA and FY physically interact and cooperate to lower not only *FLC*, but also full-length *FCA* transcript levels (Figure 1.9) (Macknight *et al.*, 2002; Quesada *et al.*, 2003; Simpson *et al.*, 2003). How FCA and FY precisely function in *FLC* regulation remains to be determined. However, the negative feedback regulation of *FCA* messenger processing by selection of a promoter-proximal polyadenylation site within the third intron of *FCA*, and subsequent cleavage and polyadenylation of a truncated *FCA* transcript, might reflect a mechanism of FCA-FY mediated *FLC* processing, although alternative *FLC* transcripts have not been described.

Like *FCA*, *FPA* and *FLK* encode RNA binding proteins, but these act genetically independently of FCA-mediated *FLC* regulation (Quesada *et al.*, 2003; Lim *et al.*, 2004). LD, on the other hand, encodes a homeodomain protein suggested

to act as a transcription factor in the process of *FLC* regulation (Figure 1.9) (Lee *et al.*, 1994). Intriguingly, LD was recently found to interact with the FRI-essential partner SUF4, possibly reflecting a mechanism by which LD titrates SUF4 from forming FRI-SUF4 complexes (Figure 1.9) (Kim *et al.*, 2006b).

Two other members of the autonomous pathway, *FLOWERING LOCUS D* (*FLD*) and *FVE*, encode chromatin regulators that directly control the status of *FLC* chromatin structure (He *et al.*, 2003; Ausin *et al.*, 2004; Kim *et al.*, 2004). FLD was initially described as being involved in histone deacetylation of the *FLC* locus (He *et al.*, 2003). However, the human FLD orthologue LSD1 was recently shown to functions as histone demethylase (Shi *et al.*, 2004, 2005; Metzger *et al.*, 2005). Possibly, lack of FLD leads to disturbance of a chromatin remodeling cascade that acts on both methylation and acetylation of *FLC* chromatin. Interestingly, FVE is a putative retinoblastoma-associated protein involved in histone deacetylation of *FLC* chromatin (Ausin *et al.*, 2004). However, whether FLD and FVE occupy positions in a single complex remains to be determined.

Notably, several autonomous pathway mutants show increased H3K4me3 of *FLC*, comparable to what was found for *FRI* containing lines. This could imply a direct competition between FRI and the autonomous pathway members on the H3K4me3 chromatin status of *FLC* (He *et al.*, 2003).

The Vernalization Pathway: How Winter Makes Flowers

Most winter annual plants require a prolonged exposure to low temperatures to enable or promote flowering later in development. During the process of cold induced floral promotion, called vernalization, the competence of a plant to respond to floral inducing signals is acquired rather than that vernalization itself induces flowering (reviewed in Chouard, 1960; Lang, 1965). The mechanism of vernalization has been considered to involve epigenetic gene regulation for some time, since cold treatment and the actual induction of flowering can be separated in time by several months (reviewed in Amasino, 2004). Such an epigenetic cellular memory of winter would rely on the mitotically stable repression of factors that normally prevent flowering. A main target of the vernalization pathway turned out to be *FLC*, explaining the great reduction in flowering time of *FRI*-containing accessions and autonomous pathway mutants upon a vernalization period (Figure 1.9) (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

The first genetic evidence for the involvement of epigenetic factors in vernalization came from cloning of mutants with an impaired vernalization response. The *VERNALIZATION2 (VRN2)* gene encodes a *Drosophila* homologue of the Su(z)12 PcG protein (Gendall *et al.*, 2001). Animal PcG proteins act in complexes that modify the chromatin status of repressed *Hox* loci to allow maintenance of the silenced state (Birve *et al.*, 2001; Cao *et al.*, 2002; Czermin *et al.*, 2002; Müller *et al.*, 2002; Fischle *et al.*, 2003). Mutations in *VRN2* render the plant insensitive to

a vernalization treatment (Chandler *et al.*, 1996), resulting in high *FLC* levels and late flowering upon vernalization (Gendall *et al.*, 2001). Like *vrn2*, *vrn1* mutants show an attenuated response to vernalization (Chandler *et al.*, 1996), but *VRN1* encodes a plant specific protein with non-specific DNA binding activity (Levy *et al.*, 2002).

Although *vrn1* and *vrn2* mutants are disturbed in the vernalization process, neither mutation affects the initial down-regulation of *FLC* during cold treatment (Gendall *et al.*, 2001; Levy *et al.*, 2002), implying that *FLC* downregulation and the stable repression of *FLC* are distinct processes. These findings are in accordance with animal models, which state that the establishment and maintenance of homeotic gene expression patterns rely on two groups of genes. The first group is involved in the initial pattern specification events, whereas the second group functions in maintaining initiated expression patterns throughout development by chromatin modifications of target loci (reviewed in Li, 2002). PcG proteins, like the *Drosophila* VRN2 orthologue Su(z)12, belong to the second group of chromatin regulators (Birve *et al.*, 2001). Accordingly, VRN1 and VRN2 were shown to mediate vernalization-induced chromatin modifications of regulatory *FLC* domains, similar to the modifications found in PcG repressed *Hox* genes (Bastow *et al.*, 2004).

Recently, two Plant Homeodomain (PHD) finger-containing proteins VERNALIZATION INSENSITIVE3 (VIN3) and VIN3-LIKE1 (VIL1 a.k.a. VRN5) were shown to be necessary for the initial down regulation of *FLC* during vernalization (Figure 1.9) (Sung and Amasino, 2004; Greb *et al.*, 2007; Sung *et al.*, 2006a). Vernalization does not affect *VIL1* levels, but *VIN3* expression is cold mediated and levels increase during prolonged cold treatments (Sung and Amasino, 2004; Greb *et al.*, 2007; Sung *et al.*, 2006a). This correlates with the quantitative effect of vernalization on *FLC* levels and flowering time (Sung and Amasino, 2004). Moreover, VIN3 is able to bind to the *FLC* locus (Sung and Amasino, 2004) and to interact with VIL1 via their conserved C-terminal domains (Greb *et al.*, 2007; Sung *et al.*, 2006a). Therefore, VIN3 and VIL1 most likely function as a heterodimer in the vernalization process. Thus, cold-induced VIN3 mediates suppression of *FLC* via recruitment of VIL1. Subsequently, VRN1 and VRN2 modify *FLC* chromatin to allow docking of LIKE HETEROCHROMATIN PROTEIN1 (LHP), which helps to maintain the repressed state of *FLC* throughout further somatic development (Figure 1.9) (Mylne *et al.*, 2006; Sung *et al.*, 2006b).

Outline of this Thesis

The shoot structure of a plant is progressively generated throughout its life cycle, allowing constant adaptation of growth to changing environmental conditions. At the start of post-embryonic development the plant body does not constitute more

than two embryonic leaves and two meristems, which are separated form each other by the hypocotyl and embryonic root. During subsequent development the two meristems grow in opposite directions and form the shoot and root structures, respectively. The shoot apical meristem (SAM) consists of a central pool of stem cells surrounded by undifferentiated stem cell daughters that form the SAM peripheral zone. Migration of peripheral zone cells away from the SAM centre coincides with their increasing competence to differentiate and become incorporated in lateral organ primordia at the flanks of the SAM. Complex genetic networks are involved in initiating and maintaining the meristem structure throughout the plants life cycle and the TALE homeodomains (HD) transcription factor STM holds critical positions within these networks.

Besides its capacity to form the initials for lateral shoot organs, the SAM passes through several distinct identity phases, of which the leaf forming vegetative and flower forming generative phases are most pronounced. Thus, next to the continuous process of self-maintenance, the SAM also participates in dictating the type of organs formed at its flanks. Although the genetic networks of meristem maintenance and phase transition are believed to be tightly linked, genetic evidence for such connections is very limited.

The work described in this thesis focuses on the roles of the BELL class TALE HD transcription factors ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) and PENNYWISE (PNY) in shoot apical meristem (SAM) function as well as the control of the vegetative to generative phase transition.

In **chapter 2** we show that the SAM depends on the redundant functioning of ATH1 and PNY for its embryonic initiation and maintenance throughout the vegetative phase, but not during the generative phase. ATH1 and PNY operate in conjunction with the TALE HD protein STM. In addition, we show that an ancient mechanism that regulates animal TALE HD protein function by controlling their subcellular distribution is likely to function in plants as well.

Chapter 3 represents a functional analysis of *ATH1* in the process of floral induction. Because this phase change is irreversible in Arabidopsis, its accurate timing is of great importance to maximize offspring success. A major player in the repression of flowering, and thus maintenance of the vegetative meristem phase, is *FLOWERING LOCUS C* (*FLC*). We found that ATH1 acts as a positive regulator of *FLC* and may so control vegetative phase identity of the SAM.

In **chapter 4** we describe the characterization of ATH1 homologues in the process of floral suppression. Since ATH1 is not fully accountable for mediating induction of high *FLC* levels, we suggested that additional members of the BELL family might act redundantly with ATH1 in this process. Surprisingly, we identified PNY as a redundantly acting factor with ATH1, not only in the process of meristem initiation and maintenance, but in floral timing by controlling *FLC* expression levels as well.

Chapter 2

The BELL Homeodomain Proteins ATH1 and PNY Are Required for Vegetative Shoot Apical Meristem Function in Arabidopsis

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The BELL Homeodomain Proteins ATH1 and PNY Are Required for Vegetative Shoot Apical Meristem Function in Arabidopsis

Summary

The KNOX TALE homeodomain protein SHOOT MERISTEMLESS (STM) is indispensable for proper shoot development in Arabidopsis. Lack of STM causes defective initiation and maintenance of the shoot apical meristem (SAM) and results in early developmental arrests. Previous genetic analyses indicated that STM function depends on related TALE homeodomain proteins of the BELL class. Moreover, STM can dimerize with a subset of BELL proteins, which results in nuclear translocation of STM protein. Here we demonstrate that two closely related BELL proteins, ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) and PENNYWISE (PNY) function redundantly during embryonic SAM initiation and vegetative meristem maintenance. Loss of ATH1 enhances shoot defects in weak *stm* backgrounds as described previously for *pny*. Moreover, combined loss of ATH1 and PNY results in *stm* phenocopies during the embryonic and vegetative phases, whereas generative development appears unaffected. Finally, we present data implying that KNOX and BELL protein function is regulated through their subcellular distribution by a mechanism highly conserved in animals and plant.

Introduction

Plant development shows tremendous plasticity, which arises from a close coupling of developmental responses to environmental stimuli. Most of the plant adult body is formed post-embryonically by the continuous activity of two pools of undifferentiated progenitor cells: the shoot and root apical meristems. Both meristems are established during embryogenesis and together with cotyledons, hypocotyl and the embryonic root make up the basic body plan of a plant. At the completion of embryogenesis the apical meristems are quiescent, but they become reactivated shortly after germination. Shoot lateral organs initiate from the peripheral zone of the shoot apical meristem (SAM). The center of the SAM consists of a cluster of slowly dividing stem cells that retain organogenic potential throughout the plants life cycle. These stem cells generate multipotent daughters that move out to the peripheral zone or downward into the interior rib zone and make up the organ founder cells that eventually become incorporated into lateral organ primordia or the main stem, respectively. 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 (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 loss-of-function *stm* alleles fail to establish and maintain a functional SAM, often do not undergo any post-embryonic development, and show fusions of the cotyledonary petioles (Barton and Poethig, 1993; Endrizzi *et al.*, 1996; Long *et al.*, 1996). Less severe mutants, however, do form a temporal SAM that initiates primordia shortly after germination. 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). Interestingly, despite initial lack of a SAM or the premature termination of the SAM, all *stm* mutants are capable of forming leaves from lateral positions in the fused cotyledonary petiole area or, in less severely affected mutants, from an apical position (Barton and Poethig, 1993; Clark *et al.*, 1996; Endrizzi *et al.*, 1996). In the latter case, often transient shoots originate in a reiterative pattern from adventitious meristem-like tissues located in leaf axils and on petiole tissue, allowing stunted vegetative and partial reproductive development (Barton and Poethig, 1993; Clark *et al.*, 1996). Moreover, *stm* floral meristems fail to produce a full complement of floral organs, resulting in flowers with a significantly reduced number of fused stamen and petals and that often lack of a central gynoecium (Clark *et al.*, 1996; Endrizzi *et al.*, 1996).

STM is widely expressed in the meristem including the stem-cell region and the peripheral zone, but is excluded from incipient primordia cells (Figure 1.6a) (Long *et al.*, 1996). Notably, down-regulation of *STM* in primordia seems essential for lateral organ formation, since in all incipient axillary and floral meristems, *STM* expression is also temporarily lost (Long and Barton, 2000).

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). This is mainly established by preventing expression of the differentiation factor *ASYMMETRIC LEAVES1* (*AS1*) in the meristem, since loss of *AS1* function rescues weak *stm* mutants (Byrne *et al.*, 2000). This suppression involves complex interactions within a network of redundant KNOX homologues (Byrne *et al.*, 2002). Two of these homologues, *BREVIPEDICELLUS* (*BP* a.k.a. *KNAT1* for *KNOTTED1-LIKE ARABIDOPSIS THALIANA1*) and *KNAT6* were recently 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).

STM belongs to the highly conserved class of three-amino-acid-loop-extension (TALE) homeodomain (HD) transcription factors (Bürglin, 1997). Besides the conserved TALE HD, KNOX proteins contain an additional conserved protein motif, the MEINOX domain. This N-terminal domain is shared with the metazoan myeloid ecotropic integration site (MEIS) class of TALE HD proteins (Bürglin, 1995, 1997). MEIS class proteins include *Drosophila* Homothorax (Hth) and vertebrate Meis and Prep HD proteins. High sequence similarity between the metazoan and plant MEINOX domains suggests that they have evolved from a common ancestral domain (Bürglin, 1997). In MEIS proteins, this MEINOX 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 (Figure 1.2) (Kamps *et al.*, 1990; Nourse *et al.*, 1990; Peifer and Wieschaus, 1990; Rauskolb *et al.*, 1993). Similar to MEIS proteins, PBC class proteins share a conserved bipartite domain N-terminal of their TALE HD, referred to as the PBC-A/B domain, (Bürglin and Ruvkun, 1992; Rauskolb *et al.*, 1993; Bürglin *et al.*, 1997, 1998). PBC and MEIS proteins are able to interact via their PBC-A and MEIS domains, respectively (Chang *et al.*, 1997; Knoepfler *et al.*, 1997; Rieckhof *et al.*, 1997; Berthelsen *et al.*, 1998b). In most cases, heterodimerization with a member of the other class is mandatory for their nuclear translocation, functionality and stability (Rieckhof *et al.*, 1997; Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999; Mercader *et al.*, 1999; Jaw *et al.*, 2000; Waskiewicz *et al.*, 2001; 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, 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. Plants contain a second group of TALE HD proteins named the BELL (BEL1-like) class in addition to the KNOX class (Reiser *et al.*, 1995; Bürglin, 1997). BELL proteins share the TALE HD and in addition two closely positioned N-terminal domains, designated SKY and BELL domains (Bellaoui *et al.*, 2001). Like PBC and MEIS proteins, BELL proteins can heterodimerize both *in vitro* and *in vivo* with KNOX proteins in a DNA independent manner (Bellaoui *et al.*, 2001; Müller *et al.*, 2001; Smith *et al.*, 2002; Byrne *et al.*, 2003; Chen *et al.*, 2003; Smith *et al.*, 2003; Bhatt *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; Müller *et al.*, 2001; Cole *et al.*, 2006). Moreover, several BELL class members have recently been shown to translocate the KNOX protein STM 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). These combined data imply a functional convergence of BELL and PBC proteins in controlling TALE HD protein localization and activity, despite lack of unambiguous evidence for evolutionary conservation of the PBC and BELL domains (Becker *et al.*, 2002),

In *Arabidopsis*, the *BELL* family consists of thirteen members. Loss-of-function mutants of most *BELL* genes have no obvious defects and for the majority of *BELL* genes their functions remain unclear as for most of the *KNOX* genes (B.R. and M.P., unpublished data). An exception is *PENNYWISE* (*PNY* a.k.a. *BELLRINGER*, *REPLUMLESS*, *LARSON* and *VAAMANA*) (Byrne *et al.*, 2003; Roeder *et al.*,

2003; Smith and Hake, 2003; Bao *et al.*, 2004, Bhatt *et al.*, 2004). *pny* mutants were identified in several screens and the most interesting phenotype is enhancement of *stm* defects, as observed for *bp* and *knat6* mutants (Byrne *et al.*, 2002, 2003; Roeder *et al.*, 2003; Smith and Hake, 2003; Bhatt *et al.*, 2004; Belles-Boix *et al.*, 2006; Kanrar *et al.*, 2006). Other *pny* phenotypes include loss of replum and patterning defects of the inflorescence (Byrne *et al.*, 2003; Roeder *et al.*, 2003; Smith and Hake, 2003; Bhatt *et al.*, 2004; Kanrar *et al.*, 2006).

PNY targets STM to the nucleus upon heterodimerization, which might partially explain the enhancement of *stm* phenotypes by *pny* mutations (Cole *et al.*, 2006). However, the *pny* SAM is not noticeable different in size or organization compared to wild type, even though PNY appears to be required for STM activity and, most likely, affects meristem function via a *KNOX* gene-specific pathway (Byrne *et al.*, 2003). Therefore, the requirement for PNY seems to be only partial. This might be explained by functional redundancy between *BELL* genes, especially since STM has the potential to bind to multiple BELL partners (Hackbusch *et al.*, 2005). The PNY paralogue POUND-FOOLISH (PNF) (Smith *et al.*, 2004), one of the BELL proteins also capable of binding to STM, seems to fulfill such a redundant role only after floral transition. PNF controls inflorescence architecture in concert with STM and PNY (Smith *et al.*, 2004; Kanrar *et al.*, 2006), but is incompetent to enhance weak *stm* phenotypes during either vegetative or reproductive phases (Kanrar *et al.*, 2006).

Here we report on ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1) and PNY as functional redundant proteins for vegetative shoot apical meristem function in *Arabidopsis*. ATH1 is capable of both interacting with class I KNOX proteins, including STM, and of directing the ATH1-STM heterodimer to the nuclear compartment (Hackbusch *et al.*, 2005; Cole *et al.*, 2006). We show that ATH1, like PNY, is required for maintenance of the vegetative meristem in the absence of fully functional STM. Importantly, combined loss of *ATH1* and *PNY* results in *stm* mutant phenocopies during embryogenesis and the vegetative growth phase, whereas inflorescence and floral development are less severely disturbed compared to *stm* plants. In addition, further evidence is provided for evolutionary conservation of a functional interaction between members of two TALE-HD families, PBC-MEIS in animals and BELL-KNOX in plants. In animal cells, CRM1/exportin-1 promotes cytoplasmic localization of monomeric PBC proteins. We demonstrate that a similar nuclear exclusion mechanism, involving a plant CRM1/exportin-1 homologue, is active in plant cells to control BELL subcellular localization. First of all, monomeric BELL proteins are localized in both the nucleus and cytoplasm of onion epidermal cells and are able to interact with *Arabidopsis* CRM1 (AtCRM1/AtXPO1a) (Haasen *et al.*, 1999), using their conserved KNOX-interacting BELL domain. Pharmacological disruption of the plant CRM1-dependent nuclear export

pathway causes consistent nuclear accumulation of BELL proteins. Moreover, many known BELL domains, both from dicot and monocot species, harbor two functionally conserved NES sequences, highly reminiscent of the two NESs found in animal PBC proteins. Mutational analysis indicates that these NES sequences are active when fused to a fluorescent marker protein and necessary for interacting with AtCRM1 and STM.

Results

ATH1 Interacts with Class I KNOX Genes

ATH1 encodes a shoot-specific BELL class transcription factor (Czechowski *et al.*, 2003) with high expression levels in the vegetative SAM (Chapter 3 of this thesis). An evolutionary tree-based analysis on full-length BELL protein sequences has positioned ATH1 in one sub-clade with PNY and PNF (Roeder *et al.*, 2003). Both PNY and PNF have been implicated in shoot meristem processes and we investigated whether ATH1 shares functions with these BELLs in the SAM. *In vitro* and *in vivo* binding, and yeast two-hybrid studies have demonstrated that PNY physically associates with STM as well as with BP and KNAT6, two KNOX proteins previously shown to contribute redundantly with STM to SAM maintenance (Byrne *et al.* 2003; Smith and Hake 2003; Bhatt *et al.* 2004; Hackbusch *et al.*, 2005; Belles-Boix *et al.*, 2006; Cole *et al.*, 2006; Kanrar *et al.*, 2006). PNF, on the other hand, is able to interact with STM and BP (Hackbusch *et al.*, 2005; Kanrar *et al.*, 2006) and functions in the generative SAM (Smith *et al.*, 2004; Kanrar *et al.*, 2006). We compared the biochemical properties of ATH1 to those of its closest relatives, PNY and PNF, and determined if there was an overlap in the interaction patterns of these three BELL proteins with the Arabidopsis KNOX proteins (Figure 2.1). For this purpose full-length proteins were fused to the GAL4 DNA-binding domain (BD) and to the GAL4 transcriptional activation domain (AD), respectively, to allow reciprocal pair wise yeast two-hybrid interaction analysis (Figure 2.1). As a negative control, we tested for interactions of the TALE HD proteins with HY5. HY5 encodes a bZIP transcription factor and functions as a positive regulator of photomorphogenesis (Oyama *et al.*, 1997; Chattopadhyay *et al.*, 1998). No interaction was detected between any of the tested TALE HD proteins and HY5 (Figure 2.1). However, the yeast two-hybrid analysis showed that ATH1 binds to STM, BP, KNAT2 and KNAT6, but not to any of the other KNOX proteins (Figure 2.1). The same results were obtained for PNY and PNF (Figure 2.1). According to phylogeny and expression patterns, *STM*, *BP*, *KNAT2* and *KNAT6* form the class I *KNOX* genes, whereas class II is formed by *KNAT3*, *KNAT4*, *KNAT5* and *KNAT7* (Fig. 1.7b) (Kerstetter *et al.*, 1994; Bharathan *et al.*, 1999; Reiser *et al.*, 2000; Bellaoui *et al.*, 2001). Thus no interactions between the three tested BELL

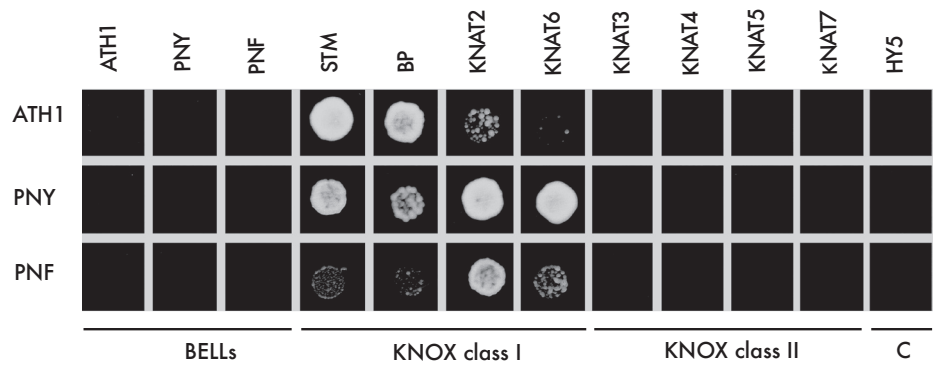

Figure 2.1 The Related BELL Proteins ATH1, PNY and PNF Interact With Class I Knox Proteins.
The BELL proteins ATH1, PNY and PNF were tested in a yeast two-hybrid system for interactions with themselves and class I and II KNOX proteins. As a negative control the bZIP protein HY5 was used.

proteins and any of the class II KNOX proteins were detected, in contrast to what was reported previously (Hackbusch *et al.*, 2005). PNY homodimerization was also not observed (Figure 2.1). In addition, the interactions of ATH1-BP, ATH1-KNAT2, ATH1-KNAT6, and PNF-KNAT6 were not previously reported.

The overlap in observed interactions in this study suggests that ATH1 might also have functional overlaps with PNY and/or PNF.

ath1 Mutations Enhance Weak and Intermediate *stm* Phenotypes

PNY and PNF have been implicated to function in association with STM based on both physical and genetic interactions. However, the requirement of STM function for PNY and PNF seems to be partial, suggesting a possible functional redundancy between related BELL proteins. Dosage experiments indicated that PNY and PNF share redundant functions together with STM during generative development (Smith *et al.*, 2004; Kanrar *et al.*, 2006), but BELL proteins that act redundantly with PNY in vegetative development have not been identified.

The role of PNY in vegetative SAM function was revealed by the enhancement of weak and intermediate *stm* loss-of-function phenotypes by introduction of *pny* mutations (Byrne *et al.* 2003; Bhatt *et al.* 2004; Kanrar *et al.*, 2006). Similarly, to address the functional relevance of the observed ATH1-STM interaction (Figure 2.1), we generated double mutants of *ath1-1* in combination with weak, intermediate and strong alleles of *STM* in both the Landsberg *erecta* (L*er*) and Columbia (Col) backgrounds.

Compared to wild type there is no noticeable difference in the size or organization of the *ath1-1* SAM during embryogenesis and vegetative development (Figure 2.5b; D.B. and M.P., unpublished data). *ath1-1* plants displayed a similar growth pattern as wild type plants when grown under long day photoperiod

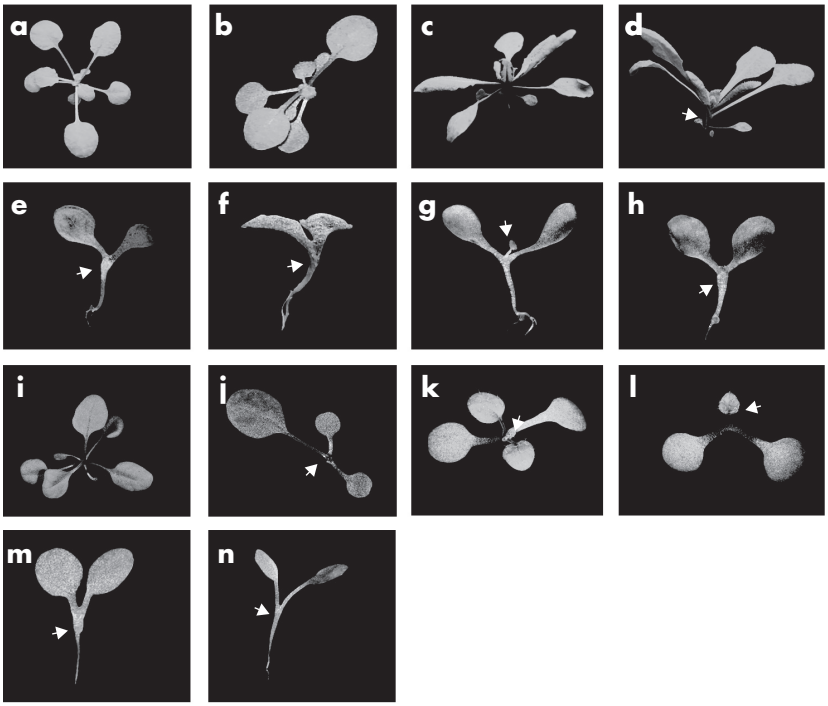

Figure 2.2 Enhancement of *stm* Phenotypes by *ath1* Mutations

(a) Rosette of a wild-type plant grown in SD (top view).

(b) Rosette of an *ath1-1* plant grown in SD (top view).

(c) Side-view of a wild-type plant grown in SD.

(d) Side-view of an *ath1-1* plant grown in SD. The arrow indicates fused tissues at the rosette base.

(e) Strong L*er stm-4* mutant with fused cotyledonary petioles (arrow).

(f) *ath1-1 stm-4* double mutant with fused cotyledonary petioles (arrow).

(g) Intermediate L*er stm-2* mutant. The arrow indicates an apical leaf escaping from the presumptive SAM area.

(h) *ath1 stm-2* double mutant. Note the increased fusion of the cotyledonary petioles (arrow; compare to *stm-2* (g) and *stm-4* (e)).

(i) Arrested *stm/bum1-1* mutant (Col background). Single mutants usually arrest after the formation of 4-8 leaves.

(j) *ath1-1 stm/bum1-1* double mutant. Double mutants display an enhanced *stm* phenotype (compare to (i)) and often arrest after the formation of a single leaf (arrow) from the presumptive SAM area.

(k) The weaker *stm/bum1-3* mutant usually completes the vegetative phase without arresting. In the plant depicted here the second leaf pair has just emerged (arrow).

(l) Arrested *stm/bum1-3 ath1-1* double mutant after formation of one single leaf (arrow), the plant is grown to the same age as the *stm/bum1-3* single mutant in (k).

(m-n) *ath1-1 pny*^{40126} double mutants in the L*er* background resemble severe *stm-4* mutants in (e). Arrows indicate fused cotyledonary petioles.

conditions. However, under short day conditions or when plants are grown in high densities, *ath1-1* plants showed a phyllotaxy defect that was expressed by an irregular leaf pattern in the basal rosette (Figure 2.2a,b). The origin of this defect seems to be physical constraints due to partial fusion of the first three to five rosette leaf petioles (Figure 2.2c,d).

Table 2.1 Effect of the *ath1-1* Mutation on Shoot Development of *stm-4* and *stm-2* Mutants

Genotype of Parent	Number of plants with initial *stm* phenotype*	Percentage rescued at 5.5 days #	Percentage rescued at 10 days #	Percentage rescued at 16 days #
Experiment 1				
ath1-1	40	100	100	100
stm-4	39	0	3	5
ath1-1 stm-4	23	0	4	14
stm-2	40	0	88	100
ath1-1 stm-2	43	0	47	81
Experiment 2				
ath1-1	40	100	100	100
stm-4	30	0	3	13
ath1-1 stm-4	42	0	0	5
stm-2	37	0	84	100
ath1 stm-2	51	0	41	80
Experiment 3				
ath1-1	40	100	100	100
stm-2	40	0	88	98
atn1-1 stm-2	38	0	50	84

Seedlings were grown on MS medium in LD conditions and initially scored for the stm phenotype at 4 days after transferring plants from 4°C to 22°C LD conditions (*). Formation of leaf tissue during the coarse of the experiment was scored as rescuing of the stm phenotype (#). Wild-type plants gave the same result as ath1-1 single mutants.

Plants that combine the *ath1-1* mutation with a strong L*er stm-4* allele were indistinguishable from *stm-4* single mutants (Figure 2.2e,f; Table 2.1). Like *stm-4*, *ath1-1 stm-4* seedlings developed cotyledons with partially fused petioles and most plants senesced without the formation of additional organs. About 20% of the plants from each genotype eventually formed so-called escape shoots, and these shoots were also highly similar to each other (data not shown). However, *ath1-1* clearly enhances the phenotype of the intermediate L*er* allele *stm-2* (Figure 2.2g,h; Table 2.1). Single *stm-2* mutants showed no or only a slight fusion at the base of the cotyledons and did form 1-3 leaves from an apical position or the fused cotyledonary petioles (Figure 2.2g). As was reported by Clark *et al.* (1996) first signs of *stm-2* leaf development were between 5.5 and 10 days after sowing and after 16 days essentially all plants had visible leaves (Table 2.1). In contrast, *ath1-1 stm-2* double mutants showed more severe fusions of the cotyledonary petioles, reminiscent to those observed in the strong *stm-4* background (Figure 2.2e,g,h). Moreover, *ath1-1 stm-2* plants formed primary leaves to a far lesser extend than *stm-2* and at later stages after germination (Table 2.1).

Remarkably, the Col *stm/bumbershoot1-3* (*stm/bum1-3*) allele (also known as *stm-10*, Kanrar *et al.*, 2006) carries the identical mutation as the L*er stm-2* allele, but conditions a much weaker phenotype (Kanrar *et al.*, 2006). *stm/bum1-3* plants

are able to initiate relatively normal leaves that form a regular rosette (Kanrar *et al.*, 2006). As such, homozygous mutants are difficult to distinguish from wild type plants until bolting. Instead of forming a proper inflorescence, *stm/bum1-3* plants enter a vegetative-inflorescence-vegetative-inflorescence development, characterized by the formation of aerial rosettes. This inflorescence defect is typical for *stm* mutants, including those in a L*er* background. The remarkable discrepancy between *stm-2* and *stm/bum1-3* vegetative phenotypes suggests that either the L*er* accession contains an enhancer of the *stm* phenotype or, opposite, that the Col background carries a suppressor of this phenotype.

All available *ath1* alleles are in the Col background. Even though the described *ath1-1 stm-2/4* mutants were backcrossed several times to L*er*, presence of the unknown enhancer/suppressor locus might have affected the interpretation of the data described above. Therefore, we also studied the genetic interaction of *ath1* with the Col *stm* alleles *stm/bum1-3* and *stm/bum1-1*. The latter being a slightly more severe allele, allowing the formation of 4-8 leaves before termination of the SAM (Figure 2.2i). Later in *stm/bum1-1* development transient shoots originate in a reiterative pattern as in most intermediate and weak *stm* plants and reproductive phenotypes of *stm/bum1-1* plants are highly similar to *stm/bum1-3* plants.

Introduction of *ath1-1* in both *stm/bum1-3* and *stm/bum1-1* plants greatly enhanced their weak *stm* Col phenotypes (Figure 2.2i-l). Shoot growth was terminated after the development of two leaves or, in a number of cases, already after a single leaf that initiated between the two cotyledons (Figure 2.2j,l), whereas *stm/bum1-3* and *stm/bum1-1* plants completed the vegetative growth phase with the formation of more or less complete rosette or arrested after the formation of 4-8 leaves, respectively (Figure 2.2i,k). Following a short developmental arrest *ath1-1 stm/bum1-3* and *ath1-1 stm/bum1-1* plants were capable of forming transient escape shoots from adventitious meristematic cells located in axils and on petioles of the previously formed leaves. Again, reproductive development of the double mutants was comparable to the individual *stm/bum1-3* and *stm/bum1-1* mutants (data not shown), implying that ATH1 primarily controls vegetative shoot development in conjunction with STM.

ATH1 was also found to interact with BP (Figure 2.1) and we also analyzed *ath1-1 bp* double mutants to determine genetic interactions between these two genes. However, *ath1 bp* double mutants were additive in all respects (data not shown).

