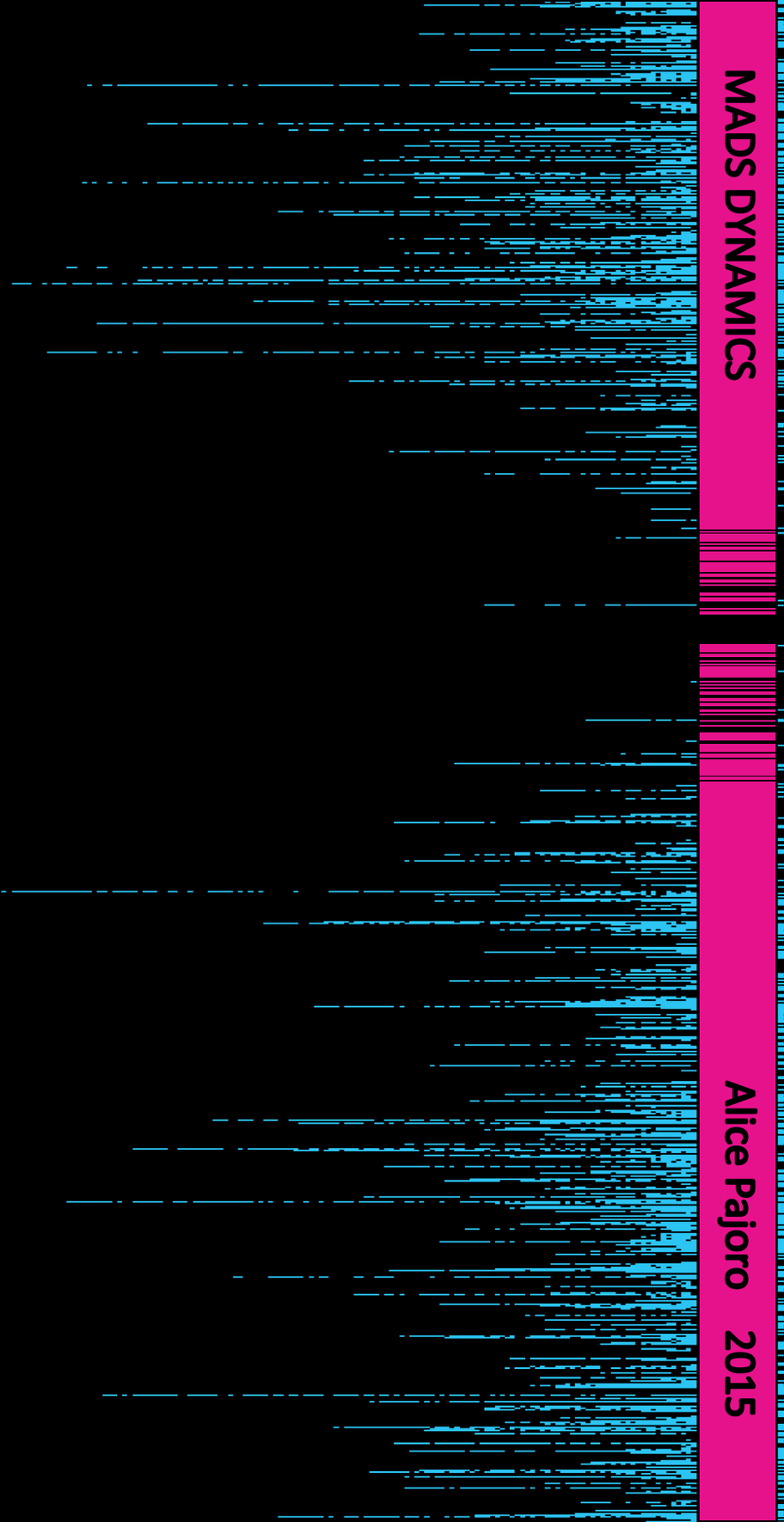

MADS DYNAMICS

Gene regulation in flower development by changes in chromatin structure and MADS-domain protein binding

Alice Pajoro

PROPOSITIONS

1. MADS-domain proteins bind to their target genes in a stage-specific combinatorial fashion.
(this thesis)

2. MADS-domain proteins act as pioneer factors.
(this thesis)

3. Since 62% of the human DNA is transcribed into RNA and only 5.5% of the RNA is translated into proteins (ENCODE Project Consortium, 2012), the central dogma, DNA-RNA-protein,represents mainly an exception.

4. Defining proteins that are involved in overall plant development as flowering time regulators is improper.

5. The conclusion that most binding events identified in ChIP-seq experiments are ineffective (Ó'Maoiléidigh et al., 2014, New Phytol. 201(1),16-30) is based on lack of information on gene regulation.

6. The European Union was the best achievement of the 20th century European governments.

7. Due to the extreme role of planning in the Dutch life style, flexibility is lost.

Propositions belonging to the thesis, entitled:

"MADS dynamics. Gene regulation in flower development by changes in chromatin structure and MADS-domain protein binding"

Alice Pajoro

Wageningen, 12 March 2015

MADS DYNAMICS

Gene regulation in flower development by changes in chromatin structure and MADS-domain protein binding

Alice Pajoro

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MADS DYNAMICS

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Alice Pajoro

Thesis

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Contents

CHAPTER 1

Introduction: When genetics meets epigenetics flowers arise

Flowers are determinate structures necessary for reproduction of angiosperms. Angiosperms such as *Arabidopsis thaliana* have lifecycles characterized by the alternation of diploid sporophytic and haploid gametophytic stages. The plant undergoes several developmental changes that can be considered as phase transitions, before the sporophyte produces the spores, which develop into male and female gametophytes. The first developmental change is the transition from the initial juvenile vegetative stage into the adult vegetative phase. Plants in the juvenile phase are able to produce leaves and axillary buds, whereas the initiation of reproductive structures only occurs in the adult vegetative phase. The next phase transition is the switch from vegetative to reproductive growth, when, upon response to environmental signals, the vegetative shoot apical meristem (SAM) acquires the identity of an inflorescence meristem (IM) that will then produce floral meristems (FM). Arabidopsis floral meristems produce four concentric whorls of floral organs: four sepals, four petals, six stamens, and two fused carpels from the periphery to the center of the floral meristem. In contrast to the indeterminate SAMs and IMs, floral meristems are genetically programmed to terminate after the primordia of the carpels have been formed. Male reproductive development takes place in the third whorl stamens, which are composed of anthers supported on filaments. Inside the anthers the male gametophytes develop by two sequential processes: microsporogenesis and microgametogenesis. Ovules, which form in the fourth whorl, provide structural support to the female gametes and enclose them until seed development is complete.

THE TRANSCRIPTIONAL REGULATION OF FLOWER DEVELOPMENT: FROM CLASSICAL GENETICS TO GENOME-WIDE APPROACHES.

Developmental processes are controlled by tightly coordinated networks of regulators, known as gene regulatory networks (GRNs) that activate and repress gene expression within a spatial and temporal context. In *Arabidopsis thaliana*, the key components of the GRNs controlling major processes in plant reproduction, such as the floral transition and floral organ identity specification, were first identified in loss-of-function mutants that affect these processes (Blazquez *et al.*, 2006). The interactions between these regulators began to be revealed later through genetic analyses, resulting in the first, mostly linear, GRN maps. These GRNs were enlarged by reverse genetics, analysis of protein-protein interactions, expression studies and protein-DNA binding profiles in wild type and mutant

plants, resulting in a hierarchical GRN in which master regulators target subsets of genes and downstream processes (Kaufmann *et al.*, 2010a).

GRN for floral transition

Endogenous and environmental signals act as input into the decision to initiate flowering. Several pathways, including photoperiod, autonomous, vernalization, and gibberellin (GA) (Baurle and Dean, 2006; Simpson and Dean, 2002) converge on a small set of central flowering regulators, including *CONSTANS* (*CO*) and *FLOWERING LOCUS C* (*FLC*), that antagonistically regulate flowering (**Figure 1**) (Samach *et al.*, 2000). *FLC* acts as a repressor of flowering and mediates the response to vernalization and autonomous pathways, while *CO* is a floral activator and controls the photoperiodic pathway. Both genes together regulate the expression of the downstream floral pathway integrator genes *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), and *LEAFY* (*LFY*) (Blazquez and Weigel, 2000; Simpson and Dean, 2002). The gibberellin pathway influences the phase transition at the SAM by promoting the expression of *SOC1* and *LFY* (Blazquez *et al.*, 1998; Moon *et al.*, 2003) and also acts upstream of *FT* in the leaf, providing evidence for crosstalk between the photoperiod and GA pathways (Porri *et al.*, 2012).

The FT protein stimulates flowering by moving from the leaf (where the light is perceived) to the SAM (where inflorescence and floral meristems are formed). Genetic and molecular studies indicate that the FT protein comprises part of the inductive signal known as *florigen*, which promotes flowering in response to photoperiod (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007). FT interacts at the SAM with the bZIP TF, FLOWERING LOCUS D (FD), to activate the expression of the floral meristem identity genes *APETALA1* (*AP1*) and *SOC1*, which in turn activate *LFY*, promoting the floral transition (Wu and Gallagher, 2012). In addition to *LFY*, other genes such as *AGAMOUS LIKE 24 (AGL24)* also contribute to the up-regulation of *AP1* (Grandi *et al.*, 2012; Pastore *et al.*, 2011). After successful initiation of the floral meristems, *AP1* and *LFY* activate the organ identity genes, initiating floral organ development.

Furthermore, the microRNAs miR156 and miR172 have been identified as important regulators of this developmental phase change. Members of the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) TF family are targeted by miR156, whereas miR172 targets 6 *APETALA2-LIKE* (*AP2-like*) transcription factors (Wu and

Poethig, 2006; Wu *et al.*, 2009). Levels of miR156 are high in the juvenile vegetative phase and decrease before onset of the adult vegetative phase, allowing the production of a subset of SPL proteins (SPL9, SPL10). These SPLs induce the expression of miR172 genes, leading to a gradual increase in miR172 levels during the adult vegetative phase (Aukerman and Sakai, 2003; Jung *et al.*, 2007; Schwarz *et al.*, 2008; Wu and Poethig, 2006; Wu *et al.*, 2009). Increased levels of miR172, in turn, cause a down-regulation of *AP2-like* genes, which otherwise repress adult traits and flowering (**Figure 1**) making the plant competent to flower (Wu *et al.*, 2009).

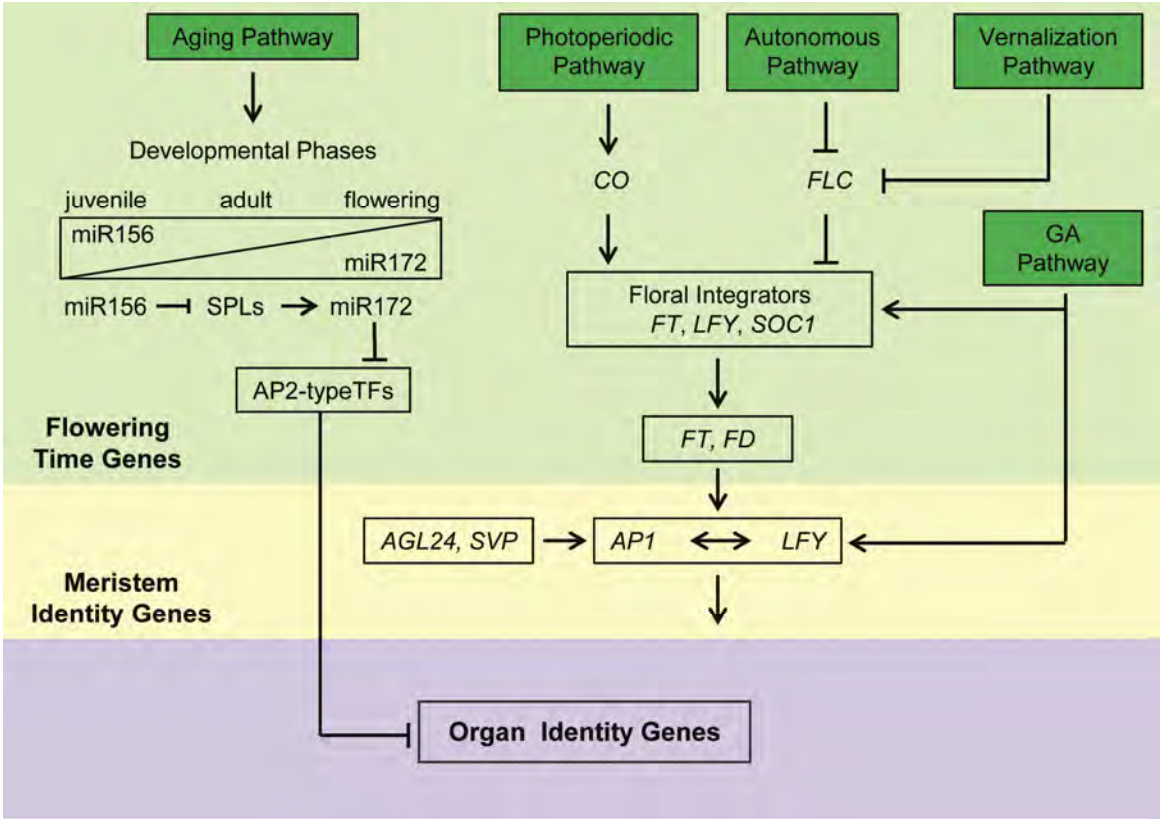

Figure 1. GRN controlling the transition to flowering. The switch from vegetative to reproductive phase is triggered by endogenous and environmental stimuli, some examples of which are illustrated here. These signals converge at the central flowering regulator genes *FLC* and *CO* that antagonistically regulate the floral integrator genes at the SAM. The floral integrators activate the meristem identity genes *AP1* and *LFY*, subsequently leading to activation of the ABCDE class genes, specifying organ identity. The endogenous aging pathway involves micro RNAs (miRNAs). At early stages of development, the level of miR156 is high, maintaining the juvenile growth phase. As the plant ages, miR156 levels decrease, allowing the production of a subset of SPL proteins. These SPL proteins induce the expression of *MIR172* genes, which are consequently expressed at low levels in the juvenile phase and steadily increase their expression levels in the adult phase. Elevated levels of miR172 cause down-regulation of *AP2-like* TF factor genes, which otherwise repress flowering. Arrows indicate activation, blocked lines indicate repression, left-right arrows indicate a positive feedback loop.

GRN for floral organ development

Floral organ identity is determined by the combinatorial action of floral homeotic genes. The way in which this limited set of genes acts to direct formation of the correct floral organs at the appropriate place in the flower is described by the classical ABC model (for reviews see: (Alvarez-Buylla *et al.*, 2010; Causier *et al.*, 2010; Immink *et al.*, 2010; O'Maoileidigh *et al.*, 2014). The ABC model was built on phenotypic observations of loss of function mutants that showed absence and conversions of floral organs. According to the ABC model, a combination of master regulators determines organ identity: the A-class genes determine sepal identity; the combination of A and B-class genes promote petal identity; the combinations of B and C-class genes determine stamen identity, and C-class genes determine carpel identity (**Figure 2**).

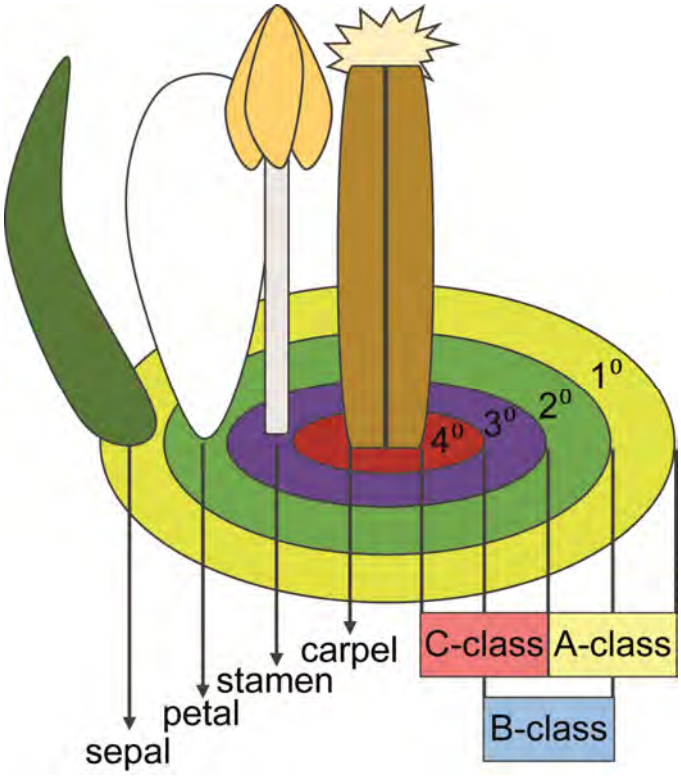

Figure 2. The ABC-model. Three classes of genes determine the identity of the 4 types of organs: sepals, petals, stamens and carpels.

In Arabidopsis, the A-class genes are the MADS-box TF-encoding *AP1* (Mandel and Yanofsky, 1995) and *APETALA2* (*AP2*), which encodes a member of the AP2/ERF (ethylene responsive factor) TF family (Jofuku *et al.*, 1994; Okamuro *et al.*, 1997). The B-class genes are *APETALA3* (*AP3*) (Jack *et al.*, 1992) and *PISTILLATA* (*PI*) (Goto and

Meyerowitz, 1994), both of which encode MADS-domain TFs. *AGAMOUS* (*AG*), another MADS-box gene, is the only member of the C-class (Yanofsky *et al.*, 1990). The original ABC model was extended by the addition of D- and E-classes, which specify ovule identity (Pinyopich *et al.*, 2003) and floral identity, respectively. The D-class genes include the MADS-box genes *SEEDSTICK (STK)* and *SHATTERPROOF1* and *2 (SHP1/2),* which are redundantly required for the specification of ovule identity (Pinyopich *et al.*, 2003). Four *SEPALLATA* genes (*SEP1-4*), all of which encode MADS-domain TFs, comprise the E-class (Ditta *et al.*, 2004; Pelaz *et al.*, 2000; Rounsley *et al.*, 1995) (**Figure 2**). Protein-protein interaction studies demonstrated that the ABCDE MADS-box TFs physically interact and bind DNA as tetramers, possibly establishing DNA loops in the promoters of their target genes to activate or repress their transcription (Egea-Cortines *et al.*, 1999; Melzer and Theissen, 2009; Smaczniak *et al.*, 2012b). The E-class SEP proteins are necessary for the formation of higher order complexes involving the A-, B- and C-class TFs and hence mediate their organ identity functions (Honma and Goto, 2001; Pelaz *et al.*, 2001). The resulting hierarchical GRN formed the basis of our understanding of flower development until the advent of new technologies described below.

Novel insights into GRN structure and function.

Recently, genome-wide molecular approaches, such as protein interaction assays, expression profiling and DNA-protein interaction studies have connected the nodes in the GRNs and added novel regulators, interactors and downstream targets (Hawkins *et al.*, 2010). In contrast to the earlier genetic networks described above, more recent versions show molecular interactions that are independent of single mutant phenotypes and are potentially more dynamic in time and space. The molecular interactions that were added are for example protein-protein interactions, which allow combinatorial action of multiple regulatory factors. In the case of TF complexes, their composition can be an important determinant of DNA binding specificity and affinity and thus interactions will affect target gene regulation. Another layer of molecular interactions recently added to the networks comprises genomic TF binding profiles, i.e. protein-DNA interactions. Large-scale expression analyses, preferably performed with inducible systems (Kaufmann *et al.*, 2010c; Wellmer *et al.*, 2006), add transcriptional activity to the connections (‘*edges*’) between nodes in the network. These large-scale data sets have substantially increased the

network connectivity and revealed the shortcomings of the classical hierarchical networks. Current GRNs are composed of TFs regulating subsets of genes in a combinatorial fashion and contain multiple regulatory feedback loops, which blur the hierarchical structure (Kaufmann *et al.*, 2010a).

Remarkably, TFs act as process integrators and connect other developmental processes as hubs in the network (Pose *et al.*, 2012). The TF target gene analyses have revealed unexpected connections between processes previously considered to be unrelated or only indirectly related, which could not have been predicted by classical genetic approaches. For example, target gene analysis of the SEP3 floral organ identity transcription factor revealed a connection with auxin signalling (Kaufmann *et al.*, 2009). As another striking example, a recent study addressing the role of LFY revealed that in addition to its function in reproductive transition, it is also involved in pathogen responses (Winter *et al.*, 2011).

In the 1990s, binding of TFs to DNA sequences was studied by *in vitro* or heterologous *in vivo* methods, such as electrophoretic mobility shift assays (EMSA) and yeast 1-hybrid studies. Research performed in the group of Suzanna Schwarz-Sommer (Trobner *et al.*, 1992) showed that the *Antirrhinum* class B homeotic proteins DEFICIENS and GLOBOSA interact with each other and bind *in vitro* to their own promoters, thereby forming an auto-regulatory loop. More such auto-regulatory loops have been recently identified in GRNs, particularly associated with transcriptional regulation of MADS-box genes (Gomez-Mena *et al.*, 2005). For instance, expression of the MADS-box gene *SOC1* is controlled by the SOC1 protein, which forms heterodimers with AP1 that suppress *SOC1* expression following the successful transition to floral meristem identity (Immink *et al.*, 2012).