Taken together, the observed genetic interactions indicate that ATH1 is required for SAM function when there is reduced STM activity. Moreover, since both *ath1* and *pny* mutations enhance weak and intermediate *stm* phenotypes during the vegetative growth phase, ATH1 and PNY might well act redundantly in conjunction with STM during SAM maintenance and/or initiation.

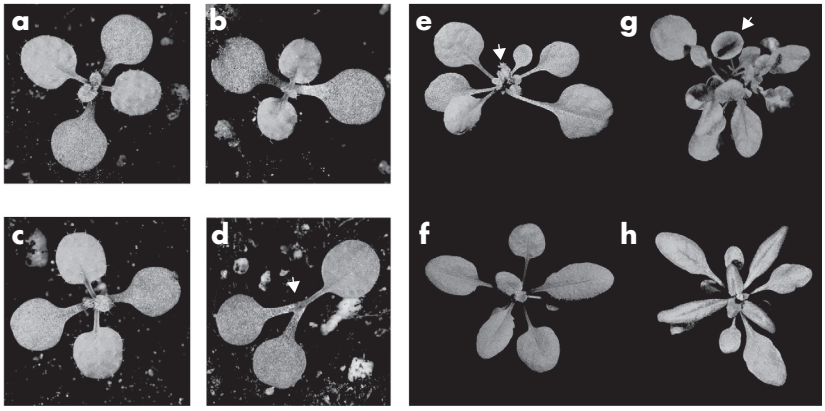

Figure 2.3 *ath1 pny* Double Mutants Are *stm* Phenocopies
(a-d) 10-day-old wild-type **(a)**, *ath1-1* **(b)**, *pny*^{40126} **(c)** and *ath1-1 pny*^{40126} **(d)** plants grown in LD conditions.
(e) After arresting, *ath1 pny*^{40126} plants start forming escape shoots in an unorganized way (arrow) from the presumptive SAM area.
(f) Wild-type plant of same age as plant in **(e)**, with leaves formed in a regular pattern from the central positioned SAM.
(g) Later in *ath1 pny*^{40126} development, leaf-like organs form also from petioles of existing leaves, and include trumpet-shaped leaves (arrow). Overall, the plants get a very busy appearance
(h) Wild-type plant of the same age as plant in **(g)**.

ATH1 and PNY Act as Redundant Partners in Vegetative SAM Initiation and Maintenance

To determine whether ATH1 and PNY share functions during SAM initiation and maintenance, *ath1-1* was crossed to the *pny*^{40126} mutant (Smith and Hake, 2003) and double mutants were analyzed for defects in SAM function. Most *ath1-1 pny*^{40126} seedlings started forming the first leaf primordia at the same time as either single mutant or wild type control plants (Figure 2.3a-d). However, *ath1-1 pny*^{40126} shoot meristems terminated after the formation of 1-5 leaves (Figure 2.3d), whereas single mutants continued with the formation of a normal rosette. After a developmental arrest, *ath1-1 pny*^{40126} mutants reinitiated leaf formation from mostly transient meristematic tissues in the axils of cotyledons and pre-existing leaves, as well as from petiole tissue (Figure 2.3e-h). This disturbed leaf initiation pattern resulted in very bushy plants (Figure 2.3e-h). Moreover, newly formed leaves were often aberrant in shape and included trumpet-shaped leaves (Figure 2.3g) and adaxial leaf-leaf fusions along the petioles and leaf blades. Overall, the *ath1-1 pny*^{40126} adult vegetative phenotype strongly resembled that of Col *stm/bum1-1* plants.

Some *ath1-1 pny*^{40126} plants were able to complete a more wild type looking vegetative growth phase. The central SAM of these plants did not terminate and was capable of undergoing floral transition. However, similar to the more severely affected double mutants, these plants eventually gained a bushy appearance due to

the initiation of numerous axillary leaves from the axils of older rosette leaves upon flowering. The majority of the offspring of these plants again showed the more severe *stm*-like vegetative phenotype, indicating that the *ath1-1 pny*^{40126} vegetative phenotype is not fully penetrant.

Like in *stm/bum1-1* plants, a number of transient shoots of the more severely affected *ath1-1 pny*^{40126} plants were capable of forming an inflorescence. In long day photoperiods, *ath1-1 pny*^{40126} plants flowered slightly later than wild type plants and the corresponding single mutants, which is likely to be caused by the reduced SAM function in the double mutant. In both mild and severe double mutants, *ath1-1 pny*^{40126} inflorescences had an almost wild type appearance, only showing the mild *pny* single mutant inflorescence phenotype observed in the Col background. Reiterative shoot formation on developing inflorescences, characteristic of *stm* mutants, was never observed. Also in contrast to most *stm* mutants, *ath1-1 pny*^{40126} flowers were often able to produce a full complement of floral organs.

Combinations of *ath1-1* with *pnf* ^{96116} (Smith *et al.*, 2004), on the other hand, did not reveal any apparent aberrant phenotypes (data not shown). a*th1-1 pnf* ^{96116} double mutants looked identical to *ath1-1* plants, suggesting a lack of functional overlap between these two genes in shoot development.

To determine the fate of the main SAM in *ath1-1 pny*^{40126} plants, we introduced an *STM* promoter-*GUS* reporter construct (*STM*$_{pro}$*:GUS*) in the double mutant. *STM* is expressed in the SAM founder cells of the embryo (Long *et al.*, 1996; Long and Barton, 1998), and continues being expressed throughout the SAM during the life span of the plant. Moreover, *STM* transcription is initiated early on in all types of lateral shoot meristems, including axillary, secondary inflorescene and floral meristems (Long and Barton, 2000). Therefore, *STM*$_{pro}$*:GUS* activity can be used as a marker of shoot meristem identity (Long and Barton, 2000).

In wild-type seedlings, *STM*$_{pro}$*:GUS* expression was confined to the SAM and excluded from all lateral organs (Figure 2.4a,b), as reported before (McConell and Barton, 1998). A similar pattern of expression was observed in *ath1-1* and *pny*^{40126} single mutants (Figure 2.4a-d), in accordance with the wild type appearance of both single mutant plants. However, double mutants that terminated, completely lacked reporter gene expression at the presumptive SAM location (Figure 2.4a,e,i), indicating lack of a main SAM. Furthermore, regions of GUS staining, indicative of early steps in meristem-tissue formation, were found ectopically on petioles, in leaf axils and on hypocotyls (Figure 2.4a,e,f,g). These regions were ranging in size, and often coincided with positions where ectopic tissues formed (Figure 2.4a,e,g). In wild type plants, axillary meristems always developed at the junction between the stem and the leaf base (Long and Barton, 2000), and ectopic meristematic tissues were never observed, nor in either of the corresponding single mutants (Figure 2.4a,b,c,d).

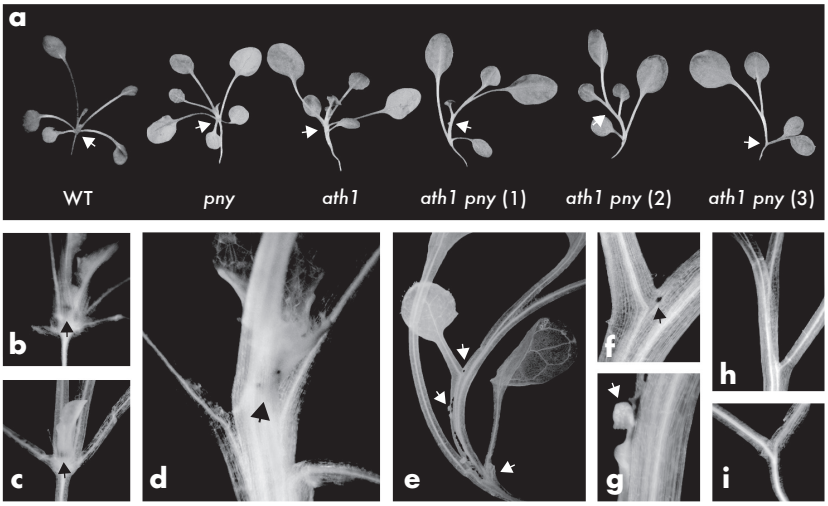

Figure 2.4 Expression of an *STM*$_{PRO}$*:GUS* Marker in *ath1 pny* Double Mutants
(a) From left to right: Densely grown wild-type (WT), *pny*^{40126} (*pny*), *ath1-1* (*ath1*) and *ath1-1 pny*^{40126} (*ath1 pny*) (1-3) plants carrying the *STM*$_{PRO}$*:GUS* construct. White arrows mark areas depicted as close-ups in **(b-i)** after GUS staining. Note fused petiole area in the *ath1-1* rosette, when compared to WT and *pny*.
(b) Wild-type plant showing a single area of *STM*$_{PRO}$*:GUS* activity (arrow) at the base of the rosette were the SAM is located.
(c) As in **(b)** for a *pny*^{40126} single mutant.
(d) *STM*$_{PRO}$*:GUS* activity in *ath1-1* plant. Due to partial fusions of leaf and stem tissues in the *ath1* single mutant rosette, the SAM is shifted to a more apical position, but according to GUS expression, still represents a single constrained domain.
(e) Stripe of ectopic *STM*$_{PRO}$*:GUS* activity on the surface of fused petiole-like tissue of an *ath1-1 pny*^{40126} (1) double mutant (middle arrow) (see **(g)** for a close-up). The upper arrow marks a single dot of GUS staining at a junction of fused tissue. The presumptive SAM area lacks *STM*$_{PRO}$*:GUS* activity (lower arrow).
(f) Close-up of **(e)** showing the single ectopic dot of GUS accumulation. The *GUS* expressing cells are located on the tissue surface.
(g) Ectopic leaf-like primordia (arrow) can be recognized as outgrowths that lack *STM*$_{PRO}$*:GUS* activity.
(h) *ath1-1 pny*^{40126} (2) plant. GUS staining is not observed in all leaf axils or junctions of fused tissues.
(i) *ath1-1 pny*^{40126} (3) plant showing a complete lack of marker line expression at the presumptive SAM area.

Since all *ath1-1 pny*^{40126} plants are capable of forming at least 1 leaf at an apical position, we suspected that progressive post-embryonic consumption of the initial SAM by developing leaf primordia might be an explanation for the observed *ath1-1 pny*^{40126} SAM phenotype.

Failing meristem maintenance, lack of SAM initiation during embryogenesis or a combination of both, could be the cause of the *ath1-1 pny*^{40126} vegetative phenotype. This was investigated by examining mature *ath1-1 pny*^{40126} embryos for morphological SAM defects using confocal laser scanning microscopy (Figure 2.5). As expected, *ath1-1*, *pny*^{40126} and wild-type embryos initiate a SAM of similar dimensions at a position just above the junction of the cotyledon and hypocotyl vascular strands (Figure 2.5a-c). In contrast, *stm-4* mutants lack any signs of an organized embryonic SAM (Figure 2.5f). The small, densely stained cells typical of

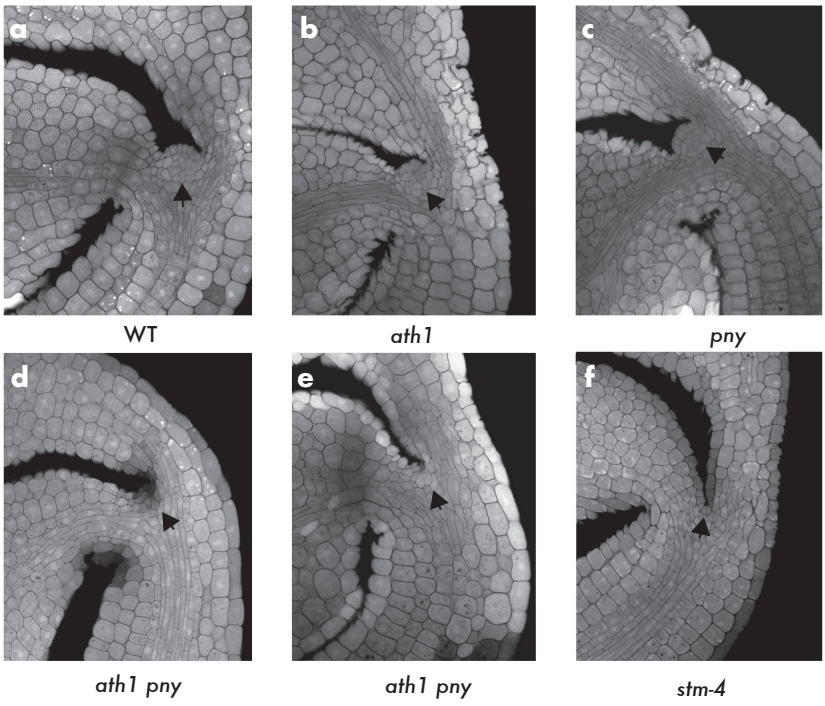

Figure 2.5 Confocal Laser Scanning Microscopy Images of Arabidopsis Embryos
Confocal Z-stacks through the SAM region of wild-type (WT) (Col-8) (a), *ath1-1* (*ath1*) (Col-8) (b), *pny*^{40126} (*pny*) (Col-8) (c), *ath1-1 pny*^{40126} (*ath1 pny*) (Col-8) (d-e) and *stm-4* (L*er*) (f) embryos. Arrows mark the (presumptive) SAM areas.

meristematic zones are completely absent from the apex of these mutants (Figure 2.5f). Rather, larger vacuolated cells were often present above the junction of the vascular elements (Figure 2.5f). Progeny of severe-looking *ath1-1 pny*^{40126} plants displayed a range of embryonic SAM defects that sometimes were nearly as severe as *stm-4* mutants (Figure 2.5d-f). This range in embryonic SAM phenotypes is consistent with the observed diversity in post-germination *ath1-1 pny*^{40126} phenotypes, and, once more, suggests that the double mutant phenotype is not fully penetrant.

Taken together, these data show that *ath1-1 pny*^{40126} plants, like *stm* mutants, display both SAM initiation and SAM maintenance defects. Therefore, we conclude that ATH1 and PNY act as redundant proteins in both SAM initiation and vegetative maintenance.

The *ath1 pny* SAM Phenotype Is Enhanced in the L*er* Background

The original *STM*$_{pro}$*:GUS* line is in the L*er* background and was crossed into Col *ath1-1 pny*^{40126} plants. The experiments described above were performed with Col/L*er ath1-1 pny*^{40126} *STM*$_{pro}$*:GUS* plants that were back-crossed three times to Col *ath1-1 pny*^{40126} and the resulting progeny was indistinguishable from the original Col

ath1-1 pny^{40126} parents. However, when crossed back several times to the L*er STM*$_{pro}$*:GUS* line, surprisingly, the *ath1-1 pny*^{40126} vegetative SAM phenotype was severely enhanced (Figure 2.2e,m,n). Most plants arrested without forming any primary apical leaves and developed fused cotyledonary petioles, very similar to *stm-4* plants (Figure 2.2e,m,n). Moreover, this phenotype was fully penetrant. Similar results were obtained by crossing back the original Col *ath1-1 pny*^{40126} double mutant to wild type L*er* plants (data not shown). After a developmental arrest of 1-3 weeks all terminated L*er ath1-1 pny*^{40126} plants developed ectopic shoots, very similar to those observed in Col *ath1-1 pny*^{40126} plants, and eventually all plants flowered with inflorescences indistinguishable from their Col double mutant counterpart (data not shown).

These observations are very similar to the discrepancy mentioned previously between Col *stm/bum1-3* vs. L*er stm-2*, and again imply the existence of a meristem activity suppressor in L*er*, or an enhancer of meristem function in the Col accession. The class I KNOX proteins BP and KNAT6 act redundantly with STM in meristem function and STM as well as BP and KNAT 6 have the potential to bind to multiple BELL partners (Hackbusch *et al.*, 2005). Therefore, it will interesting to test whether natural variation in any of the corresponding loci might be at the basis of this observation.

Nuclear Accumulation of ATH1 and STM in Onion Epidermal Cells Depends on Their Dimerization

The plant *BELL* and *KNOX* genes and their respective animal counterparts, *PBC* and *MEIS* genes, were proposed to have evolved from a single ancestral TALE homeobox gene that already existed in a common ancestor of animals and plants (Bürglin, 1997, 1998; Bellaoui *et al.* 2001; Becker *et al.*, 2002; Cole *et al.*, 2006). The evolutionary conservation of both structure and functional heterodimerization between members of each class of TALE HD proteins in both plants and animals supports this suggestion (Bellaoui *et al.* 2001; Cole *et al.*, 2006).

In metazoan embryos, dimerization of PBC and MEIS TALE HD class transcription factors is a prerequisite for nuclear accumulation of both protein types and, as a result, for exercising their regulatory functions on target gene transcription (Chang *et al.*, 1997; Rieckhof *et al.*, 1997; Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999). In embryos and undifferentiated cell types, monomeric PBC proteins are localized to the cytoplasm despite the presence of a conserved and functional nuclear localization signal (NLS) in their HD (Rieckhof *et al.*, 1997; Abu-Shaar *et al.*, 1999). MEIS proteins, on the other hand, lack a canonical NLS and are, accordingly, also localized in the cytosol (Berthelsen *et al.*, 1999). However, dimerization of PBC and MEIS proteins, via their respective PBC-A and MEINOX domains, results in nuclear translocation of the transcription factor pair. These data suggest that

the cytoplasmic localization of PBC proteins is overcome by heterodimerization, allowing the PBC NLS to direct the dimer to the nucleus.

The split-YFP system was used to determine whether the observed heterodimerization of ATH1 and STM in yeast also occurs in plant cells (Hu *et al.*, 2002; Walter *et al.*, 2004). This system relies on emission of YFP fluorescence upon reconstitution of the N- and C-terminal YFP subdomains into a functional YFP protein by the interaction of proteins that are fused to the different YFP subdomain. Chimeric proteins, consisting of ATH1 or STM translationally fused to either the N- or C terminal subdomains of the enhanced YFP protein ($eYFP_{N/C}$), were transiently expressed in onion epidermal cells using gold-particle bombardment (Figure 2.6a). Neither single ATH1- nor single STM-$eYFP_{N/C}$ fusions resulted in detectable signals when transiently expressed (data not shown). Also combinations of these ATH1- and STM-chimers with complementing non-chimeric eYFP-halves or non-interacting $eYFP_{N/C}$-fused proteins failed to yield a detectable signal (data not shown). However, combinations of STM-YFP_N with ATH1-YFP_C and vice versa always resulted in a strong, nuclear localized fluorescent signal (Fig. 2.6a). These data demonstrate that, in addition to the observed interaction in a yeast system, ATH1 and STM are also capable of physically associating in plant cells. Moreover, fluorescence is located almost exclusively in the nucleus, indicating that ATH1-STM heterodimers are efficiently incorporated into the nuclear compartment.

To test whether monomeric ATH1 and STM proteins have a similar cellular localization, we made both N- and C-terminal translational fusions of ATH1 and STM proteins with full-length enhanced GFP (eGFP). Interestingly, ATH1-eGFP fluorescence can be detected both in the cytoplasm and in the nucleus (Figure 2.6b), whereas STM-eGFP fusion proteins remained exclusively in the cytoplasm (Figure 2.6c), the latter results being consistent with those recently reported by Cole *et al.* (2006).

ATH1 likely is the driving force behind the efficient targeting of the ATH1-STM dimer to the nucleus since ATH1-eGFP fusions are partially nuclear localized, and STM, like its orthologous MEIS proteins, is devoid of a functional NLS (Abu-Shaar and Mann, 1998; Kurant *et al.*, 1998: Haller *et al.*, 2004; Cole *et al.*, 2006) (Figure 2.6a). Indeed, co-expression of untagged ATH1 with STM-eGFP fusion proteins resulted in a complete nuclear localization of the fusion proteins (Figure 2.6d), as opposed to the cytoplasmic retention of solitary expressed STM-eGFP fusions (Figure 2.6c). Similarly, co-expression of untagged STM with ATH1-eGFP fusion protein resulted in a full nuclear translocation of ATH1 chimeras (Figure 2.6e), suggesting that an interaction between ATH1 and STM efficiently translocates cytoplasmic ATH1 protein to the nucleus. Thus, both ATH1 and STM can redirect each other to the nuclear compartment in plant cells.

Similar results were obtained for the PNY-STM interaction (Bhatt *et al.*, 2004; Cole *et al.*, 2006), indicating that ATH1 and PNY might be redundantly required for

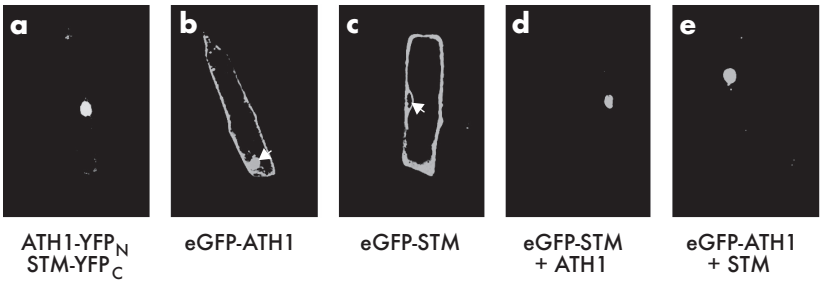

Figure 2.6 Transient Expression of TALE Homeodomain Proteins in Onion Epidermal Cells Reveals their Sub-cellular Distribution

(a) Reconstitution of YFP as a result of ATH1 and STM heterodimerization in onion epidermal cells. STM was fused to the C-terminal halve of YFP (STM-YFP$_C$) and ATH1 to the N-terminal halve (ATH1-YFP$_N$). Strong fluorescence in the nucleus indicates that the STM-ATH1 dimer has a prevailed preference for the nucleus.

(b) Both nuclear and cytoplasmic localized eGFP- ATH1 fusion protein in onion epidermal cells. Arrow marks the nucleus.

(c) Cytoplasmic localized eGFP-STM fusion protein in onion epidermal cells. The arrow marks the eGFP-depleted nucleus.

(d) Nuclear localization of eGFP-STM fusion protein when co-expressed with non-tagged ATH1 in onion epidermal cells.

(e) Nuclear localization of eGFP-ATH1 fusion protein when co-expressed with non-tagged STM in onion epidermal cells.

nuclear accumulation of STM. Importantly, this mechanism of heterodimerization to control nuclear import of ATH1-STM and PNY-STM dimers might underlie the observed redundant activities of ATH1, PNY and STM in SAM initiation and vegetative SAM maintenance.

ATH1 is Actively Exported from the Nucleus by a Conserved Leptomycin-B-Sensitive Mechanism

The observed conservation of nuclear import control between animals and plants of MEIS and KNOX TALE HD proteins through heterodimerization with the other class of TALE HD proteins, PBC and BELL, respectively, suggests the existence of a conserved mechanism.

In the absence of a MEIS interacting partner, PBC monomers essentially are excluded from the nucleus by the nuclear export receptor CRM1/exportin-1, which recognizes leucine-rich nuclear export signal (NES) sequences present in the PBC-A domain (Berthelsen *et al.*, 1999; Kilstrup-Nielsen *et al.*, 2003). CRM1/exportin-1 nuclear export activity can be specifically inhibited by leptomycin B (LMB) (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Wolff *et al.*, 1997). LMB blocks CRM1/exportin-1 activity by covalent modification of a conserved cysteine motif in the central region of the receptor (Kudo *et al.*, 1998, 1999). Arabidopsis contains two genes encoding highly similar CRM1/Exportin-1 orthologues, designated

AtXPO1a/AtCRM1 and AtXPO1b (Haasen *et al.*, 1999; Merkle, 2001), the first of which was shown to function as a LMB-sensitive nuclear export receptor in Arabidopsis (Haasen *et al.* 1999).

Our finding that solitary ATH1-eGFP does not end up solely in the nucleus of onion epidermal cells, as was found for ATH1-STM dimers (Figure 2.6a,d), might indicate the presence of an active export mechanism that relocates nuclear localized ATH1-fusions back into the cytoplasm. The significant fluorescence still observed in nuclei of ATH1-eGFP expressing cells, might be caused by saturation of the system with chimeric ATH1 protein due to strong CAMV:35S promoter activity. Alternatively, our findings might be explained by the presence of currently unknown additional mechanisms interfering with a nuclear export mechanism.

Interestingly, in cells expressing ATH1-eGFP, LMB causes a complete translocation of the fluorescent ATH1-fusion protein to the nucleus (Figure 2.7a,b), similar to the situation with co-expressed STM (Figure 2.6a,e). On the other hand, eGFP by itself did not accumulate in the nucleus upon LMB treatment (Figure 2.7c). Thus, an LMB-sensitive nuclear export mechanism seems to be active in translocating ATH1 protein out of plant cell nuclei.

BELL Domains Harbor Two Conserved Putative Leucine-rich NES Sequences and Interact with the Arabidopsis Nuclear Export Receptor AtCRM1

An intriguing aspect of PBC proteins is their capacity to interact with both MEIS proteins and CRM1/exportin-1 via a single conserved contact surface in their PBC-A domain (Berthelsen *et al.*, 1998; Kilstrup-Nielsen *et al.*, 2003). The latter interaction results in active translocation of PBC proteins from the nucleus into the cytoplasm, whereas interactions with MEIS partners cause nuclear accumulation of PBC-MEIS dimers by physically masking leucine-rich NESs in the PBC-A domain from CRM1 (Figure 1.4) (Kilstrup-Nielsen *et al.*, 2003).

The evidence for a functional conservation in animals and plants of heterodimerization to control nuclear accumulation of PBC-MEIS and BELL-KNOX protein dimers, respectively, as well as the observed effect of LMB on the cellular localization of the BELL protein ATH1, prompted us to test whether BELL proteins contain a NES sequence and, whether BELL proteins are capable of interacting with the Arabidopsis nuclear export receptor AtCRM1.

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 amino acid). Usually, bulky hydrophobic amino acids can be present instead of leucine at key positions, including isoleucine, valine, methionine and phenylalanine (Haasen *et al.*, 1999; Kotak *et al.*, 2004; la Cour *et al.*, 2004; Subramanian *et al.*, 2004).

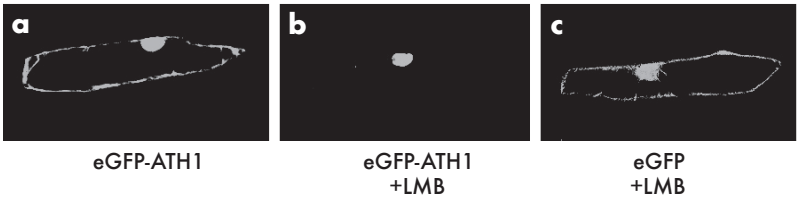

Figure 2.7 The Subcellular Distribution of ATH1 Protein is Sensitive to Leptomycin-B (LMB)
(a) eGFP-fused ATH1 localizes in both nucleus and cytoplasm of onion epidermal cells.
(b) Complete nuclear localization of eGFP-fused ATH1 in onion epidermal cells in the presence of 100 nM LMB.
(c) The sub-cellular distribution of single eGFP is not affected by 100 nM LMB. Compare to Figure 2.9 (a)

Members of the BELL family show very weak homology outside the conserved SKY-, BELL- and homeodomains (Bellaoui *et al.*, 2001; Becker *et al.*, 2002). Hence, if present, a conserved leucine-rich NES was expected to be located in one of these three domains. Moreover, from a mechanistic point of view, such a conserved NES sequence is expected to coincide with the KNOX interacting domain (Kilstrup-Nielsen *et al.*, 2003). This leaves the SKY and BELL domains, the latter of which was shown to be both required and sufficient to interact with the KNOX domain (Cole *et al.*, 2006). Therefore, the BELL domain is the most likely domain in BELL proteins to harbor a conserved NES. 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 2.8a). This, together with the LMB mediated nuclear translocation of ATH1, suggests that BELL proteins, like PBC proteins, are targets of a CRM1-dependent nuclear export mechanism.

This hypothesis was further explored by testing the capacity of ATH1 and PNY to interact with AtCRM1. Full length *AtCRM1/AtXPO1a* was cloned from the Col ecotype and translationally fused to both GAL4-AD and GAL4-BD for yeast-two-hybrid analysis. As can be seen in Figure 2.8b, both PNY and ATH1 were able to interact with AtCRM1. Moreover, since the discovered putative NES sequences are located in the conserved BELL domains, we tested for interactions between AtCRM1 and the ATH1 and PNY BELL domains (ABD and PBD). Indeed, the BELL domains also interacted with AtCRM1 like full length ATH1 and PNY proteins (Figure 2.8b). In addition, the ABD and PBD were also sufficient for interaction with STM, indicating that BELL domains harbor both the AtCRM1 interaction site and the KNOX binding site (Figure 2.8b).

Thus, the highly conserved BELL domain harbors not only two putative NESs, but is also the docking site of both STM and AtCRM1. This corresponds to the situation in the CRM1- and MEIS-interacting animal PBC-A domains, further corroborating an evolutionary conserved function of these MEINOX interacting domains.

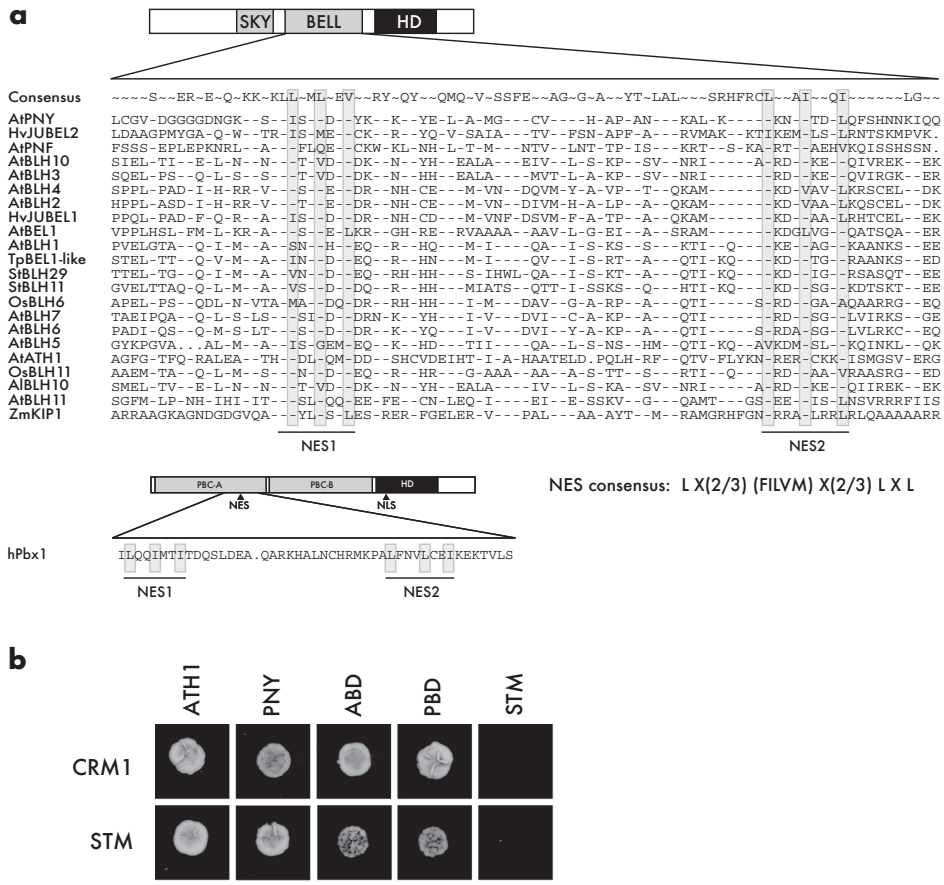

Figure 2.8 BELL Proteins Harbor Two Conserved Nuclear Export Signals (NES) in their BELL Domain and Have the Capacity to Interact with the Plant Nuclear Export Receptor AtCRM1/AtXPO1a

(a) 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. (~) mark positions within the BELL consensus with amino acid variation. Amino acids corresponding to the consensus are marked with (-). Plant proteins used in the alignment are: *Arabidopsis thaliana* (At) BEL1 (At5g41410), ATH1 (At4g32980), BLH1 (At2g35940), BLH2 (AT4g36870), BLH3 (At1g75410), BLH4 (At2g23760), BLH5 (At2g27220), BLH6 (At4g34610), BLH7 (At2g16400), PNF (At2g27990), PNY (At5g02030), BLH10 (At1g19700), BLH11 (At1g75430); *Hordeum vulgare* (Hv) HvJUBEL1 (AF334758) and HvJUBEL2 (AF334759); *Trifolium pratense* (Tp) TpBEL1-like (BAE71188); *Solanum tuberosum* (St) StBLH29 (AAN03626) and StBLH11 (AF406698); *Oryza sativa* (Os) OsBLH6 (NM_001055602) and OsBLH11 (ABA96532); *Arabidopsis lyrata* (Al) AlBLH10 (AAZ73651); *Zea mays* (Zm) ZmKIP1 (AY082396).

(b) Analysis of BELL-AtCRM1/AtXPO1a (CRM1) interactions in yeast. Both full-length proteins and the BELL domains of ATH1 (ABD) and PNY (PBD) are able to interact with AtCRM1/AtXPO1a. The BELL domains are also sufficient for interaction with STM. The KNOX protein STM is not capable of binding to AtCRM1/AtXPO1a.

Taken together, these data indicate that formation of BELL-KNOX heterodimers causes nuclear translocation of the interacting proteins by interfering with CRM1 recognition of the BELL protein.

The Two ATH1 BELL Domain NES Sequences Are Functional in Plant Cells

The *ATH1 BELL* domain (*ABD*) coding sequence was fused to the *eGFP* coding sequence to determine whether the two conserved BELL NES sequences in the ABD are functional. Constructs were expressed transiently in onion epidermal cells and cellular localization of the protein studied. The size exclusion limit for bidirectional diffusion through nuclear pore complexes is estimated to be 40–60 kDa (Görlich and Mattaj, 1996). Therefore, both single eGFP protein (29 kDa) and ABD-eGFP fusion protein (42 kDa) potentially can diffuse over the nuclear membrane. As expected, single eGFP protein was evenly distributed over the cytoplasm and the nucleus because of diffusion (Figure 2.9a). However, addition of the AB domain to eGFP impaired nuclear localization of the fusion protein (Figure 2.9b). Point-mutations disrupting both NES consensus sequences ($ABD_{NESm1+2}$) (Figure 2.9d) resulted in redistribution of the fluorescence pattern of $ABD_{NESm1+2}$–

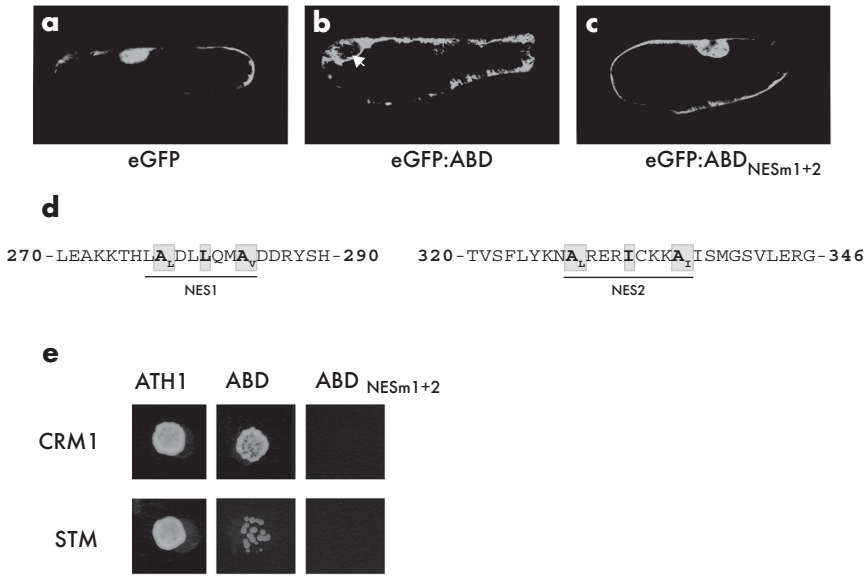

Figure 2.9 The ATH1 BELL Domain Functions in Nuclear Export

(a) Onion epidermal cells expressing solitary eGFP. Due to its small size the fluorescent protein can diffuse across the nuclear membranes without any constraints.

(b) 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 (c)), eGFP-ATH1 resides in the cytoplasm. The arrow indicates the eGFP-ATH1 depleted nucleus.

(c) Sub-cellular distribution of an eGFP-fused ABD with mutations in both NES motifs (eGFP:$ABD_{NESm1+2}$). Mutations are as depicted in **(d)**.

(d) Partial protein sequence of the mutated ABD indicating the amino acid changes that were introduced to impair NES function of the two conserved motifs (gray boxes) in the ABD. Numbers are corresponding to the the first and last amino acid of the fragments, $A_{(x)}$ represents a modified amino acid, were X shows the original amino acid replaced by alanine (A).

(e) Yeast two-hybrid interactions of full-length ATH1, the ATH1 BELL domain (ABD), and the ABD with both NES 1 and 2 mutated ($ABD_{NESm1+2}$) with AtCRM1/AtXPO1a (CRM1) and STM proteins.

eGFP over the cytoplasm and the nucleus, resembling that of non-fused eGFP (Figure 2.9a,c). Therefore, the conserved NESs present in the ATH1 BELL domain subjected to nuclear export and addition of the ABD by itself to eGFP did not impair the nuclear diffusion of the hybrid protein.

Next, we tested whether these NES mutations also affect the interaction between the ATH1 BELL domain with AtCRM1. The $ABD_{NESm1+2}$ mutant was unable to interact anymore with AtCRM1 (Figure 2.9e). Moreover, these mutations in the two NES sequences similarly impaired interactions between the ABD and STM (Figure 2.9e), indicating that the interaction domains of STM and AtCRM1 in the ATH1 BELL domain physically overlap.

Discussion

During animal embryogenesis, when body plan specification and pattern formation are prominent processes, TALE HD proteins play fundamental roles (recently reviewed in Moens and Selleri, 2006). In contrast, plant pattern formation does not end upon embryogenesis but is an ongoing process that lasts the entire life span of the plant. As a result most of the plant adult body is formed post-embryonically by continued organogenic potential of the root and shoot apical meristems. During shoot development, there is a constant delivery of cells from the shoot apical meristem (SAM) to developing organ primordia initiating at the meristem flanks. Maintenance of the SAM requires a tightly controlled balance between renewal of the central positioned stem cell pool and the division rate of stem cell daughters that provide the organ founder cells. In Arabidopsis, the WUSCHEL-CLAVATA feedback loop continuously adjusts the size of the shoot stem cell pool (recently reviewed in Williams and Fletcher, 2005), whereas the KNOX class 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 (Long *et al.*, 1996; Long and Barton, 2000; Byrne *et al.*, 2000; Lenhard *et al.*, 2002). STM has an essential role in SAM maintenance throughout development, but is also essential for initial formation of the SAM during embryogenesis (Barton and Poethig, 1993; Long *et al.*, 1996; Clarck *et al.*, 1996; Endrizzi *et al.*, 1996). Analogous to its animal counterparts, the MEIS proteins, STM is able to physically interact with members of a closely related second class of TALE HD transcription factors, the BELL class. In animals the formation of such heterodimers is often indispensable for TALE HD functionality (Rieckhof *et al.*, 1997; Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999; Mercader *et al.*, 1999; Jaw *et al.*, 2000; Waskiewicz *et al.*, 2001; Longobardi and Blasi, 2003; Stevens and Mann, 2007). One of the STM-interacting BELL proteins, PNY, is expressed in a STM overlapping domain and enhances meristem defects of weak to intermediate *stm* loss-of-function mutants (Byrne *et al.*, 2003). PNY itself,

however, is not essential for meristem function, possibly due to redundancy with other members of the BELL family.

Here we show that ATH1, a related BELL class protein is functionally redundant with PNY and STM in SAM initiation and its maintenance during the vegetative phase. Like *PNY* and *STM*, *ATH1* is highly expressed in the vegetative SAM (Chapter 3 of this thesis), but loss of ATH1 function seems not to be detrimental to meristem function as described for *pny* mutants (Byrne *et al.*, 2003). Moreover, *ath1* mutations enhance weak and intermediate *stm* alleles, as was previously observed for *pny* mutants (Byrne *et al.*, 2003; Bhatt *et al.*, 2004; Kanrar *et al.*, 2006). Most importantly, combined lesions in both *ATH* and *PNY* result in a partial phenocopy of *stm* mutants. Whereas *ath1 pny* double mutants are affected in both embryonic SAM initiation and its maintenance during the vegetative growth phase, reproductive development seems not to be affected in *ath1 pny* mutants. Interestingly, a third homologous BELL protein, PNF, has been shown to act redundantly with PNY and STM during the generative part of the plant life-cycle (Smith *et al.*, 2004; Kanrar *et al.*, 2006) and PNF has no apparent function in SAM initiation or vegetative meristem maintenance (Kanrar *et al.*, 2006). Since all three BELL proteins are capable of forming heterodimers with STM, this suggests that during the Arabidopsis life cycle STM likely recruits different sets of BELL proteins to ensure proper meristem function during all facets of above-ground plant development, with the redundantly acting cofactors ATH1 and PNY active during embryonic and vegetative development, and PNY and PNF later in development.

STM provides the critical KNOX class function for SAM development, but two STM homologues, BP and KNAT6, act redundantly with STM in this process (Byrne *et al.*, 2002; Douglas *et al.*, 2002, Venglat *et al.*, 2002; Dean *et al.*, 2004; Belles-Boix *et al.*, 2006). Our data show that both ATH1 and PNY can also physically interact with these KNOX proteins. Therefore we cannot exclude that part of the observed meristem phenotype in *ath1 pny* plants is due to reduced function of these two redundantly acting STM relatives.

A likely explanation for the observed *stm*-like phenotypes in *ath1 pny* double mutants might be the lack of nuclear localized STM-ATH1 or STM-PNY dimers in the vegetative SAM. In animals, MEIS-PBC heterodimerization is usually mandatory for stability, nuclear translocation, and, hence, functionality of both MEIS and PBC transcription factor proteins (Rieckhof *et al.*, 1997; Abu-Shaar *et al.*, 1999; Berthelsen *et al.*, 1999; Mercader *et al.*, 1999; Jaw *et al.*, 2000; Waskiewicz *et al.*, 2001; Longobardi and Blasi, 2003; Stevens and Mann, 2007). Our data, and those of others (Bhatt *et al.*, 2004; Cole *et al.*, 2006), show that both ATH1 and PNY can interact with STM in plant cells and that the resulting heterodimers are nuclear localized. In the system used, STM protein was completely cytoplasmic in the absence of interacting BELL proteins. This is in accordance with observations by Cole *et al.* (2006) that STM, like orthologous MEIS proteins, is devoid of an

efficient NLS, suggesting that STM resides in the cytoplasm by default. In addition, solitary expressed ATH1 and PNY proteins both end up in the cytosol and nucleus, as was shown here and by Cole *et al.* (2006). Moreover, our results indicate that an active nuclear export mechanism operates on ATH1, thereby explaining the subcellular distribution of the monomer. Taken together, these results imply that STM, on the one hand, and ATH1 or PNY on the other hand, are interdependent for stable nuclear localization, and, as a consequence, for functionality. Our data further show that ATH1 directs the dimer with STM to the nucleus, as do its animal PBC counterparts. Cole *et al.* (2006) showed a similar function for PNY and mapped the PNY nuclear localization sequence N-terminal of the conserved BELL domain.

Despite lack of unambiguous evidence for evolutionary conservation of PBC and BELL domains (Becker *et al.*, 2002), it has been suggested that the animal PBCA/B domain finds its counterpart in the BELL domain of plant BELL proteins (Bellaoui *et al.*, 2001; Cole *et al.*, 2006). This is based on the mechanistic conservation of MEIS/KNOX with PBC/BELL protein-protein interactions and the interdependence of both these animal and plant TALE HD proteins for heterodimerization for nuclear translocation. The data presented here show that this mechanistic conservation goes even further. In animals, the nuclear exclusion mechanism involves the recognition of leucine-rich NES sequences by the CRM1/exportin-1 nuclear export receptor which translocates monomeric PBC proteins to the cytosol. This mechanism is active also in plant cells to control the cellular localization of BELL proteins. Thus, the differential nucleocytoplasmic distribution of PBC/BELL proteins is a functionally conserved mechanism in both animals and plants.

The situation in animals suggests that it is unlikely for ATH1 and PNY or additional BELL proteins simply to function as chaperones that guide STM to the nucleus (e.g. Stevens and Mann, 2007). Most likely, BELL-KNOX dimers operate as functional units, where different combinations might have unique as well as overlapping targets. This is corroborated by overexpression experiments in transgenic Arabidopsis plants, which imply that different STM-BELL heterodimers contribute different functions (Cole *et al.*, 2006).

Moreover, we have observed *pny* mutant inflorescence phenotypes in plants overexpressing ATH1, which might imply that titration of STM by ectopic ATH1 results in reduced PNY-STM dimer formation and loss of PNY-STM specific functions (B.R. and M.P., unpublished data).

Although ATH1 and PNY were found to act redundantly during embryonic SAM formation, the contribution of PNY to this specific aspect seems more significant than that of ATH1, as inferred from the stronger effect of *pny* than *ath1* on weak *stm* phenotypes (This chapter; Byrne *et al.*, 2003; Bhatt *et al.*, 2004; Kanrar *et al.*, 2006). Also the observed differences in *ath1* and *pny* single mutant phenotypes point into the direction of overlapping and specific functions of the corresponding

BELL proteins. *pny* mutants display an increased leaf initiation rate, suggesting that PNY functions in delaying the specification of lateral organs in the peripheral zone of the SAM (Byrne *et al.*, 2003). This corresponds to part of the STM function in SAM maintenance and is not evident in *ath1* mutants, suggesting that this particular part in the process of meristem maintenance is mainly a function of cooperative action of STM and PNY. In *ath1* mutants we observed partial fusions of leaf petioles to each other, reminiscent of the fused cotyledonary petioles in strong *stm* loss-of-function mutants. The fused cotyledon phenotype is due to the loss of STM control of, and consequent aberrant expression of the *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2* genes in the embryonic cotyledon boundary region (Aida *et al.*, 1999; Takada *et al.*, 2001). Hibara *et al.* (2006) recently showed that *CUC* genes also function in boundary specification of various post-embryonic shoot organs, including leaves. Thus, the mechanism that prevents the margins of the leaf petioles from fusing and that suppresses cotyledonary petiole fusion earlier in development may be similar, and likely involves the combined action of ATH1 and STM. Such a fusion phenotype has not been observed in *stm* single mutants, probably because most *stm* mutants are too severely affected to complete vegetative development to a stage where this *ath1* associated phenotype is displayed. Alternatively the fusions might be most obvious in *ath1-1* mutants due to specific, but unknown conditions in the *ath1* background.

STM seems responsible for differences between meristematic and primordial cell identity (Byrne *et al.*, 2000, 2002; Cole *et al.* (2006). Differentially localized BELL partners in the SAM most likely account for the zone-specific functions of STM in meristem maintenance. In this light, it will be, both technically and scientifically, challenging to determine the exact localizations of specific BELL-KNOX dimers in the SAM and to identity their specific and overlapping targets. Such information is needed to understand how TALE HD proteins are employed in the different aspects of meristem function.

Experimental Procedures

Plant Materials and Growth Conditions

The wild types used in this study were the *Arabidopsis thaliana* Columbia-8 (Col-8; N60000) and Landsberg *erecta* (L*er*) strains. Mutant alleles and marker lines were described before: *ath1-1* (Proveniers *et al.*, submitted; Chapter 3 of this thesis), *pny-40126* (Smith *et al.*, 2003), *pnf-33879* and *pnf-96116* (Smith *et al.*, 2004), *stm-2* (L*er*) (Clark *et al.*, 1996), *stm-4* (L*er*) (Endrizzi *et al.*, 1996), *stm/bumbershoot1-1* (*bum1-1*) Col(*gl1*) (Jasinski *et al.*, 2005), *stm/bum1-3* (Col) (Kanrar *et al.*, 2006), STM_{pro}*:GUS* transgenic lines (L*er*) (McConell and Barton, 1998). Combinations of mutations originally present in different accessions were at least two times

backcrossed to either one or both of the original accessions. Plants were grown in long days (16 h of light/8 h of dark), unless stated differently, under cool white fluorescent lights (150 μmol·m^{-2}·s^{-1}) at 22°C with 70% RH.

Plasmid Construction and Yeast Two-Hybrid Analysis

ATH1, PNY, PNF, STM, BP, KNAT2-7 (generated within the European Community REGIA project) and AtCRM1 open reading frames, as well as the mutated version of ATH1, the ATH1 and PNY BELL domains and the mutated ATH1 BELL domain were flanked by Gateway linkers and inserted in the pDONR201 entry vector (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. Destination clones were created in the pDEST™22 transcription activation domain (AD) and pDEST™32 DNA DNA binding domain (BD) vectors following manufacturer's instructions. Resulting plasmids were transformed into the yeast strain PJ694A (James *et al.* 1996) using a lithium acetate/polyethylene glycol protocol (Schiestl *et al.*, 1993). The pDEST™32 DB-fusion constructs were tested for nonspecific activation of the reporters on selective drop-out medium (SD) (Sigma-Aldrich) lacking adenine and/or leucine, and histidine (–ade/–leu, –his) supplemented with increasing concentrations of 3-amino-1',2'-triazol (3-AT) (0-100mM final concentration). Positive interactions were identified by testing for growth on SD –ade/ –leu –his –tryptophan, supplemented with the appropriate amount of 3-AT (final concentrations ranging from 0-50 mM).

Confocal Microscopy of Mature Arabidopsis Embryos

Mature embryos were stained with aniline blue and treated as described in Bougourd *et al.* (2000) to allow high-resolution confocal imaging of individual cells within the embryo prior to germination.

Transient Expression in Onion Epidermal Cells

Fluorescent protein fusions were created using Gateway™ Technology (Invitrogen) in combination with the vectors pK7WGF2 and pK7FWG2 (Karimi *et al.*, 2002) for N- and C-terminal GFP-fusions, respectively, and with pARC233 (N-terminal YFP-halve) and pARC234 (C-terminal YFP-halve) for split-YFP fusions.

For transient expression in onion epidermal cells, gold particles (1.0 μm 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. The bombarded tissue was kept in Petri dishes on damp filter paper for 12–16 h in the dark. Onion epidermal cells were incubated in PBS on microscope glass slides. For Leptomycin B (LMB) (1mM in ethanol, LC Laboratories) experiments, PBS-buffer was supplemented with 100nM LMB and epidermal tissue was incubated in the dark for an additional 2 hours.

Using a Zeiss Confocal Laser Scanning Microscope, median Z-stack projections were made through nuclei of fluorescing cells. DAPi-staining of nuclei was used to verify Z-stack projections.

Acknowledgements

We thank Enric Belles-Boix and Jan Traas (INRA Versailles, France) for the gift of the STM, BP, KNAT 2/3/4/5/6 and HY5 two-hybrid clones, the Nottingham Arabidopsis Stock Center and the Arabidopsis Biological Resource Center for providing mutant seeds. We are grateful to The Netherlands Organisation for Scientific Research (STW-NWO) for funding this research.

Chapter 3

The Arabidopsis TALE Homeobox Gene *ATH1* Controls Floral Competency through Positive Regulation of *FLC*

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Chapter 3

The Arabidopsis TALE Homeobox Gene *ATH1* Controls Floral Competency through Positive Regulation of *FLC*

Summary

Floral induction is controlled by a plethora of genes acting in different pathways that either repress or promote floral transition at the shoot apical meristem (SAM). During vegetative development high levels of floral repressors maintain the Arabidopsis SAM incompetent to respond to promoting factors. Among these repressors, FLOWERING LOCUS C (FLC) is the most prominent. The processes underlying down-regulation of *FLC* in response to environmental and developmental signals have been elucidated in considerable detail. However, the basal induction of *FLC* and its up-regulation by FRIGIDA (FRI) are still poorly understood. Here we report the functional characterization of the *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*) gene. A function of ATH1 in floral repression is suggested by a gradual down-regulation of *ATH1* in the SAM prior to floral transition. The vernalization-sensitive late flowering of plants that constitutively express ATH1 provides further evidence for such a function of *ATH1*. Analysis of lines that differ in *FRI* and/or *FLC* allele strength show that this late flowering is caused by up-regulation of *FLC* as a result of synergism between ATH1 and FRI. Consistently, *ath1* mutants flower early in short days and display attenuated *FLC* levels, whereas other floral repressors of the FLC-clade are not affected. Moreover, *ath1* mutations partially suppress FLC-mediated late flowering of both a *FRI*-expressing line and that of *fca-1* and *fve-1* autonomous pathway mutants. Therefore, we conclude that ATH1 controls floral competency as a general regulator of *FLC* expression.

Introduction

The transition from vegetative to reproductive growth is a highly plastic developmental process that requires continuous monitoring of environmental cues and endogenous signals. Antagonistic action of promoting and repressing pathways prevents floral transition until the plant has reached a certain age or size and growth conditions are favourable for sexual reproduction and seed maturation. At a certain time, the balance of promoting and repressing factors is such that the by then competent vegetative shoot apical meristem (SAM) is 'evoked' in a florally determined state by the activation of a set of so-called floral pathway integrator (FPI) genes, including *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *FD*, (Reviewed in Bernier, 1988; Mouradov *et al.*, 2002; Boss *et al.*, 2004).

Floral induction is antagonized by members of the floral repression pathway, which is considered to render the SAM incompetent to floral inductive signals by repressing the FPI genes (Boss *et al.*, 2004). The most prominent floral repressor is the MADS box transcription factor FLOWERING LOCUS C (FLC) (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *FLC* is expressed in both the SAM and vasculature (Michaels and Amasino, 2000; Sheldon *et al.*, 2002) and high *FLC* levels delay flowering by directly repressing transcription of the floral pathway integrators *FT* and *SOC1* in leaves and SAM, respectively. In addition, FLC prevents up-regulation 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).

Given its major contribution to the reproductive switch, precise temporal expression of *FLC* is of utmost importance. The two major pathways that are responsible for repression of *FLC* are the vernalization and autonomous pathways. Vernalization, the response to winter temperatures, promotes flowering by lowering *FLC* levels (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Initial down-regulation of *FLC* is mediated by the PHD domain proteins VERNALIZATION INDEPENDENT3 (VIN3)(Sung and Amasino, 2004) and VIL1 (a.k.a VERNALIZATION 5 (VRN5))(Greb *et al.*, 2006; Sung *et al.*, 2006). After a prolonged cold exposure *FLC* levels are epigenetically maintained low by the combined action of the chromatin modifying proteins VERNALIZATION1 (VRN1), VRN2, and LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) rendering plants responsive to the perceived photoperiod in the season that is optimal to commit to flowering (Gendall *et al.*, 2001; Levy *et al.*, 2002; Bastow *et al.*, 2004; Mylne *et al.*, 2006; Sung *et al.*, 2006).

Chromatin-associated proteins also make up part of the second *FLC* repression pathway, the autonomous pathway. A second group of autonomous pathway components, including FCA, FY, FPA, and FLOWERING LATE WITH KH MOTIFS (FLK), are potential RNA binding or processing factors (Macknight *et*

al., 1997; Schomburg *et al.*, 2001; Simpson *et al.*, 2003; Lim *et al.*, 2004; Mockler *et al.*, 2004), and the seventh member, *LUMINIDEPENDENS* (*LD*) encodes a homeodomain protein with unknown function (Lee *et al.*, 1994). A mutation in any of the autonomous pathway components results in increased expression of *FLC* and late flowering under all photoperiods. Since loss-of-function *flc* mutations completely suppress the effects of the autonomous pathway mutants, the autonomous pathway components most likely affect flowering solely by the down-regulation of *FLC* (Michaels and Amasino, 2001).

Next to mutations in the autonomous pathway, the presence of a dominant allele of the *FRIGIDA* (*FRI*) gene also causes increased *FLC* mRNA levels (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). In fact, the early flowering growth habit of many Arabidopsis accessions results from natural loss-of-function mutations in *FRI* (Johanson *et al.*, 2000; Le Corre *et al.*, 2002; Gazzani *et al.*, 2003; Werner *et al.*, 2005). Most likely, FRI acts solely to up-regulate *FLC* expression levels, since the late-flowering phenotype of *FRI*-carrying plants is completely eliminated in the absence of FLC (Michaels and Amasino, 2001). In the absence of functional FRI, the autonomous pathway keeps *FLC* levels low, conferring to these plants their early flowering behavior (Michaels and Amasino, 2001). FRI activity is epistatic to the *FLC*-repressing activity of the autonomous pathway. However, as in autonomous-pathway mutants, FRI-mediated delayed flowering is eliminated by vernalization (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *FRI* encodes a plant-specific, coiled coil-domain protein (Johanson *et al.*, 2000), but its molecular mode of action in the up-regulation of *FLC* is still unclear.

In screens for mutants that suppress FRI-mediated late flowering three more proteins have been identified that act in conjunction with FRI to specifically promote *FLC* expression. Two of them are FRI homologues, FRIGIDA-LIKE 1 and 2 (FRL1 and 2) (Michaels *et al.*, 2004; Schläppi, 2006). The third one, FRIGIDA ESSENTIAL 1 (FES1), is a protein with a CCCH zinc finger (Schmitz *et al.*, 2005). All three are necessary for the promotion of *FLC* expression in a FRI dependent manner. However, at least in case of FRL1 and FES1, elevated *FLC* expression in autonomous pathway mutants does not depend on these factors, indicating that they might act specifically with FRI to promote *FLC* expression. How FRI, FRL1, FRL2 and FES1 increase *FLC* expression is not known, but epistatic analyses indicate that they act cooperatively to up-regulate *FLC* rather than function in a linear pathway (Michaels *et al.*, 2004; Schmitz *et al.*, 2005).

Moreover, expression of *FLC* and its five relatives is controlled by the Paf1 complex, and a likely SWIR1/SRCAP-complex containing PIE1, ARP6/SUF3/ESD1 and SEF (Soppe *et al.*, 1999; Zhang and van Nocker, 2002; Noh and Amasino, 2003; He *et al.*, 2004; Oh *et al.*, 2004; Choi *et al.*, 2005; Deal *et al.*, 2005; He and Amasino, 2005; Kim *et al.*, 2005; Zhao *et al.*, 2005; March-Díaz *et al.*, 2006; Martin-

Trillo *et al.*, 2006). Both complexes are involved in chromatin remodeling and promote transcription of, likely among others, members of the *FLC* clade.

Here, we report the characterization of the BEL1-like (BELL) family homeobox gene *ARABIDOPSIS THALIANA HOMEOBOX 1* (*ATH1*). ATH1 was originally isolated in a screen for light-regulated transcription factors (Quaedvlieg *et al.*, 1995). In perennial ryegrass, ectopic expression of Arabidopsis *ATH1* cDNA results in significant later heading, but also in an increased leaf biomass due to both an extended vegetative growth phase and increased tillering (van der Valk *et al.*, 2004). The putative role of ATH1 in the flowering process was investigated using expression analysis and a reversed genetics approach. Results show that ATH1 functions as a floral repressor by specifically affecting *FLC* expression levels.

Results

Developmental Regulation of ATH1 Promoter Activity

In a previous study it was found that *ATH1* mRNA is most abundant in light-grown seedlings (Quaedvlieg *et al.*, 1995). Here, we studied the spatio-temporal localization of *ATH1* expression during Arabidopsis development in more detail using a Pro_{ATH1}*:GUS* reporter gene construct. To test whether *GUS* gene expression driven by a 2.6 kb genomic *ATH1* promoter-containing fragment corresponds with endogenous *ATH1* expression, *GUS* mRNA abundance was measured during seedling dark-adaptation and consequent de-etiolation. The same responses as seen for endogenous *ATH1* expression were observed (Figure 3.1f, compare to Figure 7 in Quaedvlieg *et al.*, 1995), demonstrating the presence of the main regulatory elements in the Pro_{ATH1}*:GUS* construct. Consistent with previous data on *ATH1* expression (Quaedvlieg *et al.*, 1995), *ATH1* promoter activity was first detectable in two-day-old light-grown seedlings (data not shown). High levels of GUS activity were present in the SAM, emerging leaf primordia and the vasculature of the cotyledons (Figure 3.1a,c). No GUS expression was found in roots. When true leaves developed, strong *ATH1* promoter activity was seen in these leaves (Figure 3.1c,d). In young leaves GUS expression was evenly distributed over the tissue, whereas it became confined to the vascular tissue as the leaves matured (Figure 3.1d).

We previously observed that in seedlings *ATH1* mRNA peaks two to three days after germination and then gradually declines when they grow older (Quaedvlieg *et al.*, 1995). Such a decline was also found for GUS activity in developing Pro_{ATH1}*:GUS* plants. The same GUS staining pattern as seen in four-day-old light-grown seedlings was found up to seven days after germination (Figure 3.1a). However, in the SAM of older plants no visible GUS activity was detectable anymore (Figure 3.1b). The expanded and dome shaped appearance indicates that the SAM has developed from

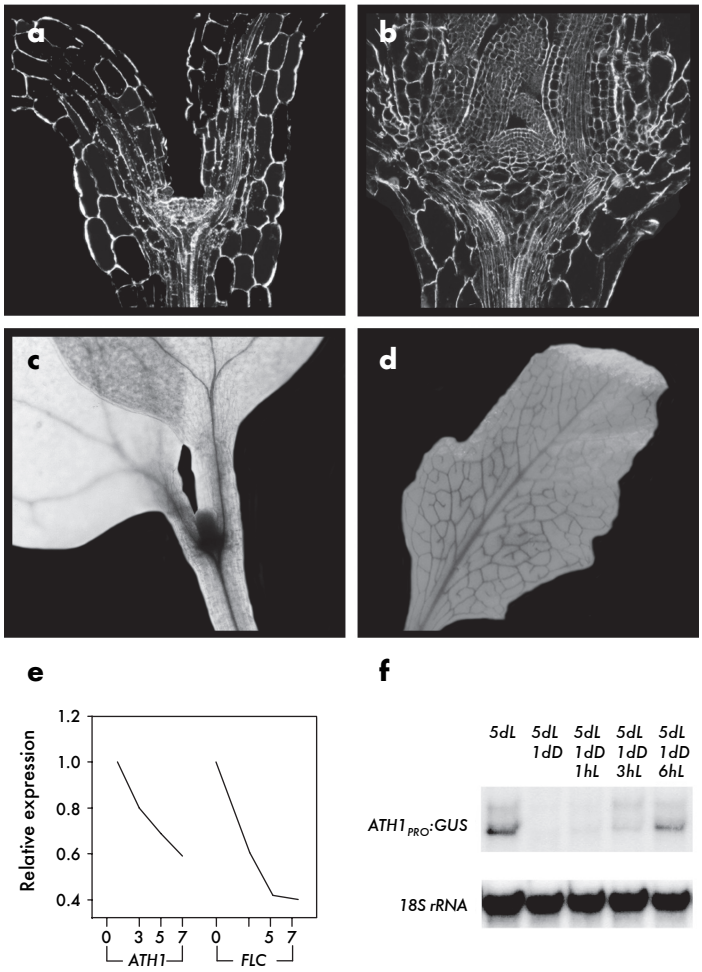

Figure 3.1 Spatiotemporal Localization of *ATH1* Expression During Arabidopsis Development.
(a) Whole mount histochemical localization of *ATH1*$_{pro}$*:GUS* activity in a median longitudinal section of a 4-day-old seedling SAM using dark-field optics (GUS activity is visible as a bright red precipitate).
(b) As in **(a)** for an 8-day-old seedling SAM lacking detectable *ATH1*$_{pro}$*:GUS* activity in the SAM.
(c) GUS stained 6-day-old seedling showing *ATH1*$_{pro}$*:GUS* activity throughout the meristem and vasculature of the cotyledons (GUS activity visible as a blue precipitate).
(d) Young rosette leaf showing *ATH1*$_{pro}$*:GUS* activity in the vasculature.
(e) Relative expression levels of *ATH1* and *FLC* in micro-dissected meristems of Arabidopsis wild-type plants (Col-8). Plants were grown for 30 non-inductive short days (SD) and transferred to floral inducing long day (LD) conditions. Meristems were isolated at 0, 3, 5 and 7 days upon shifting to LD conditions (Schmid et al., 2005). Data were obtained from Genevestigator (Zimmermann et al., 2004).
(f) RNAse protection analysis of *GUS* transcript levels in *ATH1*$_{pro}$*:GUS* plants. Seedlings were grown for 5 days in continuous light (5dL), transferred to the dark for 1 day (5dL1dD) and returned to the light for 1, 3 or 6 hours, respectively (5dL1dD1hL, etc.). Samples contained 10µg of total Arabidopsis RNA and 10µg of tRNA. The same samples were used in an RNA gel blot analysis to probe for 18S rRNA.

a juvenile into an adult form (Figure 3.1b). This developmental stage marks the final phase of vegetative development and ends with the transition to an inflorescence

meristem (Medford *et al.*, 1992, 1994). Intriguingly, the moment of *ATH1* down-regulation in the SAM coincides with the commitment time of inflorescence development (Bradley *et al.*, 1997; Mockler *et al.*, 1999). The commitment time is referred to as the developmental stage after which plants are committed to floral initiation and the quality and/or quantity of light has little effect on the flowering time. Analysis of expression data collected from Genevestigator (Zimmermann *et al.*, 2004) showed that *ATH1* is repressed in the SAM upon floral induction in both Col and L*er* accessions (Figure 3.1e, data not shown). These micro-array experiments were done with 30-day-old SD grown plants in which flowering was induced by a photoperiod shift to LD (Schmid *et al.*, 2005). This indicates that *ATH1* down regulation in the SAM is not just age dependent but coincides with floral induction. Taken together, these results indicate that ATH1 might function in the floral transition process.

Altered Expression of ATH1 Affects Flowering Time in a Accession-Dependent Manner

The proposed function of ATH1 in floral transition was tested by constitutively expressing *ATH1* cDNA in transgenic C24 Arabidopsis plants in either sense (Pro_{35S}:*ATH1*) or antisense (Pro_{35S}:*asATH1*) orientation. Interestingly, plants with reduced *ATH1* mRNA levels (Pro_{35S}:*asATH1* #7) showed a pronounced acceleration of flowering under both LD and SD conditions (Figure 3.2a,b).

Ectopic overexpression of *ATH1* had the opposite effect (Figure 3.2a,b). Lateness in these plants coincided with the formation of aerial rosettes (Figure 3.2e). These rosettes were established after floral transition at the first three to four nodes of the primary stem instead of cauline leaves bearing secondary inflorescences. The aerial rosettes formed up to 7 leaves and were similar in structure to the original basal rosette. Later in development the aerial rosettes gave rise to inflorescences with cauline leaves and flowers. Additional rosettes also developed in axils of basal rosette leaves. However, as a result of their extreme lateness, Pro_{35S}:*ATH1* plants formed a very compact, dome-shaped rosette of leaves, which made it very difficult to recognize these additional rosettes (Figure 3.2a).

Increased *ATH1* levels severely delay flowering in the C24 accession. Most remarkably, ectopically expressed *ATH1* had hardly any affect on flowering time in the Col-0 and L*er* accessions. LD grown Col-0 Pro_{35S}:*ATH1* plants flowered 22±2 days after germination, compared with 21±4 days for control plants. Similar results were obtained when using the L*er* accession to express the *HA*-tagged *ATH1* gene (Figure 3.3a). This suggests that ATH1 alone is not sufficient to delay the floral transition and depends on one or more loci that naturally vary among these accessions.