The development of chromatin immunoprecipitation (ChIP) was a major breakthrough in the study of DNA-protein interactions, since it allowed the identification of *in vivo* physical interactions between a TF and its target DNA (Gomez-Mena *et al.*, 2005; Wang *et al.*, 2002). The subsequent introduction of genome-wide arrays (ChIP-CHIP) (Zheng *et al.*, 2009) or large-scale sequencing (ChIP-seq) (Kaufmann *et al.*, 2010b) led to the identification of thousands of target genes for specific TFs. In the past 5 years many genome-wide TF-DNA binding profiles have been generated for TFs involved in plant reproduction (**Table 1**). The high numbers of interactions that were detected in ChIP-seq experiments revealed a much higher network complexity than expected and demonstrated

The ChIP-seq data also confirmed that most TFs involved in plant reproduction bind to their own locus, reinforcing the concept that auto-regulatory loops are a common mechanism of regulation in GRNs (**Figure 3**). For example, identification of the direct targets of LFY (Winter *et al.*, 2011) and AP1 (Kaufmann *et al.*, 2010c) revealed that LFY is able to promote *AP1* transcription through direct regulation, and AP1 binds to *LFY* to form a positive feedback loop, leading to a strong and rapid up-regulation of both genes.

Table 1. Overview of genome-wide protein-DNA binding profile studies for TFs involved in plant reproduction.

Gene		Family	Function	Tissue	Most relevant targets	Reference
AGAMOUS	AG	MADS-box transcription factor	floral organ development	flower buds stage 5	*AG, CRC, SHP2, SPL, JAG, SEP3, AP1, AP3, SHP1, SUP, HEC1, HEC2, VDD*	Ó'Maoiléidigha *et al* ., Plant Cell 2013
AGAMOUS-LIKE 15	AGL15	MADS-box transcription factor	floral transition	embryonic culture tissue	*FLC, SVP, LEC2, FUS3, ABI3, IAA30*	Zheng *et al* ., Plant Cell 2009
APETALA1	AP1	MADS-box transcription factor	Repressor of floral transition	inflorescence meristem; flowers at stage 2, 4 and 8	*FD, FDP, LFY, SNZ, TOE1, TOE3, TEM1, TEM2, TFL1, SPL9, SPL15, SEP3, AP2*	Kaufmann *et al.*, Science 2010; ; Pajoro et al., 2014 Genome Biology
APETALA2	AP2	AP2-like family	floral transition and floral organ development	inflorescences	*AG, SOC1, SEP3, AP1, TOE3, AGL15, ARF3, SHP1, SHP2, AGL44, TOE1, RGA-like1, miR156, and miR172*	Yant *et al.,* Plant Cell 2010
APETALA3	AP3	MADS-box transcription factor	floral organ development	flower buds stage 5	*CRC, SEP3, SPL, AP1, SUP, AG, UFO, SHP2, RBE, HEC1, HEC2, ALC*	West *et al* ., PNAS 2012
FLOWERING LOCUS C	FLC	MADS-box transcription factor	flowering time	12 days old seedlings	*SOC1, FT, SEP3, CBF1, JAZ6, AGL16, SPL15, DIN10, SVP, SPL3, SMZ, TOE3, TEM1, FRI, CIR1, FIO1, LCL1, COL1*	Deng *et al* ., PNAS 2011
FLOWERING LOCUS M	FLM	MADS-box transcription factor	flowering time	15 days old seedlings	*SOC1, ATC, TEM2, SMZ, SEP3, AP3, PI, RVE2, FIO1, SHP2, MIR156, AP2, MIR172, AP1*	Pose *et al.* , Science 2013
LEAFY	LFY		floral transition	inflorescences	*TFL1, AP1, AG, SEP4, LFY, SOC1, PRS, BB, GIS, GOA, STY2, ARR3, GA3OX2*	Moyroud *et al* ., Plant Cell. 2011
PISTILLATA	PI	MADS-box transcription factor	floral organ development	flower buds stage 5	*CRC, SEP3, SPL, AP1, SUP, AG, UFO, SHP2, RBE, HEC1, HEC2, ALC*	West *et al* ., PNAS 2012
SCHLAFMUTZE	SMZ	AP2-like family	repressor of flowering	seedlings	*FT, SMZ, SNZ, AP2, TOE3, SOC1, AP1, TEM1, FRI*	Mathieu *et al.,* PLOS Biology 2009
SEPALLATA3	SEP3	MADS-box transcription factor	flower development	inflorescences (stage1-12); flowers at stage 2, 4 and 8	*AP1, AP3, SEP1, SEP2, SEP4, AG, SHP1, SHP2, GA1, PIN4, PID, ARF3, ARG8, IAA4*	Kaufmann *et al*., PLOS Biology 2009; Pajoro *et al.* , 2014 Genome Biology
SHORT VEGETATIVE PHASE	SVP	MADS-box transcription factor	flowering time	2 weeks old seedlings	*GI, PRR7,FLK, FLD, CLF,SWN,VNR2, PHYA, STIP, SVP, CLV1, CLV2, PHB, PHV, REV, ATHB8*	Gregis *et al* ., Genome Biology 2013
SHORT VEGETATIVE PHASE	SVP	MADS-box transcription factor	floral organ development	inflorescences (stage1-11)	*SVP, CLV1, PHB, KAN1, ARF3, PIN1, WDR55*	Gregis *et al* ., Genome Biology 2013
SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1	SOC1	MADS-box transcription factor	flowering time	transition apices	*SOC1, CBF1, CBF2, CBF3, mir156, SVP, AGL15, AGL18, TEM2, TOE3, SMZ, SNZ, SEP3, SHP2, AP3, PI, SUP, ARF3*	Immink *et al* . Plant Physiology 2012

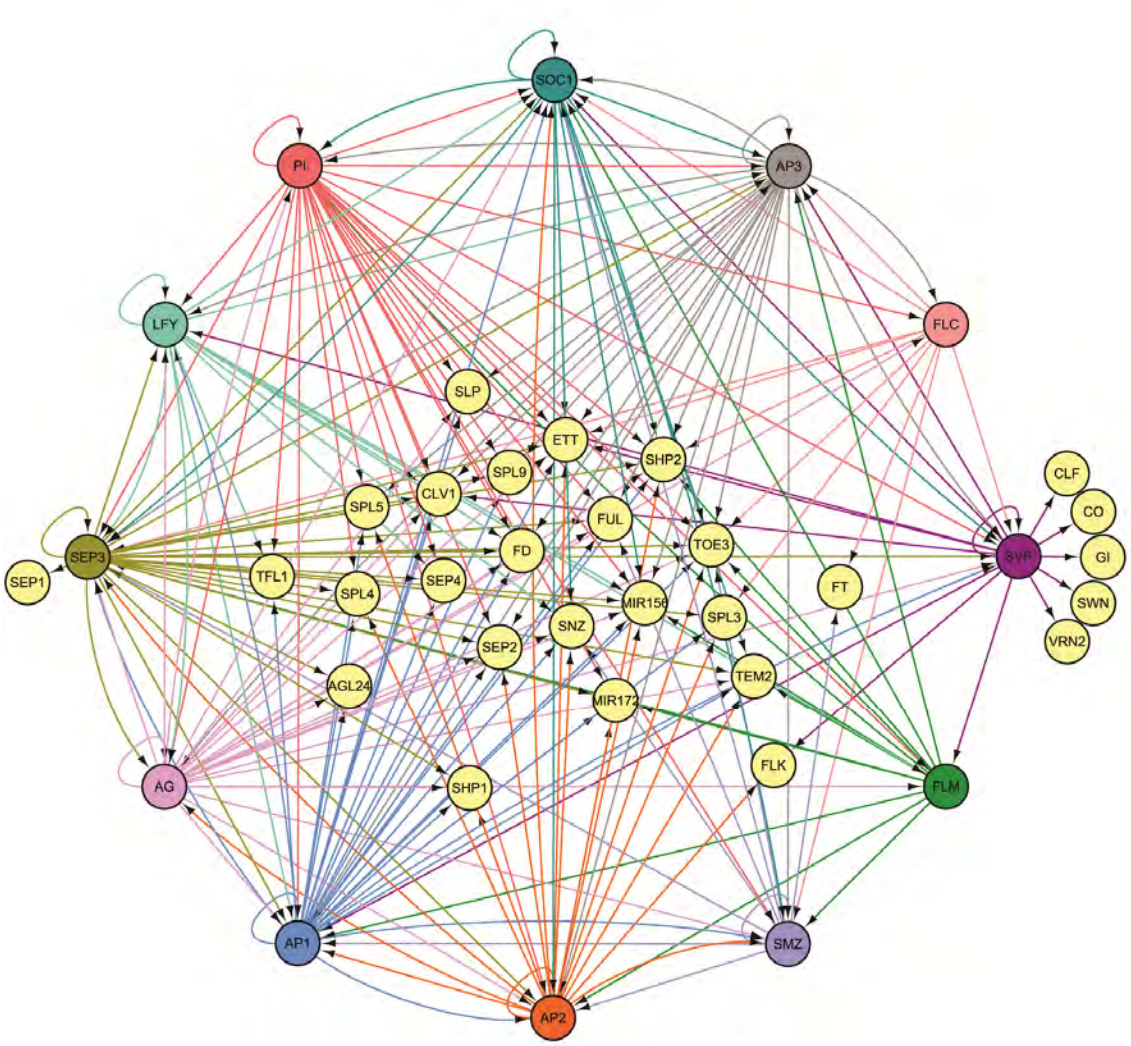

Figure 3. Gene interaction network based on DNA-binding profiles. TFs involved in plant reproduction (Table 1) show a high overlap in target genes. Most TFs bind to their own locus, suggesting that auto-regulatory loops are a common mechanism of regulation. Many pairs of TFs show a reciprocal binding suggesting a mechanism of inter-regulation.

Through these genome-wide studies, in particular in combination with transcriptome analysis (Kaufmann *et al.*, 2010c; Wuest *et al.*, 2012), it also became apparent that some TFs act as both activators and repressors. For example, at early stages AP1 represses genes controlling flowering time, whereas at later stages it mainly acts as an activator of floral homeotic genes (Kaufmann *et al.*, 2010c; Pajoro *et al.*, 2014). A similar observation was made for the B-class genes *AP3* and *PI*, which activate genes involved in organogenesis and repress those required for carpel development (Wuest *et al.*, 2012). It is likely that the composition of the TF complexes and their ability to recruit co-factors act together to determine the DNA-binding specificity and the mode of transcriptional

action. Although the consensus binding site for MADS-domain proteins, the so-called CArG-box, was found at the centre of virtually all binding peaks obtained in ChIP experiments, certain sequence motifs within and flanking the CArG-box were preferentially enriched. This corroborates the idea that the DNA binding specificity is determined to a large extent by the composition of the TF complex. Therefore, protein-protein studies will be required to enable us to understand TF-DNA interaction specificity and hence why specific target genes are recognised by specific transcription factors. Recent large-scale protein-protein and proteomics studies have elucidated the composition of many MADS-domain protein complexes involved in flowering time control and floral organ development (Immink *et al.*, 2009; Smaczniak *et al.*, 2012a). The next challenge in these studies will be to unravel the binding specificity of these complexes and how this influences the dynamic control of target gene regulation.

THE EPIGENETIC REGULATION OF FLOWER DEVELOPMENT.

In the nucleus DNA is packed into chromatin and therefore, all functions of the genome occur in the context of chromatin, whose fundamental building block is the nucleosome. A nucleosome is constituted by 147 bp of DNA wrapped around a histone octamer, formed by two of each core histone H2A, H2B, H3 and H4, and one histone linker H1 which is critical to the higher-order compaction of chromatin (**Figure 4**). Nucleosome stability and compaction are regulated by the combined effects of nucleosome-positioning sequences, histone chaperones, ATP-dependent nucleosome remodelers, histone variants and post-translational histone modifications. Dynamic post-transcriptional modifications of histones and histone tails, such as methylation, acetylation or ubiquitination, lead to a more or less compact chromatin structure that affects gene expression.

Recent advances in genome-wide methods are revealing increasingly detailed profiles of the genomic distribution of nucleosomes, their modifications and their modifiers. The overall picture that is now emerging is one in which chromatin accessibility, directed by contributions from chromatin remodelers and chromatin modifiers, highly contributes to the regulation of transcription. Moreover, transcription factors have been found to interact with chromatin modifiers and chromatin remodelers suggesting that transcriptional and

epigenetic regulation may be interconnected (Efroni *et al.*, 2013; Smaczniak *et al.*, 2012b; Vercruyssen *et al.*, 2014).

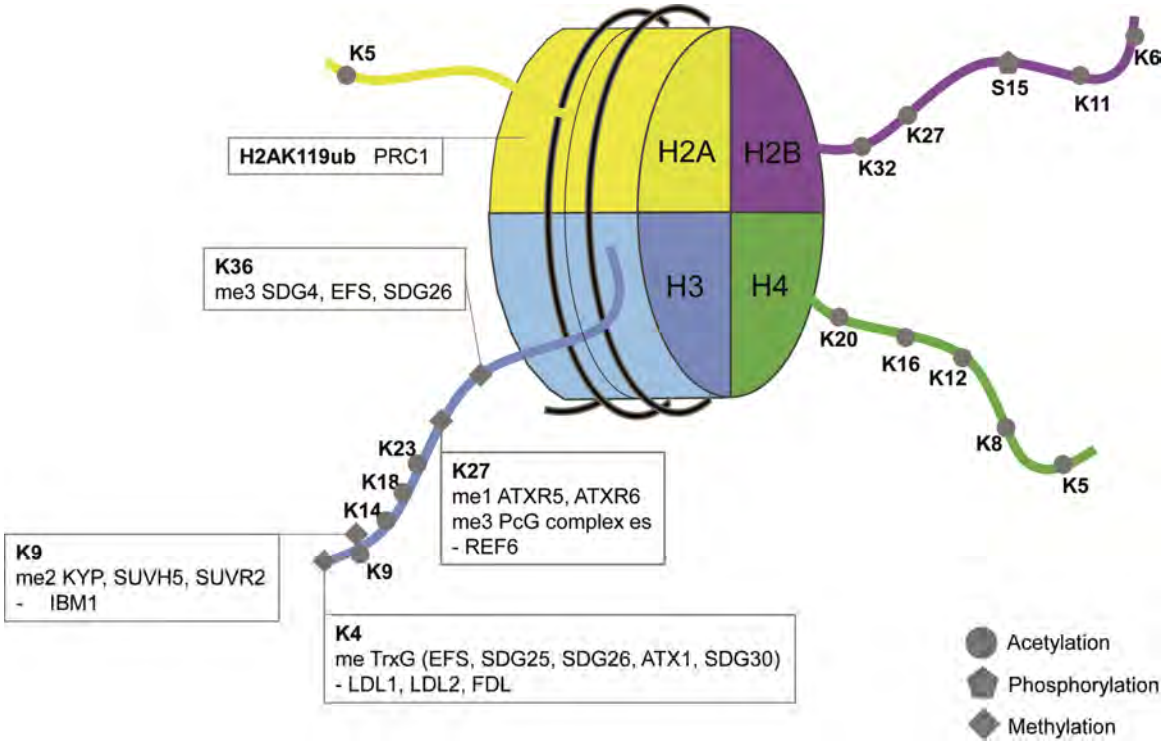

Figure 4. The histone code. The histone modifications identified in Arabidopsis are indicated. For each histone modification the known writers, readers and erasers are shown (Johnson *et al.*, 2004; Lanouette *et al.*, 2014; Zhang *et al.*, 2007).

The histone code and histone modifications in flower development.

Most research on the role of chromatin in epigenetic processes has focused on histone modifications and their regulation, leading to the so-called histone code (Strahl and Allis, 2000) (**Figure 4**). Although the role of many histone modifications has been unravelled, the function of some is still unknown. Moreover, the analysis of histone modifications in Arabidopsis revealed that although the modifications are quite conserved among eukaryotes some difference can be observed (Johnson *et al.*, 2004; Lanouette *et al.*, 2014; Zhang *et al.*, 2007). Post-transcriptional modification of histone tails has been shown to affect gene regulation (Strahl and Allis, 2000). In Arabidopsis, the best characterised modifications are the histone lysine methylations that occur at Lys4 (K4), Lys9 (K9), Lys27 (K27), and Lys36 (K36) of histone 3 (H3). For example H3K4me2 and H3K36me3 were found to be associated with active chromatin status, while H3K9me2 and H3K27me3 are repressive marks (Berr *et al.*, 2011; Liu *et al.*, 2010).

Several *Arabidopsis* proteins involved in the regulation of histone modification have been characterised (**Figure 4**). The main actors are the Polycomb group (PcG) and Trithorax group (TrxG) proteins, which act antagonistically in gene regulation: PcG proteins maintain a repressive chromatin state, while TrxG proteins maintain an active chromatin state (Pien and Grossniklaus, 2007).

The Polycomb Repressive Complex 2 (PRC2) catalyses the trimethylation of H3K27, while PRC1 recognises the H3K27me3 modified histones and catalyses H2AK119 ubiquitination that drives a more compact chromatin status. In Arabidopsis, three PRC2-like complexes have been identified, each acting at different developmental stages: the EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN) and FERTILIZATION INDEPENDENT SEED (FIS) complexes (Hennig and Derkacheva, 2009; Pien and Grossniklaus, 2007). The EMF-complex plays a role during the vegetative phase, where it represses *FT* and *AG* (Goodrich *et al.*, 1997; Jiang *et al.*, 2008). The EMF-complex is composed of the proteins EMF2, FERTILIZATION INDEPENDENT ENDOSPERM (FIE), an MSI homolog and CURLY LEAF (CLF) or SWINGER (SWN). *emf2* mutants skip the vegetative phase and flower soon after germination (Sung *et al.*, 1992; Yang *et al.*, 1995), consistent with a premature expression of flowering time genes. In the *clf* mutant, aberrations in flowers were observed, due to ectopic *AG* expression (Goodrich *et al.*, 1997). Consistent with their antagonistic action to PcG complexes, two Arabidopsis Trx proteins, ATX1 (Alvarez-Venegas *et al.*, 2003) and ULTRAPETALA1 (ULT1) (Carles *et al.*, 2005), are involved in the activation of *AG* (Carles and Fletcher, 2009; Saleh *et al.*, 2007). ATX1 is a histone methyltransferase and it establishes H3K4me3, a histone modification associated with transcriptionally active regions (Alvarez-Venegas *et al.*, 2003). Loss-of-function of *ATX1* leads to homeotic conversions of floral organs, such as the formation of stamenoid petals and carpelloid stamens, due to the down-regulation of homeotic genes, such as *AP1*, *AP2*, *PI*, and *AG* (Alvarez-Venegas *et al.*, 2003). Homeotic conversions of floral organs were also observed in mutants of SDG8, a H3K36 tri-methyltransferase (Kim *et al.*, 2005), showing carpelloid sepals and stamenoid petals. In this mutant a down-regulation of the homeotic genes *AP1*, *AP3* and *PI* was observed (Grini *et al.*, 2009). ChIP experiments revealed a lower level of H3K36me3 at the *AP1* locus, suggesting that *SDG8* plays a role in the regulation of *AP1* via the deposition of the active histone mark H3K36me3.

The action of chromatin remodeling complexes during flower development.

Packaging of DNA into a nucleosome restricts DNA accessibility for regulatory proteins, but also provides an opportunity to regulate genomic processes, as transcription or DNA replication, through modulating nucleosome positions and local chromatin structure. To enable dynamic access to DNA and to alter nucleosome composition in chromosomal regions, cells have evolved a set of specialized chromatin remodelling complexes (chromatin remodelers). Chromatin remodelers are ATP-driven protein complexes that can alter the chromatin structure by sliding histone octamers, altering histone-DNA interactions or changing histone variants, leading to an increased/decreased accessibility of regulatory proteins to DNA elements.

Based on the presence of distinct domains, chromatin remodelers can be divided into four families: the SWI/SNF (switching defective/sucrose non-fermenting) family, the ISWI (imitation switch) family, the CHD (chromo-domain, helicase, DNA binding) family and the INO80 (inositol requiring 80) family (Becker and Horz, 2002; Clapier and Cairns, 2009; Tsukiyama, 2002). Although all families contain an ATP-domain, each family has a unique set of domains that allows specialisation in their functions. The members of the SWI/SNF family contain an HSA (helicase-SANT) and a C-terminal bromo-domain and are able to form different complexes, which have a broad range of functions in development. They can slide and eject nucleosomes, but lack roles in chromatin assembly (Mohrmann and Verrijzer, 2005). The ISWI family remodelers (Langst and Becker, 2001) are characterised by the presence of a SANT domain and a SLIDE domain (SANT-like ISWI), which together form a nucleosome recognition unit with the SANT domain interacting primarily with unmodified histone tails (Boyer *et al.*, 2004). They are involved in transcriptional repression and activation, formation or maintenance of higher-order chromatin structure and DNA replication (Corona and Tamkun, 2004). The CHD family remodelers contain a tandem chromo-domain (chromatin organization modifier) and, in some cases, a DNA-binding domain (Marfella and Imbalzano, 2007; Woodage *et al.*, 1997). The CHD remodelers are involved in nucleosome spacing and sliding (Sims and Wade, 2011), for example, CHD1 (Konev *et al.*, 2007) binds to H3K4me3 and is involved in transcription elongation and nucleosome assembly (Flanagan *et al.*, 2005). The INO80 family remodelers (Bao and Shen, 2007) are characterised by the presence of a “split” ATPase domain with a long insertion in the middle (Clapier and Cairns, 2009). Members of this family are the INO80 complex, involved in promoting transcriptional

activation and DNA repair, and the SWR1 complex, which replaces the H2A-H2B dimers with H2A.Z-H2B dimers (Mizuguchi *et al.*, 2004).