Analysis of different Arabidopsis accessions has shown that natural variation in flowering behavior is to a large extend conferred by allelic variation at two

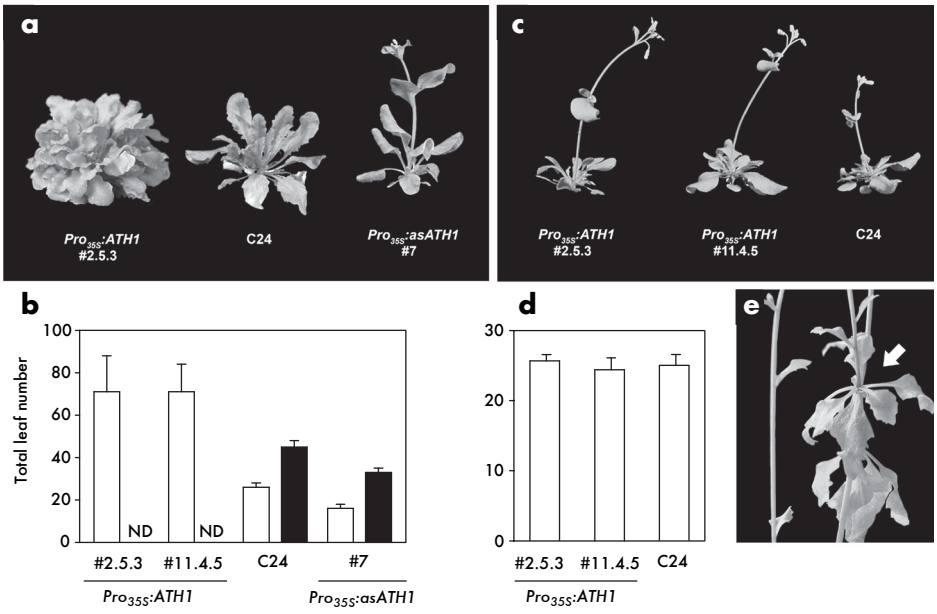

Figure 3.2 Deregulated *ATH1* Expression Results in a Vernalization Sensitive Flowering Phenotype in C24

(a) Wild type C24, *Pro$_{35S}$:ATH1*and *Pro$_{35S}$:asATH1* plants grown in LD. Pictures were taken after 5 (middle and right) or 8 weeks (left) of growth.

(b) C24 plants expressing *ATH1* from the 35S CaMV promoter (*Pro$_{35S}$:ATH1*) show an extreme delay of flowering in both LD (white bars) and SD (black bars). Expression of an anti-sense *ATH1* construct (*Pro$_{35S}$:asATH1*) driven by the same promoter leads to early flowering under both light regimes.

(c) Vernalization acts downstream of *ATH1* since *ATH1*-induced late flowering can be fully overcome by vernalization. Plants were grown in LD for 5 weeks.

(d) as in **(c)** but quantified.

(e) *ATH1* overexpression in C24 results in the formation of aerial rosettes (arrow). Primary inflorescence of a wild-type C24 plant (left) and *Pro$_{35S}$:ATH1* plant (right).

Error bars in **(b)** and **(d)** indicate SD based on means obtained for at least 15 plants for each line.

loci, *FRI* and *FLC* (Michaels and Amasino, 2000; Schläppi, 2001; Gazzani *et al.*, 2003). Interestingly, for both *FLC* and *FRI* a significant difference in allele strength between C24, Col-0, and L*er* accessions has been reported before (Koornneef *et al.*, 1994; Lee *et al.*, 1994; Johansson *et al.*, 2000; Le Corre *et al.*, 2002; Shindo *et al.*, 2005). In C24 a functional *FRI* allele that can cause late flowering is combined with an *FLC* allele that is considered to be genetically weak due to its attenuated response in the presence of an active *FRI* allele (Sanda and Amasino, 1995). L*er* harbors a *fri* null allele, and a weak *FLC* allele as a result of the presence of a non-autonomous Mutator-like transposon in its first intron (Johansson *et al.*, 2000; Gazzani *et al.*, 2003; Michaels *et al.*, 2003). The Col-0 *FLC* allele, on the other hand, is a strong allele (Michaels and Amasino, 1999). However, due to a recessive *FRI* allele Col-0, like L*er*, lacks considerable *FLC* activity (Johanson *et al.*, 2000).

The formation of an enlarged basal rosette and aerial rosettes similar to the ones observed in *Pro$_{35S}$:ATH1*C24 plants has been reported to require specific

modulation of *FLC* expression by a synergistic activity of either a dominant *FRI* allele or mutations that disrupt the FLC-repression pathway, and an active *AERIAL ROSETTE 1* (*ART1*) locus (Poduska *et al.*, 2003). The *ATH1* spatial expression pattern largely overlaps that of *FLC* (Figure 3.1a-d; Michaels and Amasino, 2000; Sheldon *et al.*, 2002). Moreover, very similar to *ATH1, FLC* is repressed in the SAM upon transfer from short to long days (Figure 3.1e). Therefore, it was tested whether ATH1 might function as a positive regulator of FLC. As expected, C24 Pro_{35S}*:ATH1* plants have increased *FLC* mRNA levels (Figure 3.4d). Furthermore, ATH1-induced late flowering in this accession can be eliminated by vernalization, a treatment which is known to stably repress *FLC* expression, irrespective of the presence of FRI or an autonomous pathway mutation (Figure 3.2c,d) (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

Taken together, the late-flowering effect of ATH1 in a C24 background is likely due to an ATH1-dependent increase of *FLC* expression. L*er*, like C24, contains a weak, but functional allele of *FLC*, whereas Col-0 contains a strong *FLC* allele. However, both L*er* and Col-0 nevertheless do not respond to *ATH1* over-expression. Therefore, ATH1 by itself seems not be sufficient to induce *FLC* expression to high levels and might require the further presence of an active *FRI* allele.

ATH1 Ectopic Expression and FRI Synergistically Affect FLC Expression Levels

To address this question, *ATH1* was ectopically expressed in backgrounds that differ in *FRI* and/or *FLC* allele strengths and flowering times in LD photoperiods were determined. For this purpose Pro_{35S}*:HA-ATH1* lines were generated in L*er*: FLC^{Col} (strong Col-0 *FLC* allele, loss-of-function L*er fri* allele) and in L*er*:FRI^{SF2} (weak L*er FLC* allele, active SF-2 *FRI* allele), in addition to L*er* Pro_{35S}*:HA-ATH1* lines that combine a weak *FLC* allele with a loss-of-function *fri* allele, and that are flowering like L*er* control plants (Figure 3.3a). Introduction of Pro_{35S}*:HA-ATH1* in a background carrying the strong Col-0 *FLC* allele did not result in any extra delay of flowering when compared to the parental line. In contrast, overexpression of *ATH1* in combination with the active SF-2 *FRI* allele dramatically affected floral transition (Figure 3.3a). Some plants never flowered within the duration of the experiment, and all plants that did flower formed >60 leaves before the transition. Importantly, the delay could be suppressed by a 4 weeks vernalization treatment as in C24 Pro_{35S}*:ATH1* plants (Figure 3.3a).

Ectopic expression of *ATH1* affected *FLC* expression levels, as observed before. In L*er* Pro_{35S}*:HA-ATH1* plants *FLC* expression was about 20-fold increased compared to control plants. However, this increase was clearly not sufficient to cause a significant delay in flowering. Similarly, presence of an active *FRI* allele in a L*er* background caused a 40-fold rise in *FLC* mRNA without dramatically effecting

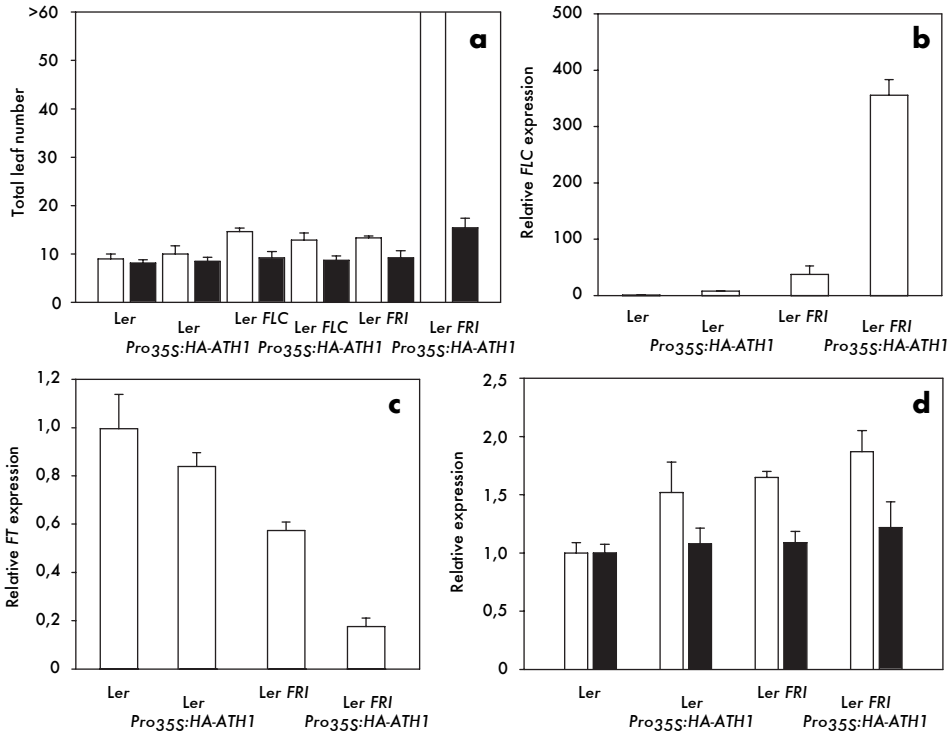

Figure 3.3 Ectopic *ATH1* Expression and *FRI* Synergistically Delay Flowering by Increasing *FLC* Levels
(a) Constitutive expression of *ATH1* in L*er* does not result in late flowering unless an active *FRI* allele is present (white bars). Delayed flowering due to ectopic expression of *ATH1* can be suppressed by vernalization (black bars). Error bars indicate SD based on means obtained for at least 15 plants for each line. A number of non-vernalized Pro_{35S}*:ATH1* L*er*:*FRI*^{SF2} plants never flowered within the duration of the experiment, and all plants that did flower formed >60 leaves before the transition.
(b) Real-time RT-PCR results for steady-state transcript levels of *FLC* in 10-day-old L*er* wild-type plants (whole seedlings) and plants containing Pro_{35S}*:ATH1*, introgressed *FRI*^{SF2}, or both. For each cDNA sample, the expression level of a given gene was normalized to the *ACTIN-8* (*ACT8*) control. Each reaction was made in duplicate, and each experiment was repeated three times. All plants were harvested at the same time of the day. Error bars indicate SD.
(c) as in **(b)** for *FT*.
(d) as in **(b)** for *FLM* (white bars) and *MAF2* (black bars).
(a) to **(d)** L*erFLC* indicates L*er*:*FLC*^{Col}, L*erFRI* indicates L*er*:*FRI*^{SF2}; all plants were grown in LD.

flowering time (Figure 3.3b), suggesting that there might be some kind of threshold for FLC to display a substantial effect. In case of Pro_{35S}*:HA-ATH1* L*er*:*FRI*^{SF2} plants this putative threshold is exceeded as a result of a synergistic action of ATH1 and FRI, causing a 350-fold increase in *FLC* levels accompanied by a severe reduction in mRNA levels of its direct target, the floral promoter *FT* (Figure 3.3c). Compared to *FLC*, expression of *FLM* and *MAF2*, the two other members of the FLC clade of MADS-box genes that have been reported to function in floral transition, was hardly, if at all, affected by ATH1 in any of the genetic backgrounds (Figure 3.3d).

Thus, ATH1 alone is not sufficient to delay the floral transition despite its capacity to induce *FLC* expression to some extent. *ATH1*-induced late flowering was only observed in a C24 background and in the L*er*:*FRI*^{SF2} introgression line, both of which contain an active *FRI* and a *FLC* allele with an attenuated response to this *FRI* allele. This strongly suggests that ATH1 functions as a floral repressor by activation of *FLC* in a FRI-mediated manner.

Basal Levels of *FLC* Are Attenuated by *ath1* Mutations

Most results obtained so far follow from experiments in which *ATH1* was ectopically expressed. Thus the possibility remains that the biological function of ATH1 is somehow masked. Therefore two mutants, *ath1-1* and *ath1-3* were obtained from publicly available collections of Arabidopsis T-DNA insertion lines (Alonso *et al.*, 2003; Li *et al.*, 2003). ATH1 contains the in BELL proteins conserved SKY and BELL domains and the three–amino acid loop extension (TALE) homeodomain (Bürglin, 1997; Bellaoui *et al.*, 2001) in the C-terminal half of the protein. The T-DNA insertion in *ath1-1* is located in the third exon, just before the part of the gene that encodes these three conserved domains (Figure 3.4a). The T-DNA insertion in *ath1-3* is located in exon 4, immediately after the start of the homeobox. Reverse transcription PCR using ATH1-specific primers showed that in neither of the mutants full-length *ATH1* mRNA is expressed (Figure 3.4a). Therefore, most likely both T-DNA insertions create null mutants. However, preliminary phenotypic analyses indicate that the *ath1-1* allele is somewhat stronger than *ath1-3* (M. Proveniers and B. Rutjens, unpublished data). Therefore *ath1-1* was used in most of our experiments.

Under LD, the flowering time of *ath1-1* is indistinguishable from that of wild-type Col-8 (Figure 3.4b). In contrast, in SD, *ath1-1* plants flower with approximately 20% less leaves than wild type controls (Figure 3.4b) similar to what has been observed before for *flc* loss-of-function mutants in a Col background (Michaels and Amasino, 2001). When compared to *flc*-7 plants under these conditions, *ath1-1* mutants flower somewhat earlier. However, introduction of the *flc*-7 mutation in the *ath1-1* background had no additive effect on flowering time (Figure 3.4b). Therefore, lack of FLC is the major contributor to the *ath1-1* flowering phenotype.

Mutations that affect expression of both *FLC* and other members of the FLC-clade of MADS-box genes (*FLM*, *MAF2-5*) flower significantly earlier than *flc* single mutants in SD (He *et al.*, 2004; Oh *et al.*, 2004; Deal *et al.*, 2005; Martin-Trillo *et al.*, 2006). In accordance with the observed flowering time phenotype, *ath1-1* mutants showed a 95% reduction in *FLC* expression and a resulting increase of *FT*, whereas *ath1* loss-of-function, like *ATH1* ectopic expression, had no significant effect on the expression of *FRI* (Figure 3.4c,d). Importantly, expression of *FLM* and *MAF2*, the two other reported floral repressors in the FLC-clade of MADS-box genes, was

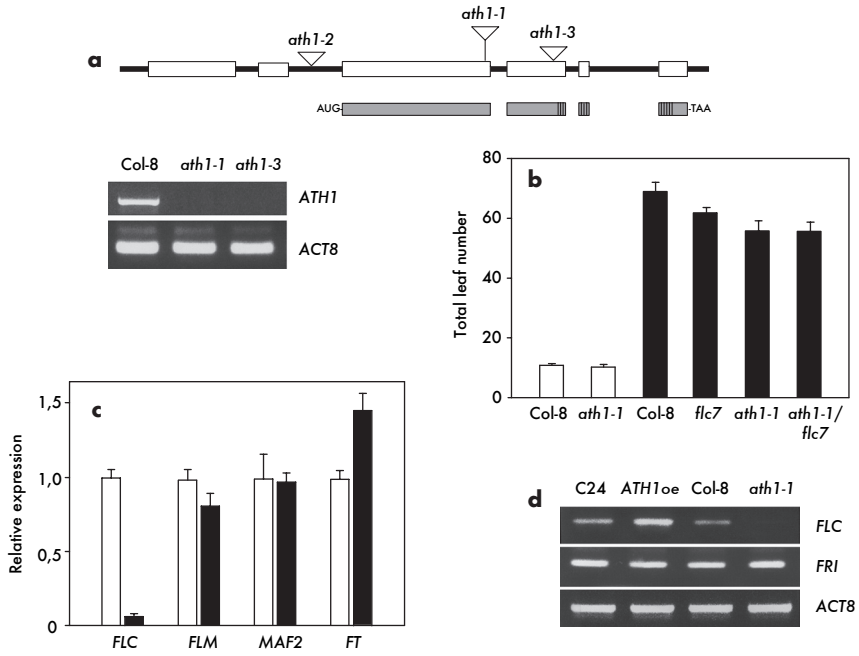

Figure 3.4 Attenuated *ATH1* Expression Results in Early Flowering by Reducing *FLC* Levels
(a) Upper part: *ATH1* gene structure showing the T-DNA insertions sites of the *ath1-1* and *ath1-3* mutants. White boxes indicate exons and lines mark introns and intergenic regions. Translated exons are depicted as gray boxes with the homeodomain-coding region marked as shaded. Lower part: RT-PCR showing the absence of detectable full-length *ATH1* messenger in the two mutants.
(b) Flowering time is not affected by an *ath1-1* mutation in LD conditions (white bars), in SD (black bars) *ath1-1* mutants flower significantly earlier than WT and slightly earlier than *flc-7* mutants. In *ath1-1flc-7* double mutants no additive effect of the *flc-7* mutation on *ath1-1* flowering time was observed. Error bars indicate SD based on means obtained for at least 15 plants for each line.
(c) Real-time RT-PCR results of flowering time genes in 10-day-old WT (white bars) and *ath1-1* (black bars) LD grown plants. For each cDNA sample, the expression level of a given gene was normalized to the *ACT8* control. Each reaction was made in duplicate, and each experiment was repeated three times. Error bars indicate SD.
(d) RT-PCR results showing that *ATH1* affects *FLC* expression without modifying *FRI* levels. *ATH1*oe indicates C24 Pro_{35S}*:ATH1*, *ACT8* was used as internal control.

not, or, in the case of FLM, only slightly affected by the *ath1-1* mutation (Figure 3.4c). Similar results were obtained when measuring the expression of these genes in plants grown in SD (data not shown). Under this condition a small reduction in *FLM* mRNA levels in *ath1-1* plants was observed and this might explain why these plants flower slightly earlier than *flc-7* mutants in SD.

In conclusion, our data suggest that ATH1 does not affect the entire FLC clade and functions as a repressor of floral transition almost entirely by affecting *FLC* expression levels.

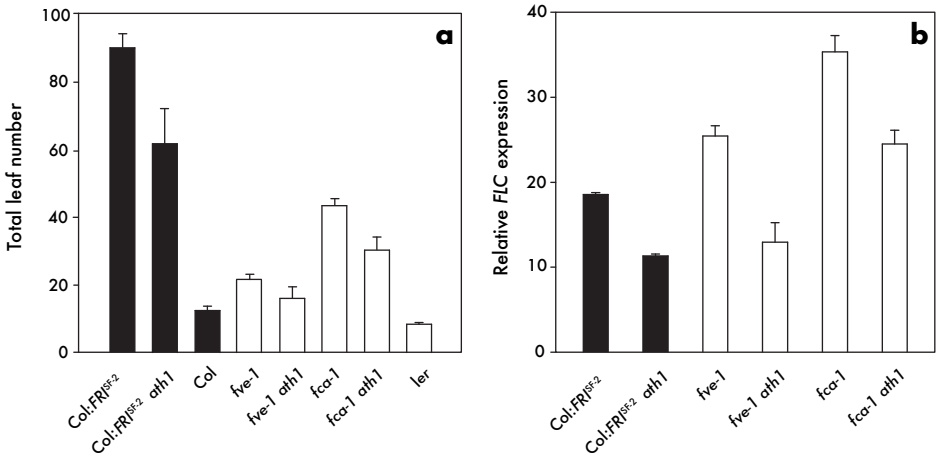

Figure 3.5 *ath1-1* Partially Suppresses both *FRI*-Dependent Late Flowering and that of *fve-1* and *fca-1* Autonomous Pathway Mutants

(a) Plants were grown in LD. Black and white bars represent Col and L*er* backgrounds, respectively. Error bars indicate SD.

(b) Real-time RT-PCR results for steady-state transcript levels of *FLC* in 10-day-old plants represented in **(a)**. Note that corresponding wild-type levels were set at a value of 1.

Late Flowering of Both FRI-Containing Plants and Autonomous Pathway Mutants Depends on ATH1

The dependency on ATH1 for basal *FLC* expression, in combination with the synergism between *FRI* and *ATH1* overexpression in delaying floral transition prompted us to test whether ATH1 is necessary for proper FRI function. Although the *ath1-1* mutation caused a significant reduction in flowering time in a Col:*FRI*^{SF2} introgression line, these plants were still considerably later than Col-8 plants that carry a non-functional *fri* allele (Figure 3.5a). Thus FRI is able to delay flowering independently of ATH1 activity. This may indicate that FRI controls flowering through both ATH1-dependent and ATH1-independent pathways. Alternatively, *ath1* mutants may not represent a total loss in activity due to functional redundancy with other related *BELL* genes.

FLC levels show a moderate decline in Col:*FRI*^{SF2} *ath1-1* plants (Figure 3.5b), which, because of the quantitative effects of *FLC* on flowering, corresponds to the observed reduction in flowering time (Figure 3.5a).

As is the case for FRI-containing lines, late flowering of autonomous pathway mutants is the result of increased *FLC* levels. To test whether ATH1 is a general regulator of *FLC* levels and is also required for the late flowering of autonomous pathway mutants, flowering time and *FLC* levels of *ath1-1 fca-1* and *ath1-1 fve-1* mutants was determined (Figure 3.5a,b). In both mutant backgrounds, a significant though not complete reduction in flowering time was observed (Figure 3.5a) and a corrsponding reduction in *FLC* transcript levels was found (Figure 3.5b). Thus, as

in Col:*FRI*^{SF2} *ath1-1* plants, autonomous pathway function is also affected by *ath1-1* mutations. These data suggest that indeed ATH1 functions as a general regulator of *FLC*-mediated floral repression.

Discussion

In Arabidopsis the antagonistic action of promotion and repression pathways prevents the switch from vegetative to reproductive growth until the developmental state of the plant and favourable environmental conditions allow floral transition. The different floral promotive pathways induce flowering by activating the expression of the floral pathway integrator genes. This activity is antagonized by floral repressors, of which FLC is the most prominent. High levels of the floral repressors prevent the SAM to respond to the promotive signals. Therefore, as reviewed by Boss *et al.* (2004), the factors that regulate the expression of these floral repressors eventually enable flowering. These factors can be regarded as regulators of meristem competence, since they control the susceptibility of the SAM to the promotive floral factors.

Here, we propose that the *BELL* gene *ATH1* is a specific, positive regulator of the floral repressor *FLC* and functions as a competency modulator of the vegetative SAM. The observed high levels of *ATH1* expression in the vegetative SAM and subsequent down regulation prior to floral transition both in LD and after SD to LD transfer is consistent with such a function. Gradual down-regulation of *ATH1* expression is accompanied with a similar decrease in *FLC* expression (Figure 3.1e) and as a result the inhibition will be progressively released. In this way down regulation of *ATH1* allows the meristem to become more susceptible to floral stimuli. The suggestion that ATH1 functions in the regulation of meristem competency is further corroborated by the formation of aerial rosettes in C24 Pro_{35S}:*ATH1* plants. Aerial rosettes are also found in Sy-0, a late-flowering accession of Arabidopsis, and in transgenic Arabidopsis plants that ectopically express *TERMINAL FLOWER 1* (*TFL1*) (Grbic and Bleecker, 1996; Ratcliffe *et al.*, 1998). In both genotypes this characteristic phenotype is proposed to be the result of a prolonged vegetative phase of axillary meristems. In Arabidopsis these meristems usually undergo an obligatory, but short-lived, vegetative phase before converting to reproductive development (Grbic and Bleecker, 1996). In Sy-0 plants an increased insensitivity to floral stimuli prolongs this period and results in the production of vegetative structures at positions where normally cauline leaves develop (Grbic and Bleecker, 1996; Poduska *et al.*, 2003). This requires specific modulation of *FLC* expression by a cooperative action of a dominant *FRI* allele and an active *ART1* locus (Poduska *et al.*, 2003). Analogous, in C24 Pro_{35S}:*ATH1* plants a synergistic interaction of continuous CaMV 35S promoter-driven *ATH1* expression in the

axillary meristems and a dominant *FRI* most likely result in substantially elevated *FLC* expression levels in these meristems. As a result, such meristems are less sensitive to floral stimuli and undergo a prolonged vegetative phase.

Recently, two other, redundant Arabidopsis BELL genes, *PENNYWISE* (*PNY*; also known as *BELLRINGER*, *REPLUMLESS*, *LARSON* and *VAAMANA*) (Byrne *et al.*, 2003; Roeder *et al.*, 2003; Smith and Hake, 2003; Bao *et al.*, 2004; Bhatt *et al.*, 2004) and *POUND-FOOLISH* (*PNF*) (Smith *et al.*, 2004) were also proposed to function as competency regulators of the SAM in the process of floral evocation. Unlike ATH1, PNY and PNF act as positive regulators of meristem competence. Moreover, *pny pnf* plants appear to respond normally to floral inductive signals, since early floral markers such as *SOC1* and *FRUITFULL* are expressed after floral induction. Nevertheless, the *pny pnf* SAM fails to complete floral evocation, as internode and floral patterning events do not take place (Smith *et al.*, 2004), indicating that these two *BELL* genes function at a later stage in meristem competence than ATH1. Possibly, *BELL* genes play a broader role in meristem competence at various stages of development.

Our results strongly suggest that ATH1 functions as a floral repressor by promoting both basal and FRI-mediated FLC expression. Introduction of Pro_{35S}:*HA-ATH1* into a *fri* mutant background does not affect flowering, making it unlikely that ATH1 levels normally are limiting for basal *FLC* induction and that ATH1 functions as a sole downstream component in a FRI pathway. In addition, *ATH1* expression levels are unaffected by FRI (M. Proveniers and B. Rutjens, unpublished data). A model in which ATH1 promotes expression of *FRI* can also be excluded, since FRI expression remains unaffected by both ATH1 overexpression and an *ath1* mutation. Therefore, ATH1 and FRI do not appear to act in a simple linear pathway to promote *FLC* expression. Likely, ATH1 and FRI somehow act in parallel, for example as components of a common protein complex. However, yeast two-hybrid analysis did not provide evidence for an interaction between ATH1 and FRI (M. Proveniers and B. Rutjens, unpublished data). Our findings that both *ath1* loss-of-function and *ATH1* gain-of-function in a *fri* background can still affect *FLC* mRNA levels further support parallel activities of FRI and ATH1.

The fact that FRI and autonomous pathway mutations are able to significantly delay flowering independently of ATH1 activity also points into this direction, since it indicates that both pathways act to inhibit flowering through both ATH1-dependent and ATH1-independent pathways. Alternatively, *ath1* mutants may not represent a total loss in activity due to functional redundancy with related *BELL* genes. The Arabidopsis genome contains a total of thirteen *BELL* genes and functional redundancy between paralogous BELL genes has been reported for *PNY* and *PNF* in the process of floral evocation and inflorescence development, and for *SAWTOOTH 1* and *2* in leaf development (Smith *et al.* 2004; Kumar *et al.*, 2006).

Therefore, we are investigating whether ATH1 functions as a regulator of *FLC* expression together with other *BELL* genes.

Currently it is unclear how ATH1 increases *FLC* expression levels. As mentioned before, *ATH1* encodes a transcription factor of the BELL family of homeodomain proteins. BELL proteins comprise one of two three amino acid loop extension (TALE)-homeodomain protein subfamilies in plants, the other one being formed by members of the *Knotted1*-like homeobox (KNOX) family (Bürglin, 1997). The plant *BELL* and *KNOX* genes and their animal counterparts, the pre-B cell (PBC) and myeloid ecotropic integration site (MEIS) genes, respectively, were proposed to have evolved from a single ancestral TALE homeobox gene. This is supported by their evolutionary conserved structure and a conserved mechanism of nuclear import control of one class of TALE-homeodomain proteins through interaction with the other class of TALE-homeodomain proteins in both plants and animals (Bürglin, 1997, 1998; Bellaoui *et al.* 2001; Becker *et al.*, 2002; Cole *et al.*, 2006). Members of the PBC and MEIS families were initially identified as cofactors that enhance the function of a third class of homeodomain transcription factors, the Hox proteins, by improving their affinity and specificity for specific promoter elements. In plants, no Hox orthologs have been found, but it has become clear that PBC and MEIS proteins function more broadly to modulate the activity of other transcription factors, including non-Hox homeodomain proteins and non-homeodomain proteins (reviewed in Moens and Selleri, 2006). PBC proteins have been found in complexes that can function either as activator or as repressor of transcription depending on its differential interactions with co-regulators, such as the chromatin modifiers histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Saleh *et al.*, 2000). *FLC* expression is regulated by many pathways in which chromatin modification is a recurring theme (Figure 1.9) (for recent reviews see He and Amasino, 2005; Sung and Amasino, 2005, 2006). Activation of *FLC* transcription by either the presence of FRI or the loss of autonomous-pathway genes coincides with increased levels of H3-K4 tri-methylation around the *FLC* transcription start site, and requires a suite of proteins that are involved in chromatin remodeling, such as the Arabidopsis PAF1 complex and the EFS methyl transferase (Kim *et al.*, 2005; He *et al.*, 2004). It will be interesting to investigate whether ATH1 has a function similar to its animal counterparts in the activation of *FLC* expression.

An interesting link between MADS-box gene regulation and BELL proteins was recently provided for PNY. In addition to its function in meristem competence, PNY has also been identified as a transcriptional repressor of the floral homeotic gene *AG* in floral and inflorescence meristems. It was proposed that PNY might serve as an initiating factor for *AG* repression by recruiting transcriptional co-repressors to the *AG* chromatin (Bao *et al.*, 2004). Remarkably, striking parallels

exist between the regulation of *FLC* expression and that of *AG*. *FLC* and *AG* both encode a type II MADS-box transcription factor, and both genes appear to be regulated both epigenetically and by proteins involved in RNA metabolism, e.g. HUA2 (reviewed in Quesada *et al.*, 2005; Simpson, 2004). Moreover, in either case many of the *cis*-regulatory sequences that are important for transcriptional control are found in an unusually long intron that is characteristic for these two genes (3.5 kb intron 1 of *FLC*, 3.0 kb intron 2 of *AG*) (Sheldon *et al.*, 2002; Hong *et al.*, 2003). Knowing that PNY was found to act upon this long second intron of *AG in vivo* and to directly bind to it *in vitro* (Bao *et al.*, 2004), it will be interesting to determine whether ATH1 directly binds to *FLC* chromatin.

Experimental Procedures

Plant Materials and Growth Conditions

The wild types used in this study were the *Arabidopsis thaliana* C24, Columbia-0 (Col-0), Col-8 (N60000), and Landsberg *erecta* (L*er*) strains. FRI^{SF-2} and FLC^{Col} introgression lines used in this study have been described previously: lines homozygous for FLC^{Col} in Ler (Ler:FLC^{Col}) (Koornneef *et al.*, 1994), FRI^{SF2} in Ler (L*er*:FRI^{SF2}), and FRI^{SF2} in Col (Col:FRI^{SF2}) (Lee *et al.* 1994; Michaels and Amasino, 1999). The effect of ectopic expression of *ATH1* in L*er* introgression backgrounds was tested by introducing the Pro_{35S}*:HA-ATH1* transgene in the respective backgrounds.

The *ath1-1* (GK line 114A12) and *ath1-3* (SALK_113353) T-DNA insertion lines were obtained from GABI-KAT (MPI for Plant Breeding Research, Cologne, Germany) and NASC (University of Nottingham, UK), respectively. Both alleles were backcrossed two times into the Col-8 ecotype and lines were selected for single insertions. *ath1-1* was used in most of our phenotypic and molecular analyses. The *flc*-7 allele (SALK_092716) was obtained from NASC. In SD *flc*-7 plants flowered like the *flc-3* null mutant.

Plants mutant for both *ath1-1* and *fca-1* were made by crossing Col-8 *ath1-1* with L*er fca-1*. L*er* looking F2 plants were genotyped for both mutations and the L*er FLC* allele. Selected plants were subsequently backcrossed twice to the L*er fca-1* mutant background. Flowering time of *ath1-1/fca-1* plants was compared to *fca-1* single mutants that segregated from the second backcross population. Two independent lines were used.

Plants were grown in long days (16 h of light/8 h of dark) or short days (8 h of light/16 h of dark) under cool white fluorescent lights (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C with 70% RH. For vernalization, seeds were imbibed and allowed to germinate on full strength Murashige and Skoog medium at 4°C in low light short days for 4 weeks in the absence of sucrose. Flowering time was measured by counting the total leaf number.

Plasmid Construction

For the *ATH1* promoter-*GUS* fusion a 2.6 kb SpeI-NcoI genomic fragment of *ATH1* (Quaedvlieg *et al.*, 1995) was isolated, and after filling in the NcoI restriction site with Klenow polymerase, it was inserted into the unique SmaI/XbaI sites of pBI101.1 (Jefferson *et al.*, 1987). The fragment contains approximately 1.3 kb of promoter sequence, the entire, 700 nt long 5' untranslated region (5' UTR), and the 42 most N-terminal amino acids encoding part of *ATH1*. In this way a translational fusion between the ATH1 promoter and the GUS gene was created. Fifteen representative homozygous lines in the C24 background were chosen for further analysis. Histochemical assays for GUS activity at different stages of development showed similar results for all lines, with only the levels of GUS expression differing. Three lines (#1.3, #6.5, and #9.6) with intermediate levels of GUS-expression were chosen for detailed analysis. GUS histochemistry was performed as described in Dockx *et al.* (1995).

For antisense expression, the entire *ATH1* cDNA sequence (Quaedvlieg *et al.*, 1995) was fused in antisense orientation to the 35S Cauliflower Mosaic Virus (CaMV) promoter (Odell *et al.*, 1985). For ectopic overexpression of *ATH1*, a 1573 bp cDNA fragment encoding ATH1 was fused in sense orientation to the 35S CaMV promoter. The resulting chimeric genes (*Pro_{35S}:asATH1* and *Pro_{35S}:ATH1*, respectively) were introduced into Arabidopsis C24 plants. Nineteen independent *Pro_{35S}:asATH1* and ten independent *Pro_{35S}:ATH1* lines segregating for a single T-DNA insertion, as determined by kanamycin resistance, were selected and further analyzed in the T3 generation. *Pro_{35S}:asATH1* lines #3 and #7, which have undetectable *ATH1* levels, and *Pro_{35S}:ATH1* lines #2 and #11, which show significantly higher ATH1 expression than wild type control plants, were selected for further phenotypic analysis. Results are shown for lines #7, and #2 and #11, respectively.

For ectopic expression of a *HA*-tagged version of *ATH1* (*Pro_{35S}:HA-ATH1*) *ATH1* cDNA flanked by Gateway linkers (generated within European Community REGIA project) was inserted in the pAlligator2 vector (Benshimen *et al.*, 2004) by Gateway recombination following Invitrogen recommendations. Transgenic L*er* seeds were selected using a Leica MZFLIII stereomicroscope equipped with GFP3 (470 nm/525±50 nm) and YFP (510 nm/640±50 nm) filter sets.

Analysis of Gene Expression

RNeasy minikit columns (Qiagen) were used to isolate RNA for Q-PCR and RT-PCR analysis. Total RNA was DNaseI treated (RNase-free; Fermentas GMBH, Germany) 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. Q-PCR analyses were performed as described before (van Dijken *et al.*, 2004). Primers used for Q-PCR expression

analysis were as described in Czechowski *et al.* (2004) (*FLC*, *FLM*, *MAF2*) and El-Din El-Assal *et al.* (2003) (*FT*). Primers used for RT-PCR analysis were as described in Kumaran *et al.* (2002) (ACT8) and Michaels *et al.* (2004) (*FLC, FRI*). For *ATH1* RT-PCR the primers *ATH1* RT FWD (5'-TCCTCCACTTCATCCTTTGG-3') and *ATH1* RT REV (5'-CGTTGGGTTGAATGTGACTG-3') were used. For each mRNA under study the exponential range of amplification was determined. *GUS* RNase protection assays were done as in Rook *et al.* (1998).

Genotyping

Genomic DNA was isolated using the quick-prep method (Cheung *et al.*, 1993). Genotypes of *ath1-1* and *ath1-3* plants were determined using primers to the 35S CaMV promoter 35S-mini (5'-CTGCAGCAAGACCCTTCCTCTAT-3') and the left border LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3'), respectively, and *ATH1* gene specific primers ATH1TAG3.3 FWD (5'-GCTCGGAGATAAGTCTT TGTGCAGCTA-3'), and SALK_113353 LP (5'-TTTGTAGTTCAAGAGAAAAG CTTGA-3'), respectively. *ATH1* wild type alleles were identified using the primer combination SALK_113353 LP (5'-TTTGTAGTTCAAGAGAAAAG CTTGA-3') and SALK_113353 RP (5'-GGCGGGTTTCGGATCTACATT-3'). The *flc-7* insertion was detected using the LBb1 primer in combination with SALK_092716 RP (5'-CTTCTGTCCCTTTTTCATGGG-3'). Presence of a wild type *FLC* allele was determined using the primer combination SALK_ 092716 LP (5'-TCCCTTAACTCTAACCAGCCG-3') and SALK_092716 RP (5'-CTTCTGTCCCTTTTTCATGGG-3'). *FRI* allelic variation (Col, L*er* or SF-2) was scored as described by Johanson *et al.* (2000). *FLC* alleles (Col or L*er*) were genotyped using the primers designed by Gazzani *et al.* (2003) to detect the Mutator-like transposon in *FLC* in L*er*. The *fca-1* and *fve-1* mutations were genotyped using the respective primer combinations *fca-1* FWD (5'-AAAAACCTCTTCACAGTCCACA-3') and *fca-1* REV (5'-AGTTAAAA CAACACAATAGCAGCTGAA-3'), and *fve-1* FWD (5'-GGATGTTGAAACC CAACCAA-3') and *fve-1* REV (5'-GCTGAATGCCACATCTTCAA-3'). The *fca-1* mutation creates a Tru9I restriction polymorphism, whereas the *fve-1* mutation creates an HpyF10VI polymorphism.

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Chapter 4

BELL Genes Dictate Vegetative and Generative Meristem Phase Identity

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Chapter 4

BELL Genes Dictate Vegetative and Generative Meristem Phase Identity

Summary

Plant floral competence is facilitated mainly by downregulation of the floral repressor *FLOWERING LOCUS C* (*FLC*). We have previously shown that *ARABIDOPSIS THALIANA HOMEOBOX1* (*ATH1*), a *BELL* class TALE homeobox gene, acts as a suppressor of floral competence by positive regulation of *FLC*. From a screen to identify additional *BELL* genes involved in the control of flowering time we isolated *PENNYWISE* (*PNY*) as a second *BELL* gene involved in floral repression. Like *ath1* mutants, strongly reduced basal *FLC* transcript levels and an early flowering phenotype in non-inductive short days characterize *pny* loss-of-function. In the presence of dominant *FRI* alleles, *pny* partially suppresses late flowering, as observed for *ath1*. The *ath1 pny* double mutants fully suppress FRI-mediated late flowering, consistent with the notion that ATH1 and PNY can function redundantly in plant development and that both single mutants partially suppress FRI-mediated late flowering. In addition, we found that ATH1 interacts with the essential FRI homologue FRIGIDA-LIKE1 (FRL1), whereas PNY dimerizes with FRI. Intriguingly, no role for the *BELL* gene *POUND-FOOLISH* (*PNF*) was found in *FLC*-mediated flowering time control. *PNF* is an established redundant partner of *PNY* in inflorescence and flower development, suggesting that throughout plant development different *BELL* combinations might dictate developmental phase identity by controlling key components of phase identity.

Introduction

In Arabidopsis, post-embryonic development can be divided in two major phases reflecting the identity of the shoot apical meristem (SAM), the vegetative phase and the reproductive phase. All primordia formed during these two phases initially execute a common developmental program that divides the primordia into two domains with distinct fates (reviewed in Grbic, 2005). The adaxial domain is competent to give rise to an axillary meristem, while the abaxial domain correlates with organ primordium formation. During the vegetative phase of the SAM, a series of juvenile and later adult leaves are initiated, which together form the basal rosette. In addition, each rosette leaf bears a small quiescent axillary meristem in its axil, which can be activated during the floral transition and then develops into an axillary flower-bearing inflorescence. Following the floral transition, the SAM, now called inflorescence meristem (IM), rises from the rosette due to strong elongation of organ internodes, a process called bolting. Lower nodes on the developing inflorescence gain generative identity and give rise to expanded axillary meristems subtended by cauline leaves. These axillary meristems form secondary inflorescences that reiterate the developmental programs executed by the central inflorescence. Later in inflorescence development, floral primordia are characterized by out-balanced adaxial floral meristem formation at the expense of abaxial cauline leaf development. In Arabidopsis floral primordia, cauline leaves are normally reduced to a cryptic leaf region that is later on subsumed by the developing solitary flower (Long and Barton, 2000). Thus, primordial fate, which is determined by signals derived from both SAM and established lateral organs, progressively changes during plant development. For example, leaf identity is marked by primordial expression of the leaf differentiation factor *ASYMMETRIC LEAVES1* (*AS1*) (Byrne *et al.*, 2000). *AS1* is expressed throughout vegetative leaf primordia and in the domain of secondary inflorescence primordia that develops into the subtending cauline leaf. Moreover, *AS1* expression is observed even in the cryptic cauline region of floral primordia (Byrne *et al.*, 2000). On the other hand, flower identity is marked by high primordial expression of the floral meristem identity (FMI) genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) upon floral transition (recently reviewed in Parcy, 2005; Komeda, 2004).

Floral induction is a classic example of the interplay between the SAM and established leaves in determining primordial fate (recently reviewed in Bäurle and Dean, 2006). Initial signals that stimulate the floral switch originate from the leaves and travel to the SAM via phloem tissue (reviewed in Bernier, 1988). The floral pathway integrator (FPI) *FLOWERING LOCUS T* (*FT*) was recently identified as one of these signals. In long day photoperiods, FT transcript, originating from the leaf vasculature, moves via phloem tissue to the SAM. Here, translated FT

together with its partner, the FPI protein FD, triggers floral induction by activating expression of the FMI genes *LFY* and *AP1* (recently reviewed in Imaizumi and Kay, 2006). Interestingly, once floral meristem identity is established, Arabidopsis plants are committed to flowering and maintain the generative state throughout further development (reviewed in Boss *et al.*, 2004). As a consequence, proper timing of the vegetative to reproductive phase change is of uttermost importance to maximize reproductive success. The competence of plants to respond to floral inducing signals is a major determinant in flowering time control (reviewed in Bernier *et al.*, 1988). Promoting floral competence can be achieved by lowering transcript levels of the floral repressor *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *FLC* encodes a MADS-box transcription factor that is expressed in both the SAM and leaf vasculature (Michaels and Amasino, 2000; Bastow *et al.*, 2004). FLC directly binds the FPI genes *FT*, *FD* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) to facilitate their downregulation (Searle *et al.*, 2006). In Arabidopsis, two parallel acting pathways, the autonomous pathway and the vernalization pathway mainly achieve downregulation of FLC expression. The autonomous pathway comprises a set of seven genes, either encoding chromatin regulators or RNA binding/processing factors, which collectively reduce *FLC* levels (reviewed in Simpson, 2004; Bäurle and Dean, 2006). Late flowering of plants impaired in any one of the autonomous pathway components suggests that they function in parallel and share *FLC* as a common target. Secondly, the vernalization pathway stably suppresses *FLC* expression by epigenetic silencing of the *FLC* locus upon prolonged growth at winter temperatures (reviewed in Henderson and Dean, 2004; Sung and Amasino, 2005). Consequently, vernalized plants maintain low levels of *FLC* throughout further somatic development, allowing them to flower in favorable long day (LD) photoperiods.

A primary role for vernalization is to override the winter-annual flowering behavior of plants carrying dominant alleles of *FRIGIDA* (*FRI*) (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Johanson *et al.*, 2000). *FRI* acts as a strong activator of *FLC* and outbalances the autonomous pathway, resulting in high *FLC* levels and delayed flowering in the absence of vernalization. Although the molecular mechanism underlying *FLC* activation by FRI has remained elusive, several essential cofactors in this process have been isolated recently. The emerging picture is that FRI probably acts in a complex containing either its homologue FRIGIDA-LIKE1 (FRL1) or FRL2 (Michaels *et al.*, 2004; Schläppi, 2006) and SUPPRESSOR OF FRI4 (SUF4) (Kim *et al.*, 2006a; Kim *et al.*, 2006b). SUF4, a putative zinc-finger transcription factor, is most likely accountable for DNA-binding activity of such a FRI-containing complex, as SUF4 has been found to physically interact with both FRI and FRL1 and is capable of binding to *FLC* chromatin (Kim *et al.*, 2006b).

We reported that the *BEL1-like* (*BELL*) class three amino acid loop extension (TALE) homeobox gene *ARABIDOPSIS THALIANA HOMEOBOX1* (*ATH1*) acts as a positive regulator of *FLC* expression (Chapter 3 of this thesis). However, although basal *FLC* expression was eliminated by *ath1* mutations in a *fri* background, late flowering and expression of *FLC* were only partially affected in *FRI* plants and autonomous pathway mutants. Here we report on the characterization of a homologous *BELL* class gene *PENNYWISE* (*PNY*) as a partially redundant factor of *ATH1* in controlling *FLC* induction. In a *fri* background, *pny* mutants display reduced *FLC* transcript levels comparable to those previously observed in *ath1* mutants. As a result, *pny* plants flower early in short day photoperiods. Moreover, *FRI* containing plants impaired in PNY function show a slight reduction in flowering time, whereas loss of both *ATH1* and *PNY* results in a flowering time resembling that of *fri* and *ath1 pny fri* plants, suggesting that ATH1 and PNY act redundantly in FRI-dependent *FLC* induction. In addition, we show that ATH1 is capable of associating with the FRI homologue FRL1 in a heterologous yeast system, whereas PNY is able to interact with FRI itself. These observations suggest a mechanism in which BELL proteins may serve as transcription factors for both basal and FRI mediated *FLC* induction. Contrary to PNY, the BELL protein POUND-FOOLISH (PNF), an established partner of PNY in inflorescence patterning and floral development (Smith *et al.*, 2004), does not seem to be involved in controlling *FLC* levels and flowering time. This implies that *PNY* fulfills different roles during the vegetative and generative phases of plant development and activity of specific BELL combinations likely dictates the phase identity of the SAM.

Results

Identification of PNY as a Positive Regulator of the Floral Repressor FLOWERING LOCUS C

Lowering expression levels of the floral repressor *FLOWERING LOCUS C* (*FLC*) greatly contributes to floral competence of Arabidopsis plants (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Searle *et al.*, 2006). We previously demonstrated that the BELL-class TALE homeodomain (HD) protein ARABIDOPSIS THALIANA HOMEOBOX1 (ATH1) determines floral competence by acting as a positive regulator of *FLC* expression (Chapter 3 of this thesis). Lesions in *ATH1* cause a dramatic reduction of basal *FLC* levels in Col (*fri*) plants, and flowering time of *ath1* mutants in short day (SD) conditions resembles that of *flc* loss-of-function mutants (Chapter 3 of this thesis). However, disturbed ATH1 function in combination with *FRI* or an *fca* or *fve* autonomous pathway mutation, only moderately affected *FLC* levels and flowering time (Chapter 3 of this thesis).

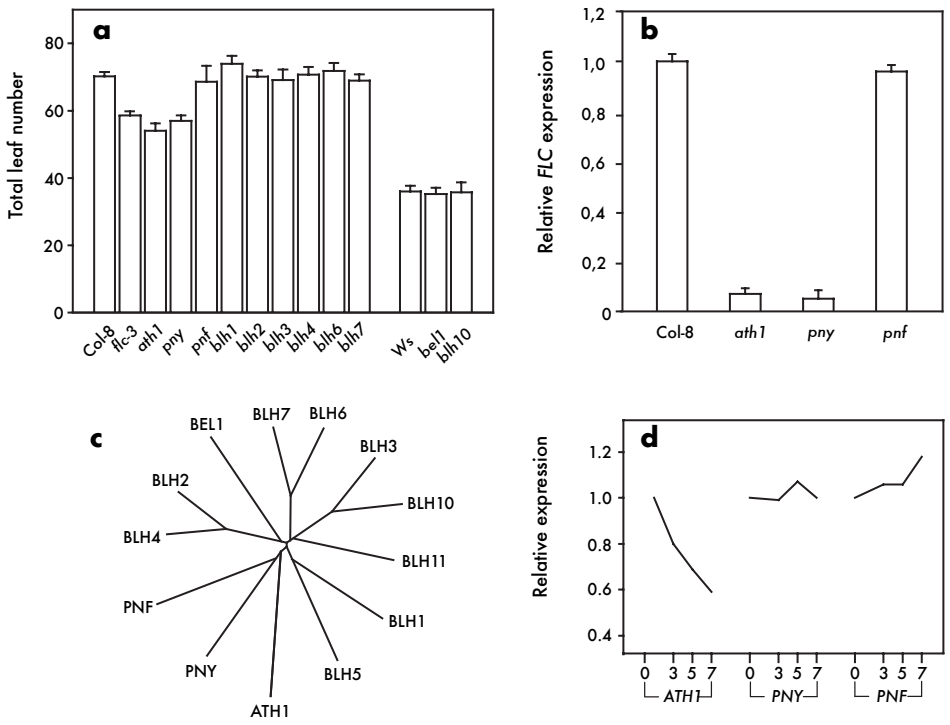

Figure 4.1 Identification of PNY as a Floral Repressor

(a) Flowering time of *BELL* class mutants compared to respective wild type control plants (Col-8 or Ws) under short day (SD) photoperiods. Only *pny*^{40126} mutants are early flowering like *ath1-1* and *flc-3* null mutants. Error bars indicate SD based on means obtained for at least 7 plants for each line.

(b) Real-time RT-PCR results for *FLC* in 10-day-old WT, *ath1-1*, *pny*^{40126} and *pnf*^{96116} mutants grown in LD conditions. For each cDNA sample, the *FLC* expression level was normalized to the *UBQ* control. Each reaction was done in duplicate, and each experiment was repeated three times. Error bars indicate SD.

(c) Evolutionary relationship of Arabidopsis BELL class proteins based on full-length protein sequences (also see Roeder *et al.*, 2003).

(d) Relative expression levels of *ATH1*, *PNY* and *PNF* in micro-dissected meristems of Arabidopsis wild-type plants (Col). Plants were grown for 30 non-inductive short days (SD) and transferred to floral inducing long day (LD) conditions. Meristems were isolated at 0, 3, 5 and 7 days upon shifting to LD conditions (Schmidt *et al.*, 2005). Data were obtained from Genevestigator (Zimmermann *et al.*, 2004).

ATH1 belongs to a family of thirteen Arabidopsis BELL proteins and functional redundancy among these BELL proteins has been described for several developmental processes (Smith *et al.*, 2004; Kumar *et al.*, 2006; Chapter 2 of this thesis). We investigated whether other BELL proteins act redundantly with ATH1 in the control of floral competence through regulation of *FLC* expression. Initially *BELL* mutants were analyzed for their flowering behavior under SD photoperiods (Figure 4.1a). T-DNA insertion mutants for *BELL* members were collected from publicly available collections and tested.

Only lack of functional *PNY* caused a significant flowering time phenotype in SD conditions. *pny*^{40126} mutants (Smith and Hake, 2003) flowered significantly earlier than wild type control plants, comparable to *flc-3* and *ath1-1* loss-of-function mutants (Figure 4.1a). No effect on flowering time was observed in LD grown *pny*^{40126} plants, similar to *flc-3* and *ath1-1* mutants. None of the other tested *bell* mutants showed an effect in LD either (data not shown). The early flowering of loss-of-function *pny* mutants in SD, comparable to *flc* null mutations, combined with the absence of a flowering time phenotype under LD conditions, suggests that PNY acts as a positive regulator of *FLC* expression as observed for ATH1.

This hypothesis was tested by comparing the *FLC* transcript levels in 10-day-old Col-8, *ath1-1* and *pny*^{40126} seedlings. For comparison, the POUND-FOOLISH (PNF) loss-of-function mutant *pnf*^{96116} (Smith *et al.*, 2004) was also analyzed. PNF was previously characterized to act redundantly with PNY, as a positive regulator of inflorescence and flower development by controlling expression of at least the floral meristem identity (FMI) genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Smith *et al.*, 2004). Loss of *PNY* results in a strong attenuation of *FLC* messenger levels in agreement with the observed flowering time defect (Figure 4.1b). In contrast, *pnf* plants showed no significant changes in *FLC* transcript accumulation (Figure 4.1b).

In addition, phylogenetic analysis based on full-length BELL protein sequences, places ATH1 in one evolutionary sub-clade with PNY and PNF (Fig. 4.1c, also see Roeder *et al.*, 2003).

Together, these findings suggest that PNY is the only likely BELL family member to act redundantly with ATH1 in flowering time control via regulations of *FLC* expression.

ATH1-Subclade Genes Are Differentially Expressed in the SAM During the Plant Life-Cycle

We previously reported that *ATH1* mRNA levels in the SAM are down regulated just prior to the floral transition, and proposed that this reduction might contribute to a reduction in *FLC* levels, allowing transition to the generative phase (Chapter 3 of this thesis).

Data retrieved from the Genevestigator database (www.genevestigator.ethz.ch) (Zimmermann *et al.*, 2004) showed that *PNY* mRNA levels in the meristematic region remain relatively unchanged during the vegetative to generative switch, whereas *PNF* expression shows a slight increase during the floral transition (Fig. 4.1d). Thus, *PNY* and *PNF* expression is in agreement with the reported functions of these proteins. PNY fulfills essential roles in both vegetative and generative meristem function (Byrne *et al.*, 2003; Smith and Hake, 2003; Bhatt *et al.*, 2004; Smith *et al.*, 2004; Kanrar *et al.*, 2006; Chapter 2 of this thesis) and controls the expression of corresponding phase identities genes (Smith *et al.*, 2004). PNF, as mentioned above,

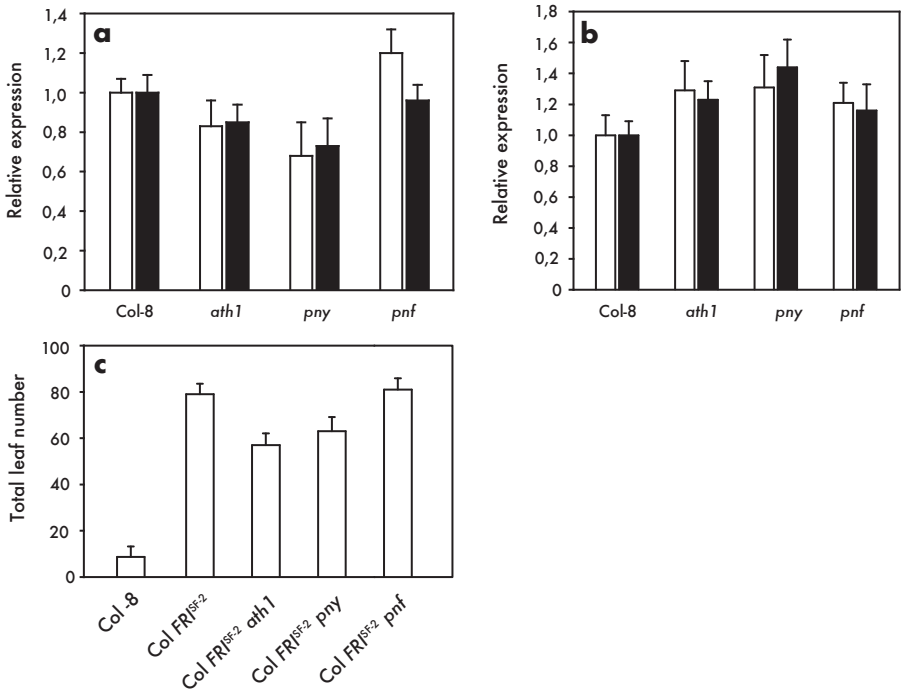

Figure 4.2 PNY Regulates Flowering Time in an FLC Specific Manner and Partially Suppresses *FRI*-Dependent Late Flowering

(a) Real-time RT-PCR results for *FLM* (white bars) and *MAF2* (black bars) in 10-day-old LD grown Col-8 wild type, *ath1-1*, *pny*^{40126} and *pnf*^{96116} plants. For each cDNA sample, the expression level of a given gene was normalized to the *UBQ* control. Each reaction was made in duplicate, and each experiment was repeated three times. Error bars indicate SD.

(b) Real-time RT-PCR results for the *FCA* (white bars) and *FPA* (black bars) autonomous pathway genes in 10-day-old LD grown wild-type controls (Col-8) and *ath1-1*, *pny*^{40126} and *pnf*^{96116} plants. For each cDNA sample, the expression level of a given gene was normalized to the *UBQ* control. Each reaction was made in duplicate, and each experiment was repeated three times. Error bars indicate SD.

(c) Mutations in either *ATH1* or *PNY* cause a partial reduction of *FRI*-mediated late flowering, whereas lack of functional *PNF* has no effect on flowering time. Plant were grown in LD conditions and values represent total leaf number. Error bars indicate SD.

specifically functions in inflorescence and floral meristem development (Smith *et al.*, 2004; Kanrar *et al.*, 2006).

PNY Acts Specifically via *FLC* and Partially Suppresses FRI-Mediated Late Flowering

The autonomous pathway and FRI act specifically on *FLC*. However, members of the Arabidopsis PAF1 and SWIR1 complexes control floral transition by regulating both *FLC* and related family members (Soppe *et al.*, 1999; Zhang and van Nocker, 2002; Noh and Amasino, 2003; He *et al.*, 2004; Oh *et al.*, 2004; Choi *et al.*, 2005; Deal *et al.*, 2005; He and Amasino, 2005; Kim *et al.*, 2005; Zhao *et al.*, 2005; March-Díaz *et al.*, 2006; Martin-Trillo *et al.*, 2006).

To better understand the role of PNY in controlling flowering time, we assayed transcript levels of the two *FLC*-related genes that have an established role as floral repressors, *FLOWERING LOCUS M* (*FLM*) and *MADS-BOX AFFECTING FLOWERING2* (*MAF2*) (Ratcliffe *et al.*, 2001, 2003; Scortecci *et al.*, 2003). No substantial effects on *FLM* or *MAF2* expression was observed in the *pny*^{40126} mutant background (Fig. 4.2a), as was previously found for *ath1-1* mutants. Therefore, likely *PNY* does not function as a general regulator of *FLC*-clade transcription, but rather acts specifically via *FLC*.

One explanation for the *FLC* specificity of ATH1 and PNY might be that they are involved in the regulation of autonomous pathway genes. The autonomous pathway forms an intrinsic group of seven proteins whose parallel actions converge at the *FLC* locus. All members appear essential and negatively affect transcript levels of *FLC*. Some act through chromatin modification of the *FLC* locus, whereas others are proposed to function as RNA binding/processing factors involved in *FLC* transcript processing. For the putative RNA-binding protein FCA it was shown that an increase in expression causes an additional decrease in *FLC* mRNA levels, even in the presence of dominant *FRI* alleles (Macknight *et al.*, 2002). However, when analyzed in 10-day-old *ath1-1* and *pny*^{40126} seedlings, the mRNA levels of *FCA* and *FPA*, which encodes a second RNA binding protein of the autonomous pathway, were unaffected (Fig. 4.2b).

FRI also acts specifically via *FLC* and we previously observed a partial suppression of FRI-mediated late flowering by *ath1* mutations (Chapter 3 of this thesis). To test whether the same is true for PNY, the *pny*^{40126} mutation was crossed into Col plants carrying dominant *FRI* alleles (Col *FRI*^{SF2}). As expected, under LD photoperiods also lack of *PNY* resulted in a partial reduction of flowering time in this background (Fig. 4.2c). Despite this partial reduction in flowering time, Col *FRI*^{SF2} *pny*^{40126} plants, like Col *FRI*^{SF2} *ath1-1* plants, were still substantially later than *fri* loss-of-function plants (Col-8) (Figure 4.2c). Thus, from these single mutant analyses it can be concluded that ATH and PNY fulfill highly similar roles in *FLC* regulation, suggesting that they might operate as functional redundant proteins in flowering control.

FRI-Mediated Late Flowering Is Redundantly Controlled by *ATH1* and *PNY*

The presence of dominant *FRI* alleles accounts for most of the winter-annual flowering habit amongst Arabidopsis accessions (Shindo *et al.*, 2005). Since *ath1* and *pny* single mutants partially affect late flowering in a *FRI*-containing background, we tested whether ATH1 and PNY act redundantly in proper FRI functioning. As described in Chapter 2 of this thesis, ATH1 and PNY have functional redundant roles in vegetative SAM function. As a result, in many *ath1-1 pny*^{40126} double mutants

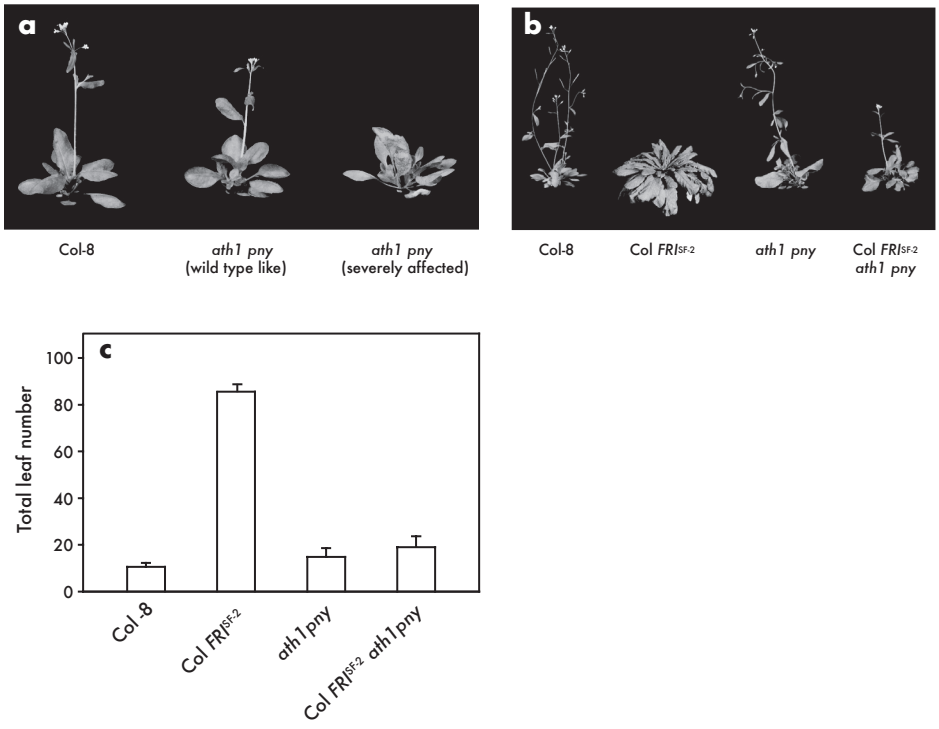

Figure 4.3 ATH1 and PNY Redundantly Control FRI-Mediated Late Flowering

(a) Wild type (WT) Col-8 and two *ath1-1 pny*^{40126} mutants grown in long days (LD). Shown *ath1 pny* mutants display the weak (left) or severe (right) phenotypes usually observed within the mutant population. Weak phenotype plants flowered slightly later than WT and were used for flowering time analysis, whereas severely affected plants always flowered later.

(b) Flowering time of Col-8 WT, *ath1-1 pny*^{40126}, Col *FRI*$^{SF-2}$ and Col *FRI*$^{SF-2}$ *ath1-1 pny*^{40126} plants grown in LD conditions. Photographed plants were of the same age, except for the Col *FRI*$^{SF-2}$ plant, that was pictured at a later stage during development, at a time that Col-8 WT, *ath1-1 pny*^{40126}, and Col *FRI*$^{SF-2}$ *ath1-1 pny*^{40126} plants had already died.

(c) Histogram representing flowering time of the in (b) described plants expressed as the total leaf number formed at flowering. Ten plants per genotype were scored. Error bars indicate SD.

the SAM terminates before completing the vegetative growth phase, whereas the individual mutants show no obvious signs of meristem malfunction. This aspect of ATH1 and PNY function obviously hampers research into their combined contribution to flowering time control. However, in the Col-8 background studied here, the meristem phenotype is not fully penetrant and can be found in a varying severity (Figure 4.3a). In the least affected plants the central SAM does not terminate and is capable of undergoing floral transition, although often somewhat later than wild type control plants (Figure 4.3b,c; Chapter 2 of this thesis). Most likely this is a secondary effect attributable to reduced meristem function, since *FLC* levels in *ath1-1 pny*^{40126} plants are even further reduced compared to either single mutants,

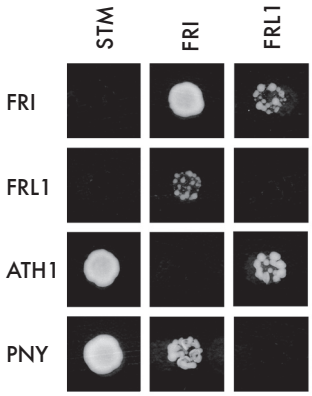

Figure 4.4 Yeast Two-Hybrid Interactions between ATH1 and PNY and FRI-family Proteins
As positive controls we used the interactions between the two BELL proteins and STM (Chapter 2 of this thesis) and the recently published FRI-FRI interaction (Kim *et al.*, 2006b).

and more severely affected plants consistently showed a delay in flowering (Fig. 4.3a).

The least affected plants were selected and their total leaf number upon flowering in LD conditions was determined (Fig. 4.3b,c). Flowering time of Col-8 *ath1-1 pny*^{40126} plants was compared to Col:*FRI*^{SF2} *ath1-1 pny*^{40126} plants. Both lines flowered at nearly identical leaf numbers under these conditions (Fig. 4.3b-c), indicating that lack of both *ATH1* and *PNY* essentially annihilate FRI-mediated late flowering (Fig. 4.3b-c). Similar results were obtained when plants were grown in SD photoperiods (data not shown).

Therefore, we conclude that ATH1 and PNY act redundantly in the FRI-mediated delay of flowering time in Arabidopsis.

ATH1 and PNY Interact with Members of the FRI Family

In the process of FRI-mediated *FLC* induction, no factors acting genetically downstream of FRI have been described. FRI was suggested to act in parallel with its relatives FRL1 and FRL2, and the zinc finger proteins, FRIGIDA ESSENTIAL 1 (FES1) and SUF4 (Michaels *et al.*, 2004; Schmitz *et al.*, 2005; Kim *et al.*, 2006a; Kim *et al.*, 2006b; Schläppi, 2006), in regulating expression of the *FLC* locus. Lack of a canonical DNA-binding motif in FRI and its close relatives prevent a direct binding to *FLC*, implying dependance on additional DNA binding proteins for proper functioning. Likely, SUF4 has such a function, since it is capable of both binding to *FLC* chromatin and of associating with both FRI and FRL1 (Kim *et al.*, 2006b). In the case of FES1, however, no evidence was found for an interaction between FRI and FES1, or FRL1 and FES1 (Schmitz *et al.*, 2005).

ATH1 and *PNY* encode putative transcription factors and we tested whether they might function as facilitators of *FLC* transcription by recruiting FRI and/or FRL1. We did not detect an interaction between ATH1 and FRI in yeast two-hybrid studies (Chapter 3 of this thesis), but a consistent interaction between ATH1 and FRL1 was found (Fig. 4.4). Interestingly, PNY was found to interact with FRI,

but not FRL1, in yeast two-hybrid experiments (Fig. 4.4). Moreover, FRI and FRL1 proteins were suggested to function in parallel and it was tested whether these proteins could interact in the yeast two-hybrid system. Beside homodimerization of FRI, heterodimerization of FRI and FRL1 was indeed observed in yeast (Figure 4.4).

Together, these results imply that ATH1 and PNY function in close proximity to both FRI and FRL1 in an *FLC* inducing complex.

Discussion

The tightly controlled switch from vegetative to generative development in Arabidopsis has emerged as a highly complex gene regulatory network. The two main sites of flowering time control are the leaves, where floral inducing signals emerge, and the shoot apex, where all signals are integrated and flowering is induced. Increasing the plants competence to respond to floral inducing signals is a primary step in reproductive development (reviewed in Bernier, 1988). Lowering levels of the floral repressor *FLC* below a certain threshold increases the plants floral competence and allows flower-inducing pathways to activate developmental programs that dictate reproductive primordial fate and commit the plant to the generative phase (recently reviewed in Bernier and Perilleux, 2005). We have previously shown that the *BELL* class TALE homeobox gene *ATH1* acts as a suppressor of floral competence by positive regulation of *FLC* (Chapter 3 of this thesis). However, since the effects of either FRI or an autonomous pathway mutation on *FLC* expression and thus flowering time are only partially suppressed by loss-of-function *ath1* mutations, it was investigated whether *ath1* mutants may not represent a total loss in activity due to functional redundancy with related *BELL* genes.