In *Arabidopsis*, there are 42 putative SWI2/SNF ATPase (Plant Chromatin Database, http://www.chromdb.org), many of which still remain uncharacterised (Kwon and Wagner, 2007; Reyes *et al.*, 2002; Verbsky and Richards, 2001; Wagner, 2003). Among the ones with a proposed role during development are members of the SWI/SNF family, such as *BRAHMA* (*BRM*), *SPLAYED* (*SYD*), *MINUSCULE1* (*MINU1*) and *MINU2*, members of the CHD family, such as *PICKLE* (*PKL*), *PKL1* and *PKL2* and two member of the INO80 family, *INO80* and *PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1* (*PIE1*) (Farrona *et al.*, 2004; Fritsch *et al.*, 2004; Noh and Amasino, 2003; Ogas *et al.*, 1999; Sang *et al.*, 2012; Wagner and Meyerowitz, 2002). Recently, two members of the ISWI family, *CHR11* and *CHR17*, have been reported to play a role in development (Li *et al.*, 2012; Smaczniak *et al.*, 2012b). Loss of function of chromatin remodeller complex genes generally causes pleiotropic developmental defects in the plant, due to the general roles of these proteins. For example, loss of *SYD* causes defects in many different developmental pathways such as phase transitions, growth, patterning and stem cell maintenance (Wagner and Meyerowitz, 2002). In addition, *SYD* has been shown to play an important role in flower development as well: before floral transition *SYD* represses *LFY*, while after transition it activates B and C-class genes (Wagner and Meyerowitz, 2002). Next to *SYD*, also *BRM* plays a role in the activation of homeotic genes. In *brm* flowers lower expression of the C-class genes was observed, while the flower phenotype resembles a weak B-class mutant with sepaloid-petals and carpelloid-stamens (Hurtado *et al.*, 2006). Defects in floral organ development were also observed in *chr11 chr17* double mutant (Smaczniak *et al.*, 2012b), suggesting a role for these ISWI family remodelers in flower development. Moreover, mutation in *PIE1*, the Arabidopsis gene most homologous to *SWR1*, leads to the formation of extra petals (Noh and Amasino, 2003), Formation of extra petals was also observed upon loss of function of other components of the SWR1 complex, such as *SUPPRESSOR OF FRIGIDA 3* (*SUF3*) (Choi *et al.*, 2005) and *AtSWC6* (Choi *et al.*, 2007; Lazaro *et al.*, 2008), suggesting that the SWR1 complex may play a role in floral organ number determination.

Although chromatin remodeler complexes can be recruited to the chromatin via specific protein domains, for example the bromo-domain binds to acetylated histones and the chromo-domain to methylated histones, these complexes usually lack specificity, as is

reflected in the pleiotropic effects observed in loss of function mutants. Thus, how can they play a role in gene regulation in a more specific manner? Recent studies showed that transcription factors can interact with chromatin remodelers and histone modifiers (Efroni *et al.*, 2013; Smaczniak *et al.*, 2012b; Vercruyssen *et al.*, 2014). These findings reveal a new picture of gene regulation in which transcription factors, chromatin remodelers and chromatin modifiers act in concert in the regulation of gene expression during development.

AIM AND OUTLINE OF THE THESIS.

The aim of this thesis was to investigate the dynamics of gene transcriptional regulation and the relationship between transcription factor DNA-binding and changes in chromatin structure during the formation of floral organs.

In **chapter 2**, we reviewed the current knowledge about the mechanisms involved in transcriptional gene regulation during developmental switches in plants. Recent advances in genome-wide identification of target genes controlled by plant master regulators of transcription and their interactions with epigenetic factors provide new insights into regulatory mechanisms controlling switches in developmental programs and cell fates in complex organisms.

In **chapter 3** and **chapter 4** we investigated the dynamics in transcriptional regulation and chromatin conformation in the context of flower development. Using genome-wide approaches we investigated the action of transcriptional master regulators during flower development. The protein DNA-binding behaviour of two MADS-domain proteins, AP1 and SEP3, was studied at different stages of flower development. AP1 and SEP3 show stage specific binding events and they mainly act as activators during floral organ development. Additionally, we investigated changes in chromatin landscape by profiling chromatin accessibility and nucleosome positioning at different stages of flower development. Transcription factors bind to low nucleosome occupied regions and their binding precedes to changes in chromatin accessibility. Our results prime the idea that transcription factors can act as pioneers in gene regulation: they bind to DNA and recruit chromatin remodelers and modifiers to specific loci leading to a change in chromatin structure and hence gene expression.

Chapter 5 and **chapter 6** describe the role of two MADS-domain protein target genes in flower development: *STERILE APETALA* (*SAP*) and *WUSCHEL-RELATED HOMEOBOX 12* (*WOX12*), respectively. *SAP* is a target of many MADS-domain proteins such as APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), AGAMOUS (AG) and SEPALLATA3 (SEP3). *SAP* is strongly expressed in meristems and loss of function of *SAP* causes strong aberrations in flowers, such as reduction in petal and stamen numbers. Preliminary results indicate that SAP interacts with proteins of the SCF ubiquitin ligase complex, suggesting that SAP could act in the ubiquitination pathway. *WOX12* acts downstream AP1 and ectopic expression of *WOX12* leads to reduction of *AG* expression, suggesting a role for *WOX12* in regulating the antagonistic interplay between the homeotic genes *AP1* and *AG*.

To conclude, in **chapter 7** I discuss the results of this thesis and the future implications the work performed in this thesis may have.

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CHAPTER 2

Regulation of transcription in plants: mechanisms controlling developmental switches

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ABSTRACT

Unlike animals, plants produce new organs throughout their life cycle using pools of stem cells that are organized in meristems. Although many master regulators of meristem and organ identities have been identified, it is still not well understood how they act at the molecular level and how they can switch an entire developmental program in which thousands of genes are involved. Recent advances in the genome-wide identification of target genes controlled by plant transcriptional master regulators and their interactions with epigenetic factors provide new insights into general transcriptional regulatory mechanisms controlling switches of developmental programs and cell fates in complex organisms.

Organ development in plants is a continuous and flexible process, which is not restricted to the embryonic phase (Walbot and Evans, 2003). This plasticity in plant development is linked to the presence of **pluripotent** cells residing in **meristems,** which acquire different 'identities' depending on the position in the plant and its developmental phase, thus leading to the production of different organ types. The switch from a vegetative **shoot apical meristem** to an **inflorescence meristem**, as well as the establishment of **floral meristem** and floral organ identities, are among the most well studied examples of developmental switches in plants (**Figure 1**), particularly in the model species *Arabidopsis thaliana*. These meristematic switches are controlled by multiple environmental and internal input pathways. Switches in cell identity do not only play a role in major phase transitions, but also occur during embryonic patterning, and more locally during organ differentiation. Positional information plays a crucial role in cell fate specification, and the plasticity of plant development is at least partially linked to the ability of cells to differentiate or dedifferentiate, depending on external signals and cell-to-cell communication, which are mediated by hormones and other moving signaling molecules (Busch and Benfey, 2010). Cell-extrinsic signals have also shown to play a role in cell specification and lineage commitment in animal development (Graf and Enver, 2009; Scheres, 2007).

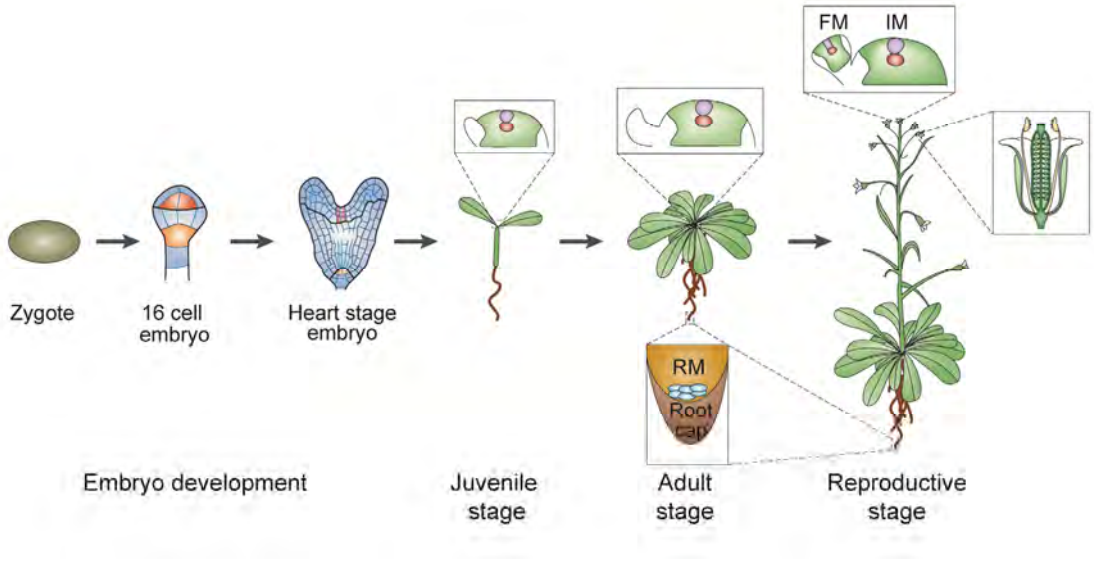


Figure 1: Phase changes in plant development. Root and shoot meristems are established during embryo development which occurs within the seed. The first shoot-meristem marker to be expressed is *WUSCHEL* (*WUS*) in the 16 cell embryo (red). *WUS* expression defines the 'quiescent centre' of the shoot meristem, which contains the stem cells. The hypophysis (orange) will give rise to the quiescent organizing centre of the root meristem. In the heart stage embryo, shoot and root meristem are already established. After germination, the shoot apical meristem (SAM) produces leaves. Within the shoot meristem (upper box),

the meristematic region is shown in green, the stem-cell marker *CLAVATA* 3 (*CLV3*) in purple, and *WUS* expression in red. The first leaves have a 'juvenile' appearance, while later produced leaves gradually develop more 'adult' characteristics (Willmann and Poethig, 2005). The change from adult to reproductive stage is more dramatic in flowering plants. In *Arabidopsis*, it is triggered by floral induction pathways converging in the upregulation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and other factors in the shoot meristem, which triggers the conversion from a vegetative to an inflorescence meristem (IM) identity (Samach *et al.*, 2000). The inflorescence meristem produces floral meristems (FMs) at its flanks, in which separate stem cell pools are established. Floral meristem identity genes repress IM identity genes and activate the expression of floral organ identity genes, which then produces the different types of floral organs: sepals and petals in the outer two whorls, respectively, as well as stamens (male reproductive organs) and carpels (female reproductive organs) in the inner two whorls. Upon floral organ differentiation, stem cell activity in the floral meristem ceases and undifferentiated cells are only maintained in specific regions within the carpels, giving rise to ovules, which upon fertilization with male pollen form the next generation zygote.

Pioneered by research in *Drosophila melanogaster* (Letsou and Bohmann, 2005), signaling cascades that control developmental switches in animals and plants have been shown to converge at the level of gene regulation; transcription factors can change entire developmental programmes, resulting in switches of cell and organ identities. Ectopic expression of key regulatory transcription factors can cause reprogramming of cell fate in animals, resulting in de-differentiation or conversion of one partially or fully differentiated cell type into another one (Graf and Enver, 2009). Aside from transcription factors, switches in cell identity and differentiation state were shown to be regulated at the level of chromatin structure. Accordingly, **histone-modifying enzymes** as well as ATP-dependent **nucleosome-remodeling enzymes** have been shown to control these processes in plants and animals (Desvoyes *et al.*, 2010; Ho and Crabtree, 2010).

The first glimpse on how developmental switches are controlled in plants appeared in the early 1990s when key regulatory genes controlling the transition from vegetative to reproductive growth were identified in the model species *Arabidopsis thaliana* and *Antirrhinum majus*. These encode transcription factors that specify the identities of IMs, FMs and floral organs (Causier *et al.*, 2010). Genetic and molecular studies revealed not only antagonistic genetic interactions between transcription factors specifying alternative meristem identities, but also cooperation and re-inforcement of factors acting in the same identity switch. These studies also uncovered roles for heteromeric protein complexes in cell and organ identity specification. For example, the crucial role of heteromeric protein interactions of floral master regulators was consolidated in 2001 (Goto *et al.*, 2001; Honma and Goto, 2001), when the 'floral quartet model' was proposed (Theissen, 2001). According to this model, floral organ identity regulators of the **MADS-box family**

assemble into organ-specific quarternary transcription factor complexes, thereby obtaining their regulatory specificity. Since then, many interactions between transcription factors acting in diverse developmental processes have been identified in different plant species, mainly using the yeast-two hybrid system. However, how transcription factors act and interact *in planta*, what their target genes are and how regulatory specificity is determined could not be resolved by these 'classical' types of studies.

Genome-wide approaches for target gene identification provided new insights into mechanisms of gene regulation by transcriptional key regulators. A number of studies used inducible versions of transcription factors and the global analysis of gene expression changes upon induction (Gomez-Mena *et al.*, 2005; Ito *et al.*, 2004; Kaufmann *et al.*, 2010; Morohashi and Grotewold, 2009; Wellmer *et al.*, 2006). Recent genome-wide analysis of *in vivo* DNA-binding sites of transcription factors using chromatin immunoprecipitation (ChIP) techniques, like **ChIP-SEQ** and **ChIP-CHIP** (Farnham, 2009), provided powerful approaches to identify potential **direct target genes**.

In this Review, we first introduce recent findings on direct downstream targets of key regulatory transcription factors controlling developmental switches in plants, and how **autoregulation** and cross-regulation in the transcriptional regulatory network controls developmental switches. We then discuss the emerging concept that developmental transitions are regulated by interplay of transcription factor complexes and proteins shaping chromatin structure. This provides insights into mechanisms underlying the action and functional specificity of different transcription factor complexes and chromatin regulators in the control of developmental switches in plants.

GLOBAL GENE REGULATION BY TRANSCRIPTION FACTORS

During developmental transitions and cell fate specification in higher eukaryotes, changes in expression of many genes need to be coordinated to initiate the correct differentiation programs and to suppress earlier or 'inappropriate' programmes. This global regulation is the task of transcriptional master regulators. The term 'master regulator' was first introduced in 1985 and applied to regulators of mating type in yeast (Herskowitz, 1985). It was later adopted to transcriptional regulators in metazoan (Halder *et al.*, 1995; Weintraub *et al.*, 1989) and plant (Meyerowitz, 2002) development. Initially, master regulators were thought to control a limited set of 'second-level' transcription factors,

which in turn regulate the expression of downstream target genes that are more involved in cellular responses, such as cell cycle, metabolic processes and intra-cellular signaling processes (**Figure 2**) (Garcia-Bellido, 1975; Gehring and Ikeo, 1999; Yu and Gerstein, 2006). The structure of regulatory networks turned out to more complex than initially anticipated after target genes of transcription factors were identified (Akam, 1998), and in particular when the results of genome-wide target gene identification approaches became available. It appeared that the master regulators directly control more genes than initially anticipated. They control not only the 'second-level' transcription factors but also genes encoding structural proteins, other signaling molecules (e.g. involved in hormonal pathways) and enzymes. Most likely, subsets of these transcriptional cascades are regulated by different combinations of transcription factors, some of which are also regulated by the 'master regulator', resulting in a more complex transcriptional network (**Figure 2**). In this model, combinatorial transcription factor interactions can create flexibility and specificity in the regulation of subsets of target genes. The transcriptional cascades are also characterized by multiple feedback and feedforward loops (Alon, 2007; Jothi *et al.*, 2009), creating a more 'democratic' network structure (Bar-Yam *et al.*, 2009).

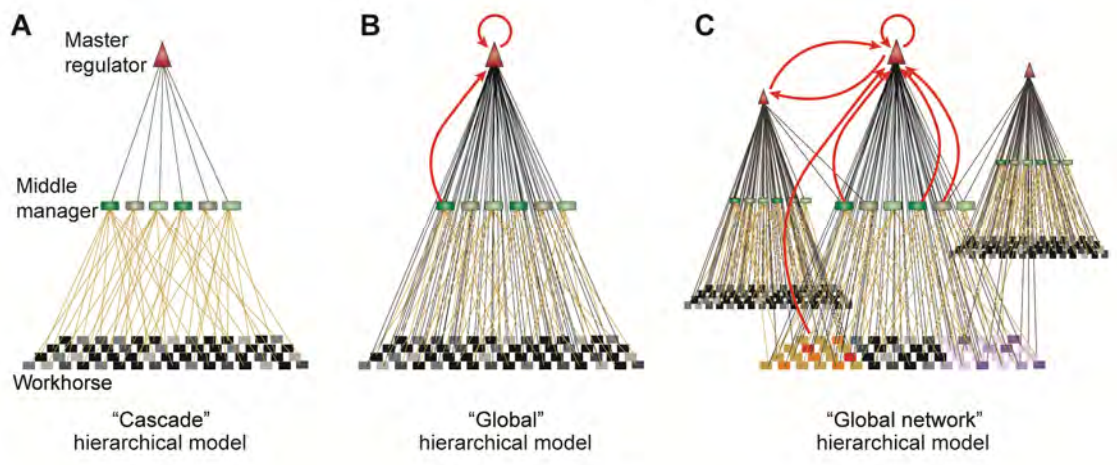


Figure 2: Models on the action of developmental 'master regulators'. A. The classical model suggested a strictly hierarchical network, in which master regulators activate a (small) set of second level regulatory genes who control the expression of genes producing distinct morphologies, e.g. encoding enzymes and structural proteins. **B**. Identification of downstream targets of individual transcription factors suggested a more complex network, in which master regulators can control genes at different levels in the hierarchy. The master regulators appear to regulate hundreds of genes directly. **C**. Genome-wide identification of targets of multiple transcription factors suggests a complex combinatorial regulation of subsets of downstream targets, as well as multiple cross-regulatory loops. Note that transcription factors control genes that can be acting either upstream or downstream in the developmental pathway.

Orchestration of gene expression during developmental switches.

Genome-wide DNA-binding sites and direct target genes of several key regulatory transcription factors with roles in plant development have been reported recently (**Table S1**). The results shed light on 'global' regulatory networks controlling switches in meristem and organ identity, as well as 'local' networks that specify cell fate and terminal differentiation during organ development.

Stem cell identity in plants and animals is controlled by the interplay of intercellular signaling and transcriptional regulation (Scheres, 2007). The homeobox transcription factor WUSCHEL (WUS) has an instructive role in stem cell identity specification in embryos and its maintenance in the stem cell niches in shoot meristems. Analysis of direct WUS target genes revealed multiple regulatory links to hormonal signaling, cell division control, as well as feedback control on the receptor complex that restricts stem cell proliferation and *WUS* expression (Busch *et al.*, 2010). The findings emphasize the complexity of intercellular communication and transcriptional feedback in stem cell specification in plants.

Transcription factors control developmental phase switches by orchestration of gene expression changes within meristems in order to repress previous developmental programs and to establish new ones. By that, they specify shoot growth and which types of lateral organs are produced. Several transcription factors have been described that act as key switches in meristem identity during transition from vegetative to reproductive growth (**Figure 3**).

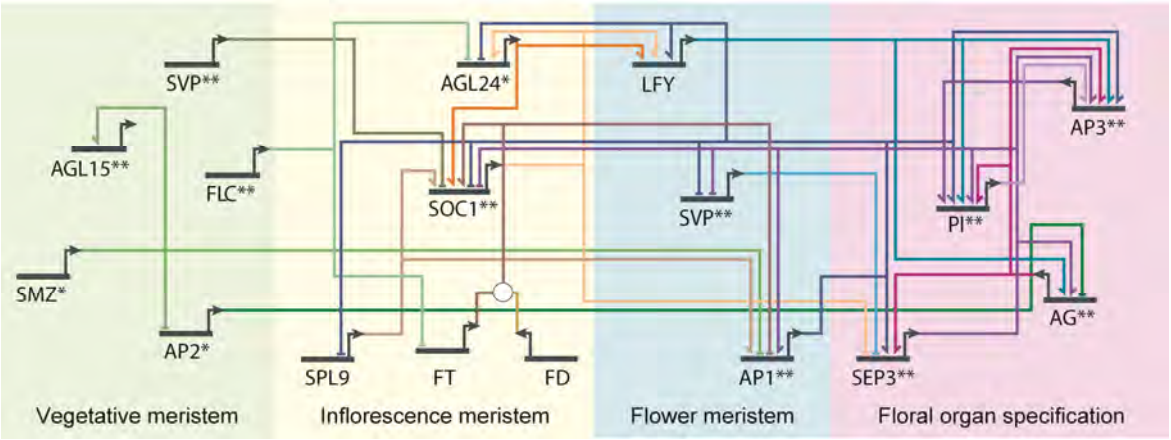


Figure 3: Regulatory network focusing on the genes that are discussed in this review. The regulatory network summarizing the major regulators acting as suppressors or activators in the consecutive developmental stages from a vegetative meristem to the formation of the flower. All factors indicated are transcription factors, except FT, which is a mobile protein produced in leaves and transported to the SAM,

where it interacts with the bZIP transcription factor FD (Corbesier *et al.*, 2007; Wigge *et al.*, 2005) to mediate the switch to IM identity. The factors are positioned in the pathway where they act for the first time, e.g AP1 is required for floral meristem identity specification and floral organ identity specification. Various external (photoperiod, temperature) and internal (autonomous, age, hormones) conditions regulate the floral transition from vegetative to inflorescence meristem. These inputs into the network are combined at the level of the so-called **floral integrators** (*SOC1*, *AGL24, FT, LFY*), and ultimately regulate the expression of the **floral meristem identity genes** (*AP1, SVP* and *LFY*). Subsequently, *SEP3* is activated by AP1 and act in the flower as the main component of protein complexes that specify floral organ identities. The floral organ identity genes (*SEP3, AP1, PI, AP3* and *AG*) are controlled by the meristem identity genes. The SVP node is duplicated in the figure because of its dual role in floral transition and meristem specificiation. **Abbreviations:** *SMZ SCHLAFMUETZE; AGL15 AGAMOUS-LIKE 15; SVP SHORT VEGETATIVE PHASE; AP2 APETALA2; FLC FLOWERING LOCUS C; SOC1 SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1; SPL9 SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9, AGL24 AGAMOUS-LIKE 24; FT FLOWERING LOCUS T; FD FLOWERING LOCUS D; LFY LEAFY; AP1 APETALA1; SEP3 SEPALLATA3; PI PISTILLATA; AG AGAMOUS; AP3 APETALA3*. * *AP2* family member. ** *MADS* family member.