The data presented here demonstrate that, *PNY*, one of the twelve remaining *BELL* genes in the Arabidopsis genome, acts redundantly with *ATH1* as a positive regulator of *FLC* expression. In the absence of *ATH1*, *PNY* loses the ability to induce wild type *FLC* levels. Reciprocally, lack of *PNY* results in attenuated *FLC* levels because *ATH1* alone is insufficient for maintaining proper *FLC* levels. Moreover, *ath1-1 pny*^{40126} double loss-of-function mutants essentially loose their ability to express *FLC* (data not shown). Most importantly, absence of both ATH1 and PNY almost fully impairs FRI-mediated late flowering, in contrast to the relatively weak effects of either single mutant.

Our yeast two-hybrid analyses indicate that ATH1 and PNY might induce *FLC* expression in association with FRI and its homologue FRL1. Since PNY and ATH1 encode putative transcription factors, this could imply that both BELL proteins bind to the *FLC* locus, thereby allowing FRI and FRL1 to dock on *FLC* chromatin. A similar function has recently been proposed for SUF4 in *FLC* induction

(Kim *et al.*, 2006a; Kim *et al.*, 2006b). Such a function would fit with a model that was recently postulated for the function of animal orthologues of ATH1 and PNY. According to this model the TALE homeodomain proteins penetrate repressive chromatin and act as a molecular 'beacon' to mark the corresponding genes for subsequent activation by master regulatory factors (Berkes *et al.*, 2004; de la Serna *et al.*, 2005). However, in contrast to SUF4, data on physical interactions between ATH1/PNY and the *FLC* locus is currently lacking. SUF4 and the BELL proteins ATH1 and PNY share common protein partners in FRI and FRL1, suggesting that all act in a single complex. However, this remains to be investigated.

ATH1 and PNY interact with FRL1 and FRI, respectively, but previous data on ATH1 (Chapter 3 of this thesis) indicate that ATH1 functions as well in FRI-independent control of *FLC* expression. Here we show that PNY is also necessary for basal *FLC* levels in the absence of *FRI*, suggesting a similar FRI-independent function for PNY. This most likely also holds for *PNY*. In addition to FRI and cofactors, also the autonomous pathway components are known to regulate the floral transition specifically through *FLC* expression regulation (Michaels and Amasino, 2001). We are currently testing if lack of *PNY* can suppress late flowering in autonomous pathway mutant backgrounds as observed for loss of of ATH1 (Chapter 3 of this thesis). Preliminary data show a moderate reduction of flowering time in *pny fca-1* plants, resembling the effect of *ath1* mutants in this background. Surprisingly, despite several attempts, we have been unable to retrieve *pny fve-1* double mutants from several independent crossing events (data not shown), suggesting embryo lethality.

In accordance with a function as activator of *FLC* gene expression, *PNY* is expressed in seedlings in a pattern similar to that of *FLC*: strong expression in the meristematic region and throughout the vasculature of cotyledons and leaves (Byrne *et al.*, 2003; Michaels and Amasino, 2000; Bastow *et al.*, 2004). We have previously shown that *ATH1* has a similar expression pattern. We also found that expression of *ATH1* in the SAM declines just before the vegetative to reproductive transition and that this gradual down-regulation of *ATH1* expression is accompanied with a similar decrease in *FLC* expression (Chapter 3 of this thesis). In contrast, expression of *PNY* remains relatively unchanged during floral transition, whereas at the same time expression of the lowly expressed third related BELL member PNF slightly increases. Recently, *PNY* and *PNF* were proposed to redundantly function as competency regulators of the SAM in the process of floral evocation (Smith *et al.*, 2004). However, whereas ATH1 and PNY act as floral repressors, PNY and PNF were proposed to be positive regulators of the generative phase. As a result, PNY can act both as a negative and positive regulator of flowering. This apparent contradiction might be explained by the temporal presence of the redundantly acting proteins ATH1 and PNF. During the vegetative phase, PNY together with ATH1 prevents premature flowering by activation of *FLC*, rendering the SAM incompetent to

respond to floral inductive signals. Associated with the floral transition ATH1 is downregulated and PNF becomes the new partner of PNY. Together, PNY and PNF enable the SAM to properly respond to floral inductive signals and to form an inflorescence. Thus, the emerging picture now is that three BELL proteins dictate meristem phase identity due to temporally partial overlapping functions.

Experimental Procedures

Plant Materials and Growth Conditions

The wild types used in this study were the *Arabidopsis thaliana* Columbia-8 (Col-8) and Wassilewskija (Ws) strains. The Col FRI^{SF-2} introgression line and *flc-3* mutant used in this study have been described previously (Michaels and Amasino, 1999).

The *ath1-1* (GK line 114A12) T-DNA insertion line was obtained from GABI-KAT (MPI for Plant Breeding Research, Cologne, Germany). Remaining *BELL* gene insertion lines/mutants were obtained from NASC (University of Nottingham, UK), except for *blh10* that was obtained from FLAGdb (INRA Versailles). *BELL* gene insertion lines and mutants used in this study are: *bel1-3* (At5g41410) (Ws) (Modrusan *et al.*, 1994), *blh1* (At2g35940) (SM_3_16405) (Col-8), *blh2* (At4g36870) (SALK_009120) (Col-8), *blh3* (At1g75410) (SM_3_38995) (Col-8), *blh4* (At2g23760) (SALK_121117) (Col-8), *blh6* (At4g34610) (SALK_018708) (Col-8), *blh7* (At2g16400) (SALK_145182) (Col-8), pnf^{96116} (At2g27990) (SALK_096116) (Col-8) (Smith *et al.*, 2004), pny^{40126} (At5g02030) (SALK_040126) (Col-8) (Smith and Hake, 2003), *blh10* (At1g19700) (INRA line 083A11) (Ws). For two independent *BLH5* (At2g27220) insertion lines, no plants homozygous for the T-DNA insertion were found. *BLH11* (At1g975430) was not included in the screen. The *ath1-1*, pnf^{96116} and pny^{40126} lines were backcrossed two times to the Col-8 ecotype.

Plants were grown in long days (16 h of light/8 h of dark) or short days (8 h of light/16 h of dark) under cool white fluorescent lights (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C with 70% RH. For germination, seeds were stratified on full strength Murashige and Skoog medium at 4°C for 2 days in the absence of sucrose. Flowering time was measured by counting the total leaf number.

Yeast Two-Hybrid Analysis

To determine the biochemical properties of ATH1 and PNY we used the ProQuest™ Two-Hybrid System with Gateway™ Technology from Invitrogen. *ATH1, PNY, FRI,* and *FRL1* open reading frames were flanked by Gateway linkers and inserted in the pDEST™22 and pDEST™32 vectors by Gateway recombination following Invitrogen instructions. FRI and FRL1 cDNAs were cloned from the

H51 and Col ecotypes, respectively. Primers used for the gateway constructs are: *FRI* FWD (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCCA ATTATCCACCGAC-3') and *FRI* REV (5'-GGGGACCACTTTGTACAAGAA AGCTGGGTCCTATTTGGGGTCTAATGATG-3'), *FRL1* FOR (5'-GGGGA CAAGTTTGTACAAAAAAGCAGGCTTCATGACGGCGAGTGAGACTATC -3'), for *FRL1* REV (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCT ACTGAGAATAATAAGGC GGGT-3').

All plasmids were transformed into the yeast strain PJ69A (James *et al.,* 1996) using a lithium acetate/polyethylene glycol protocol (Schiestl *et al.*, 1993). The pDEST™32 DB-fusion constructs were tested for nonspecific activation of the reporters on selective drop-out medium (SD) (Sigma-Aldrich) lacking adenine and/or leucine, and histidine (–ade/–leu, –his) supplemented with increasing concentrations of 3-amino-1',2'-triazol (3-AT) (0-100mM final concentration). Positive interactions were identified by testing for growth on SD –ade/ –leu –his –tryptophan supplemented with the appropriate amount of 3-AT (final concentrations ranging from 0-50 mM).

Analysis of Gene Expression

RNeasy minikit columns (Qiagen) were used to isolate RNA for Q-PCR analysis. Total RNA was DNaseI treated (RNase-free; Fermentas GMBH, Germany) 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. Q-PCR analyses were performed as described before (van Dijken *et al.*, 2004). Primers used for Q-PCR expression analysis were as described in Czechowski *et al.* (2004) (*FLC*, *FLM*, *MAF2*) and in Deng *et al.* (2007) (*FCA, FPA, UBQ*).

Genotyping

Genomic DNA was isolated using the quick-prep method (Cheung *et al.*, 1993). The genotype of *ath1-1* was determined using primers to the 35S CaMV promoter 35S-mini (5'-CTGCAGCAAGACCCTTCCTCTAT-3') and *ATH1* gene specific primers ATH1 TAG3.3 FWD (5'-GCTCGGAGATAAGTCTT TGTGCAGCTA-3'). *ATH1* wild type alleles were identified using the primer combination SALK_113353 LP (5'-TTTGTAGTTCAAGAGAAAAGCTTGA-3') and SALK_113353 RP (5'-GGCGGGTTTCGGATCTACATT-3'). The *pny* ^{40126} and *pnf* ^{96116} genotypes were determined as in Smith and Hake (2003) and Smith *et al.* (2004), respectively. Other T-DNA insertion lines were genotyped using primers designed by the SIGnAL T-DNA Verification Primer Design Tool (www.signal.salk.edu/tdnaprimers.2.html).

FRI allelic variation (Col vs. SF-2) was scored as described by Johanson *et al.* (2000).

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Chapter 5

General Discussion

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Meristem Function and Phase Identity: a Time for Integration?

One of the most prominent features of plant development is its indefinite organogenic potential provided by undifferentiated apical group of cells, termed meristems. The aerial portion of the plant is formed via the formation of organ primordia at the flanks of the shoot apical meristem (SAM), while the central region of the SAM contains pluripotent stem cells. 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. During the vegetative phase of Arabidopsis development, the SAM generates leaf primordia in a spiral phyllotaxy to form rosette leaves accompanied by small resting axillary meristems. Upon reaching floral competence, the SAM undergoes a morphological change, and from here on deposited primordia form active axillary meristems subtended by cauline leaves during early generative development and solitary floral meristems that lack any pronounced leaf development during the later stages of this developmental phase.

Although the robust and balanced self-perpetuating mechanisms that maintain the shoot apical meristem (SAM) operate seemingly autonomously, it is believed that intensive communication occurs between these basic meristem mechanisms and genetic networks that determine meristem phase identity. All primordia formed by the SAM gain identity upon reaching a certain size or competence to respond to identity signals, making it unlikely that identity is already determined during primordial initiation at the SAM (reviewed in Hempel *et al.*, 2000). The signals that eventually dictate primordial identity are believed to be derived from both established lateral organs and the SAM (reviewed in Grbic, 2005; Parcy, 2005). Whether established organs influence the identity of primordia directly, or first modify the SAM, which in turn then determines primordial identity, is still unclear. Intriguingly, it was recently shown that a major signal component for generative identity of the SAM, the *FT* gene product, is leaf-derived. *FT* mRNA is transported from leaf vascular cells to the SAM where it is translated and binds the bZIP transcription factor FD to induce floral meristem identity of SAM-derived lateral primordia, corroborating a model in which established organs modify organ identity via the SAM (Abe *et al.*, 2005; Huang *et al.*, 2005, Wigge *et al.*, 2005).

As for the main SAM, all lateral meristems depend on the basic meristem maintenance machinery including the *WUS, CLV* and *STM* genes. Moreover, floral meristem formation from lateral primordia involves the local expression of so-called floral meristem identity genes (FMI), which directly dictate generative identity of the lateral primordia. An antagonistic group of genes, including *FLC* and *TFL1*, repress expression of the FMI genes to prevent the transition to flowering or floral differentiation of the SAM, respectively (Figures 1.8 and 1.9).

How genetic networks that control meristem phase identity on the one hand, and meristem maintenance on the other hand, communicate, remains largely unknown, although recently several genes have been found to influence both processes.

One such gene, *SERRATE* (*SE*), encodes a C2H2 zinc finger protein that controls the plants responsiveness to *KNOX* genes and plays a prominent role in FRI-mediated *FLC* induction (Bezerra *et al.*, 2004; Grigg *et al.* 2005). Moreover, juvenile leaf initiation and phase length are disturbed in *se* mutants (Clarke *et al.*, 1999). It was recently shown that SE acts in fundamental steps of miRNA processing and might therefore influence transcript levels of numerous genes (Lobbes *et al.*, 2006; Yang *et al.*, 2006), making it momentarily impossible to conclude whether meristem function and flowering time processes are coordinately controlled by SE activity.

Meristem function and phase identity are also both disturbed in *embryonic flower1* (*emf1*) and *emf2* mutants (Yang *et al.*, 1995). In both single mutants, the SAM completely bypasses vegetative development and terminates directly after germination in a flower-like structure, or, in case of less severely affected alleles,

after formation of a small inflorescence. Moreover, both mutations are epistatic to mutations causing late flowering in relation to rosette leaf development (Yang *et al.*, 1995; Chen *et al.*, 1997; Haung *et al.*, 1998). EMF1 is proposed to function as a transcriptional regulator, whereas *EMF2* encodes a member of the Polycomb-group of chromatin remodeling proteins. This group further includes the VRN2 protein, implying that epigenetic control of developmental programs is a crucial aspect of meristem phase identity control (Aubert *et al.*, 2001; Yoshida *et al.*, 2001). Moreover, *emf* double mutants display lack of post-germinative shoot development (Yang *et al.*, 1995), suggesting that maintenance and phase identity of the SAM at least partially depend on similar components. Nevertheless, details on EMF targets are still lacking and do likely not include established flowering time genes, such as *FT*, *SOC1* and *LFY* (Moon *et al.*, 2003), which prevents a fruitful discussion on the networks controlled by the *EMF* genes.

Another example of a gene involved in both meristem maintenance and meristem phase identity is the *MGOUN3* (*MGO3* a.k.a. *BRUSHY1* (*BRU1*) and *TONSUKU* (*TSK*)) gene (Guyomarch *et al.*, 2004; Suzuki *et al.*, 2004; Takeda *et al.*, 2004). *mgo* mutants display disturbed root meristem organization and enlarged disorganized shoot meristems that produce fewer lateral organs. Moreover, MGO3 (Guyomarch *et al.*, 2004; Suzuki *et al.*, 2004; Takeda *et al.*, 2004), a nuclear leucine-glycine-asparagine (LGN) domain protein implicated in chromatin remodeling, has recently been shown to control, beside shoot patterning, expression of several MADS-box genes including *FLC* and the floral homeotic genes (FHG) *AG*, *PI* and *SEP3* (Guyomarch *et al.*, 2004, 2006). Intriguingly, *mgo3* mutants display attenuated levels of *FLC*, but contrary, ectopic expression of the FHG, implying that *MGO* genes could be involved in both basic meristem processes as well as phase identity specification (Guyomarch *et al.*, 2006).

The work in this thesis describing the functional characterization of two related *BELL* genes, *ATH1* and *PNY*, revealed that these two proteins are redundantly involved in both meristem function and in the specification of meristem phase identity. Moreover, our data imply that ATH1 and PNY most likely do so by direct control of key components in both processes.

Since both ATH1 and PNY proteins can interact with the KNOX protein STM, and *ath1 pny* double mutants result in an *stm* mutant phenocopy without preventing the initiation of *STM* expression, our data suggest that ATH1 and PNY act in parallel with STM in initiation and maintenance of the vegetative SAM (Chapter 2). Moreover, *ath1* and *pny* single phenotypes imply that these BELL proteins could be involved in multiple aspects of cell differentiation control within the meristem. Whether proposed STM targets, including the *KNOX* genes *BP* and *KNAT2*, and the mitotic cyclin *CycB1;1* also depend on ATH1 and/or PNY remains to be determined (Byrne *et al.*, 2002; Lenhard *et al.*, 2002). Interestingly, *ath1 pny* double mutant phenotypes cause clear *stm* phenocopies only during the

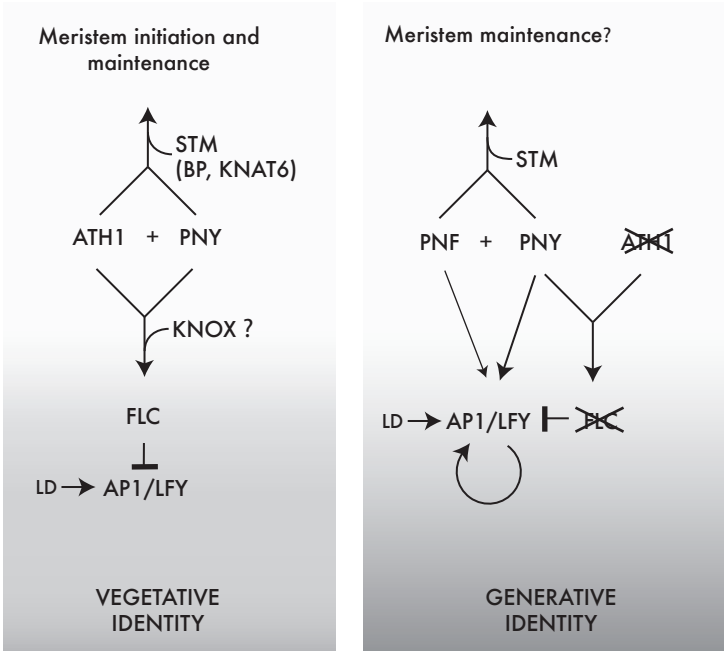


Figure 5.1 Proposed Model for the Function of *BELL* Genes in Meristem Maintenance and Phase Identity Determination.

Plant post-embryonic development can be roughly divided in two distinct SAM identity phases, the vegetative (left panel) and generative (right panel). Preventing differentiation of meristem cells throughout both phases is a key process in SAM maintenance. During the vegetative phase, ATH1 and PNY are both present and redundantly act as partners of the class I KNOX protein STM to prevent SAM differentiation. However, prior to the transition to the generative phase, *ATH1* expression is downregulated, whereas PNY expression is maintained at the same level. Simultaneously, expression of a third BELL gene, PNF, is upregulated. Most likely, during generative development PNF takes over the function of ATH1 as a redundantly acting partner with PNY in the process of meristem maintenance. Accordingly, meristem defects in ath1 pny double mutants are only observed during vegetative development.

A second function for ATH1 and PNY that has emerged from our experiments is the control of vegetative SAM phase identity. Major contributors to the generative phase identity of the SAM are the FMI genes AP1 and LFY. During vegetative development, FMI gene levels are kept low by the floral repressive activity of FLC. Surprisingly, we found that besides controlling vegetative meristem maintenance, ATH1 and PNY also act as partially redundant, positive regulators of FLC expression.

Both ATH1 and PNY are necessary for proper FLC induction (left panel). Prior to the floral transition, ATH1 levels gradually decline, accompanied by a similar decrease of FLC levels. Since PNY alone is incapable of inducing proper FLC expression, the floral transition is initiated. Moreover, PNF, was recently shown to control inflorescence and floral development by controlling expression of at least AP1 and LFY (right panel). Surprisingly, PNY was found to act redundantly with PNF in this process, which is in agreement with sustained PNY expression during both meristem identity phases. Conclusively, it appears that specific BELL combinations contribute to determining different SAM phase identities.

vegetative phase, which corroborates with the proposed redundant function of ATH1 and PNY as positive regulators of *FLC* expression to maintain vegetative meristem identity (Chapter 3 and 4). Both ATH1 and PNY are necessary for full induction of *FLC* expression in early and late flowering accessions and *ath1 pny* double mutants completely lack *FLC* transcription. Despite the evidence provided, at the moment we can not unambiguously discriminate between direct and indirect

effects of ATH1 and PNY on *FLC* transcriptions levels. However, both ATH1 and PNY transcription factors can associate with cofactors required for *FLC* induction. These are suggested to act in close proximity of *FLC* chromatin, implying that ATH1 and PNY may also exert their positive functions on *FLC* transcription in close proximity of the *FLC* locus. Nevertheless, it remains important to investigate whether ATH1 and PNY are capable of a direct interaction with the *FLC* locus. Therefore, we are currently setting up experiments that could further contribute to determining the directness of the proposed mechanism.

Conclusively, we have demonstrated that ATH1 and PNY act redundantly and at key positions in both vegetative SAM maintenance and flowering time control genetic networks and therefore we propose a model in which the overlapping as well as combinatorial actions of ATH1 and PNY are necessary for proper shoot development and maintenance of the vegetative phase identity (Figure 5.1).

TALE Proteins and Target Gene Control: Lessons from Mouse

TALE HD proteins encode transcription factors with a highly conserved DNA binding domain and for several members of the plant TALE HD class DNA binding has been demonstrated (Sakamoto *et al.*, 2001; Smith *et al.*, 2002; Bao *et al.*, 2004; Chen *et al.*, 2004). However, the precise mechanism used to regulate target gene expression remains elusive. Recently, for animal TALE HD proteins a model was proposed for their function as transcriptional regulators. Taken the significant evolutionary conservation of TALE HD function between animals and plants, this model might also help to explain the mechanism used by plant TALE HD proteins to control target gene expression.

In a study using mouse cell lines it was elegantly shown that control of a subset of targets of the non-homeobox transcription factor MyoD involves prior binding of a PBC–MEIS complex to the condensed chromatin of MyoD target loci. MyoD acts as a differentiation factor in the process of myogenesis and binds stretches, so-called E-boxes, at target loci to induce gene expression (Figure 1.3). Only upon direct binding to these E-boxes, can MyoD activate target gene induction. The sequence-specific binding of MyoD, however, can be prevented by a repressed chromatin state of its target loci. Surprisingly, it was found that MyoD is recruited to target loci through interaction with a constitutively target bound PBC-MEIS complex (Figure 1.3). This interaction is a prerequisite for subsequent recruitment of chromatin remodelers. Their activity results in clearance of the E-boxes, facilitating direct binding and activation of targets by MyoD. The authors postulate that PBC–MEIS heterodimers penetrate repressive chromatin and act as a molecular 'beacon'

to mark these genes for subsequent activation by master regulatory factors (Berkes *et al.*, 2004; de la Serna *et al.*, 2005).

Whether a similar model accounts for BELL and KNOX proteins in plants will be interesting to determine. Intriguingly, *FLC* is not the only MADS-box gene controlled by BELL proteins. PNY was recently found to control expression of the floral homeotic MADS-box gene *AG*, by binding cis-regulatory sequences in the extraordinary large second intron of *AG*. In plants, large introns (>1 Kb) are rarely found. Exceptions are, among others, found in the type II-family of MADS-box genes to which both AG and FLC belong. Like AG, the FLC locus contains an extraordinary large intron packed with cis-regulatory elements (Sheldon *et al.*, 2002; Sung *et al.*, 2006b). Whether ATH1 and/or PNY can bind this large *FLC* intron and/or promoter sequences of *FLC* to serve as molecular beacons for additional transcriptional regulators forms an interesting challenge for the future. Especially since both BELL proteins can bind the *FLC* activators FRI and FRL1, which both lack a canonical DNA binding motif and likely depend on other proteins to dock on *FLC*. Another likely candidate in this process is SUF4, a putative zinc finger transcription factor protein that is capable of binding both FRI and FRL1. Moreover, SUF4 can bind *FLC* chromatin directly. It will be interesting to test whether, analogous to the animal model, gene specific activators, such as SUF4, together with both FRI-family members use TALE beacon's to dock on *FLC*.

Moreover, it would be interesting to test whether KNOX proteins are involved in controlling *FLC,* as well as *AG,* expression in conjunction with BELL proteins. From a mechanistic point of view one would suggest that KNOX-BELL dimerization is a prerequisite for transcription factor activity (Chapter 2). However, expression of both ATH1 and PNY in the leaf vasculature, a tissue where class I KNOX gene expression is presumed to be absent, implies additional functions for these BELL proteins independently of KNOX partners. Whether in such situations other mechanisms can guide nuclear localization of BELL proteins, or BELL proteins also exert cytoplasmic functions remains currently unknown. Interestingly, like *ATH1* and *PNY*, *FLC* is expressed in both the meristem and in leaf vasculature and we are currently testing the effects of *ATH1* and *PNY* mutations on *FLC* accumulation in both tissues.

References

Abbot, M.K., Kaufman, T.C. (1986). The relationship between the functional complexity and the molecular organization of the *Antennapedia* locus of *Drosophila melanogaster*. Genetics **114**, 919-942.

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

Abu-Shaar, M., and Mann, R.S. (1998). Generation of multiple antagonistic domains along the proximodistal axis during Drosophila leg development. Development **125,** 3821-3830.

Abu-Shaar, M., Ryoo, H.D., and Mann, R.S. (1999). Control of the nuclear localization of Extradenticle by competing nuclear import and export signals. Genes Dev **13,** 935-945.

Aida, M., Ishida, T., and Tasaka, M. (1999). Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. Development **126,** 1563-1570.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-Wide Insertional Mutagenesis of Arabidopsis thaliana Science **301**, 653-657.

Alvarez-Venegas, R., Pien, S., Sadder, M., Witmer, X., Grossniklaus, U., and Avramova, Z. (2003). ATX-1, an Arabidopsis homolog of trithorax, activates flower homeotic genes. Curr Biol **13,** 627-637.

Amasino, R. (2004). Take a cold flower. Nat Genet **36,** 111-112.

An, H., Roussot, C., Suarez-Lopez, P., Corbesier, L., Vincent, C., Pineiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C.G.N., and Coupland, G. (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development **131,** 3615–3626.

Aspland, S.E., and White, R.A. (1997). Nucleocytoplasmic localisation of extradenticle protein is spatially regulated throughout development in Drosophila. Development **124,** 741-747.

Aubert, D., Chen, L., Moon, Y.H., Martin, D., Castle, L.A., Yang, C.H., and Sung, Z.R. (2001). EMF1, a novel protein involved in the control of shoot architecture and flowering in Arabidopsis. Plant Cell **13,** 1865-1875.

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat Genet **36,** 162–166.

Bao, X., Franks, R.G., Levine, J.Z., and Liu, Z. (2004). Repression of *AGAMOUS* by BELLRINGER in floral and inflorescence meristems. Plant Cell **16**, 1478–1489.

Barton, M. K. and Poethig, R. S. (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana* – an analysis of development in the wildtyp and in the *shoot meristemless* mutant. Development **119**, 823-831.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004). Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature **427,** 164–167.

Bateson, W. (1894). Materials for the study of variation treated with special regard to discontinuity in the origin of species. (London: Macmillan and Co.), pp. 209-222.

Bäurle, I., and Dean, C. (2006). The timing of developmental transitions in plants. Cell 125, 655-664.

Becker, A., Bey, M., Burglin, T.R., Saedler, H., and Theissen, G. (2002). Ancestry and diversity of BEL1-like homeobox genes revealed by gymnosperm (Gnetum gnemon) homologs. Dev Genes Evol **212,** 452-457.

Bellaoui, M., Pidkowich, M.S., Samach, A., Kushalappa, K., Kohalmi, S.E., Modrusan, Z., Crosby, W.L., and Haughn, G.W. (2001). The Arabidopsis BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. Plant cell **13,** 2455-2470.

Belles-Boix, E., Hamant, O., Witiak, S.M., Morin, H., Traas, J., and Pautot, V. (2006). KNAT6: an Arabidopsis homeobox gene involved in meristem activity and organ separation. Plant cell **18,** 1900-1907.

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

Berkes, C.A., Bergstrom, D.A., Penn, B.H., Seaver, K.J., Knoepfler, P.S., and Tapscott, S.J. (2004). Pbx marks genes for activation by MyoD indicating a role for a homeodomain protein in establishing myogenic potential. Mol Cell **14**, 465-477.

Bernier, G. (1988). The control of floral evocation and morphogenesis. Annu Rev Plant Physiol Plant Mol Biol **39**, 175–219.

Bernier, G., and Perilleux, C. (2005). A physiological overview of the genetics of flowering time control. Plant Biotech J (Print) **3,** 3-16.

Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F., and Blasi, F. (1998a). The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. EMBO J **17,** 1434-1445.

Berthelsen, J., Zappavigna, V., Mavilio, F., and Blasi, F. (1998b). Prep1, a novel functional partner of Pbx proteins. EMBO J **17,** 1423-1433.

Bertolino, E., Reimund, B., Wildt-Perinic, D., and Clerc, R.G. (1995). A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. Jour Biol Chem **270,** 31178-31188.

Bezerra, I.C., Michaels, S.D., Schomburg, F.M., and Amasino, R.M. (2004). Lesions in the mRNA cap-binding gene ABA HYPERSENSITIVE 1 suppress FRIGIDA-mediated delayed flowering in Arabidopsis. Plant J **40,** 112–119.

Bharathan, G., Janssen, B.J., Kellogg, E.A., and Sinha, N. (1999). Phylogenetic relationships and evolution of the KNOTTED class of plant homeodomain proteins. Mol Biol Evol **16,** 553-563.

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

Birve, A., Sengupta, A.K., Beuchle, D., Larsson, J., Kennison, J.A., Rasmuson-Lestander, A., and Muller, J. (2001). Su(z)12, a novel Drosophila Polycomb group gene that is conserved in vertebrates and plants. Development **128,** 3371-3379.

Blazquez, M.A., and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. Nature **404,** 889-892.

Blazquez, M.A., Green, R., Nilsson, O., Sussman, M.R., and Weigel, D. (1998). Gibberellins promote flowering of arabidopsis by activating the LEAFY promoter. Plant Cell **10,** 791-800.

Blazquez, M.A., Soowal, L.N., Lee, I., and Weigel, D. (1997). LEAFY expression and flower initiation in Arabidopsis. Development **124,** 3835-3844.

Borner, R., Kampmann, G., Chandler, J., Gleissner, R., Wisman, E., Apel, K., and Melzer, S. (2000). A MADS domain gene involved in the transition to flowering in Arabidopsis. Plant J **24,** 591-599.

Boss, P.K., Bastow, R.M., Mylne, J.S., and Dean, C. (2004). Multiple pathways in the decision to flower: Enabling, promoting, and resetting. Plant Cell **16** (suppl.), S18–S31.

Bougourd, S., Marrison, J., and Haseloff, J. (2000). Technical advance: an aniline blue staining procedure for confocal microscopy and 3D imaging of normal and perturbed cellular phenotypes in mature Arabidopsis embryos. Plant J **24,** 543-550.

Bowman, J.L., and Meyerowitz, E.M. (1991). Genetic control of pattern formation during flower development in Arabidopsis. Symposia of the Society for Experimental Biology **45,** 89-115.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., and Coen, E. (1997). Inflorescence commitment and architecture in Arabidopsis. Science **275**, 80-83.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., and Coen, E. (1997). Inflorescence commitment and architecture in Arabidopsis. Science **275,** 80-83.

Brand, U., Grunewald, M., Hobe, M., and Simon, R. (2002). Regulation of CLV3 expression by two homeobox genes in Arabidopsis. Plant Physiol **129,** 565-575.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H.H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J **8,** 701-709.

Bürglin, T.R. (1995). The Evolution of Homeobox genesin 'Biodiversity and Evolution', Arai, R., Kato, M., Doi, Y., ed (Tokyo: The National Science Museum Foundation), pp. 291-336.

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

Bürglin, T.R. (1998). The PBC domain contains a MEINOX domain: coevolution of Hox and TALE homeobox genes? Dev Genes Evol **208**, 113-116.

Bürglin, T.R., and Ruvkun, G. (1992). New motif in PBX genes. Nat Genet **1,** 319-320.

Byrne, M.E. (2006). Shoot meristem function and leaf polarity: the role of class III HD-ZIP genes. PLoS Genet **2,** e89.

Byrne, M.E., Barley, R., Curtis, M., Arroyo, J.M., Dunham, M., Hudson, A., and Martienssen, R.A. (2000). Asymmetric leaves1 mediates leaf patterning and stem cell function in Arabidopsis. Nature **408,** 967-971.

Byrne, M.E., Groover, A.T., Fontana, J.R., and Martienssen R.A. (2003). Phyllotactic pattern and stem cell fate are determined by the *Arabidopsis* homeobox gene *BELLRINGER*. Development **130**, 3941–3950.

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

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science **298,** 1039-1043.

Carballo, E., Lai, W.S., and Blackshear, P.J. (1998). Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science **281,** 1001-1005.

Carrozza, M.J., Li, B., Florens, L., Suganuma, T., Swanson, S.K., Lee, K.K., Shia, W.J., Anderson, S., Yates, J., Washburn, M.P., and Workman, J.L. (2005). Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell **123,** 581-592.

Chan, R.L., Gago, G.M., Palena, C.M., and Gonzalez, D.H. (1998). Homeoboxes in plant development. Bioch Biophys Acta **1442,** 1-19.

Chan, S.K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R.S. (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. Cell **78,** 603-615.

Chan, S.K., Ryoo, H.D., Gould, A., Krumlauf, R., and Mann, R.S. (1997). Switching the in vivo specificity of a minimal Hox-responsive element. Development **124,** 2007-2014.

Chandler, J., Wilson, A., and Dean, C. (1996). Arabidopsis mutants showing an altered response to vernalization. Plant J **10,** 637-644.

Chang, C.P., Jacobs, Y., Nakamura, T., Jenkins, N.A., Copeland, N.G., and Cleary, M.L. (1997). Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. Mol Cell Biol **17,** 5679-5687.

Chattopadhyay, S., Ang, L.H., Puente, P., Deng, X.W., and Wei, N. (1998). Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. Plant Cell **10,** 673-683.

Chen, H., Banerjee, A.K., and Hannapel, D.J. (2004). The tandem complex of BEL and KNOX partners is required for transcriptional repression of ga20ox1. Plant J **38,** 276-284.

Chen, H., Rosin, F.M., Prat, S., and Hannapel, D.J. (2003). Interacting transcription factors from the three-amino acid loop extension superclass regulate tuber formation. Plant Physiol **132,** 1391-1404.

Chen, L., Cheng, J.C., Castle, L., and Sung, Z.R. (1997). EMF genes regulate Arabidopsis inflorescence development. Plant Cell **9,** 2011-2024.

Cheng, Y., Kato, N., Wang, W., Li, J., and Chen, X. (2003). Two RNA binding proteins, HEN4 and HUA1, act in the processing of AGAMOUS pre-mRNA in Arabidopsis thaliana. Dev Cell **4,** 53-66.

Choi, K., Kim, S., Kim, S.Y., Kim, M., Hyun, Y., Lee, H., Choe, S., Kim, S.G., Michaels, S., and Lee, I. (2005). *SUPPRESSOR OF FRIGIDA3* encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in Arabidopsis. Plant Cell **17**, 2647-2660.

Chouard, P. (1960). Vernalization and its relations to dormancy. Annu Rev Plant Physiol **11,** 191–238.

Clark, S.E., Jacobsen, S.E., Levin, J.Z., and Meyerowitz, E.M. (1996). The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in Arabidopsis. Development **122,** 1567-1575.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1993). CLAVATA1, a regulator of meristem and flower development in Arabidopsis. Development **119,** 397-418.

Clarke, J.H., Tack, D., Findlay, K., Van Montagu, M., and Van Lijsebettens, M. (1999). The SERRATE locus controls the formation of the early juvenile leaves and phase length in Arabidopsis. Plant J **20,** 493-501.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: genetic interactions controlling flower development. Nature **353,** 31-37.

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

Czechowski, T., Bari, R.P., Stitt, M., Scheible, W.R., and Udvardi, M.K. (2004). Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J **38**, 366-379.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell **111,** 185-196.

De Bodt, S., Raes, J., Florquin, K., Rombauts, S., Rouze, P., Theissen, G., and Van de Peer, Y. (2003). Genomewide structural annotation and evolutionary analysis of the type I MADS-box genes in plants. Jour Mol Evol **56,** 573-586.

de la Serna, I.L., Ohkawa, Y., Berkes, C.A., Bergstrom, D.A., Dacwag, C.S., Tapscott, S.J., Imbalzano, A.N. (2005). MyoD targets chromatin remodeling complexes to the myogenin locus prior to forming a stable DNA-bound complex. Mol Cell Biol **10**, 3997-4009.

Deal, R.B., Kandasamy, M.K., McKinney, E.C., and Meagher, R.B. (2005). The nuclear actin-related protein ARP6 is a pleiotropic developmental regulator required for the maintenance of *FLOWERING LOCUS C* expression and repression of flowering in Arabidopsis. Plant Cell **17**, 2633-2646.

Deal, R.B., Topp, C.N., McKinney, E.C., and Meagher, R.B. (2007). Repression of Flowering in Arabidopsis Requires Activation of FLOWERING LOCUS C Expression by the Histone Variant H2A.Z. Plant Cell **19,** 74-83.

Dean, G., Casson, S., and Lindsey, K. (2004). KNAT6 gene of Arabidopsis is expressed in roots and is required for correct lateral root formation. Plant Mol Boil **54,** 71-84.

Deng, W.W., Liu, C.Y., Pei, Y.X., Deng, X., Niu, L.F., and Cao, X.F. (2007). Involvement of the histone acetyltransferase AtHAC1 in the regulation of flowering time via repression of FLC in Arabidopsis. Plant Physiol 10.1104/pp.107.095521.

van Dijken, A.J., Schluepmann, H., and Smeekens, S.C. (2004). Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. Plant physiol 135, 969-977.

Dockx, J., Quaedvlieg, N., Keultjes, G., Kock, P., Weisbeek, P., and Smeekens, S. (1995). The homeobox gene ATK1 of Arabidopsis thaliana is expressed in the shoot apex of the seedling and in flowers and inflorescence stems of mature plants. Plant Mol. Biol. **28**, 723-37.

Douglas, S.J., Chuck, G., Dengler, R.E., Pelecanda, L., and Riggs, C.D. (2002). KNAT1 and ERECTA regulate inflorescence architecture in Arabidopsis. Plant Cell **14,** 547-558.

Doyle, M.R., Bizzell, C.M., Keller, M.R., Michaels, S.D., Song, J., Noh, Y.S., and Amasino, R.M. (2005). HUA2 is required for the expression of floral repressors in Arabidopsis thaliana. Plant J **41**:376-3785.

Ebner, A., Cabernard, C., Affolter, M., and Merabet, S. (2005). Recognition of distinct target sites by a unique Labial/Extradenticle/Homothorax complex. Development **132,** 1591-1600.

El-Din El-Assal, S., Alonso-Blanco, C., Peeters, A.J., Raz, V., and Koornneef, M. (2001). A QTL for flowering time in Arabidopsis reveals a novel allele of *CRY2*. Nat Genet **29**, 435-440.

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

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

Ferrandiz, C., Liljegren, S.J., and Yanofsky, M.F. (2000b). Negative regulation of the SHATTERPROOF genes by FRUITFULL during Arabidopsis fruit development. Science **289,** 436-438.

Ferretti, E., Marshall, H., Popperl, H., Maconochie, M., Krumlauf, R., and Blasi, F. (2000). Segmental expression of Hoxb2 in r4 requires two separate sites that integrate cooperative interactions between Prep1, Pbx and Hox proteins. Development **127,** 155-166.

Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev **17,** 1870-1881.

Fletcher, J.C. (2002). Coordination of cell proliferation and cell fate decisions in the angiosperm shoot apical meristem. Bioessays **24,** 27-37.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. Science **283,** 1911-1914.

Fognani, C., Kilstrup-Nielsen, C., Berthelsen, J., Ferretti, E., Zappavigna, V., and Blasi, F. (2002). Characterization of PREP2, a paralog of PREP1, which defines a novel sub-family of the MEINOX TALE homeodomain transcription factors. Nuc Acids Res **30,** 2043-2051.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell **90,** 1051-1060.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature **390,** 308-311.

Gazzani, S., Gendall, A.R., Lister, C., and Dean, C. (2003). Analysis of the molecular basis of flowering time variation in Arabidopsis accessions. Plant Physiol **132,** 1107–1114.

Gebelein, B., McKay, D.J., and Mann, R.S. (2004). Direct integration of Hox and segmentation gene inputs during Drosophila development. Nature **431,** 653-659.

Gehring, W. (1967). Formation of a complete mid leg with sternopleura in the antennae region by the mutant *Nasobemia* (*Ns*) of *Drosophila melanogaster*. Arch Julius Klaus- Stift Vererbungsforsch Sozialanthropol Rassenhyg **41**, 44-54.

Gehring, W.J. (1987). Homeoboxes in the study of development. Science **236,** 1245-1252.

Gendall, A.R., Levy, Y.Y., Wilson, A., and Dean, C. (2001). The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell **107,** 525–535.

Gonzalez-Crespo, S., Abu-Shaar, M., Torres, M., Martinez, A.C., Mann, R.S., and Morata, G. (1998). Antagonism between extradenticle function and Hedgehog signalling in the developing limb. Nature **394,** 196-200.

Goodrich, J., and Tweedie, S. (2002). Remembrance of things past: chromatin remodeling in plant development. Ann. Rev. Cell Dev Biol **18,** 707-746.

Gorlich, D., and Mattaj, I.W. (1996). Nucleocytoplasmic transport. Science **271,** 1513-1518.

Goto, K., and Meyerowitz, E.M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. Genes Dev **8,** 1548-1560.

Grbic, B., and Bleecker, A.B. (1996). An altered body plan is conferred on Arabidopsis plants carrying dominant alleles of two genes. Development **122**, 2395-2403.

Grbic, V. (2005) Comparative analysis of axillary and floral meristem development. Can J Bot **83,** 343-349.

Greb, T., Mylne, J.S., Crevillen, P., Geraldo, N., An, H., Gendall, A.R., and Dean, C. (2007). The PHD finger protein VRN5 functions in the epigenetic silencing of Arabidopsis FLC. Curr Biol **17,** 73-78.

Grigg, S.P., Canales, C., Hay, A., and Tsiantis, M. (2005) SERRATE coordinates shoot meristem function and leaf axial patterning in Arabidopsis. Nature **437**, 1022-1026.

Gu, Q., Ferrandiz, C., Yanofsky, M.F., and Martienssen, R. (1998). The FRUITFULL MADS-box gene mediates cell differentiation during Arabidopsis fruit development. Development **125,** 1509-1517.

Gustafson-Brown, C., Savidge, B., and Yanofsky, M.F. (1994). Regulation of the arabidopsis floral homeotic gene APETALA1. Cell **76,** 131-143.

Guyomarch, S., Benhamed, M., Lemonnier, G., Renou, J.P., Zhou, D.X., and Delarue, M. (2006). MGOUN3: evidence for chromatin-mediated regulation of FLC expression. Jour Exp Bot **57,** 2111-2119.

Guyomarch, S., Vernoux, T., Traas, J., Zhou, D.X., and Delarue, M. (2004). MGOUN3, an Arabidopsis gene with TetratricoPeptide-Repeat-related motifs, regulates meristem cellular organization. Jour Exp Bot **55,** 673-684.

Haasen, D., Kohler, C., Neuhaus, G., and Merkle, T. (1999). Nuclear export of proteins in plants: AtXPO1 is the export receptor for leucine-rich nuclear export signals in Arabidopsis thaliana. Plant J **20,** 695-705.

Hackbusch, J., Richter, K., Muller, J., Salamini, F., and Uhrig, J.F. (2005). A central role of Arabidopsis thaliana ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. Proc Natl Acad Sci USA **102,** 4908-4912.

Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M., and Laux, T. (2004). Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in Arabidopsis thaliana. Development **131,** 657-668.

Hake, S., Smith, H.M., Holtan, H., Magnani, E., Mele, G., and Ramirez, J. (2004). The role of knox genes in plant development. Ann Rev Cell Dev Biol **20,** 125-151.

Haller, K., Rambaldi, I., Daniels, E., and Featherstone, M. (2004). Subcellular localization of multiple PREP2 isoforms is regulated by actin, tubulin, and nuclear export. Jour Biol Chem **279,** 49384-49394.

Haller, K., Rambaldi, I., Kovacs, E.N., Daniels, E., and Featherstone, M. (2002). Prep2: cloning and expression of a new prep family member. Dev Dyn **225,** 358-364.

Haung, M.D., and Yang, C.H. (1998). EMF genes interact with late-flowering genes to regulate Arabidopsis shoot development. Plant Cell Physiol **39,** 382-393.

He, Y., and Amasino, R.M. (2005). Role of chromatin modification in flowering-time control. Trends Plant Sci **10,** 30–35.

He, Y., Doyle, M.D., and Amasino, R.M. (2004). PAF1 complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in *Arabidopsis.* Genes Dev **18,** 2774–2784.

He, Y., Michaels, S.D., and Amasino, R.M. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. Science **302,** 1751–1754.

Hempel, F.D., Weigel, D., Mandel, M.A., Ditta, G., Zambryski, P.C., Feldman, L.J., and Yanofsky, M.F. (1997). Floral determination and expression of floral regulatory genes in Arabidopsis. Development **124,** 3845-3853.

Hempel, F.D., Welch, D.R., and Feldman, L.J. (2000). Floral induction and determination: where is flowering controlled? Trends Plant Sci **5,** 17-21.

Henderson, B.R., and Eleftheriou, A. (2000). A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. Exp Cell Res **256,** 213-224.

Hepworth, S.R., Valverde, F., Ravenscroft, D., Mouradov, A., Coupland, G. (2002). Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. EMBO J **21**, 4327-4337.

Hibara, K., Karim, M.R., Takada, S., Taoka, K., Furutani, M., Aida, M., and Tasaka, M. (2006). Arabidopsis CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. Plant Cell **18,** 2946-2957.

Hoey, T., and Levine, M. (1988). Divergent homeo box proteins recognize similar DNA sequences in Drosophila. Nature **332,** 858-861.

Hong, R.L., Hamaguchi, L., Busch, M.A., and Weigel, D. (2003). Regulatory elements of the floral homeotic gene *AGAMOUS* identified by phylogenetic footprinting and shadowing. Plant Cell **15**, 1296-1309.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol Cell **9,** 789-798.

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

Huang, T., Bohlenius, H., Eriksson, S., Parcy, F., and Nilsson, O. (2005). The mRNA of the Arabidopsis gene *FT* moves from leaf to shoot apex and induces flowering. Science **309**, 1694-1696.

Irish, V.F., and Litt, A. (2005). Flower development and evolution: gene duplication, diversification and redeployment. Curr Opin Gen Dev **15,** 454-460.

Irish, V.F., and Sussex, I.M. (1990). Function of the apetala-1 gene during Arabidopsis floral development. Plant Cell **2,** 741-753.

Ito, M., Sato, Y., and Matsuoka, M. (2002). Involvement of homeobox genes in early body plan of monocot. Int Rev Cyt **218,** 1-35.

Jack, T., Brockman, L.L., and Meyerowitz, E.M. (1992). The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell **68,** 683-697.

Jacobs, Y., Schnabel, C.A., and Cleary, M.L. (1999). Trimeric association of Hox and TALE homeodomain proteins mediates Hoxb2 hindbrain enhancer activity. Mol Cell Biol **19,** 5134-5142.

James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144,** 1425-1436.

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

Jaw, T.J., You, L.R., Knoepfler, P.S., Yao, L.C., Pai, C.Y., Tang, C.Y., Chang, L.P., Berthelsen, J., Blasi, F., Kamps, M.P., and Sun, Y.H. (2000). Direct interaction of two homeoproteins, homothorax and extradenticle, is essential for EXD nuclear localization and function. Mech Dev **91,** 279-291.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6**, 3901-3907.

Jeong, S., Trotochaud, A.E., and Clark, S.E. (1999). The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. Plant Cell **11,** 1925-1934.

Jofuku, K.D., den Boer, B.G., Van Montagu, M., and Okamuro, J.K. (1994). Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell **6,** 1211-1225.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. Science **290,** 344–347.

Kamps, M.P., Murre, C., Sun, X.H., and Baltimore, D. (1990). A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell **60,** 547-555.

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

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J., and Weigel, D. (1999). Activation tagging of the floral inducer FT. Science **286,** 1962–1965.

Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7,** 193-195.

Kayes, J.M., and Clark, S.E. (1998). CLAVATA2, a regulator of meristem and organ development in Arabidopsis. Development **125,** 3843-3851.

Kempin, S.A., Savidge, B., and Yanofsky, M.F. (1995). Molecular basis of the cauliflower phenotype in Arabidopsis. Science **267,** 522-525.

Kerstetter, R., Vollbrecht, E., Lowe, B., Veit, B., Yamaguchi, J., and Hake, S. (1994). Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. Plant Cell **6,** 1877-1887.

Kerstetter, R.A., Laudencia-Chingcuanco, D., Smith, L.G., and Hake, S. (1997). Loss-of-function mutations in the maize homeobox gene, knotted1, are defective in shoot meristem maintenance. Development **124,** 3045-3054.

Kilstrup-Nielsen, C., Alessio, M., and Zappavigna, V. (2003). PBX1 nuclear export is regulated independently of PBX-MEINOX interaction by PKA phosphorylation of the PBC-B domain. EMBO J **22,** 89-99.

Kim, H.J., Hyun, Y., Park, J.Y., Park, M.J., Park, M.K., Kim, M.D., Lee, M.H., Moon, J., Lee, I., and Kim, J. (2004). A genetic link between cold responses and flowering time through *FVE* in *Arabidopsis thaliana*. Nat Genet **36,** 167–171.

Kim, S., Choi, K., Park, C., Hwang, H.J., and Lee, I. (2006b). SUPPRESSOR OF FRIGIDA4, encoding a C2H2-Type zinc finger protein, represses flowering by transcriptional activation of Arabidopsis FLOWERING LOCUS C. Plant Cell **18,** 2985-2998.

Kim, S.Y., and Michaels, S.D. (2006a). SUPPRESSOR OF FRI 4 encodes a nuclear-localized protein that is required for delayed flowering in winter-annual Arabidopsis. Development **133,** 4699-4707.

Kim, S.Y., He, Y., Jacob, Y., Noh, Y.S., Michaels, S., and Amasino, R. (2005). Establishment of the vernalization-responsive, winter-annual habit in Arabidopsis requires a putative histone H3 methyl transferase. Plant Cell **17**, 3301-10.

Kizer, K.O., Phatnani, H.P., Shibata, Y., Hall, H., Greenleaf, A.L., and Strahl, B.D. (2005). A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol Cell Biol **25,** 3305-3316.

Knoepfler, P.S., and Kamps, M.P. (1995). The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. Mol Cell Biol **15,** 5811-5819.

Knoepfler, P.S., Calvo, K.R., Chen, H., Antonarakis, S.E., and Kamps, M.P. (1997). Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a-Pbx1. Proc Natl Acad Sci USA **94,** 14553-14558.

Kobayashi, M., Fujioka, M., Tolkunova, E.N., Deka, D., Abu-Shaar, M., Mann, R.S., and Jaynes, J.B. (2003). Engrailed cooperates with extradenticle and homothorax to repress target genes in Drosophila. Development **130,** 741-751.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. Science **286,** 1960–1962.

Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D., Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D., and Rine, J. (2004). A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol **2,** E131.

Komeda, Y. (2004). Genetic regulation of time to flower in Arabidopsis thaliana. Annu Rev Plant Biol **55,** 521-535.

Koornneef, M., Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C.J., and Peeters, A.J. (1998). Genetic interactions among late-flowering mutants of Arabidopsis. Genetics **148,** 885-892.

Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe, W., and Peeters, T. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild type. Plant J **6,** 911–919.

Koornneef, M., Hanhart, C.J., and van der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. Mol Gen Genet **229,** 57-66.

Kotak, S., Port, M., Ganguli, A., Bicker, F., and von Koskull-Doring, P. (2004). Characterization of C-terminal domains of Arabidopsis heat stress transcription factors (Hsfs) and identification of a new signature combination of plant class A Hsfs with AHA and NES motifs essential for activator function and intracellular localization. Plant J **39,** 98-112.

Krogan, N.J., Keogh, M.C., Datta, N., Sawa, C., Ryan, O.W., Ding, H., Haw, R.A., Pootoolal, J., Tong, A., Canadien, V., Richards, D.P., Wu, X., Emili, A., Hughes, T.R., Buratowski, S., and Greenblatt, J.F. (2003). A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. Mol Cell **12,** 1565-1576.

Kudo, N., Khochbin, S., Nishi, K., Kitano, K., Yanagida, M., Yoshida, M., and Horinouchi, S. (1997). Molecular cloning and cell cycle-dependent expression of mammalian CRM1, a protein involved in nuclear export of proteins. Jour Biol Chem **272,** 29742-29751.

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E.P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proc Natl Acad Sci USA **96,** 9112-9117.

Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E.P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp Cell Res **242,** 540-547.

Kumar, R., Kushalappa, K., Godt, D., Pidkowich, M., Pastorelli, S., Hepworth, S., and Haughn, G. (2006). The Arabidopsis homeodomain proteins SAW1 and SAW2 control leaf development through negative regulation of KNOX function. Abstract 196, 17th International Conference on Arabidopsis Research, June 28-July 2, 2006, Madison (WI), USA.

Kumaran, M.K., Bowman, J.L., and Sundaresan, V. (2002). *YABBY* Polarity Genes Mediate the Repression of *KNOX* Homeobox Genes in Arabidopsis. Plant Cell **14**, 2761-2770.

Kurant, E., Pai, C.Y., Sharf, R., Halachmi, N., Sun, Y.H., and Salzberg, A. (1998). Dorsotonals/homothorax, the Drosophila homologue of meis1, interacts with extradenticle in patterning of the embryonic PNS. Development **125,** 1037-1048.

la Cour, T., Kiemer, L., Molgaard, A., Gupta, R., Skriver, K., and Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. Protein Eng Des Sel **17,** 527-536.

Lang, A. (1965). Physiology of flower initiation. In Encyclopedia of Plant Physiology, Ruhland W., ed (Berlin: Springer-Verlag), pp. 1371–1536.

LaRonde-LeBlanc, N.A., and Wolberger, C. (2003). Structure of HoxA9 and Pbx1 bound to DNA: Hox hexapeptide and DNA recognition anterior to posterior. Gen Dev **17,** 2060-2072.

Laufs, P., Grandjean, O., Jonak, C., Kieu, K., and Traas, J. (1998). Cellular parameters of the shoot apical meristem in Arabidopsis. Plant Cell **10,** 1375-1390.

Laux, T., Mayer, K.F., Berger, J., and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development **122,** 87-96.

Le Corre, V., Roux, F., and Reboud, X. (2002). DNA polymorphism at the *FRIGIDA* gene in Arabidopsis thaliana: extensive nonsynonymous variation is consistent with local selection for flowering time. Mol Biol Evol **19**, 1261-71

Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000). The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev **14**, 2366-2376.

Lee, I., Michaels, S.D., Masshardt, A.S., and Amasino, R.M. (1994). The late-flowering phenotype of *FRIGIDA* and *LUMINIDEPENDENS* is suppressed in the Landsberg *erecta* strain of Arabidopsis. Plant J **6,** 903–909.

Lemons, D., and McGinnis, W. (2006). Genomic evolution of Hox gene clusters. Science **313,** 1918-1922.

Lenhard, M., Jurgens, G., and Laux, T. (2002). The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in Arabidopsis shoot meristem regulation. Development **129,** 3195-3206.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendal,l A.R., and Dean, C. (2002). Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. Science **297**, 243-246.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev **3,** 662-673.

Li, Y., Rosso, M.G., Strizhov, N., Viehoever, P., and Weisshaar, B. (2003). GABI-Kat SimpleSearch: a flanking sequence tag (FST) database for the identification of T-DNA insertion mutants in Arabidopsis thaliana. Bioinformatics **19**, 1441-1442.

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

Lim, M.H., Kim, J., Kim, Y.S., Chung, K.S., Seo, Y.H., Lee, I., Kim, J., Hong, C.B., Kim, H.J., and Park, C.M. (2004). A new *Arabidopsis* gene, *FLK*, encodes an RNA binding protein with K homology motifs and regulates flowering time via FLOWERING LOCUS C, Plant Cell **16**, 731–740.

Lobbes, D., Rallapalli, G., Schmidt, D.D., Martin, C., and Clarke, J. (2006). SERRATE: a new player on the plant microRNA scene. EMBO Reports **7,** 1052-1058.

Long, J., and Barton, M.K. (2000). Initiation of axillary and floral meristems in Arabidopsis. Dev Biol **218,** 341-353.

Long, J.A., and Barton, M.K. (1998). The development of apical embryonic pattern in Arabidopsis. Development **125,** 3027-3035.

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

Longobardi, E., and Blasi, F. (2003). Overexpression of PREP-1 in F9 teratocarcinoma cells leads to a functionally relevant increase of PBX-2 by preventing its degradation. The Journal of biological chemistry **278,** 39235-39241.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997). *FCA*, a gene controlling flowering time in *Arabidopsis*, encodes a protein containing RNA-binding domains, Cell **89**, 737–745.

Macknight, R., Duroux, M., Laurie, R., Dijkwel, P., Simpson, G., and Dean, C. (2002). Functional significance of the alternative transcript processing of the Arabidopsis floral promoter FCA. Plant Cell **14,** 877-888.

Mandel, M.A., and Yanofsky, M.F. (1995a). The Arabidopsis AGL8 MADS box gene is expressed in inflorescence meristems and is negatively regulated by APETALA1. Plant Cell **7,** 1763-1771.

Mandel, M.A., and Yanofsky, M.F. (1995b). A gene triggering flower formation in Arabidopsis. Nature **377,** 522-524.

Mandel, M.A., Gustafson-Brown, C., Savidge, B., and Yanofsky, M.F. (1992). Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. Nature **360,** 273-277.

Mann, R.S., and Abu-Shaar, M. (1996). Nuclear import of the homeodomain protein extradenticle in response to Wg and Dpp signalling. Nature **383,** 630-633.

Mann, R.S., and Affolter, M. (1998). Hox proteins meet more partners. Curr Opin Genet Dev **8,** 423-429.

March-Diaz, R., Garcia-Dominguez, M., Florencio, F.J., and Reyes, J.C. (2007). SEF, a New Protein Required for Flowering Repression in Arabidopsis, Interacts with PIE1 and ARP6. Plant physiol **143,** 893-901.

Marquardt, S., Boss, P.K., Hadfield, J., and Dean, C. (2006). Additional targets of the Arabidopsis autonomous pathway members, FCA and FY. Jour Exp Bot **57,** 3379-3386.

Martin-Trillo, M., Lazaro, A., Poethig, R.S., Gomez-Mena, C., Pineiro, M.A., Martinez-Zapater, J.M., and Jarillo, J.A. (2006). EARLY IN SHORT DAYS 1 (ESD1) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates FLC accumulation in Arabidopsis. Development **133**, 1241-52.

Mayer, K.F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G., and Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell **95,** 805-815.

McConnell, J.R., and Barton, M.K. (1998). Leaf polarity and meristem formation in Arabidopsis. Development **125,** 2935-2942.

McGinnis, W., Garber, R.L., Wirz, J., Kuroiwa, A., Gehring W.J. (1984a). A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. Cell **37**, 403-408.

McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A., Gehring, W.J. (1984b). A conserved DNA sequence in homoeotic genes of the *Drosophila* Antennapedia and bithorax complexes. Nature **308**, 428-433.

Medford, J.I., Behringer, F.J., Callos, J.D., and Feldmann, K.A. (1992). Normal and abnormal development in the Arabidopsis vegetative shoot apex. Plant Cell **4**, 631-643.

Medford, J.I., Callos, J.D., Behringer, F.J., and Link, B.M. (1994): Development of the vegetative shoot apical meristem. In *Arabidopsis*, Meyerowitz, E.M., and Somerville, C.R., eds., Cold Spring Harbor Laboratory Press, New York.

Meier, C., Bouquin, T., Nielsen, M.E., Raventos, D., Mattsson, O., Rocher, A., Schomburg, F., Amasino, R.M., and Mundy, J. (2001). Gibberellin response mutants identified by luciferase imaging. Plant J **25,** 509-519.

Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., Martinez, C., and Torres, M. (1999). Conserved regulation of proximodistal limb axis development by Meis1/Hth. Nature **402,** 425-429.

Merkle, T. (2001). Nuclear import and export of proteins in plants: a tool for the regulation of signalling. Planta **213,** 499-517.

Messenguy, F., and Dubois, E. (2003). Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. Gene **316,** 1-21.

Metzger, E., Wissmann, M., Yin, N., Muller, J.M., Schneider, R., Peters, A.H., Gunther, T., Buettner, R., and Schule, R. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature **437,** 436-439.

Meyerowitz, E.M. (2002). Plants compared to animals: the broadest comparative study of development. Science **295,** 1482-1485.

Meyerowitz, E.M., Bowman, J.L., Brockman, L.L., Drews, G.N., Jack, T., Sieburth, L.E., and Weigel, D. (1991). A genetic and molecular model for flower development in Arabidopsis thaliana. Dev Suppl **1,** 157-167.

Michaels, S., and Amasino, R. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949–956.

Michaels, S., and Amasino, R. (2000). Memories of winter: vernalization and the competence to flower. Plant Cell Env **23**,1145 -1154.

Michaels, S.D., and Amasino, R.M. (2001). Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell **13,** 935–941.

Michaels, S.D., Bezerra, I.C., and Amasino, R.M. (2004). FRIGIDA-related genes are required for the winter-annual habit in Arabidopsis. Proc Natl Acad Sci USA **101,** 3281–3285.

Michaels, S.D., He, Y., Scortecci, K.C., and Amasino, R.M. (2003). Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. Proc Natl Acad Sci USA **100,** 10102–10107.

Michaels, S.D., Himelblau, E., Kim, S.Y., Schomburg, F.M., and Amasino, R.M. (2005). Integration of flowering signals in winter-annual Arabidopsis. Plant Physiol **137**, 149-156.

Mizuguchi, G., Shen, X., Landry, J., Wu, W.H., Sen, S., and Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science **303,** 343-348.

Mockler, T.C., Guo, H., Yang, H., Duong, H., and Lin, C, (1999). Antagonistic actions of Arabidopsis cryptochromes and phytochrome B in the regulation of floral induction. Development **126**, 2073-2082

Mockler, T.C., Yu, X., Shalitin, D., Parikh, D., Michael, T.P., Liou, J., Huang, J., Smith, Z., Alonso, J.M., Ecker, J.R., Chory, J., and Lin, C. (2004). Regulation of flowering time in Arabidopsis by K homology domain proteins. Proc Natl Acad Sci USA **101**, 12759-12764.

Modrusan, Z., Reiser, L., Feldmann, K.A., Fischer, R.L., and Haughn, G.W. (1994). Homeotic Transformation of Ovules into Carpel-like Structures in Arabidopsis. Plant Cell **6,** 333-349.

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

Monica, K., Galili, N., Nourse, J., Saltman, D., and Cleary, M.L. (1991). PBX2 and PBX3, new homeobox genes with extensive homology to the human proto-oncogene PBX1. Mol Cell Biol **11,** 6149-6157.

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

Moon, Y.H., Chen, L., Pan, R.L., Chang, H.S., Zhu, T., Maffeo, D.M., and Sung, Z.R. (2003). EMF genes maintain vegetative development by repressing the flower program in Arabidopsis. Plant Cell 15**,** 681-693.

Morris, S.A., Shibata, Y., Noma, K., Tsukamoto, Y., Warren, E., Temple, B., Grewal, S.I., and Strahl, B.D. (2005). Histone H3 K36 methylation is associated with transcription elongation in Schizosaccharomyces pombe. Eukar Cell **4,** 1446-1454.

Mouradov, A., Cremer, F., and Coupland, G. (2002). Control of flowering time: Interacting pathways as a basis for diversity. Plant Cell **14** (suppl.), S111–S130.

Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell **111,** 197-208.

Mylne, J.S., Barrett, L., Tessadori, F., Mesnage, S., Johnson, L., Bernatavichute, Y.V., Jacobsen, S.E., Fransz, P., and Dean, C. (2006). LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of FLC. Proc Natl Acad Sci USA **103,** 5012-5017.

Neuteboom, S.T., Peltenburg, L.T., van Dijk, M.A., and Murre, C. (1995). The hexapeptide LFPWMR in Hoxb-8 is required for cooperative DNA binding with Pbx1 and Pbx2 proteins. Proc Natl Acad Sci USA **92,** 9166-9170.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol Cell **11,** 709-719.

Noh, Y.S., and Amasino, R.M. (2003). *PIE1*, an *ISWI* family gene, is required for *FLC* activation and floral repression in Arabidopsis. Plant Cell **15,** 1671–1682.

Nourse, J., Mellentin, J.D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S.D., and Cleary, M.L. (1990). Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell **60,** 535-545.

Odell, J.T., Nagy, F., and Chua, N.H. (1985). Identification of DNA sequences required for activity of the Cauliflower Mosaic Virus 35S promoter. Nature **313**, 810-812

Oh, S., Zhang, H., Ludwig, P., and van Nocker, S. (2004). A mechanism related to the yeast transcriptional regulator Paf1c is required for expression of the Arabidopsis *FLC/MAF MADS* box gene family. Plant Cell **16,** 2940–2953.

Ohshima, S., Murata, M., Sakamoto, W., Ogura, Y., and Motoyoshi, F. (1997). Cloning and molecular analysis of the Arabidopsis gene Terminal Flower 1. Mol Gen Genet **254,** 186-194.

Oyama, T., Shimura, Y., and Okada, K. (1997). The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes Dev **11,** 2983-2995.

Pai, C.Y., Kuo, T.S., Jaw, T.J., Kurant, E., Chen, C.T., Bessarab, D.A., Salzberg, A., and Sun, Y.H. (1998). The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila. Genes Dev **12,** 435-446.

Parcy, F. (2005). Flowering: a time for integration. Int Jour Dev Biol **49,** 585-593.

Parcy, F., Nilsson, O., Busch, M.A., Lee, I., and Weigel, D. (1998). A genetic framework for floral patterning. Nature **395,** 561-566.

Passner, J.M., Ryoo, H.D., Shen, L., Mann, R.S., and Aggarwal, A.K. (1999). Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. Nature **397,** 714-719.

Pearson, J.C., Lemons, D., and McGinnis, W. (2005). Modulating Hox gene functions during animal body patterning. Nat Rev **6,** 893-904.

Peifer, M., and Wieschaus, E. (1990). Mutations in the Drosophila gene extradenticle affect the way specific homeo domain proteins regulate segmental identity. Genes Dev **4,** 1209-1223.

Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E., and Yanofsky, M.F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. Nature **405,** 200-203.

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E.R., and Yanofsky, M.F. (2001). Conversion of leaves into petals in Arabidopsis. Curr Biol **11,** 182-184.

Phelan, M.L., Rambaldi, I., and Featherstone, M.S. (1995). Cooperative interactions between HOX and PBX proteins mediated by a conserved peptide motif. Mol Cell biol **15,** 3989-3997.

Pinsonneault, J., Florence, B., Vaessin, H., and McGinnis, W. (1997). A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. EMBO J **16,** 2032-2042.

Piper, D.E., Batchelor, A.H., Chang, C.P., Cleary, M.L., and Wolberger, C. (1999). Structure of a HoxB1-Pbx1 heterodimer bound to DNA: role of the hexapeptide and a fourth homeodomain helix in complex formation. Cell **96,** 587-597.

Poduska, B., Humphrey, T., Redweik, A., and Grbic, V. (2003). The synergistic activation of *FLOWERING LOCUS C* by FRIGIDA and a new flowering gene *AERIAL ROSETTE 1* underlies a novel morphology in Arabidopsis. Genetics **163**, 1457-1465.

Popperl, H., Rikhof, H., Chang, H., Haffter, P., Kimmel, C.B., and Moens, C.B. (2000). lazarus is a novel pbx gene that globally mediates hox gene function in zebrafish. Mol Cell **6,** 255-267.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. (1995). The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell **80,** 847–857.

Quaedvlieg, N., Dockx, J., Rook, F., Weisbeek, P., and Smeekens, S. (1995). The homeobox gene *ATH1* of Arabidopsis is derepressed in the photomorphogenic mutants *cop1* and *det1*. Plant Cell **7**, 117-29.