The switch from vegetative to IM identity is controlled by **floral pathway integrators**, which integrate the "input" of different environmental and internal signaling pathways. Floral integrators activate the expression of FM identity genes in the flanks of the IM. The MADS domain transcription factor APETALA1 (AP1) establishes FM identity and consequently plays a role in initiation of sepals and petals within the flower (for review, see (Causier *et al.*, 2010)). *AP1* acts partially redundantly with a closely related gene, *CAULIFLOWER (CAL)*(Kempin *et al.*, 1995). AP1 has about 2000 genomic binding sites in the earliest stages of FM initiation (Kaufmann *et al.*, 2010) and at this developmental stage it acts mostly as a transcriptional repressor to downregulate the 'previous' developmental program of the meristem. Among its many targets, AP1 downregulates its own activators, such as *FD* (Abe *et al.*, 2005), *SPL9* (Wang *et al.*, 2009) and the floral integrator *SOC1* (Liu *et al.*, 2007). However, it also upregulates genes such as the master regulator *LEAFY* (*LFY*) (Lee *et al.*, 2008), which acts together with AP1 in FM identity specification (Parcy *et al.*, 1998; Weigel *et al.*, 1992) (Kaufmann *et al.*, 2010; Liljegren *et al.*, 1999). Later in FM development, AP1 activates the expression of genes that are involved in downstream processes such as floral organ initiation, probably together with its MADS family interaction partner SEPALLATA3 (SEP3). SEP3 interacts with many MADS-domain transcription factors (Honma and Goto, 2001; Immink *et al.*, 2009) and is a global regulator of floral organ identities (Honma and Goto, 2001; Pelaz *et al.*, 2000) in a largely redundant fashion with 3 other SEP paralogs (Pelaz *et al.*, 2000). SEP3 also binds thousands of regions in the genome (Kaufmann *et al.*, 2009). All floral organ identity genes are also expressed at later stages and modulate aspects of organ

differentiation that are linked to their 'identity', which is reflected in the large functional range of their target genes (Gomez-Mena *et al.*, 2005; Ito *et al.*, 2007; Ito *et al.*, 2004; Kaufmann *et al.*, 2009).

Another example for a transcription factor that plays a role in identity specification in the meristem is APETALA2 (AP2). AP2 is the founding member of the plant-specific AP2-like transcription factor family. Next to its role as a repressor of floral transition, AP2 promotes sepal and petal identity specification in the outer two whorls of the flower, and it plays a role in stem cell maintenance by promoting *WUS* expression (Drews *et al.*, 1991; Ohto *et al.*, 2009; Wurschum *et al.*, 2006; Yant *et al.*, 2010). These roles are reflected in its genomic binding sites and direct target genes. AP2 has about 2000 genomic binding sites and can repress the floral transition by direct transcriptional repression or indirect repression through the activation of other repressor genes or miRNAs, forming a network with other key regulators (Yant *et al.*, 2010). *AP2* is member of a subfamily of AP2-like genes whose partially redundantly acting members are regulated by miR172 (Wu *et al.*, 2009) (**Box 1**). For example, its paralog SCHLAFMÜTZE (SMZ) acts as a repressor of the transition to flowering, sharing more than one quarter of its direct target genes with AP2 (Mathieu *et al.*, 2009; Schmid *et al.*, 2003).

Meristem and organ identity genes establish basic developmental decisions in meristems, whereas lateral organ development is triggered by transcription factors that repres meristematic fate and activate differentiation programmes (Ha *et al.*, 2007). During organ growth, local switches in individual cell fates then lead to organ patterning and terminal cell differentiation. The specification of cellular identities during organ growth requires a meticulous balance of cell specification versus maintenance of cell division potential (Gutierrez, 2005). Accordingly, cell cycle control genes are not only targeted by meristematic regulators, but are also identified among direct targets of transcription factors specifying **trichomes** and guard cells of **stomata** in the epidermis of above-ground organs: The R2R3 MYB transcription factor MYB FOUR LIPS appears to repress several core cell cycle genes (Xie *et al.*, 2010), while the interacting transcription factors GLABRA1 (GL1) and GLABRA3 (GL3) initiate endoreduplication and thereby terminal differentiation of trichomes by activation of cell cyle modulators (Morohashi and Grotewold, 2009). While GL1 and GL3 have both several hundred genomic binding sites, they only share about 20 direct target genes, likely reflecting additional independent functions and interaction partners. The tight links between cell division and cell fate

specification are also reflected in the target genes of the interacting GRAS transcription factors SHORTROOT (SHR) and SCARECROW (SCR). These factors together control formative cell divisions generating the ground tissue in the root and were found to activate specific core cell cycle genes (Sozzani *et al.*, 2010).

Combinatorial interactions between transcription factors.

In all genome-wide target gene analyses, many direct regulatory links between transcription factors acting in the same or somehow related developmental pathways can be found, emphasizing the role transcriptional networks in developmental switches and cell fate specification. Heteromeric protein interactions have been identified for a number of transcription factors controlling meristem identity switches (Gregis *et al.*, 2006; Lee *et al.*, 2008), meristematic functions (Cole *et al.*, 2006; Rutjens *et al.*, 2009) and differentiation (Cui *et al.*, 2007; Kim *et al.*, 2008; Wenkel *et al.*, 2007; Zhao *et al.*, 2008). The consequences of protein interactions for the regulation of target gene expression are just starting to be elucidated.

Transcription factor interactions potentially influence DNA-binding site selection *in vivo*. This has been suggested to play a role in target gene selection of SEP3 complexes based on genome-wide binding data in wildtype and a floral **homeotic mutant** (Kaufmann *et al.*, 2009). Combinatorial interactions can also influence the association with cofactors affecting the transcriptional response. For instance, AP1 interactions with SOC1, SVP and AGL24 have been proposed to be involved in gene repression via recruitment of transcriptional co-repressors in floral meristem initiation (Gregis *et al.*, 2006, 2008), whereas the interaction of AP1 and SEP3 appears to be required to activate genes in floral organogenesis.

Combinatorial interactions also play a role in development of trichomes. According to the current model, they are specified by a transcription factor complex consisting of different types of regulators (Zhao *et al.*, 2008) (TTG1 (WD40), GL1 (MYB), GL3 and/or EGL3 (bHLH)). According to the current model, this complex activates its own inhibitor, which can move to neighoring cells and competes out GL1 in the complex, rendering the complex inactive in trichome specification and thereby contributing to the regular spacing of trichomes in the leaf epidermis (Zhao *et al.*, 2008).

Box 1. Combinatorial interactions by MADS-domain proteins and promoter structure.

The spatiotemporal regulation of gene expression during eukaryotic development is controlled by a complex interplay of cis-regulatory modules in core promoters and enhancers. The current model of enhancer action involves the binding of activating transcription factors to enhancer sequence elements, subsequent recruitment of additional coactivators and loop formation towards the core promoter, by which the transcription by RNA polymerase II is activated (Visel *et al.*, 2009).

Although DNA-binding sites of transcription factors are enriched in the proximal promoter regions of genes, they can also occur more distantly upstream, within introns or even downstream of target genes. Surprisingly, many target gene loci contain multiple binding sites for the same factor, for instance covering larger regions upstream and/or downstream of the gene. This is particularly notable for DNA-binding sites of MADS-domain proteins (Kaufmann *et al.*, 2009; Kaufmann *et al.*, 2010; Zheng *et al.*, 2009). Binding of transcription factors to multiple sequence elements suggest that transcriptional control is a quantitative process allowing the modulation of transcriptional activity by varying the number of transcription factor molecules independently bound to the DNA (Gertz *et al.*, 2009; Segal and Widom, 2009). Alternatively, multiple binding sites are required to enable a conformational change of the DNA allowing the formation of DNA loops. Recent models also support a role for chromatin structure and the formation of large DNA loops in the process of transcription by RNA-polymerase complexes (Lanzuolo *et al.*, 2007), which may occur in localized 'transcription factories' (Cook, 2010; Fraser and Bickmore, 2007).

DNA-looping has been observed for MADS-domain protein complexes composed of two dimers that bind two binding sites in the same promoter region (Egea-Cortines *et al.*, 1999; Theissen, 2001). *In vitro* DNA binding studies have shown that a minimum distance between the binding sites is required (Melzer *et al.*, 2009), and that the formation of heteromeric higher-order complexes can stabilize the binding to DNA (Egea-Cortines *et al.*, 1999). While loop formation has only been tested for DNA sites within appr. 100 bp distance of each other *in vitro*, loops could potentially also bridge larger distances *in vivo*.

The flexibility of the DNA that allows the looping depends on the nucleosome density of the chromatin. The optimal separation distances for looping-mediated interactions *in vivo* is estimated to be in the range of several tens of kb in condensed chromatin fibers (Rippe, 2001). Accordingly, nucleosome density around the transcription factor binding sites needs to be low to facilitate the looping at shorter distances. Besides the distance between the interacting sites, also the helical phasing of the DNA is critical to ensure the proper orientation of the DNA-bound interacting proteins facing to each other (Melzer *et al.*, 2009).

Characteristics of loop formation could also contribute to the functional specificity of different MADS-domain protein complexes, as well as differences in DNA-bending by MADS dimers (Riechmann *et al.*, 1996; West *et al.*, 1998). Reorganization of promoter structure by MADS-domain transcription factors is thus one possible mechanism by which these proteins fundamentally change the expression of developmentally tightly controlled genes.

While individual members of the complexes can potentially bind to target gene promoters on their own, all members of the complex are required to control the expression of a specific set of target genes. Small changes in the composition of these protein complexes may drastically affect the binding to promoters of target genes or the transcriptional response and thereby promote or repress a specific developmental program. Understanding the dynamics of these interactions and their trancriptional 'output' is a major challenge for future studies.

Combinatorial regulation of gene expression does not require a strong direct interaction between transcription factors in target gene promoters, also proteins that bind their sites individually or cooperate upon DNA-binding can influence gene expression in a combinatorial fashion. Identification of *cis*-regulatory modules that specify distinct spatiotemporal expression patterns, similar to the way that has been used in *Drosophila* (Zinzen *et al.*, 2009), is expected to contribute to our understanding of interconnections between different types of regulators of cell identity in plants.

Feedback and feedforward regulation.

A remarkable feature of developmental regulatory networks in animals and plants is the presence of multiple and complex auto- and cross-regulatory loops of master regulators (Graf and Enver, 2009). These loops, which often require formation of heteromeric transcription factor complexes, can confer robustness to switches in developmental programs (Kitano, 2004; Lenser *et al.*, 2009). Cross-antagonism of competing transcription factors has been proposed to be a driving force of cell lineage specification in animals (Graf and Enver, 2009). Also in plants, negative cross-regulation between antagonistically acting transcription factors acting in identity specification appears to be a common theme. Examples are the antagonism of transcription factors specifying the root and the shoot pole in the embryo (Smith and Long, 2010), antagonistic regulation of FM an IM identity genes (Liljegren *et al.*, 1999), the mutual repression of genes maintaining meristematic identity and genes triggering differentiation (Ha *et al.*, 2003; Jun *et al.*, 2010) and the mutual repression of genes specifying abaxial and adaxial organ identities (Wu *et al.*, 2008). Remarkably, coordinated regulation by microRNAs and their target transcription factors has important roles in cross-antagonistic loops during developmental transitions and cell type specification (**Box 2**).

In positive autoregulatory loops, the gene products activate the expression of the corresponding genes, ensuring maintenance of gene expression. A classical example is the autoregulation of the obligate heterodimers of class B homeotic proteins that specify petal and stamen identities (Schwarzsommer *et al.*, 1992). Also, other transcription factors specifying meristem and organ identities can control their own transcription (Gomez-Mena *et al.*, 2005; Kaufmann *et al.*, 2009).

Cross-regulatory interactions between proteins can be accompanied by direct protein interactions, particularly among members of the MADS-box transcription factor family. The requirement for two or even more proteins to interact with each other in order to establish a positive feedback loop ensures that a specific developmental program is only activated in the presence of all involved factors.

A common feature of master regulators acting in the same switch is that they can upregulate each other's expression, e.g. SOC1 and AGL24 (which interact at protein level) (Lee *et al.*, 2008; Liu *et al.*, 2008; Michaels *et al.*, 2003); AP1 and LFY (Kaufmann *et al.*, 2010; Liljegren *et al.*, 1999). Re-inforcing positive feedforward loops are found during meristem identity specification as well as linking meristem and organ identity genes. When one or more FM or IM meristem identity genes become mutated, the balance in the regulatory network becomes disturbed, and the meristem can switch from FM/IM identity back to a more vegetative state during development (Melzer *et al.*, 2008; Okamuro *et al.*, 1996), often depending on environmental condition. This process is called **floral reversion**.

Another type of regulation found among transcription factors controlling developmental switches are negative feedback loops of consecutively acting proteins. These loops ensure the suppression of the preceding program once the activator of the next program is induced. By this, a switch in developmental programs is established and reversion to the previous stage is blocked. Examples are negative feedback loops during establishment of the FM (Kaufmann *et al.*, 2010; Liu *et al.*, 2007). Negative feedback loops can also dampen effects of stochastic fluctuations in protein levels, for instance in stem cell homeostasis (Kim *et al.*, 2008).

Box 2: Regulation of developmental switches and cell fate specification by plant microRNAs

MicroRNAs control major developmental phase transitions in plant life as well as cell fate specification in different tissues. One important feature is their ability to simultaneously target several, often evolutionarily related, transcription factors. Another emerging feature is their ability to move between cells, thereby controlling cell fate in a non-cell autonomous manner.

For example, the juvenile-to-adult phase transition in *Arabidopsis* is regulated by the sequential action of miR156, which targets 10 SPL transcription factors, and miR172, which targets six AP2 transcription factors. miR156 and miR172 show complementary temporal expression patterns in *Arabidopsis* and in maize (Chuck *et al.*, 2007; Wang *et al.*, 2009; Wu *et al.*, 2009). miR156 acts during the juvenile phase to repress the transition to adult phase. In contrast, miR172 shows abundant expression in the adult phase. Two targets of miR172, TARGET OF EAT1 and 2, repress adult characteristics of leaf morphology. In order to trigger transition from juvenile to adult phase, two targets of miR156, SPL9 and SPL10, directly activate the expression of a locus encoding miR172, while at the same moment negatively regulating their own expression by direct upregulation of miR156. This negative regulation can be overcome by input from the photoperiodic flowering pathway, which results in a rapid increase of SPL expression. Next to the activation of miR172, SPL transcription factors directly contribute to the transition to flowering by direct activation of floral integrators and floral meristem identity genes (Yamaguchi *et al.*, 2009).

MicroRNAs also participate in cross-antagonistic regulation of transcription factors specifying different cell fates. For instance, radial patterning of the shoot and abaxial/adaxial patterning of the leaf are regulated by miR165/166, which target members of the HD-ZIP family of transcription factors(Emery *et al.*, 2003; Williams *et al.*, 2005). Data from root development show that miR156/166 expression is upregulated by SHR/SCR in the endodermis, from where it moves to the stele where it forms a gradient to repress HD-ZIP family members in a dose-dependent manner (Carlsbecker *et al.*, 2010). Different levels of HD-ZIP activity appear to specify different cell fates in the vasculature. This movement of miR165/166 is opposite to that of SHR, which is produced in the central vascular tissue and moves to the endodermis, where it is transported to the nucleus by its interaction partner SCR. This bi-directional gradient reminds of morphogen gradients establishing and consolidating cell fate decisions in *Drosophila* embryo development (Scheres, 2010). Another example of a microRNA function is the establishment of boundaries between meristematic growth and organogenesis by miR164 (Laufs *et al.*, 2004; Mallory *et al.*, 2004).

MicroRNA gradients also play roles in organ patterning in the flower. Next to its role in floral transition in *Arabidopsis*, miR172 accumulates in the centre of floral meristems, where it restricts the accumulation of *AP2* and thereby specifies the boundary between perianth and reproductive organs (Aukerman and Sakai, 2003; Wollmann *et al.*, 2010). In *Antirrhinum* and *Petunia*, the conserved miR169 is required for threshold-dependent activation of MADS-box genes that specify reproductive organ identities. miR169 accumulates in the centre of the floral meristem, and represses NF-YA, which is a repressor of reproductive MADS-box gene expression (Cartolano *et al.*, 2007).

Several other roles of microRNAs in hormonal responses and morphogenesis have been described, and have been reviewed comprehensively elsewhere (Nag and Jack, 2010).

INTERPLAY WITH CHROMATIN ASSOCIATED-PROTEINS

The transcriptional activity of eukaryotic genes is determined by the combined action of transcription factors and chromatin modifying proteins. Accordingly, transcription factors, histone-modifying enzymes as well as ATP-dependent nucleosome remodeling complexes have been shown to coordinate differentiation programs during plant and animal development. Major classes of chromatin organizing proteins are conserved between plants and animals (www.chromdb.org). Linked to the evolution of complex body plans and developmental programs in plants and animals, chromatin-regulating protein families have expanded independently in these two groups of higher eukaryotes (Chen *et al.*, 2009; Flaus *et al.*, 2006; Hennig and Derkacheva, 2009). Global changes of chromatin structure have been associated with initiation of differentiation or re-programming, and with local chromatin changes during cell fate specification within developing organs (Exner and Hennig, 2008). However, the regulatory interplay between transcription factors and chromatin regulators is only starting to be unraveled. Below, we will give examples for molecular links between transcription factors and chromatin regulators in the control of developmental switches and differentiation in plants.

Regulatory interplay of chromatin organizers and transcription factors.

Chromatin-associated proteins play roles in the tissue-specific activation and repression of genes, as well as in maintenance of expression states across cell divisions ('cellular memory'). The role of chromatin organizers in coordinating developmental switches and cellular specification can often be related to their role in repressing or activating identity-instructive transcription factors.

Nucleosome remodeling and assembly complexes have diverse roles in the transcriptional regulation of patterning genes in plants (**Table S2**), since nucleosomes modulate the accessibility of DNA for transcription factors and the basic transcriptional machinery. During DNA replication, proper nucleosome deposition is essential for maintaining the right expression of patterning genes. Mutants of core components of the nucleosome assembly factor CAF-1 (*fas-1* and *fas-2*) show de-regulation of expression of the stem cell factors *WUS* and *SCR* in the shoot and root meristems, respectively (Exner *et al.*, 2006; Kaya *et al.*, 2001; Ono *et al.*, 2006). Nucleosome sliding by SWI/SNF-type remodelers BRAHMA and SPLAYED has roles in meristem maintenance, major developmental phase transitions and the regulation of floral organ identity genes (Bezhani

et al., 2007; Farrona *et al.*, 2004; Hurtado *et al.*, 2006; Kwon *et al.*, 2005; Tang *et al.*, 2008; Wagner and Meyerowitz, 2002). Deposition of the histone variant H2A.Z by the conserved SWR1 complex mediates globally developmental response to changes in temperature, for instance by triggering floral induction via regulation of the floral inducer FT (Kumar and Wigge, 2010).

At the 'local' level of a single cell, the end of mitosis and beginning of G1 of the next cell cycle are important moments for cell fate decisions, this is the moment when cell fate regulators are induced or remain switched off (Desvoyes *et al.*, 2010). Accordingly, study of the *GLABRA2* locus indicated local changes in chromatin structure associated with activation of this locus triggering the induction of root hairs (Costa and Shaw, 2006). These are likely linked with changes in nucleosome deposition by CAF-1 (Costa and Shaw, 2006) and in the histone methylation state at the *GL2* locus (Caro *et al.*, 2007).

The 'cellular memory' of gene expression states is provided by histone-modifying enzymes, which play important roles in global and local changes in cell fate during plant development. Histone acetylation is a dynamic and reversible modification and high levels of acetylation are linked with activation of gene expression via changes in DNA accessibility. Acetylated histones are also recognized by certain types of nucleosome remodelers. The important roles of histone acetylation in cell fate decisions are for instance reflected in mutants of the histone acetyl transferase GCN5, which forms SAGA-like complexes in *Arabidopsis* (for review, see (Servet *et al.*, 2010b)). Among other functions, GCN5 is required for *PLETHORA*-mediated root stem cell regulation (Kornet and Scheres, 2009), for restricting the expression domains of *WUS* and of floral organ identity genes (likely indirect), as well as for cell differentiation in leaves (Bertrand *et al.*, 2003; Servet *et al.*, 2010b).