Quesada, V., Dean, C., and Simpson, G.G. (2005). Regulated RNA processing in the control of Arabidopsis flowering. Int J Dev Biol **49**, 773-780.

Quesada, V., Macknight, R., Dean, C., and Simpson, G.G. (2003). Autoregulation of FCA pre-mRNA processing controls Arabidopsis flowering time. EMBO J **22,** 3142-3152.

Ratcliffe, O.J., Amaya, I., Vincent, C.A., Rothstein, S., Carpenter, R., Coen, E.S., and Bradley, D.J. (1998). A common mechanism controls the life cycle and architecture of plants. Development **125**,1609-1615.

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J., and Riechmann, J.L. (2003). Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: *MAF2* prevents vernalization by short periods of cold. Plant Cell **15,** 1159–1169.

Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L., and Riechmann, J.L. (2001). Regulation of flowering in Arabidopsis by an *FLC* homologue. Plant Physiol **126,** 122–132.

Rauskolb, C., Peifer, M., and Wieschaus, E. (1993). extradenticle, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene pbx1. Cell **74,** 1101-1112.

Reeves, P.H., and Coupland, G. (2001). Analysis of flowering time control in Arabidopsis by comparison of double and triple mutants. Plant physiol **126,** 1085-1091.

Reiser, L., Sanchez-Baracaldo, P., and Hake, S. (2000). Knots in the family tree: evolutionary relationships and functions of knox homeobox genes. Plant Mol Biol **42,** 151-166.

Riechmann, J.L., and Meyerowitz, E.M. (1997). MADS domain proteins in plant development. Biol Chem **378,** 1079-1101.

Rieckhof, G.E., Casares, F., Ryoo, H.D., Abu-Shaar, M., and Mann, R.S. (1997). Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. Cell **91,** 171-183.

Robinson-Beers, K., Pruitt, R.E., and Gasser, C.S. (1992). Ovule Development in Wild-Type Arabidopsis and Two Female-Sterile Mutants. Plant Cell **4,** 1237-1249.

Roeder, A.H., Ferrandiz, C., and Yanofsky M.F. (2003). The role of the REPLUMLESS homeodomain protein in patterning the *Arabidopsis* fruit. Curr Biol **13**, 1630–1635.

Roguev, A., Schaft, D., Shevchenko, A., Pijnappel, W.W., Wilm, M., Aasland, R., and Stewart, A.F. (2001). The Saccharomyces cerevisiae Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. EMBO J **20,** 7137-7148.

Rook, F., Gerrits, N., Kortstee, A., van Kampen, M., Borrias, M., Weisbeek, P., and Smeekens, S. (1998). Sucrose-specific signalling represses translation of the Arabidopsis ATB2 bZIP transcription factor gene. Plant J **15**, 253-63.

Ruhl, D.D., Jin, J., Cai, Y., Swanson, S., Florens, L., Washburn, M.P., Conaway, R.C., Conaway, J.W., and Chrivia, J.C. (2006). Purification of a human SRCAP complex that remodels chromatin by incorporating the histone variant H2A.Z into nucleosomes. Biochem **45,** 5671-5677.

Ryoo, H.D., and Mann, R.S. (1999). The control of trunk Hox specificity and activity by Extradenticle. Genes Dev **13,** 1704-1716.

Ryoo, H.D., Marty, T., Casares, F., Affolter, M., and Mann, R.S. (1999). Regulation of Hox target genes by a DNA bound Homothorax/Hox/Extradenticle complex. Development **126,** 5137-5148.

Sablowski, R. (2004). Plant and animal stem cells: conceptually similar, molecularly distinct? Trends Cell Biol **14,** 605-611.

Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S., and Matsuoka, M. (2001). KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes Dev **15,** 581-590.

Saleh, M., Huang, H., Green, N.C., and Featherstone, M.S. (2000). A conformational change in PBX1A is necessary for its nuclear localization. Exp Cell Res **260,** 105-115.

Saleh, M., Rambaldi, I., Yang, X.-J., and Featherstone, M.S. (2000). Cell signaling switches HOX–PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases Mol Cell Biol **20**, 8623-8633.

Saleh, M., Rambaldi, I., Yang, X.J., and Featherstone, M.S. (2000). Cell signaling switches HOX-PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. Mol Cell Biol **20,** 8623-8633.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288,** 1613–1616.

Sanda, S.L., and Amasino, R.M. (1995). Genetic and physiological analysis of flowering time in the C24 line of Arabidopsis thaliana. Weeds World **2**, 2-8.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. Nature **419,** 407-411.

Savidge, B., Rounsley, S.D., and Yanofsky, M.F. (1995). Temporal relationship between the transcription of two Arabidopsis MADS box genes and the floral organ identity genes. Plant Cell **7,** 721-733.

Schiestl, R.H., Dominska, M., and Petes, T.D. (1993). Transformation of Saccharomyces cerevisiae with nonhomologous DNA: illegitimate integration of transforming DNA into yeast chromosomes and in vivo ligation of transforming DNA to mitochondrial DNA sequences. Mol Cell Biol **13,** 2697-2705.

Schläppi, M. (2001). RNA levels and activity of *FLOWERING LOCUS C* are modified in mixed genetic backgrounds of *Arabidopsis thaliana*. Int. J. Plant Sci. **162**, 527-537.

Schläppi, M.R. (2006). FRIGIDA LIKE 2 is a functional allele in Landsberg erecta and compensates for a nonsense allele of FRIGIDA LIKE 1. Plant Physiol **142,** 1728-1738.

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

Schmitz, R.J., Hong, L., Michaels, S., and Amasino, R.M. (2005). *FRIGIDA-ESSENTIAL 1* interacts genetically with *FRIGIDA* and *FRIGIDA-LIKE 1* to promote the winter-annual habit of Arabidopsis thaliana. Development **132**, 5471-5478.

Schnewly, S., Klemenz, R., Gehring, W.J. (1987). Redesigning the body plan of *Drosophila* by ectopic expression of the homeotic gene *Antennapedia*. Nature **325**, 816-818.

Schomburg, F.M., Patton, D.A., Meinke, D.W., and Amasino, R.M. (2001). *FPA*, a gene involved in floral induction in *Arabidopsis*, encodes a protein containing RNA-recognition motifs. Plant Cell **13**, 1427–1436.

Schultz, E.A., and Haughn, G.W. (1991). LEAFY, a Homeotic Gene That Regulates Inflorescence Development in Arabidopsis. Plant Cell **3,** 771-781.

Scortecci, K., Michaels, S.D., and Amasino, R.M. (2003). Genetic interactions between *FLM* and other flowering-time genes in *Arabidopsis thaliana*. Plant Mol Biol **52,** 915–922.

Scortecci, K.C., Michaels, S.D., and Amasino, R.M. (2001). Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. Plant J **26,** 229–236.

Scott, M.P., Weiner, A.J. (1984). Structural relationships among genes that control development: sequence homology between the *Antennapedia*, *Ultrabithorax*, and *fushi tarazu* loci of *Drosophila*. Proc Natl Acad Sci USA **81**, 4115-4119.

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

Selleri, L., Depew, M.J., Jacobs, Y., Chanda, S.K., Tsang, K.Y., Cheah, K.S., Rubenstein, J.L., O'Gorman, S., and Cleary, M.L. (2001). Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation. Development **128,** 3543-3557.

Selleri, L., DiMartino, J., van Deursen, J., Brendolan, A., Sanyal, M., Boon, E., Capellini, T., Smith, K.S., Rhee, J., Popperl, H., Grosveld, G., and Cleary, M.L. (2004). The TALE homeodomain protein Pbx2 is not essential for development and long-term survival. Mol Cell Biol **24,** 5324-5331.

Serikawa, K.A., and Zambryski, P.C. (1997). Domain exchanges between KNAT3 and KNAT1 suggest specificity of the kn1-like homeodomains requires sequences outside of the third helix and N-terminal arm of the homeodomain. Plant J **11,** 863-869.

Serikawa, K.A., Martinez-Laborda, A., Kim, H.S., and Zambryski, P.C. (1997). Localization of expression of KNAT3, a class 2 knotted1-like gene. Plant J **11,** 853-861.

Shannon, S., and Meeks-Wagner, D.R. (1991). A Mutation in the Arabidopsis TFL1 Gene Affects Inflorescence Meristem Development. Plant Cell **3,** 877-892.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The *FLF MADS* box gene: A repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11,** 445–458.

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

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell **119,** 941-953.

Shi, Y.J., Matson, C., Lan, F., Iwase, S., Baba, T., and Shi, Y. (2005). Regulation of LSD1 histone demethylase activity by its associated factors. Mol Cell **19,** 857-864.

Shim, S., Kim, Y., Shin, J., Kim, J., and Park, S. (2007). Regulation of EphA8 gene expression by TALE homeobox transcription factors during development of the mesencephalon. Mol Cell Biol **27,** 1614-1630.

Shindo, C., Aranzana, M.J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M., and Dean, C. (2005). Role of FRIGIDA and FLOWERING LOCUS C in determining variation in flowering time of Arabidopsis. Plant Physiol **138**, 1163-73.

Simpson, G.G. (2004). The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. Curr Opin Plant Biol **7**, 570-574.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., and Dean, C. (2003). FY is an RNA 3' end-processing factor that interacts with FCA to control the *Arabidopsis* floral transition. Cell **113**, 777–787.

Smith, H.M.S, and Hake, S. (2003). The interaction of two homeobox genes, *BREVIPEDICELLUS* and *PENNYWISE*, regulates internode patterning in the *Arabidopsis* inflorescence. Plant Cell **15**, 1717–1727.

Smith, H.M.S, Campbell, B.C., and Hake, S. (2004). Competence to respond to floral inductive signals requires the homeobox genes *PENNYWISE* and *POUND-FOOLISH*. Curr Biol **14**, 812–817.

Soppe, W.J., Bentsink, L., and Koornneef, M. (1999). The early-flowering mutant *efs* is involved in the autonomous promotion pathway of *Arabidopsis thaliana*. Development **126,** 4763–4770.

Squazzo, S.L., Costa, P.J., Lindstrom, D.L., Kumer, K.E., Simic, R., Jennings, J.L., Link, A.J., Arndt, K.M., and Hartzog, G.A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. EMBO J **21,** 1764-1774.

Stevens, K., and Mann, R. (2007). A balance between two NLSs and a NES governs Extradenticle subcellular localization. Genetics 10.1534/Genetics106.0664499.

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

Subramanian, C., Kim, B.H., Lyssenko, N.N., Xu, X., Johnson, C.H., and von Arnim, A.G. (2004). The Arabidopsis repressor of light signaling, COP1, is regulated by nuclear exclusion: mutational analysis by bioluminescence resonance energy transfer. Proc Nat Acad Sci USA **101,** 6798-6802.

Sung S., and Amasino R.M. (2006) Molecular genetic studies of the memory of winter. J Exp Bot **57**, 3369–3377.

Sung, S., and Amasino, R.M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature **427,** 159–164.

Sung, S., and Amasino, R.M. (2005). Remembering Winter: Toward a Molecular Understanding of Vernalization. Annu Rev Plant Biol **56**, 491-508.

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

Sung, S., Schmitz, R.J., and Amasino, R.M. (2006a). A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. Genes Dev **20,** 3244-3248.

Suzuki, T., Nakajima, S., Inagaki, S., Hirano-Nakakita, M., Matsuoka, K., Demura, T., Fukuda, H., Morikami, A., and Nakamura, K. (2005). TONSOKU is expressed in S phase of the cell cycle and its defect delays cell cycle progression in Arabidopsis. Plant Cell Physiol **46,** 736-742.

Takada, S., and Goto, K. (2003). TERMINAL FLOWER2, an Arabidopsis homolog of HETEROCHROMATIN PROTEIN1, counteracts the activation of *FLOWERING LOCUS T* by CONSTANS in the vascular tissues of leaves to regulate flowering time. Plant Cell **15,** 2856–2865.

Takada, S., Hibara, K., Ishida, T., and Tasaka, M. (2001). The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. Development **128,** 1127-1135.

Takeda, S., Tadele, Z., Hofmann, I., Probst, A.V., Angelis, K.J., Kaya, H., Araki, T., Mengiste, T., Mittelsten Scheid, O., Shibahara, K., Scheel, D., and Paszkowski, J. (2004). BRU1, a novel link between responses to DNA damage and epigenetic gene silencing in Arabidopsis. Genes Dev **18,** 782-793.

Trotochaud, A.E., Hao, T., Wu, G., Yang, Z., and Clark, S.E. (1999). The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. Plant Cell **11,** 393-406.

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

Tsiantis, M., and Hay, A. (2003). Comparative plant development: the time of the leaf? Nature Rev **4,** 169-180.

van der Valk, P., Proveniers, M.C.G., Pertijs, J. H., Lamers, J.T.W.H., van Dun, C.M.P., and Smeekens, J.C.M. (2004). Late heading of perennial ryegrass caused by introducing an Arabidopsis homeobox gene. Plant Breeding **123**, 531-535.

Venglat, S.P., Dumonceaux, T., Rozwadowski, K., Parnell, L., Babic, V., Keller, W., Martienssen, R., Selvaraj, G., and Datla, R. (2002). The homeobox gene BREVIPEDICELLUS is a key regulator of inflorescence architecture in Arabidopsis. Proc Natl Acad Sci USA **99,** 4730-4735.

Vollbrecht, E., Reiser, L., and Hake, S. (2000). Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, knotted1. Development **127,** 3161-3172.

Vollbrecht, E., Veit, B., Sinha, N., and Hake, S. (1991). The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature **350,** 241-243.

Wagner, D., Sablowski, R.W., and Meyerowitz, E.M. (1999). Transcriptional activation of APETALA1 by LEAFY. Science **285,** 582-584.

Wagner, K., Mincheva, A., Korn, B., Lichter, P., and Popperl, H. (2001). Pbx4, a new Pbx family member on mouse chromosome 8, is expressed during spermatogenesis. Mech Dev **103,** 127-131.

Walter, M., Chaban, C., Schutze, K., Batistic, O., Weckermann, K., Nake, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K., and Kudla, J. (2004). Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J **40,** 428-438.

Waskiewicz, A.J., Rikhof, H.A., and Moens, C.B. (2002). Eliminating zebrafish pbx proteins reveals a hindbrain ground state. Dev Cell **3,** 723-733.

Waskiewicz, A.J., Rikhof, H.A., Hernandez, R.E., and Moens, C.B. (2001). Zebrafish Meis functions to stabilize Pbx proteins and regulate hindbrain patterning. Development **128,** 4139-4151.

Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F., and Meyerowitz, E.M. (1992). LEAFY controls floral meristem identity in Arabidopsis. Cell **69,** 843-859.

Weigel, D., and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. Nature **377,** 495-500.

Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B., and Miller, A.D. (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci USA **86,** 5434-5438.

Werner, J.D., Borevitz, J.O., Uhlenhaut, N.H., Ecker, J.R., Chory, J., Weigel, D. (2005). FRIGIDA-independent variation in flowering time of natural Arabidopsis thaliana accessions. Genetics **170**, 1197-1207.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., and Weigel, D. (2005). Integration of spatial and temporal information during floral induction in Arabidopsis. Science **309**, 1056-1059.

Willemsen, V., and Scheres, B. (2004). Mechanisms of pattern formation in plant embryogenesis. Annu Rev Gen **38,** 587-614.

William, D.A., Su, Y., Smith, M.R., Lu, M., Baldwin, D.A., and Wagner, D. (2004). Genomic identification of direct target genes of LEAFY. Proc Natl Acad Sci USA **101,** 1775-1780.

Williams, L., and Fletcher, J.C. (2005). Stem cell regulation in the Arabidopsis shoot apical meristem. Curr Opin Plant Biol **8,** 582-586.

Williams, T.M., Williams, M.E., and Innis, J.W. (2005). Range of HOX/TALE superclass associations and protein domain requirements for HOXA13:MEIS interaction. Dev Biol **277,** 457-471.

Wilson, R.N., Heckman, J.W., and Somerville, C.R. (1992). Gibberellin Is Required for Flowering in Arabidopsis thaliana under Short Days. Plant Physiol **100,** 403-408.

Wolff, B., Sanglier, J.J., and Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chem Biol **4,** 139-147.

Yang, C.H., Chen, L.J., and Sung, Z.R. (1995). Genetic regulation of shoot development in Arabidopsis: role of the EMF genes. Dev Biol **169**, 421-435.

Yang, L., Liu, Z., Lu, F., Dong, A., and Huang, H. (2006). SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. Plant J **47,** 841-850.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., and Meyerowitz, E.M. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. Nature **346,** 35-39.

Yanovsky, M.J., and Kay, S.A. (2002). Molecular basis of seasonal time measurement in *Arabidopsis*. Nature **419,** 308–312.

Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R., and Takahashi, S. (2001). EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. Plant Cell **13,** 2471-2481.

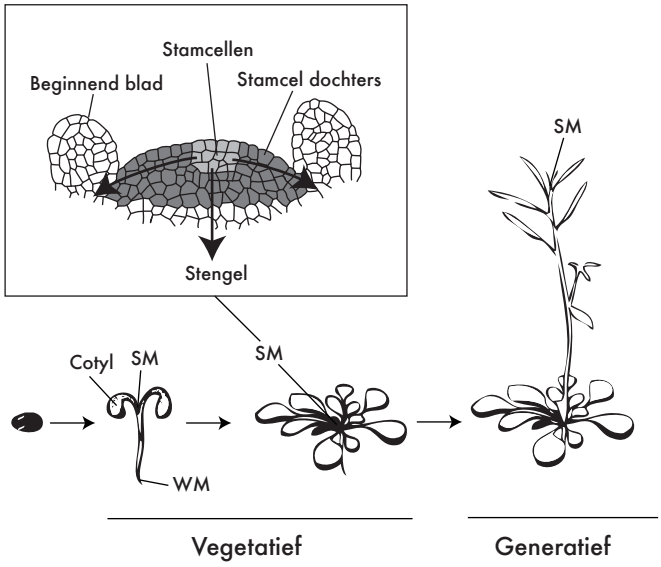
Zhang, H., and van Nocker, S. (2002). The *VERNALIZATION INDEPENDENCE 4* gene encodes a novel regulator of *FLOWERING LOCUS C.* Plant J **31,** 663–673.

Zhao, Z., Yu, Y., Meyer, D., Wu, C., and Shen W.H. (2005). Prevention of early flowering by expression of *FLOWERING LOCUS C* requires methylation of histone H3 K36. Nat Cell Biol **7**,1256-1260.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol **136**, 2621-2632.

Samenvatting

Planten zijn sessiel, met andere woorden, zij brengen hun hele leven door op één plek. Om te overleven moeten planten direct kunnen inspelen op een constant veranderend leefmilieu, wat betreft onder andere de omgevingstemperatuur en lichtcondities. Om dit te realiseren beschikken planten over een zeer uitgebreid sensorisch systeem en een uiterst flexibele ontwikkelingsstrategie. Na de embryonale fase bestaat de plant dan ook uit niet meer dan een embryonale wortel, twee kiemblaadjes (cotylen) en twee meristemen, die respectievelijk in de wortelpunt en tussen de cotylen zijn gesitueerd (Figuur S1). Meristemen bestaan uit stamcellen waarvan de dochtercellen worden gebruikt als bouwstenen om organen (bijvoorbeeld bladeren en bloemen) van te vormen. Vanuit deze meristemen zullen alle weefsels ontstaan die de plant gedurende zijn leven vormt (Figuur S1). Logischerwijs is het instandhouden van de meristemen van essentieel belang om een constante stroom van nieuwe cellen voor ontwikkeling van de plant te waarborgen.



Figuur S1 De Levenscyclus van een Arabidopsis Plant
Na kieming groeit de zaailing, die primair bestaat uit twee kiemblaadjes (cotylen), een embryonale wortel en twee meristemen (scheutmeristeem (SM) en wortelmeristeem (WM)) uit tot een complete plant door de continue aanmaak van scheut- en respectievelijk wortelweefsel vanuit de twee meristemen. De gehele periode van kieming tot initiatie van bloei wordt de vegetatieve ontwikkelingsfase genoemd, terwijl de generatieve fase bestaat uit bloemvorming en zaadontwikkeling. Tijdens de vegetatieve fase vormt Arabidopsis een roset van bladeren vanuit het centraal gelegen SM. Na de transitie tot bloei vindt er naast de aanleg van bloemen ook stengelgroei plaats en stuwt het SM zichzelf de hoogte in. Ter verduidelijking is het SM vergroot weergegeven in het kader. De stamcellen (lichtgrijs) zijn centraal gepositioneerd in het SM. Door continue deling van de stamcellen ontstaat er een perifeer gebied van stamceldochtercellen (donkergrijs). Deze stamceldochters differentiëren niet direct en vormen samen met de stamcellen het meristeem. De stamceldochters delen snel zodat aan de vraag naar cellen vanuit de zich ontwikkelende organen (witte cellen) kan worden voldaan. Eenmaal gemigreerd uit het meristeem differentiëren de stamceldochters en nemen ze een specifieke orgaanidentiteit aan. De zwarte pijlen geven aan welke weg een cel af kan leggen om uiteindelijk als bouwsteen voor de plant te fungeren.

Er bestaat een dynamisch evenwicht tussen de aanmaak van nieuwe stamceldochters (bouwstenen) en de vraag naar bouwstenen vanuit de zich ontwikkelende organen. Eén van de genen die betrokken is bij de instandhouding van dit evenwicht in het scheutmeristeem is het *SHOOTMERISTEMLESS* (*STM*) gen. Dit gen codeert voor een transcriptiefactor-eiwit dat DNA kan binden en direct betrokken is bij de aan- en uitschakeling van ontwikkelingsgerelateerde genen in het meristeem. *STM* is vooral betrokken bij het voorkomen van differentiatie van stamcellen en waarborgt zo het behoud van het scheutmeristeem. Daarnaast zorgt *STM* voor een snelle celdeling van de stamceldochters zodat aan de vraag naar nieuwe cellen kan worden voldaan door zich ontwikkelende organen (Figuur S1). Mutaties in het *STM*-gen resulteren in een vertraagde deling en het differentiëren van de stamcellen, waardoor planten vroeg in hun ontwikkeling stagneren (Figuur S1 en voor de die-hards Figuren 2.2, 2.3 en 2.5 uit hoofdstuk 2). Zeer interessant is het feit dat alle meercellige organismen *STM*-achtige genen bevatten. Ook bij dieren vervullen deze genen belangrijke taken tijdens de gehele ontwikkeling. Vooral in de

embryonale fase werken deze zogenaamde MEINOX-genen (MEINOX staat voor de groep van genen waartoe *STM* en zijn dierlijke verwanten behoren) samen met andere regulerende eiwitten en zij bepalen de identiteit van organen over de gehele kop-staart-as van een zich ontwikkelend dier (Hoofdstuk 1, Figuur 1.1). Daarnaast zijn MEINOX-genen intensief betrokken bij het reguleren van celdelingen en men heeft ontdekt dat vele vormen van kanker veroorzaakt worden door ondermeer mutaties in deze genen.

Een van de mechanismen om de activiteit van MEINOX-eiwitten te controleren is het beïnvloeden van hun positie in de cel. Om als transcriptiefactor te fungeren moeten deze eiwitten in de celkern verblijven en het daar aanwezige DNA binden. Opvallend genoeg kunnen MEINOX-transcriptiefactoren van zichzelf niet de kern in, maar verblijven zij in het cytoplasma (celvloeistof waarin de kern ligt en allerlei belangrijke processen plaatsvinden). Om de kern in te komen moeten MEINOX eiwitten een binding aangaan met MEINOX-achtige transcriptiefactor-eiwitten die normaal gesproken ook buiten de kern verblijven. Een binding tussen beide eiwitten zorgt voor een eiwitcomplex dat de kern in zal gaan. Het is dus noodzakelijk voor het functioneren van deze twee typen transcriptiefactoren dat zij samen in een cel aanwezig zijn. In planten heten deze MEINOX-achtige eiwitten BELL-eiwitten. ATH1 en PENNYWISE (PNY) behoren tot de familie van de BELL-eiwitten en wij hebben aangetoond dat deze een binding aan kunnen gaan met het MEINOX-eiwit STM. Bovendien zorgt een binding tussen STM- en ATH1- of PNY-eiwitten voor de verplaatsing van beide naar de celkern (hoofdstuk 2). Verder hebben we onderzocht wat de functies van de *BELL*-genen *ATH1* en zijn naaste verwant *PNY* zijn in relatie tot het functioneren van het meristeem. Omdat de *ATH1*- en *PNY*-genen coderen voor bijna identieke eiwitten, vervullen zij nagenoeg dezelfde taken. Het interessante van het hebben van een dergelijk sterk verwant gen is dat een mutatie in één van de twee genen hoogstwaarschijnlijk geen drastische gevolgen zal hebben voor de ontwikkeling van een organisme. Inderdaad resulteert een mutatie in *ATH1* of *PNY* slechts in kleine afwijkingen tijdens de ontwikkeling. Fascinerend is het echter om een plant te creëren die én ATH1 én PNY mist, zodat hun specifieke en overlappende functies aan het licht kunnen komen. Wij hebben aangetoond dat verlies van zowel een functioneel *ATH1*- als *PNY*-gen leidt tot planten die hun meristeem verliezen, net zoals in *stm*-mutanten (hoofdstuk 2). Kennelijk zorgen STM, ATH1 en PNY gezamenlijk voor de instandhouding van het meristeem. De volgende uitdaging zal zijn om erachter te komen welke genen in hun activiteit worden gereguleerd door STM en ATH1/PNY.

Naast de basale meristeemfuncties, zoals aanmaak en behoud van de stamcellen, maakt een scheutmeristeem meerdere identiteitswisselingen door tijdens zijn leven. Deze transities hebben grote gevolgen voor de ontwikkeling van de plant aangezien alle scheutorganen ontstaan vanuit dit meristeem. De meest opvallende

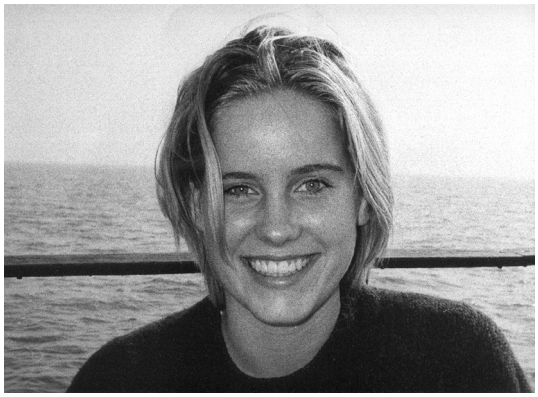
transitie is die van de vegetatieve (bladvorming) naar generatieve (bloemvorming) ontwikkeling (zie Figuur S1). Deze transitie wordt in veel planten gekenmerkt door zijn irreversibele karakter. Met andere woorden, wanneer bloei eenmaal is geïnitieerd, vindt er geen vegetatieve ontwikkeling meer plaats. De omschakeling van blad- naar bloemvorming moet dus zeer nauwkeurig gebeuren in het juiste seizoen. Alleen dan is er een grote overlevingkans voor de nakomelingen van de plant. Tijdens de vroege ontwikkelingsfasen wordt het initiëren van bloei geremd. De plant moet zich, net als een dier, eerst ver genoeg ontwikkelen om tot voortplanting over te kunnen gaan. Een zeer belangrijk gen in de remming van bloei is *FLOWERING LOCUS C* (*FLC*). Het *FLC*-gen codeert voor een transcriptiefactor-eiwit dat de activiteit van verschillende bloei-inducerende genen remt. Normaliter wordt het *FLC*-gen minder actief naarmate de plant ouder wordt, zodat bloei kan worden geïnduceerd. Planten die hun levenscyclus voltooien binnen een jaar (van lente tot najaar) hebben vaak een lage *FLC*-gen activiteit, omdat zij hun korte levenscyclus volbrengen gedurende de optimale seizoenen voor voortplanting. Daar staat tegenover dat planten die meerjarig zijn en dus overwinteren vaak een zeer actief *FLC*-gen hebben dat voor sterke remming van bloei zorgt. Dit moet gebeuren, omdat zulke planten niet mogen bloeien tijdens een warm najaar. In dat geval zouden de gevormde zaden nog voor het aanbreken van de winter kunnen kiemen, waarna de jonge plantjes de barre winteromstandigheden niet zouden overleven. Om dit te voorkomen heeft de plant twee mechanismen ontwikkeld. Allereerst moet het *FLC*-gen superactief worden gemaakt. Dit wordt onder andere veroorzaakt door een eiwit dat FRIGIDA (FRI) heet. Hierdoor bevatten de overwinterende planten een zulke grote hoeveelheid aan FLC eiwit, dat vroegtijdige bloei uitgesloten is. Tijdens de winter wordt het *FLC*-gen op een zeer vernuftige wijze steeds iets minder actief naarmate de kou aanhoudt. Na ongeveer drie maanden is de *FLC*-genactiviteit zo laag, alsof FRI nooit actief is geweest. Het vernuftige van dit systeem zit hem in het feit dat de *FLC* activiteit na de winter laag blijft, ondanks de aanwezigheid van FRI. De plant onthoudt (op genetisch niveau) feitelijk dat de winter voorbij is en het voorjaar in zicht is, met andere woorden, bloei mag plaatsvinden. Samen met de bloei-inducerende eigenschappen van de toenemende daglengte in het voorjaar wordt de plant snel aangezet om zich voort te planten.

Aangezien alle scheutorganen, dus ook bloemen, uit scheutmeristemen ontstaan, vindt het hierboven beschreven regulatiemechanisme voor bloei voornamelijk plaats in het scheutmeristeem. Hier worden de belangrijke beslissingen genomen om over te schakelen van vegetatieve naar generatieve ontwikkeling.

In hoofdstuk 3 en 4 beschrijven wij hoe ATH1 en PNY naast hun functies in de basale mechanismen voor meristeembehoud, ook werkzaam zijn in het reguleren van de bloei-inductie. Zij doen dit door het activeren van het *FLC*-gen en helpen zo bij het bepalen van de vegetatieve identiteit van het scheutmeristeem. Hoewel we nog weinig begrijpen van het mechanisme hoe ATH1 en PNY precies

functioneren, hebben we een eerste en belangrijke stap gezet in het ontrafelen van de complexe interacties tussen genetische netwerken die voor de continue en fasespecifieke ontwikkeling van planten zorgen.

Ik dank



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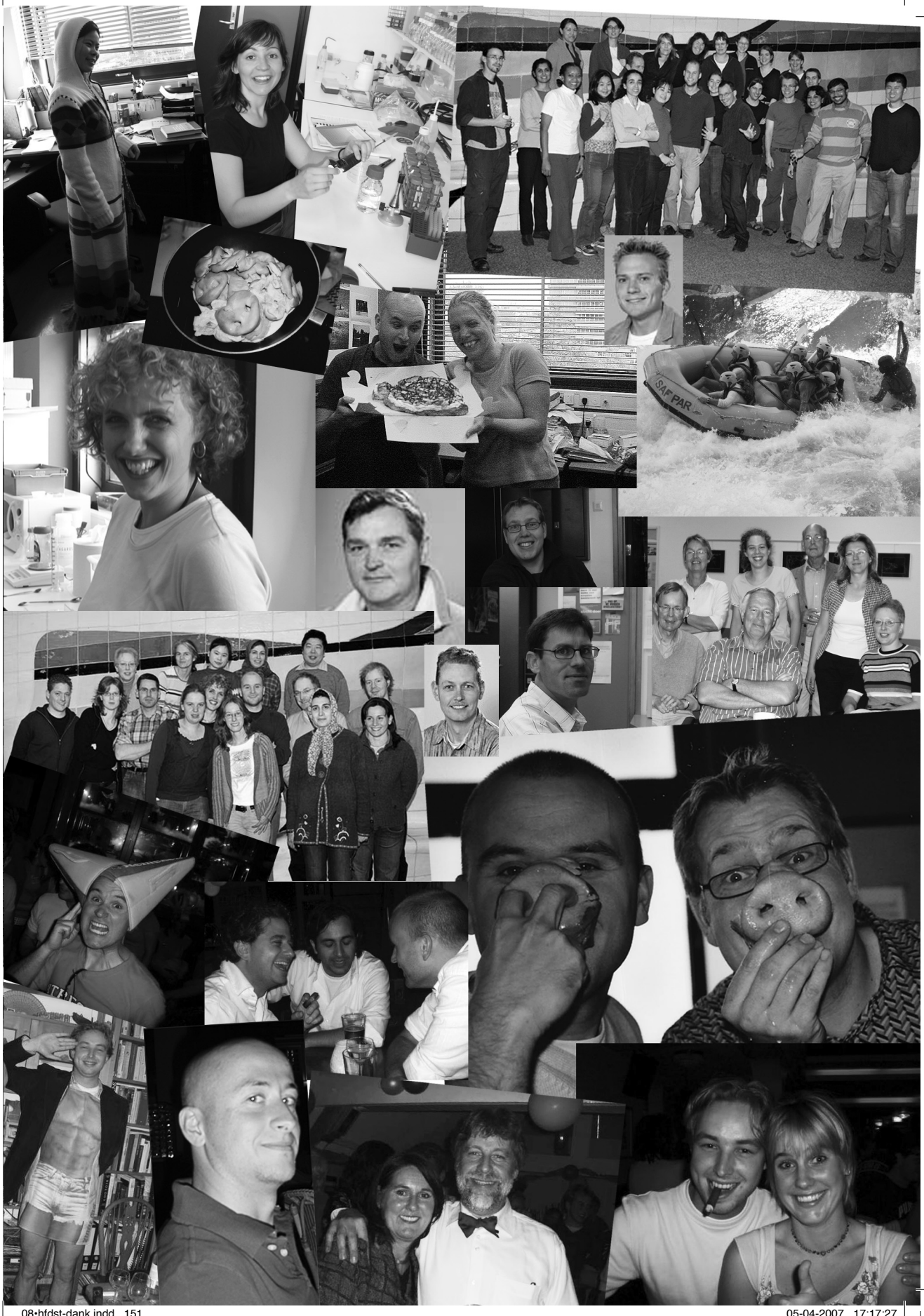
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