The PcG protein complex PRC2 mediates trimethylation of histone H3 lysine 27 (H3K27me3) (Kuzmichev *et al.*, 2002), a mark that is recognized by PRC1 in animals and a TERMINAL FLOWER 2 (TFL2)/LHP1 -containing complex in plants. This mark labels transcriptionally repressed genes and thereby creates a cellular memory. PcG complexes are required for the repression of meristematic factors in differentiating organs (Schubert *et al.*, 2006). Their role appears thus to be different from that in animals (Aichinger *et al.*, 2009), where PcG proteins are required for maintenance of the pluripotent state. PcG complexes were also shown to play roles in the maintenance of major developmental phases (**Table S2**) (Hennig and Derkacheva, 2009).

During vegetative phase and in the switch to reproductive phase, PcG protein complexes do not only repress (precocious) expression of reproductive identity genes, but they also down-regulate the expression of floral repressors upon retrieval of environmental stimuli. Genetic and biochemical studies support the existence of several PRC2-like complexes with partially overlapping biological functions (Chanvivattana *et al.*, 2004). PcG repression might also create a threshold for transcriptional activation which ensures that genes are not induced under low inductive conditions and therefore play a more dynamic role in the regulation of at least some of their targets (Adrian *et al.*, 2009).

In animals, the repressive action of PcG complexes is counteracted by trithorax group (TrxG) proteins (Poux *et al.*, 2002), which set H3K4me3 (Klymenko and Muller, 2004), a mark that is mostly found around the start site of transcriptionally active genes. The occurence and potential roles of 'bivalent' H3K27me3/H4K4me3 marks, which poise genes for activation in animal stem cells, remain to be further investigated in plants (Zhang *et al.*, 2009). The TrxG homolog ATX1 (Alvarez-Venegas and Avramova, 2001; Tamada *et al.*, 2009) , which has shown histone methyltransferase activity (Saleh *et al.*, 2008), and related proteins (Tamada *et al.*, 2009) have been associated with a TrxG-like functions in plants. Two other factors have been suggested to act as TrxG proteins: the SAND-domain transcriptional regulator ULTRAPETALA1 (ULT1), which interacts with ATX1 (Carles and Fletcher, 2009) and the **(CHD)-type nucleosome remodelers** PICKLE (PKL) and PICKLE-RELATED 2 (PKR2) (Aichinger *et al.*, 2009) (**Figure 4**). Loss of the nucleosome remodeling factor PKL results in reduced expression of many PcG target genes (Aichinger *et al.*, 2009). PKL has multiple roles in development, it acts as a repressor of embryonic traits and appears to be involved in the activation of floral organ identity genes. In line with a TrxG-like function of PKL, floral organ identity genes appear to be directly antagonistically regulated by PKL and PcG proteins. At a mechanistic level, PKL might shift nucleosomes in target gene core promoters in the absence of PcG proteins and thereby allow transcriptional activators to access their binding sites in opened chromatin. Chromatin-modifying enzymes and core members of nucleosome remodeling complexes often have broad expression patterns during plant development. However, changes in the expression of specific subunits of chromatin organizer complexes can drastically change their activity. For instance, the **vernalization**-induced addition of a single subunit is required for efficient repression of the floral repressor *FLOWERING LOCUS C* (*FLC*) (De Lucia *et al.*, 2008). Genes encoding certain

members of PcG protein complexes are themselves under epigenetic control, which suggest that cross-regulatory mechanisms can control chromatin organizer activity (Aichinger *et al.*, 2009; Vielle-Calzada *et al.*, 1999).

Protein interactions between transcription factors and chromatin organizers.

If chromatin regulators control partially different sets of target genes during distinct developmental phases, how are they 'actively' recruited to specific genomic loci? The currently most widely accepted view is that chromatin regulatory proteins are recruited by the action of tissue-specific transcription factors (for review, see (Ho and Crabtree, 2010; Schwartz and Pirrotta, 2007)) and/or RNA molecules (for review, see (Henderson and Jacobsen, 2007)).

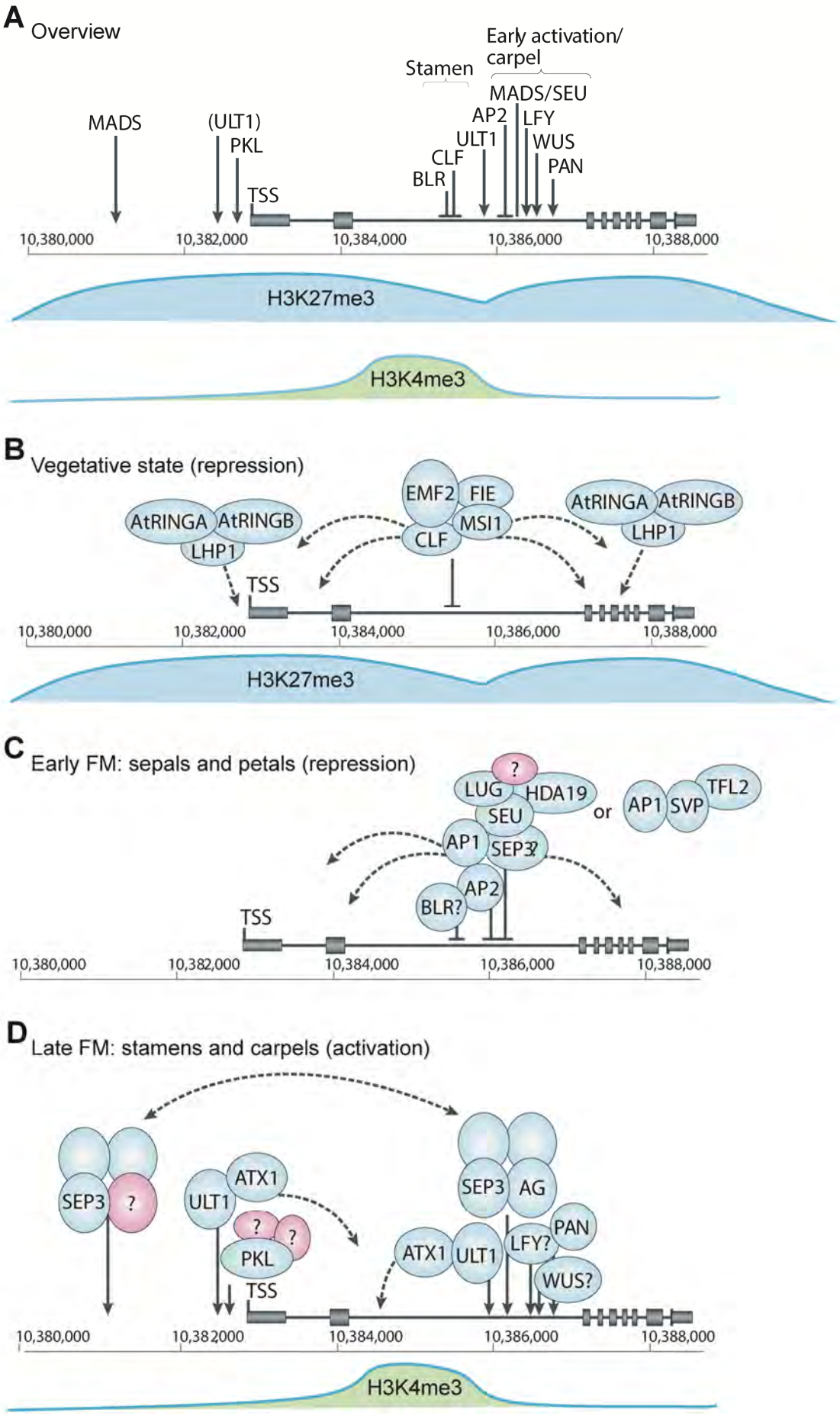
All except 3 of the loci of transcriptional regulators indicated in **Figure 3** have been found to be marked by H3K27me3 (Zhang *et al.*, 2007b). In order to establish the negative and positive regulatory interactions between master regulators controlling meristematic switches in plants, interplay with chromatin organizers thus appears to be likely. Several protein interactions between plant transcription factors and histone modifying enzymes have been described so far. MADS-box transcription factors controlling the transition to flowering have been shown to interact with chromatin regulators (Hill *et al.*, 2008; Liu *et al.*, 2009b): SVP can interact with the PcG protein TFL2/LHP1. SOC1, AGL24 and AGL15 can interact with a member of the Sin3/histone deacetylase (HDAC) complex (Hill *et al.*, 2008; Liu *et al.*, 2009a). The repressive activities of complexes consisting of transcription factors and chromatin modifiers were shown to prevent the premature activation of the floral organ identity factors *SEP3* in early floral meristems, in that way inhibiting premature differentiation of cells in the floral meristem (Liu *et al.*, 2009a). These interactions could also play a role in the global repression of target genes during floral meristem initiation by AP1 (Gregis *et al.*, 2006, 2008), since AP1 has been shown to interact with SVP, SOC1 and AGL24 (de Folter *et al.*, 2005; Gregis *et al.*, 2006, 2008) (**Box 1**). In addition, AP1 gradually downregulates the expression of *SVP, SOC1* and *AGL24*, thereby eventually releasing the inhibitory effects of these proteins on *SEP3* and possibly other target genes (Kaufmann *et al.*, 2010).

A second mechanism by which the MADS-domain protein complexes can repress the expression of target genes in early flower development is via their interaction with a general co-repressor complex consisting of SEUSS (SEU) and LEUNIG (LUG) (Gregis

et al., 2006; Sridhar *et al.*, 2006). SEU shares homology with transcriptional co-regulators in animals (Franks *et al.*, 2002). The LUG protein is similar to Groucho (Gro)-like co-repressors in animals and yeast (Conner and Liu, 2000; Liu and Karmarkar, 2008). These cofactors are recruited to their regulatory targets by interacting with DNA-bound transcription factors, since they do not possess a DNA-binding domain. LUG has been shown to directly interact with components of the MEDIATOR complex and with the class 1 histone deacetylase HDA19, and its repressor activity depends on histone deacetylase activity (Gonzalez *et al.*, 2007). SEU/LUG transcriptional co-repressor complexes could thus mediate target gene repression via histone modifications, which appears to be a general mechanism of eukaryotic co-repressor function (Perissi *et al.*, 2010). The best studied target of a MADS/co-repressor complex is the *AG* locus (Sridhar *et al.*, 2006) (**Figure 4**).

Groucho-type co-repressors have shown to have a variety of roles in cell fate specification and developmental patterning. For instance, co-repressor TOPLESS (TPL) has a basic role in specification of the shoot pole by repression of root 'identity' genes in embryos, forming a complex with the AUX/IAA factor IAA12/BODENLOS (BDL) and the auxin response factor MONOPTEROS (MP) (Szemenyei *et al.*, 2008). TPL interacts, like LUG, with HDA19 (Long *et al.*, 2006). LUG and its close homolog, on the other hand, also interact with YABBY transcription factors specifying abaxial cell identities and morphology of developing organs (Navarro *et al.*, 2004; Stahle *et al.*, 2009). General co-repressors are thus major components of antagonistic relationships of transcription factors specifying that specify cell fates during plant development.

While interactions of transcription factors and chromatin modifiers in target gene repression have been described, the interplay between these two types of regulators in gene activation during plant development is much less well understood. One example indicating a possible direct combined action of transcription factors and chromatin regulators is the activation of *KNUCKLES* (*KNU*) by AG, which apparently requires rounds of cell division after initial AG binding to the locus (Sun *et al.*, 2009). A related mechanism involving the requirement of cell divisions might in part account for the observed delay of gene activation during early floral meristem development. Understanding the molecular dynamics of epigenetic changes and the role of transcription factors in epigenetic programming and re-programming in plants remains a major challenge for the future.


A
Overview
Early activation/
carpel
Stamen
MADS/SEU
AP2
LFY
ULT1
WUS
CLF
PAN
BLR
MADS
(ULT1)
PKL
TSS
10,380,000
10,382,000
10,384,000
10,386,000
10,388,000
H3K27me3
H3K4me3
B
Vegetative state (repression)
EMF2
FIE
MSI1
CLF
AtRINGA
AtRINGB
LHP1
AtRINGA
AtRINGB
LHP1
TSS
10,380,000
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10,386,000
10,388,000
H3K27me3
C
Early FM: sepals and petals (repression)
?
LUG
HDA19
SEU
or
AP1
SVP
TFL2
AP1
SEP3?
AP2
BLR?
TSS
10,380,000
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D
Late FM: stamens and carpels (activation)
SEP3
?
ATX1
ULT1
?
?
PKL
TSS
SEP3
AG
ATX1
ULT1
LFY?
PAN
WUS?
10,380,000
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10,384,000
10,386,000
10,388,000
H3K4me3

Figure 4: Interplay between transcription factors and chromatin regulatory proteins in the regulation of *AGAMOUS* expression. **A.** Most regulatory elements controlling *AG* expression are located in its 2nd intron (Sieburth and Meyerowitz, 1997). Early studies have identified two enhancers, one acting in stamens and another one acting in the early activation and in carpels (Deyholos and Sieburth, 2000). *AG* is repressed in vegetative tissues, in early floral meristems (stage 1-2) and the outer whorl organs. *AG* is activated at later stages of floral meristem development in the central domain that give rise to the stamens and the carpels. **B.** In vegetative tissues, *AG* is stably repressed by the action of a PRC2-like PcG complex containing CURLY LEAF (CLF) (Schubert *et al.*, 2006), FERTILISATION INDEPENDENT ENDOSPERM (FIE), EMBRYONIC FLOWER2 (EMF2), MULTICOPY SUPPRESSOR OF ira1 (MSI1) and possibly other, yet to be identified components. The PCR2 complex catalyses trimethylation of histone H3 at lysine 27 (H3K27me3). H3K27me3 is recognized by LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER LIKE 2 (LHP1/TFL2) (Gaudin *et al.*, 2001; Turck *et al.*, 2007), which then binds to the *AG* locus (Turck *et al.*, 2007). LHP1 is essential for the repression of at least a subset of the PcG target genes, suggesting a PcG-like function. It has been proposed to be the functional analog of PRC1-analogous complexes, which in animals catalyse H2AK119u (for review, see (Hennig and Derkacheva, 2009)). **C.** In early floral meristems and in petals and sepals, *AG* is repressed by the combined action of several transcription factors. The MADS-domain transcription factors AP1 and SEP3 can interact with the co-repressor SEUSS (SEU) which forms a complex with LUG (Sridhar *et al.*, 2006) to recruit histone deacetylase 19 (HDA19) (Gonzalez *et al.*, 2007). A MADS-domain protein complex containing AP1 and SVP has been proposed to repress precocious *AG* expression in early stages of meristem development via recruitment of TFL2 (Gregis *et al.*, 2008). The transcription factor *APETALA2* is also required for *AG* repression in young floral meristems as well as in petals and sepals (Drews *et al.*, 1991),(Yant *et al.*, 2010). Also the BELL1-like homeobox protein BELLRINGER (BLR) is required for *AG* repression in inflorescence and early floral meristems (Bao *et al.*, 2004). BLR binds to sequence elements overlapping with the region most strongly bound by CLF *in vitro*, however *in vivo* binding has not been shown so far. Other factors have been described as *AG* repressors, however it is not known whether this regulation is direct. **D.** At the stage when the organ identities are being determined, *AG* expression is activated in parts of the floral meristem that develop into stamens and carpels. SEP3 activates *AG* expression and may also interact with the AG protein conferring a positive autoregulatory control of *AG* expression (Gomez-Mena *et al.*, 2005),(Kaufmann *et al.*, 2009). In wild-type inflorescences, SEP3 has two binding sites in the *AG* locus (Kaufmann *et al.*, 2009): one binding site is located upstream the transcription start site and another one is present in the second intron at about 5 kb distance. This makes it possible that a loop between these two sites is formed by quarternary complexes containing SEP3 and AG, ultimately leading recruitment of activating facors close to the transcriptional start site. The SAND-domain protein ULT1 contributes to the early activation of *AG* in floral stem cells by antagonizing the repressive action of PcG proteins (Carles and Fletcher, 2009). Since ATX1 and ULT1 interact *in planta*, ULT1 could recruit ATX1 to target loci, for instance *AG*, via its DNA-binding SAND domain and thus enable ATX1 to set H3K4me3 (Carles and Fletcher, 2009; Sommer et al., 1990) (Alvarez-Venegas et al., 2003). The CHD3-type chromatin remodeling factor PKL (Ogas et al., 1999) and its paralog PKR2 have also been shown to contribute to AG activation, also antagonizing the action of PcG proteins (Aichinger et al., 2009). The bZIP transcription factor PERIANTHIA (PAN) contributes to AG activation in specific regions of the floral meristem (Das et al., 2009; Maier et al., 2009). Also the floral meristem identity factor LEAFY (LFY) and the stem cell regulator WUSCHEL (WUS) have been proposed the contribute to AG activation (Lohmann et al., 2001), however the binding of these transcription factors to the AG locus was not shown in vivo so far.

CONCLUSIONS AND PERSPECTIVES

Recent technological advances enable us to study the action of transcription factors in the direct regulation of gene expression. These led to novel understanding of developmental processes in plants and animals. Transcription factor networks are highly organized: Complex regulatory loops between transcription factors and combinatorial interactions control the expression of target genes. Regulatory loops in transcription factor networks ensure 'sharp' transitions in developmental programs, but also the stable maintenance of developmental programs by feedforward and autoregulatory loops.

Developmental switches require changes in chromatin organization. Recent results indicate that transcription factors and chromatin regulators act in union in the control of gene expression during development. Yet, many questions in this field remain to be answered. How generally important are direct protein interactions between transcription factors and chromatin regulators? While at least some plant transcription factors have shown to interact with histone-modifying proteins to repress gene expression, the role of such interactions in gene activation remains to be elucidated. Dissection of the transcriptional responses linked to *cis*-regulatory elements and chromatin status at specific genomic loci, as done for the FT locus recently (Adrian *et al.*, 2010), is one step towards unraveling the molecular mechanisms of target gene regulation by transcription factors and chromatin regulators.

Transcription factors acting in developmental transitions directly control the expression of a large number of genes, only a fraction of which also appear as targets of the PcG/TrxG system. Thus, it can be expected that there are multiple mechanisms by which these transcription factors can control gene expression, involving involve nucleosome remodeling or other processes.

The first global comprehensive studies on *in vivo* DNA-binding sites and target genes of key developmental transcription factors have provided a wealth of information about the topology of the transcriptional networks. However, how combinatorial interactions between proteins affect DNA-binding and transcriptional regulation need to be studied further. To decipher this regulatory code, more systematic studies of transcription factor binding sites and transcriptional response are needed. Technically challenging *in vivo* approaches such as **sequential ChIP** (Geisberg and Struhl, 2004) and/or the use of mutants in which one of the interacting factors is eliminated can shed light on the role of combinatorial transcription factor interactions. The assembly of these complexes and

hence the transcriptional output is highly dynamic and cell-type specific. Approaches to analyze gene regulation in a cell-type specific manner are just beginning to be explored (Deal and Henikoff, 2010) (Jiao and Meyerowitz, 2010; Sozzani *et al.*, 2010). In order to understand the nature of combinatorial interactions, a challenge is to identify interaction partners of endogenously expressed transcription factors *in planta*. Both biochemical and sophisticated imaging-based technologies offer potential to study the developmental dynamics of protein interactions.

The important role for the higher-order organization of chromatin in the control of eukaryotic gene expression has also emerged in recent years. Biochemical techniques to study chromatin loops such as 3C- and ChIP-based technologies are just starting to be used in plants (Louwers *et al.*, 2009). In the future, these methods will help us understanding the dynamics of chromatin organization during developmental switches, as well as the role of master regulatory transcription factors in this process.

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GLOSSARY

Pluripotent cell: An undifferentiated cell that has the potential to adopt different identities. In plants, pluripotent cells are found in meristems and there are stem-cell like populations in shoots, roots and leaves.

Meristem: A tissue in plants consisting of pluripotent cells. In apical meristems, cell-to-cell signaling establishes and maintains a zone that contains the stem cells, which is separated from the peripheral zone in which differentiation is eventually initiated. Other types of meristems give rise to the vascular and epidermis, or enable secondary growth.

Shoot apical meristem: is the meristem that forms all major above-ground plant organs. It is established during embryogenesis. During plant development, it changes from a juvenile to a vegetative, and then to an inflorescence and floral identity.

Inflorescence meristem: Type of **shoot apical meristem** that gives rise to floral meristems at its flanks.

Floral meristem: shoot meristem that gives rise to floral organs: sepals, petals, stamens and carpels.

Histone-modifying enzyme: Enzymes that can modify specific sites in histones. Common modifications are (de-) methylation, acetylation, ubiquitination, sumoylation, phosphorylation or proline isomerization.

Nucleosome-remodeling enzyme: protein complexes complexes that can establish, remove, or change the positions of nucleosomes on the DNA.

MADS-box family: A family of transcription factors that is present in all major groups of eukaryotes. The family is named after the founding members MCM1 from *Saccharomyces cerevisiae*, AGAMOUS from *Arabidopsis thaliana*, DEFICIENS from *Antirrhinum majus* and SRF from humans.

ChIP-SEQ: Chromatin immunoprecipitation followed by next generation sequencing. Technique that is used to identify DNA-binding sites of transcription factors or other DNA-binding proteins (e.g. histones). After crosslinking of protein-DNA interaction, isolation and fragmentation of the chromatin, genomic regions that are bound by a protein are isolated using specific antibodies. Protein-bound DNA is then sequenced.

ChIP-CHIP: See **ChIP-SEQ**. Instead of sequencing of immunoprecipitated DNA, it is hybridized to genomic tiling arrays to identify DNA-binding sites of the protein.

Direct target gene: A gene whose expression at any moment or in any tissue is controlled by a transcription factor via direct binding of the factor to *cis*-regulatory elements of that gene.

Autoregulation:A mechanism in which a molecule (such as a. transcription factor) regulates its own production. The process can involve interaction with other molecules/proteins.

Floral pathway integrator: A protein that can integrate the inputs of the different enviromental and internal floral induction pathways and transmit the information to their downstream targets, such as floral meristem identity genes, at the shoot apex. Their combined action controls flowering time. The transcriptional regulators SOC1, LFY, FT and FD are ‘classical’ floral pathway integrators.

Trichome: epidermal outgrowths (hairs) of different types and functions. In *Arabidopsis*, trichomes are unicellular.

Stomata: A pore found in the epidermis of leaves and in several other above-ground plant organs. Stomata are surrounded by pairs of specialized epidermal cells called guard cells.

Homeotic mutant: A mutant in which one organ type is replaced by a different organ type.

Floral reversion: reversion of a meristem from a reproductive state back to a vegetative state caused by mutations in regulatory genes, e.g. a floral meristem to an inflorescence meristem or an inflorescence meristem back to a vegetative meristem. Leads to the formation of shoots instead of flowers and 'aerial' rosettes instead of shoots.

(CHD3)-type nucleosome remodeler: ATP-dependent chromatin remodeling factor of the chromodomain/helicase/DNA-binding domain (CHD3) subfamily. Usually act as part of multi-subunit complexes. In mammals and flies, they are involved in transcriptional repression by nucleosome remodeling and histone deacetylation. Also have been shown to be involved in activation of transcription.

Vernalization: Induction of the transition from vegetative to reproductive plant growth by a prolonged period of cold (winter).

Sequential ChIP: Identification of binding sites that are common to two proteins (e.g. two types of modified histones or transcription factors). Involves two rounds of immunoprecipitation using separate antibodies against the proteins of interest.

DESCRIPTION OF ADDITIONAL TABLES

The following additional tables are available online.

http://www.nature.com/nrg/journal/v11/n12/suppinfo/nrg2885.html

Table S1. Genome-wide target gene studies of developmental transcription factors in *Arabidopsis*.

Table S2: Examples for chromatin modifying and remodeling complexes with roles in developmental switches

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CHAPTER 3

Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development

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ABSTRACT

Development of eukaryotic organisms is controlled by transcription factors that trigger specific and global changes in gene expression programs. In plants, MADS-domain transcription factors act as master regulators of developmental switches and organ specification. However, the mechanisms by which these factors dynamically regulate the expression of their target genes at different developmental stages are still poorly understood.

We characterized the relationship of chromatin accessibility, gene expression and DNA-binding of two MADS-domain proteins at different stages of *Arabidopsis* flower development. Dynamic changes in APETALA1 and SEPALLATA3 DNA-binding correlated with changes in gene expression, and many of the target genes could be associated with the developmental stage in which they are transcriptionally controlled. We also observe dynamic changes in chromatin accessibility during flower development. Remarkably, DNA-binding of APETALA1 and SEPALLATA3 is largely independent of the accessibility status of their binding regions and it can precede increases in DNA accessibility. These results suggest that APETALA1 and SEPALLATA3 may modulate chromatin accessibility, thereby facilitating access of other transcriptional regulators to their target genes.

Our findings indicate that different homeotic factors regulate partly overlapping, yet also distinctive sets of target genes in a partly stage-specific fashion. By combining the information from DNA-binding and gene expression data, we are able to propose models of stage-specific regulatory interactions, thereby addressing dynamics of regulatory networks throughout flower development. Furthermore, MADS-domain TFs may regulate gene expression by alternative strategies, one of which is modulation of chromatin accessibility.

INTRODUCTION

Stem cells residing in meristems enable plants to produce new organs throughout their lives. Vegetative meristems in the shoot apex produce leaves, while reproductive meristems produce flowers or floral organs. The identities of different types of floral organs (sepals, petals, stamens and carpels) are established by homeotic MADS-domain transcription factors (TFs) via modification of the leaf developmental programme (Honma and Goto, 2001). Homeotic genes become activated in floral meristems through regulators that specify floral meristem identity. An important regulator of floral meristem identity in Arabidopsis is the MADS-box gene *APETALA1* (*AP1*), which has an additional role as homeotic regulator of sepal and petal identity (Mandel *et al.*, 1992). Homeotic proteins specify different floral organ identities in a combinatorial fashion, mediated by protein interactions and formation of heteromeric quaternary protein complexes (Coen and Meyerowitz, 1991; Smaczniak *et al.*, 2012b; Theissen, 2001) . Homeotic genes can also enhance or repress each other's expression, resulting in a complex transcriptional regulatory network. Mediators of higher-order complex formation are the largely redundantly acting members of the SEPALLATA MADS-domain subfamily, SEPALLATA 1 to 4 (SEP1-4) (Ditta *et al.*, 2004; Honma and Goto, 2001; Pelaz *et al.*, 2000). Therefore, these proteins have an important role in the specification of floral organ identities. Members of the MADS-domain TF family also act in many other developmental processes in plants, regulating directly and indirectly the expression of thousands of genes in the genome (for review, see (Kaufmann *et al.*, 2010a; Smaczniak *et al.*, 2012a)). Floral MADS-domain TFs are found in larger protein complexes together with chromatin remodelling and modifying proteins, as well as with general transcriptional co-regulators (Smaczniak *et al.*, 2012b; Sridhar *et al.*, 2006). These interactions are important for the regulation of gene expression by the MADS-domain factors (Smaczniak *et al.*, 2012b; Sridhar *et al.*, 2006; Wu *et al.*, 2012). The expression of floral homeotic MADS-box genes is also regulated at the level of chromatin structure: outside the flower and at the earliest stages of floral meristem development, these genes are repressed by Polycomb group (PcG) protein complexes that act in concert with earlier acting MADS-domain TFs and other transcriptional regulators (Liu *et al.*, 2009). The physical and genetic interactions between MADS-domain proteins and chromatin regulatory factors suggest an important role of these TFs in controlling

chromatin dynamics during plant development. To gain a genome-wide perspective on the developmental dynamics of gene regulation in plants, we studied MADS-domain TF occupancy, chromatin accessibility and gene expression changes at different stages of Arabidopsis flower development. Our findings suggest that MADS-domain TFs may induce changes in chromatin accessibility, and thereby they are able to set appropriate chromatin landscapes for following regulatory processes leading to meristem and organ differentiation during flower development. By combining DNA-binding data and expression data, we established stage-specific gene regulatory interactions in floral morphogenesis.

RESULTS

Developmental dynamics of floral gene regulation.

We studied global changes in chromatin accessibility, gene expression and DNA-binding of two MADS-domain TFs at different stages of flower development (**Figure 1**). To obtain sufficient stage-specific plant material, we used an inducible system for synchronized flower development based on a chemically inducible version of the AP1 TF expressed under the control of its own promoter in the *ap1 cal* mutant background (*pAP1*:AP1-GR *ap1 cal* line).

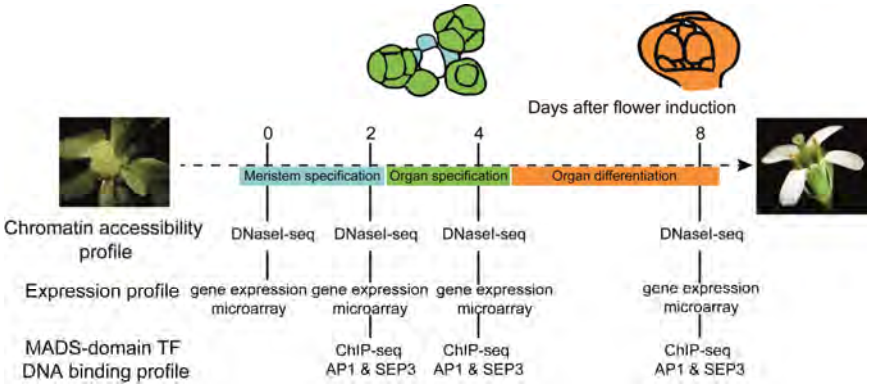


Figure 1. Overview of the experimental set up. Using a system for synchronized floral induction (*pAP1*:AP1:GR *ap1 cal*), different developmental stages were analysed: meristem specification (stage 2; 2 days after induction), organ specification (stage 4-5; 4 days after induction) and organ differentiation (stage 7-8; 8 days after induction). Around day 4, organ identity genes specify the floral whorls within the meristem, and sepal growth has been initiated. At day 8, sepals are largely differentiated, and the organs in the inner whorls are being formed. The experimental techniques used at each time point are indicated in the lower part of the figure. For illustrative purposes, images of wild type floral meristems of the respective stages (colour) are indicated above the graph.

We analysed different floral stages during which floral meristem specification (day 0-2), floral organ specification (day 4) and floral organ differentiation (day 8) take place (Smyth *et al.*, 1990). In order to study chromatin accessibility at these different stages, we made use of DNase-seq (Song and Crawford, 2010). Furthermore, we performed ChIP-seq experiments to identify stage-specific DNA-binding sites (BSs) of the two MADS-domain TFs, AP1 and SEP3. *SEP3* is a direct target gene of AP1 and becomes strongly expressed around floral stage 3, when the sepal primordia arise (Kaufmann *et al.*, 2010c). Genome-wide expression analyses were performed in order to detect changes in gene activity between different floral stages.

The ChIP-seq experiments generated high confidence sets of TF-bound regions for each factor and timepoint (see **Table S1** for an overview and a list of TF-bound regions). Many TF binding events were common to the different timepoints (**Figure 2A**), this result also holds when we analysed each biological ChIP-seq replicate independently (**Figure S1**). For example, 67% of AP1 target genes and 90% of SEP3 target genes identified at day 4 are also present in the day 8 dataset **(Figure S2A).** We also observed a number of stage-specific binding events and potential direct target genes, for example 21% of putative AP1 target genes at day 4 were not found at any other timepoint (**Figure S2A**). DNA-binding of TFs is not a none-or-all phenomenon; rather, quantitative differences in TF occupancy can influence transcriptional behaviour (Segal and Widom, 2009). Therefore, we studied quantitative changes in AP1 and SEP3 binding levels between different timepoints (Bardet *et al.*, 2012; He *et al.*, 2011). By comparing ChIP-seq peak scores as a measure of relative binding levels, we identified several hundred genomic regions with changes in TF occupancy (fold-change (FC) 2; **Figure 2B** and **Table S2**). In case of significant AP1 binding sites, 26% show differences between days 2 and 4, and 42% between days 4 and 8. For SEP3 binding levels, 1118 (17%) genomic regions showed changes between days 2 and 4 and 1003 (12%) between days 4 and 8 (**Figure 2B**).

To investigate whether differences in AP1 and SEP3 binding are associated with stage-specific functions of these TFs, we analysed the overrepresentation of GO categories in the different datasets. GO enrichment analysis revealed that genes involved in pattern formation, meristem maintenance, organ formation and polarity are mostly bound by AP1 and SEP3 at early developmental stages (**Figure S2B**). For example, *STERILE APETALA* (*SAP*) a regulator of floral organ patterning (Byzova *et al.*, 1999), and *FILAMENTOUS FLOWER* (*FIL*) (Sawa *et al.*, 1999) and ASYMMETRIC LEAVES 1 and 2 (Xu *et al.*,

2003), genes controlling axis specification, are among those genes. On the other hand, genes involved in hormonal signalling are more strongly bound at later developmental stages (**Figure S2B**). The results of stage-specific ChIP-seq experiments, in combination with gene expression data, therefore allow to identify stage-specific regulatory interactions.

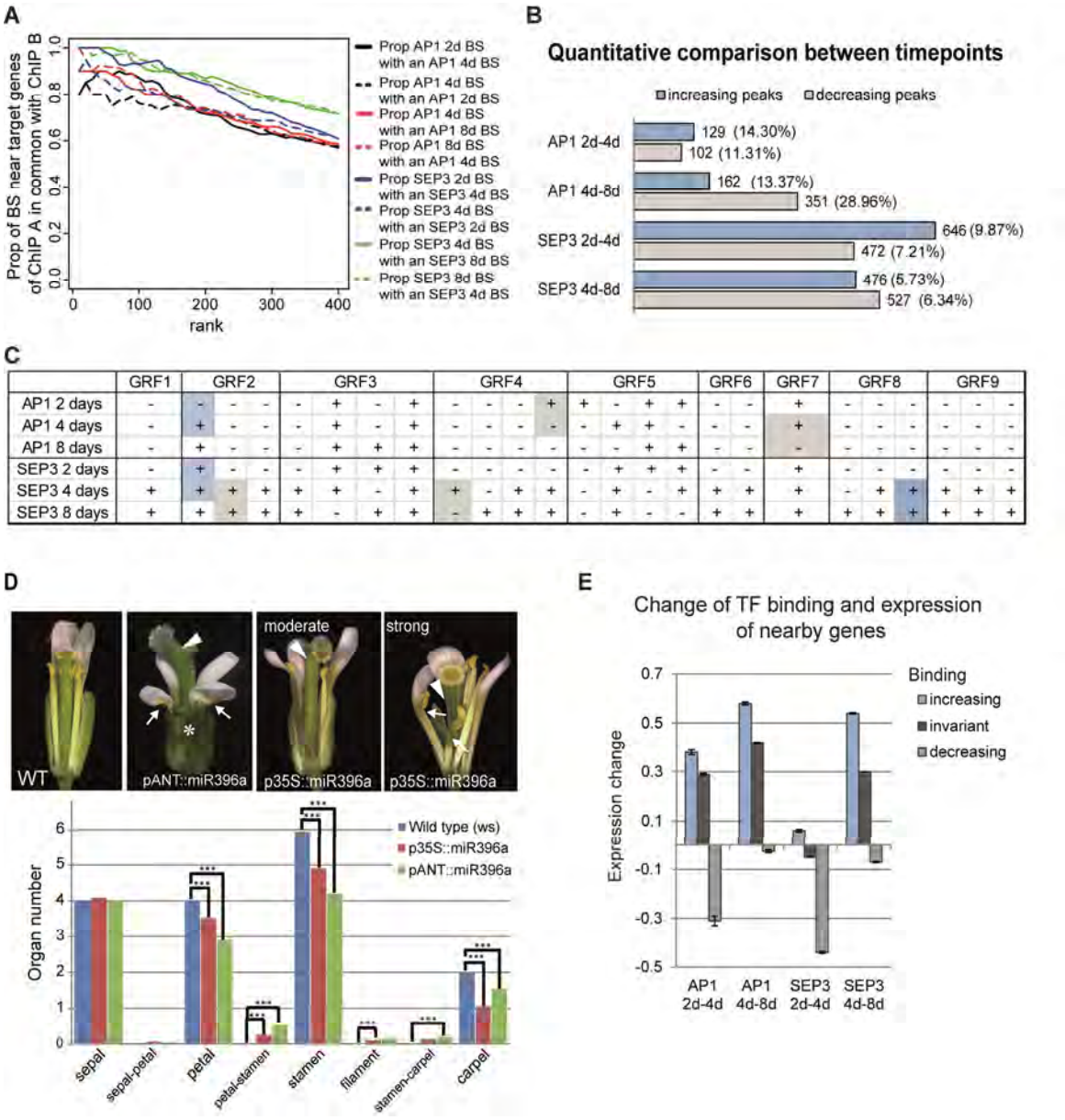


Figure 2. Developmental dynamics of MADS-domain TF-bound genomic regions. **A**. Proportion of overlapping AP1 or SEP3 BSs between different timepoints depending on their rank (1=highest rank). Only peaks 3 kb upstream to 1 kb downstream of genes were considered. **B**. Changes in AP1 and SEP3 BSs between consecutive timepoints. "Increasing peaks" are genomic regions with a peak score at least 2-fold higher while "decreasing peaks" are genomic regions with a peak score at least 2-fold lower when compared with the previous timepoint. Only significant peaks (FDR<0.001) near genes in at least one of the timepoints are considered. **C**. Summary of AP1 and SEP3 BSs at GRF loci. Each locus has a number of

columns depending on the number of different AP1 or SEP3 BSs at any timepoint. For each column, "–" indicates that the region was not bound and "+" that it was bound (FDR<0.001); two consecutive timepoints are coloured in grey when the ChIP-seq score of the earlier timepoint is at least 2-fold higher than at later timepoint and in blue when it is 2-fold lower. **D.** Floral phenotypes of miRNA396 overexpression lines using 35S promoter or p*ANT* promoter, which drives strong expression in floral organ primordia. One sepal and petal were removed to uncover organs from inner whorls. Arrow indicates petal-stamen organs, asterisk indicates conversion of floral organs into filament, arrowhead indicates ovary composed of a single valve in the mutant flowers. In the column chart, data are represented as means, 100 flowers of each genotype were assessed. *** indicates significant difference at p-value < 0.001 by unpaired Student's *t* test. **E.** Mean change of log10 fold expression of genes in vicinity (up to 1 kb upstream of start or in the gene body) of different classes of AP1- and SEP3-bound genomic regions. Only genes that were differentially expressed among the time points considered were used (Table S4). Bars correspond to standard error of mean.

Among the potential direct target genes of AP1 and SEP3, there is overrepresentation of specific TF families, and the degree of overrepresentation for a given family may vary between timepoints (**Table S3**), suggesting stage-specific regulatory interactions. A family that is overrepresented among both AP1 and SEP3 targets at 2, 4 and 8 days (p-value < 0.05) is the GROWTH REGULATING FACTOR (GRF) family (**Table S3**).

In particular, all 9 GRF family genes are significantly bound by SEP3 (FDR < 0.001), although a quantitative difference in binding levels was observed, and 5 of them are bound by AP1 (**Figure 2C**).*GRF* genes have well-known roles in leaf growth (Rodriguez *et al.*, 2010), but no known function in the determination of flower organ identity. Seven out of the nine Arabidopsis *GRF* genes (*GRF1,2,3,4,7,8,9*) contain a target site for miR396 (Jones-Rhoades and Bartel, 2004; Rodriguez *et al.*, 2010). The floral phenotypes of plants overexpressing miR396a from the 35S or p*ANT* promoters largely resemble the phenotype of a weak *ap1* mutant allele, *ap1-3*, suggesting a role of these genes down-stream of AP1. In *ap1-3* flowers, as well as in miR396a overexpression lines (**Figure 2D**), the second floral whorl is often occupied by petal-stamen mosaic structures (Bowman *et al.*, 1993; Mandel *et al.*, 1992). Plants overexpressing miR396a show also a reduction in carpel number (**Figure 2D**). Severity of the mutant phenotype directly correlates with the level of reduction in GRF transcript abundance (**Figure 2D** and **Figure S2C**). In summary, these results indicate that, apparently redundant GRF family members are regulated in different ways, and that the phenotype that was observed in the miRNA-directed knockdown lines probably reflects the combined function of these family members in floral meristem patterning and in floral organ differentiation.

We next investigated the relationship at genome-wide level between changes of MADS-domain TF binding and changes in the expression of closely adjacent genes (i.e. genes with a binding site within a region 1 kb upstream of the start of the gene or in the gene body) (**Figure 2E**). We observed a correlation between changes in binding and changes in expression. Genes located near regions with decreasing TF-binding preferentially showed a reduction in their expression level, whereas increased TF binding was associated with an increase in the expression of nearby genes (**Figure 2E**).

In summary, AP1 and SEP3 binding sites overlap substantially between timepoints, but there is also an important number of BSs specific for each TF at each timepoint. Moreover, we observed that dynamic changes in AP1 and SEP3 DNA-binding correlate with changes in gene expression.

Overlap and differences between AP1 and SEP3 binding and potential direct target genes.

We found a significant overlap for AP1 and SEP3 target genes (**Figure 3A** and **Figure S3A**), which is in agreement with previous observations that were made using different plant materials, antibodies for the AP1-GR fusion protein, and timepoints (Kaufmann *et al.*, 2010c). In agreement with the fact that SEP3 and AP1 form higher-order protein complexes with the B-class homeotic proteins APETALA3 (AP3) and PISTILLATA (PI), we observed a clear overlap between sets of potential direct target genes (**Figure S3B**) (Wuest *et al.*, 2012).

Results from Drosophila have shown that while many TFs have common binding sites in the genome, quantitative differences in binding levels correlate with the specific biological functions of different factors (MacArthur *et al.*, 2009). Quantitative comparison of genomic regions that are bound by both AP1 and SEP3 at the same timepoint shows that between 70% and 80% of the regions have peaks of similar height for both TFs (**Table S2**).

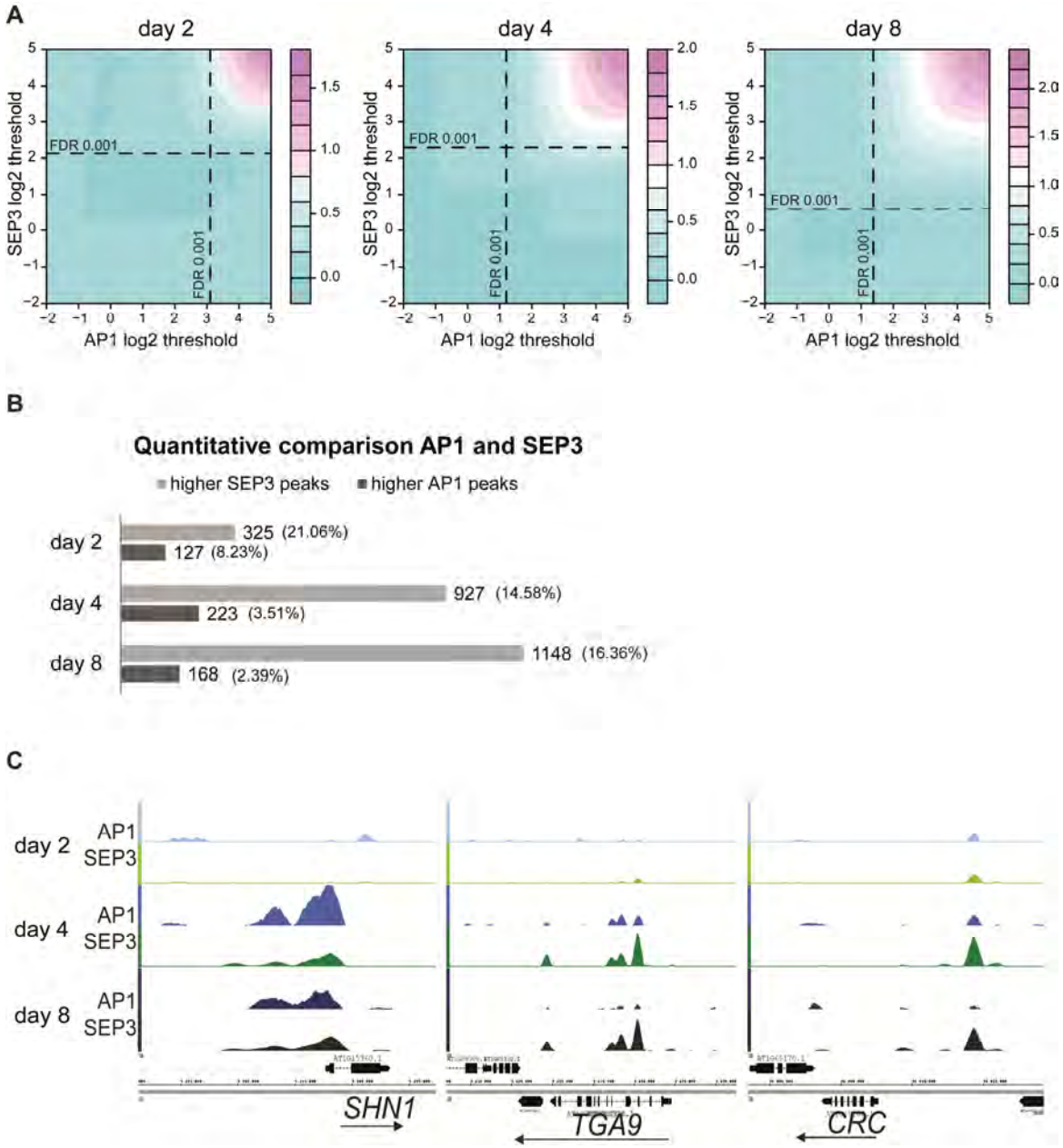


Figure 3. Overlap and differences between AP1 and SEP3 binding. A. Overview of AP1 and SEP3 common target genes obtained from the ChIP-seq datasets at the same timepoints. The figure shows the ratio between the observed number of common target genes divided by the expected number when the location of AP1 and SEP3 BSs are independent, this expected number was estimated by multiplying the proportion of AP1 BSs by the proportion of SEP3 BSs and by the total number of BSs. The x-axis and y-axis represent the threshold values for declaring a given region as significantly bound by AP1 and SEP3, respectively. **B**. Changes in AP1 and SEP3 binding at common timepoints. "Higher AP1 peaks" are genomic regions with AP1 peaks that are at least 2-fold higher than the SEP3 peaks, while "higher SEP3 peaks" are genomic regions with AP1 peaks at least 2-fold lower than the SEP3 peaks. Only significant peaks (FDR<0.001) located in a region comprising 3 kb upstream and 1 kb downstream of a gene are considered. (Table S2). **C.** AP1 and SEP3 binding profiles for selected target genes. *SHN1* is an example of an AP1 target gene that is most strongly bound at day 4, whereas *TGA9*, a gene involved in anther development, and *CRC,* a gene involved in carpel development, are preferentially SEP3 targets.

Nevertheless, depending on the time point, from about 8% to 2% of all bound regions are preferentially bound by AP1 while a higher number of regions are more strongly occupied by SEP3 (FC 2; **Figure 3B**). For example, *SHINE 1* (*SHN1*), a regulator of epidermal cell morphology of floral organs (Shi *et al.*, 2011), is preferentially bound by AP1 at day 4. In contrast, *CRABS CLAW* (*CRC*), which is involved in specifying abaxial cell fate in carpels and in nectary formation (Bowman and Smyth, 1999), and *TGACG (TGA) MOTIF-BINDING PROTEIN 9* (*TGA9)*, which is involved in anther formation (Murmu *et al.*, 2010) are preferentially bound by SEP3 (**Figure 3C**). These genes are significantly up-regulated throughout all stages of flower development in the gene expression microarray data (**Table S4**) and in quantitative PCR experiments (**Figure S3C**). Thus, differences in quantitative levels of TF occupancy may help to explain target-gene specificity of floral homeotic protein complexes.

Dynamics of chromatin accessibility during flower development.

Mapping of DNase I hypersensitive sites (DHSs) is a well-established method to identify the location of active gene regulatory elements (Wu, 1980). The DNase I enzyme preferentially digests DNA in regions of low nucleosome occupancy, and DNase I digest of chromatin followed by deep sequencing identifies open or accessible genomic regions at genome-wide scale. DHSs have been found to be correlated with genomic regulatory features such as transcription start sites (TSSs), enhancers and TF binding sites (Natarajan *et al.*, 2012). Focussing on genomic regions nearby genes (3 kb upstream of the start of the gene and 1 kb downstream of the end of the gene), we found that the overall number of high-confidence (FDR<0.01) DHSs at the different timepoints after AP1 induction varies between 5680 and 8789 (**Table S5**). We observed a high overlap (98.7%) between the DHSs identified at day 8 compared with the 41193 previously identified DHSs in wild-type inflorescences (stages 1-11) (Zhang *et al.*, 2012), the larger number of DHSs in wild-type inflorescences may be a consequence of using tissue that represents a mixture of different stages. Whereas the majority of DHSs were invariant across consecutive timepoints (FC< 2), 1370 quantitative changes in chromatin accessibility (measured as changes in DHS peak score) were detected. While there were only a small number of changes in DHS peak score comparing the different meristematic stages, the transition to organ differentiation (day 4 to 8) was found to be associated with the most changes in chromatin accessibility. There were significantly more differences between day 4 to 8

than between the earlier timepoints ($p < 2.2e-16$, ^{2} test) (**Table S6**). 1304 DHSs (11.8% of all DHSs detected at days 4 and 8) show quantitative differences in DHS peak score between days 4 and 8, with a slight preponderance of changes leading to increased accessibility (**Figure 4A**). Distinct clusters of differentially expressed genes were identified: those specific to early meristematic stages (cluster 1 and 2), transiently activated (cluster 6) or repressed (cluster 4) and genes that are specific to later floral stages (cluster 5) (**Figure 4B**). The trends in gene expression are reflected in concordant changes in chromatin accessibility: for example, genes that are expressed predominantly during meristematic stages of flower development (cluster 2), show over-representation of decreasing DHSs towards later stages (day 8). On the other hand, genes that are specifically activated later during floral organ development (cluster 5) show preferentially concordant increase in accessibility (**Figure S4A**). These data support the idea that changes in accessible genomic regions are linked with different sets of genes being active in meristematic cells versus differentiating tissues.

Next, we studied the relationship between changes in accessibility level of AP1- or SEP3-bound regions and expression of closely adjacent genes. Change in chromatin accessibility between meristematic tissues and differentiating floral organs is related with a corresponding change of expression of nearby genes (**Figure 4C**). This relation is statistically significant for both AP1- and SEP3-bound loci comparing days 2 to 4 and days 4 to 8 ($p<0.001$; ^{2} test), where the proportions of up-regulated genes are larger for regions with increased accessibility, and the proportions of down-regulated genes are correspondingly smaller.Using members of the GRF family as an example, we analysed how variations in chromatin accessibility were associated with differences in spatiotemporal gene activity. *GRF8* shows an increased SEP3 BS between days 4 and 8 and GRF8 chromatin becomes more accessible in differentiating floral organs (day 8) (**Table S6**). GFP reporter gene analyses show that the GRF8 protein is, in contrast to other factors such as GRF2 and 5, not expressed in flower meristems, and its expression increases in differentiating organs (**Figure 4D,** and **Figure 4E**).

General meristematic regulators are found among genes with a decrease in both accessibility and expression, such as *SHOOT MERISTEMLESS (STM)* (**Figure 4E**).

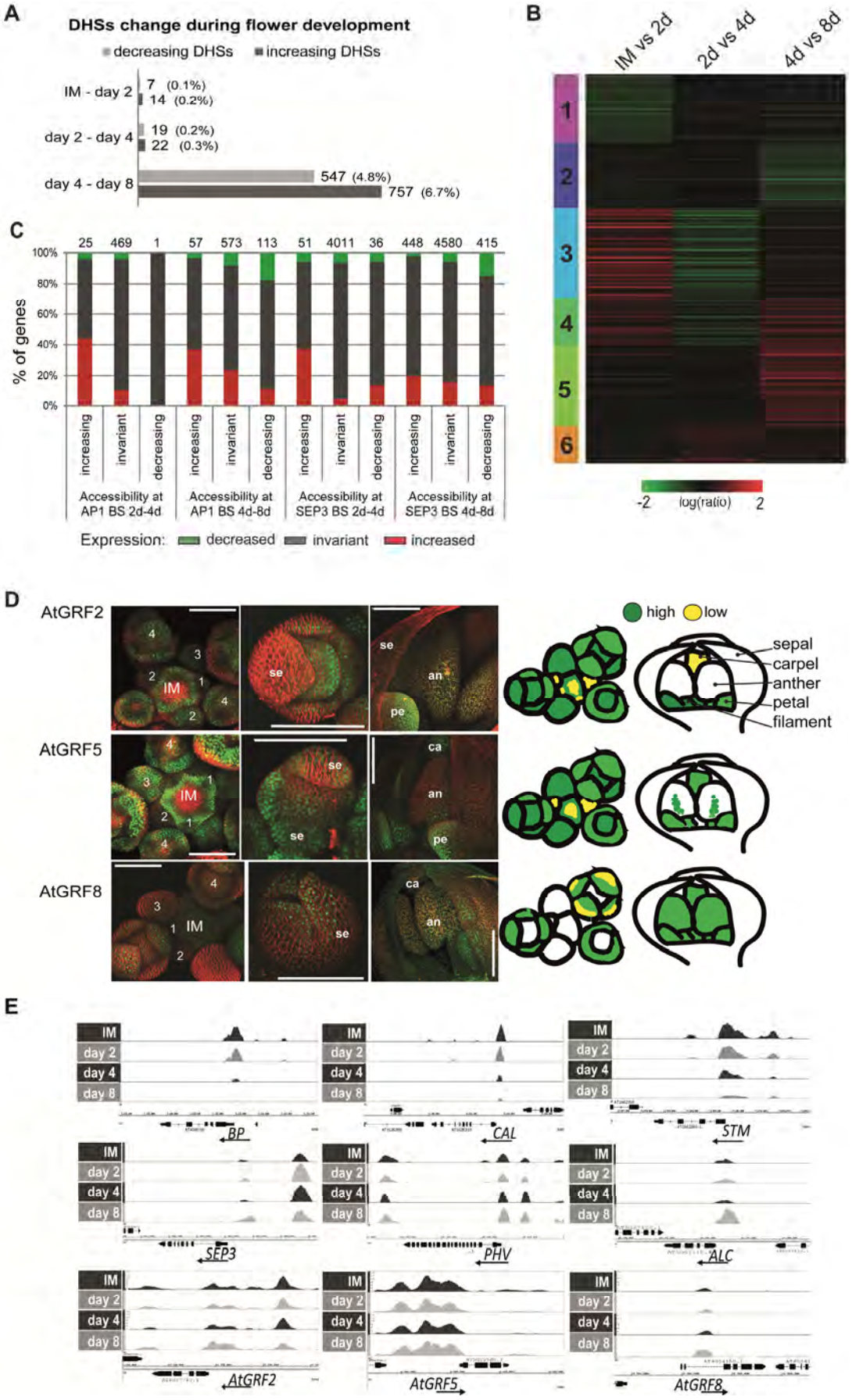

A
DHSs change during flower development
decreasing DHSs
increasing DHSs
IM - day 2
7 (0.1%)
14 (0.2%)
day 2 - day 4
19 (0.2%)
22 (0.3%)
day 4 - day 8
547 (4.8%)
757 (6.7%)
B
IM vs 2d
2d vs 4d
4d vs 8d
1
2
3
4
5
6
-2
log(ratio)
2
C
25 469 1 57 573 113 51 4011 36 448 4580 415
100%
80%
60%
40%
20%
0%
% of genes
increasing
invariant
decreasing
Accessibility at AP1 BS 2d-4d
Accessibility at AP1 BS 4d-8d
Accessibility at SEP3 BS 2d-4d
Accessibility at SEP3 BS 4d-8d
Expression:
decreased
invariant
increased
D
AtGRF2
AtGRF5
AtGRF8
IM
se
an
pe
ca
high
low
sepal
carpel
anther
petal
filament
E
IM
day 2
day 4
day 8
BP
CAL
STM
SEP3
PHV
ALC
AtGRF2
AtGRF5
AtGRF8

Figure 4. Dynamics of chromatin accessibility in *Arabidopsis* flower development. **A**. Quantitative changes in DHSs between IM and day 2, 4 and 8 after flower induction for genomic regions detected as open chromatin at any timepoint and located nearby genes (3 kb upstream to 1 kb downstream of a gene). **B**. K-means cluster analysis of differentially expressed genes. All the genes detected as differentially expressed (BH < 0.05 and FC >1.8) in at least one timepoint comparison (IM vs 2d, 2d vs 4d, and 4d vs 8d) are represented. **C.** Percentage of genes in vicinity of AP1 or SEP3 and different classes of DHS, classified according to their expression change between days. Numbers above the bars show the total number of genes in the group. In all four cases there is a significant change of fractions across categories of DHS ($\chi 2$ test, $p < 0.001$). **D.** Confocal images of expression patterns of *pGRF*::*GRF-GFP* fusions in inflorescence meristems and during flower development. Expression patterns are summarized in schematic drawings on the right. Numbers indicate floral stage according to Smyth et al. 1990 (Smyth et al., 1990), IM: inflorescence meristem, se: sepal, pe: petal, an: anther and ca: carpel. **E**. Examples of TF gene loci that have DHSs with decreasing accessibility (top) and increasing accessibility (center) after flower induction. Genes involved in meristem identity like *STM*, *BREVIPEDICELLUS* (*BP*) and *CAL* show decreasing DHS peaks. On the other side, genes involved in flower organ initiation and determination like *SEP3*, *PHV* and *ALC* show increased DHSs. In the bottom part of the figure are shown the accessibility profiles for *AtGRF2*, *AtGRF5* and *AtGRF8* loci are shown. DNaseI hypersensitivity profiles at *AtGRF2* and *AtGRF5* loci don't change during time while an increase in accessibility is found for *AtGRF8* locus between day 4 and 8.

These data are consistent with previous findings, which report *STM* expression mainly in meristems, while the expression is later restricted to cells in the gynoecium, which give rise to ovules (Long *et al.*, 1996). A decrease in chromatin accessibility and expression is also found for loci that control early patterning processes in floral meristems, such as *AINTEGUMENTA-LIKE 6* (*AIL6*), *CAULIFLOWER* (*CAL*) (**Figure 4E**) and *STERILE APETALA (SAP)*. These data are corroborated by previous studies that reported predominant expression of *AIL6* (Nole-Wilson *et al.*, 2005), *SAP* (Byzova *et al.*, 1999) and *CAL* (Kempin *et al.*, 1995) in meristems and young developing floral organ primordia (**Table S6** and **Table S4**).Among the genes that show an increase in accessibility during flower development are a number of genes with specific roles in floral organ development, as well as more general regulators of organogenesis and growth. For example, the *SEPALLATA3* locus is among the earliest genes with increased accessibility (day 2). Other examples for genes with increased accessibility at day 4 include patterning genes like *PHAVOLUTA* (*PHV*) (**Figure 4E**). All these genes show a corresponding increase in expression. Among the genes that show predominantly increased accessibility from day 4 to day 8 (**Table S6**) are for example TFs known to be involved in the formation of carpels, ovules and seeds, like *ALCATRAZ* (*ALC*) and *NGATHA3* (*NGA3*) (**Figure 4E** and **Table S6**). In accordance with the idea that different promoter elements may control different aspects of gene regulation, we found that at a subset of those loci,

individual DHSs change in opposite fashion: some DHS peaks increase, while others in the same promoter decrease (**Figure S4B**).

In summary, we found that changes in chromatin accessibility occur mainly between days 4 and 8 and that they correlate with changes in gene expression.

Footprints of MADS-domain TF binding sites in flower development.

The binding of a TF protects the DNA from DNase I digestion, creating a specific 'footprint' (Neph et al., 2012). We analysed footprint patterns caused by protection of DNA upon AP1 or SEP3 binding. The time-series ChIP-seq data indicate that AP1 and SEP3 show quantitative differences in TF occupancy levels at different developmental stages (**Figure 2**). As MADS-domain TFs assemble into protein complexes in a combinatorial fashion, these differences may reflect changes in complex composition resulting in changes in DNA-binding specificity. In line with previous results (Kaufmann *et al.*, 2009; Kaufmann *et al.*, 2010c), *de novo* identification of DNA sequence motifs in genomic regions bound by AP1 and SEP3 resulted mainly in motifs representing CArG boxes (**Figure S4C**). The generic CArG-box motif (hereafter named 'CArG box 1'), which was identified both in the AP1 and the SEP3 datasets, possesses [A/T] stretches of variable length outside the central CC$[A/T]_6$GG core. Thus, for AP1 and SEP3 we identify a longer consensus sequence than the canonical CArG-box motif: TTxCC[A/T]6GGxAA. A second CArG motif, lacking an [A/T] stretch on one side of the CArG-box, was identified in SEP3-bound regions (hereafter named 'CArG box 2'). The generic CArG-box 1 has a footprint with a central dip corresponding to the region that is highly protected to the cutting of DNase I, indicating a possible contact between the protein and the nucleotide at that position (**Figure 5A**). In contrast, CArG-box 2 shows a footprint that suggests exposure of the DNA in the centre of the CArG-box (**Figure 5B**). By comparing the frequency of footprints at different developmental stages (**Figure 5**, left panels), we found that the genomic sequences corresponding to CArG-box 1 are similarly bound at all developmental stages. In contrast, those corresponding to CArG-box 2 show increasing frequencies of footprints at day 8 compared to earlier timepoints. This suggests that CArG-box 2 is more predominantly (though not exclusively) bound by SEP3 complexes lacking AP1 later in flower development. Indeed, among genes with the CArG box 2, we found an overrepresentation of GO categories involved in late reproductive processes, such as carpel, stamen and anther development (**Figure S4D**).

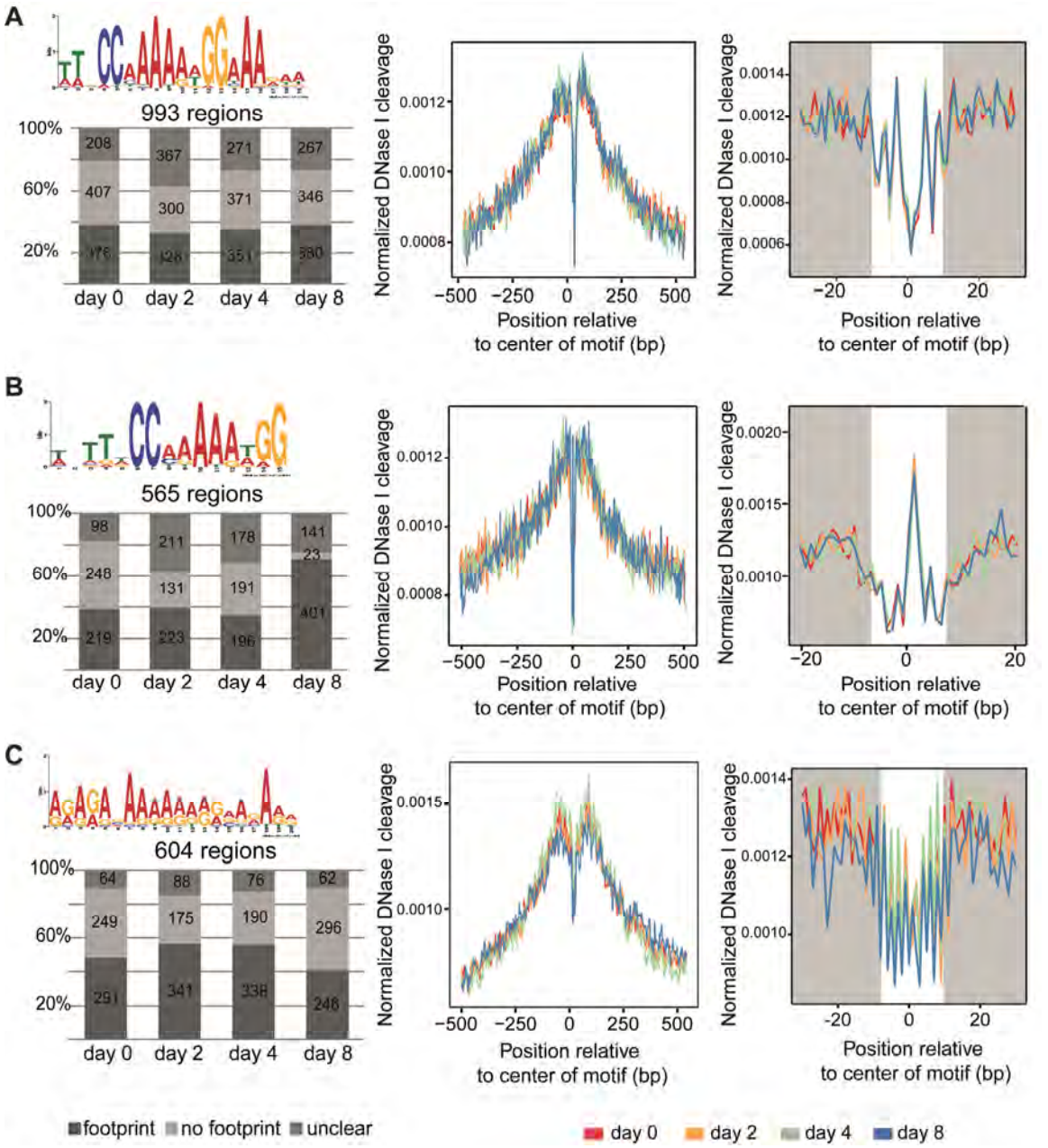


Figure 5. **DNase I footprints created by TF binding at different timepoints of flower development.** CArG box motifs were identified by MEME-ChIP in the AP1 and SEP3 peak regions (full list of motifs identified by MEME-ChIP in Figure S4C). Footprints for selected motifs are shown in the right part of the figure. **A.** CArG-box 1 produces footprints at similar frequency at every timepoint. **B**. CArG-box 2, identified only in the SEP3 ChIP-seq data, shows an increased footprint frequency at day 8. **C**. An example of GA-rich motif, which produces more frequently footprints at early timepoints of flower development.

In summary, our data suggest that different CArG motifs are characterized by different footprint profiles and show temporal differences in their occupancy in flower development. The stage-specific enrichment of CArG motifs suggests a role in of

combinatorial protein interactions in the spatiotemporal dynamics of AP1 and SEP3 DNA-binding.

In agreement with previous findings (Zhang *et al.*, 2012), we also identified GA-rich sequence motifs in the genomic regions bound by AP1 and SEP3 (**Figure S4C**). Candidate proteins that bind to this motif are the BASIC PENTACYSTEINE (BPC) transcriptional regulators, which control multiple aspects of plant development (Monfared *et al.*, 2011). Recently it was shown that BPC proteins interact with MADS-domain proteins to regulate their target genes (Simonini *et al.*, 2012). For this motif, footprints are most frequently detected in the day 2 and 4 datasets ($p \quad 0.01$, ^{2} test), i.e. during early stages of floral meristem development (**Figure 5C**). Thus, our data suggest a developmentally dynamic function of the GA-rich motif. However, its exact role and which factors bind to this motif remain to be determined.

MADS-domain TFs can bind in poorly accessible chromatin regions and their binding precedes changes in chromatin accessibility.

In order to understand the dynamic relationship between chromatin accessibility and MADS-domain TF binding, we tested whether TF-bound genomic regions reside within DHSs (**Figure 6**). At the earliest timepoint after floral induction, day 2, the vast majority of AP1 and SEP3 bound regions (73% and 68%, respectively) do not reside in DHSs. However, the overlap increases at later timepoints as development progresses. At day 4, over 50% of the sites bound by AP1 or SEP3 reside in DHSs, a fraction that increases to about 75% at day 8 (**Figure 6A**). We see a significant relation between change in binding and accessibility of sites between 4 and 8 days. This relation is mainly explained by an overrepresentation of sites with both decreased binding and decreased accessibility (**Table S7**).

Under the hypothesis that MADS-domain TFs have a role in the modulation of chromatin accessibility, we should expect that quantitative changes in MADS-domain TF DNA-binding should precede corresponding changes in chromatin accessibility during development (but not *vice versa*).

In agreement with this idea, we found that increase in levels of DNA-binding by AP1 or SEP3 from day 2 to day 4 correlates more strongly with corresponding changes in chromatin accessibility from day 4 to day 8, rather than simultaneous changes in accessibility from day 2 to day 4 (**Figure 6B** and **Table S7**).

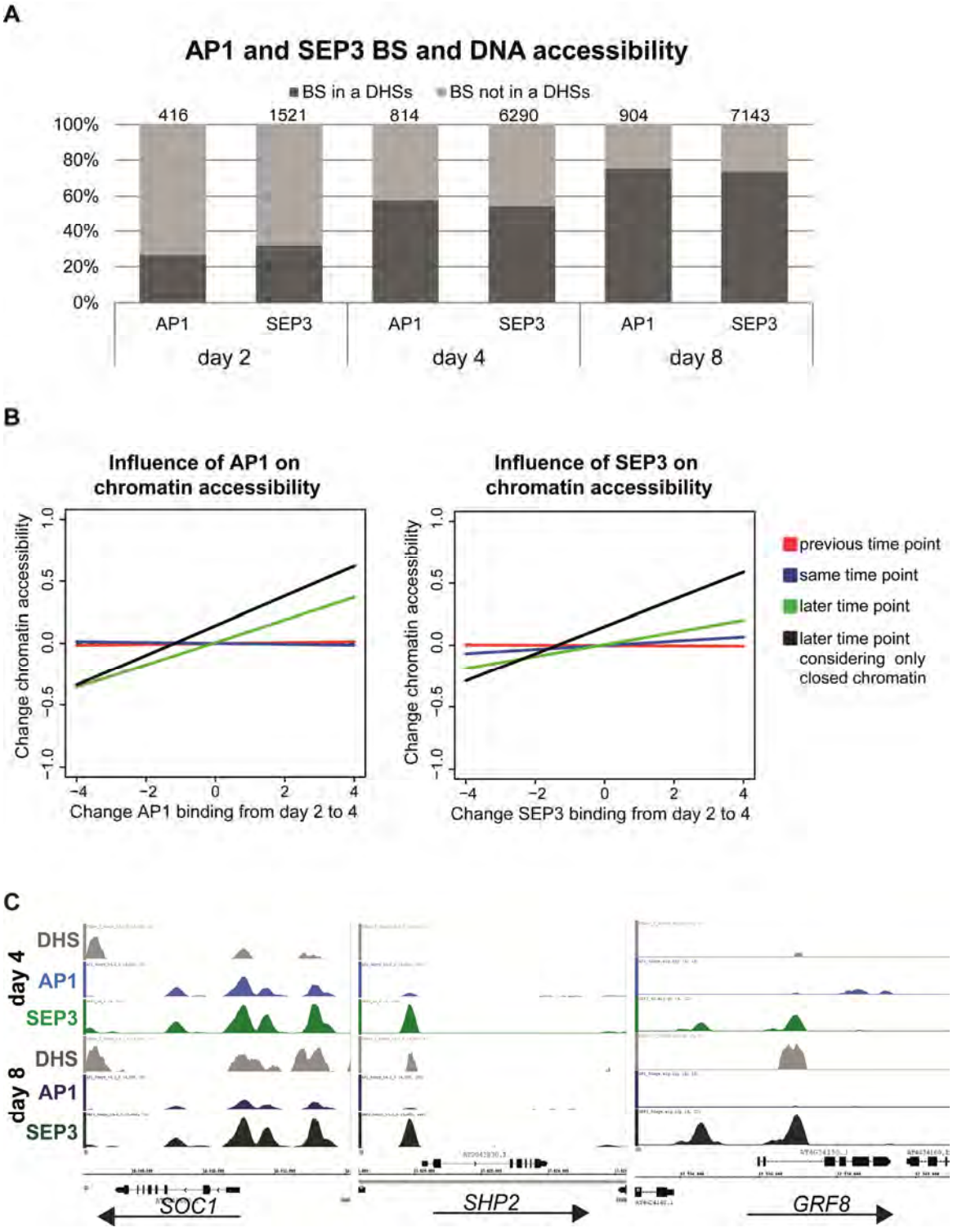


Figure 6. MADS-domain TF binding determines chromatin accessibility changes. A. Overlap between AP1 and SEP3-bound genomic regions and DHSs at the different timepoints after floral induction. Graph shows percentage of bound regions. While at day 2 most of the BSs identified at that timepoint are in closed chromatin, at day 8 most BSs are in open chromatin regions. Significant AP1 and SEP3 BSs located 3 kb upstream and 1 kb downstream of genes are considered. **B.** Change in AP1 and SEP3 binding precedes change in chromatin accessibility. Regression lines with regression coefficients (Pearson correlation)

between change in AP1 and SEP3 binding from day 2 to day 4 and change in DHSs between the different timepoints. A correlation is found only between change in AP1 and SEP3 binding from day 2 to day 4 and change in DHS from day 4 to day 8. The correlation is stronger when only closed regions (FDR > 0.04) at day 4 are considered**.** Correlation is obtained considering AP1 or SEP3 BSs located in a range of 3 kb upstream and 1 kb downstream of at least one gene. **C.** Examples of AP1 and SEP3 targets where DNA-binding events in closed chromatin at day 4 precede a more open chromatin state at the later stage.

The same result was observed when we analysed each biological ChIP-seq replicate independently (**Figure S5**). This delay in change in chromatin accessibility suggests that MADS-domain TFs may act as pioneer factors (Zaret and Carroll, 2011) that directly or indirectly trigger changes in chromatin state during flower development.

Among the genes for which AP1 and/or SEP3 may act as 'pioneer factors' are *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), *SHATTERPROOF 2* (*SHP2*) and *GRF8* (**Figure 6C**). In all three gene loci at day 4, regions are bound by AP1 and/or SEP3, while these regions are hardly or not accessible but become accessible at a later timepoint. *SOC1* is a special case since it is active in IMs, repressed in young floral meristems (stage 1-4) and later becomes expressed again in whorls 3 and 4, and it maintains expression during differentiation of stamens and carpels (Samach *et al.*, 2000). Also, the expression of *SHP2* and *GRF8* increases at later developmental stages (**Table S4** and **Figure 4D**).

In conclusion, we observed that DNA-binding of APETALA1 and SEPALLATA3 can occur in regions of low accessibility (DHS), and that it can precede increase in DNA accessibility.

DISCUSSION

Plant development is controlled by the combined action of chromatin regulators and transcription factors. Here, we address the question of how this dynamic interplay is achieved at the molecular level using flower development as a model system. We characterize changes in MADS-domain TF occupancy, chromatin accessibility and gene expression. Our results provide insights into the mechanisms by which MADS-domain TFs exert their master regulatory functions in meristem and organ differentiation in plants.

Developmental regulation of gene expression at the chromatin level.

Data from the animal field show that developmental control of gene expression is tightly linked with dynamic changes in chromatin accessibility. Given that multicellular development originated independently in plants and animals, we aimed to understand how dynamic the chromatin accessibility landscape is during plant development, and how this reflects changes in developmental gene expression that are associated with this process. In summary, we observed a number of quantitative changes in chromatin accessibility in the course of flower development, mostly in the transition from meristematic stages to floral organ differentiation. These changes can reflect the establishment of multiple new cell types during flower differentiation, and be linked with the activation of regulatory regions driving cell-type specific expression patterns of genes. It can also be related to the fact that during floral organ morphogenesis, gene activation is more frequent than down-regulation of genes (Kaufmann *et al.*, 2010c; Wellmer *et al.*, 2006). Changes in DHSs globally correlate with changes in gene expression, although not all gene expression changes are associated with a change in chromatin accessibility. These findings suggest that there are multiple mechanisms by which developmental changes in gene expression are controlled, and that developmental changes in gene expression are partly manifested in changes in chromatin structure in plants.

MADS-domain TFs regulate target gene expression in a dynamic fashion.

Although many MADS-domain TF-bound regions are occupied by these factors throughout flower development, we did observe dynamic quantitative changes in occupancy levels at a number of binding sites. Binding site dynamics reflect regulatory dynamics of genes with stage-specific functions in flower development, such as floral meristem patterning and organ growth. In line with previous results (Kaufmann *et al.*, 2009; Kaufmann *et al.*, 2010c; Wuest *et al.*, 2012), our data suggest that floral MADS-domain TFs can act as repressors or as activators of gene expression. Given that many genes show no quantitative change in MADS-domain TF binding but they are differentially expressed throughout flower development, it appears that MADS-domain TF binding alone *per se* is not sufficient to explain changes in their gene expression, or that there is a delay in the regulatory response, e.g. due to the mechanisms by which gene expression is regulated. It is possible that promoter binding by MADS-domain TFs is a prerequisite for regulatory response, but that additional factors are needed to generate

cell-type or stage-specific gene expression patterns. This finding is supported by the fact that SEP3 and AP1, like other MADS-domain TFs, show relatively broad expression patterns in meristems and developing floral organs, and are thereby expressed in a variety of cell types, while the gene expression patterns of their targets need to be more tightly controlled, as we could show for GRF genes.

DNA-binding of MADS-domain TFs may trigger changes in chromatin accessibility.

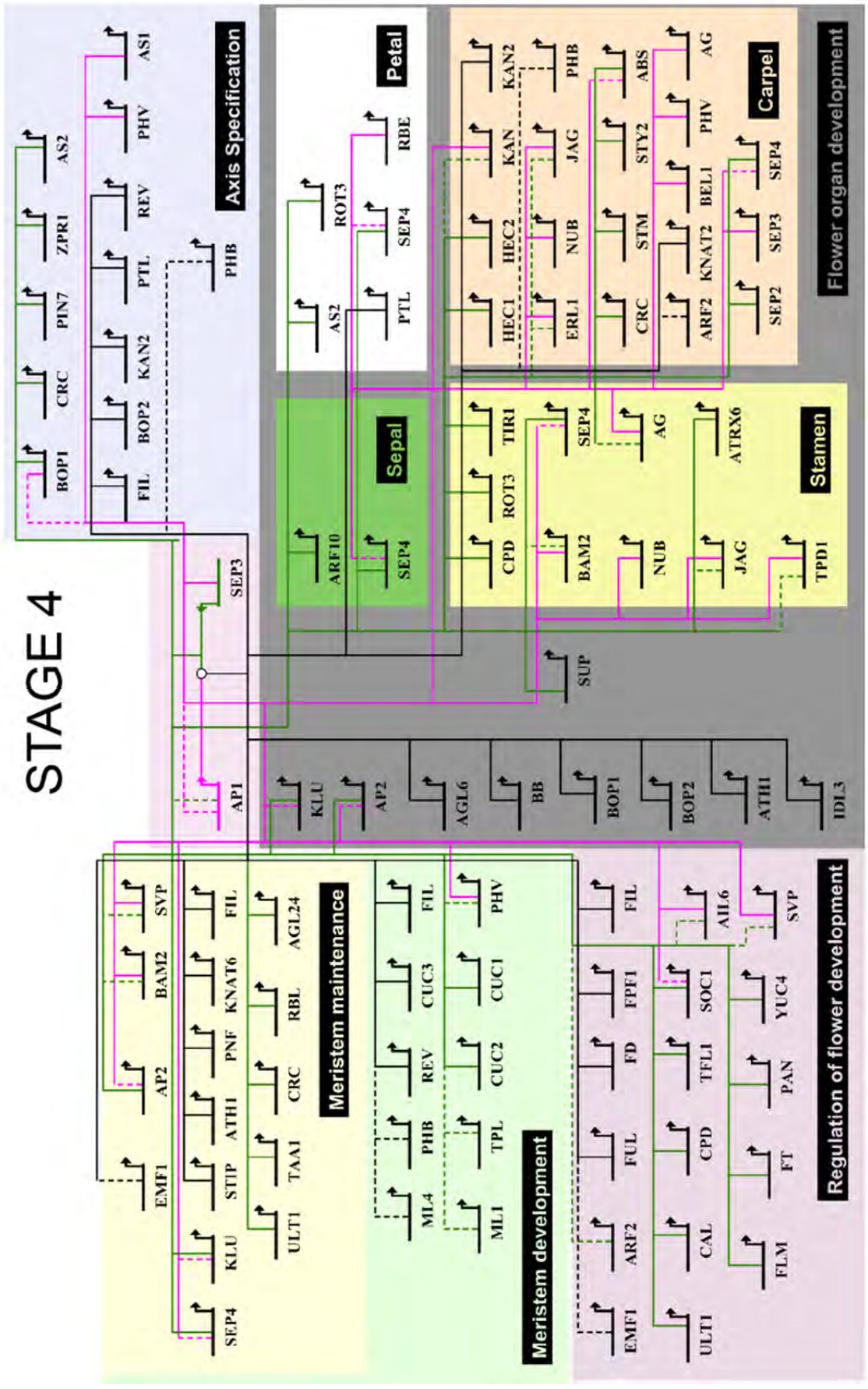
A result of the combined analysis of MADS-domain TF binding dynamics and chromatin accessibility is that MADS-domain TFs select their binding sites largely independently of chromatin accessibility, and that binding of AP1 to DNA precedes opening of the chromatin. These results suggest that a mechanism by which AP1 regulates gene expression is through increasing accessibility of cis-regulatory regions. While this is the first report proposing such a mode of action for a plant TF, a similar mode of action has been previously described for animal TFs that trigger reprogramming of cell fate, such as Oct4, Sox2, Klf4 and c-Myc (Soufi *et al.*, 2012). Previous results have shown that floral homeotic MADS-domain proteins form larger complexes together with ATP-dependent nucleosome remodelers and with histone-modifying enzymes *in planta* (Smaczniak *et al.*, 2012b; Wu *et al.*, 2012). Taken together, MADS-domain proteins may act as 'pioneer factors' that trigger changes in chromatin accessibility. Given the important roles of MADS-domain proteins as master regulators of developmental switches and floral organ specification, this is an intriguing mode of action. But how do these proteins target different regulatory regions at different stages of development? Based on the different properties of CArG boxes that we found for SEP3 and AP1, we propose that different higher-order MADS-domain protein complexes have different affinities for specific 'types' of CArG boxes. Thereby, changing MADS-domain TF occupancy at individual sites could modulate chromatin accessibility in a stage- or organ-specific manner.

CONCLUSION

In conclusion, our work represents a first step to a better understanding of the dynamics of regulatory networks in plants. By combining the information from DNA-binding and gene expression data, we are able to propose models of stage-specific regulatory interactions (**Figure 7**). Our findings suggest that different homeotic factors regulate

partly overlapping, yet also distinctive sets of target genes in a partly stage-specific fashion. Furthermore, MADS-domain TFs may regulate gene expression by alternative strategies, one of which is modulation of chromatin accessibility. Future research needs to reveal which target genes are specifically regulated by a certain homeotic protein complex, and by which exact molecular modes of action different sets of target genes can be modulated in specific ways.

Figure 7. Stage-specific regulatory networks. Putative target gene networks at different floral stages reflecting preferential binding of AP1 and SEP3 at different timepoints (Figure 2) and making use of GO category enrichment analysis for differentially bound genes across the time points (Figure S2B). Here, we focused on a selection of representative GO categories: meristem development, meristem maintenance, regulation of flower development, axis specification and floral organ development (sepal, petal, stamen and carpel development). We included only genes that belong to these categories and that were found to be preferentially bound by either AP1 or SEP3 on a comparison of floral stages 4 and 7/8 (corresponding to day 4 and day 8 in our data). Black line indicates common targets, while pink line indicates AP1-specific targets, and green line indicates SEP3 targets. Dashed lines are used to indicate gene with significant (FDR < 0.001) TF-binding peak, while solid lines for genes with higher peak respectively at stage 4 or stage 8. In grey are genes not bound at the specific stage. In red are represented up-regulated genes while in blue down-regulated genes from day 4 to 8.


STAGE 4
Axis Specification
Petal
Sepal
Carpel
Stamen
Flower organ development
Meristem maintenance
Meristem development
Regulation of flower development
BOP1
CRC
PIN7
ZPR1
AS2
FIL
BOP2
KAN2
PTL
REV
PHV
AS1
PHB
AS2
ROT3
PTL
SEP4
RBE
ARF10
SEP4
HEC1
HEC2
KAN
KAN2
ERL1
NUB
JAG
PHB
CRC
STM
STY2
ABS
ARF2
KNAT2
BEL1
PHV
AG
SEP2
SEP3
SEP4
CPD
ROT3
TIR1
BAM2
SEP4
AG
NUB
ATRX6
JAG
TPD1
SUP
SEP3
AP1
KLU
AP2
AGL6
BB
BOP1
BOP2
ATH1
IDL3
EMF1
AP2
BAM2
SVP
SEP4
KLU
STIP
ATH1
PNF
KNAT6
FIL
ULT1
TAA1
CRC
RBL
AGL24
ML4
PHB
REV
CUC3
FIL
ML1
TPL
CUC2
CUC1
PHV
EMF1
FIL
ARF2
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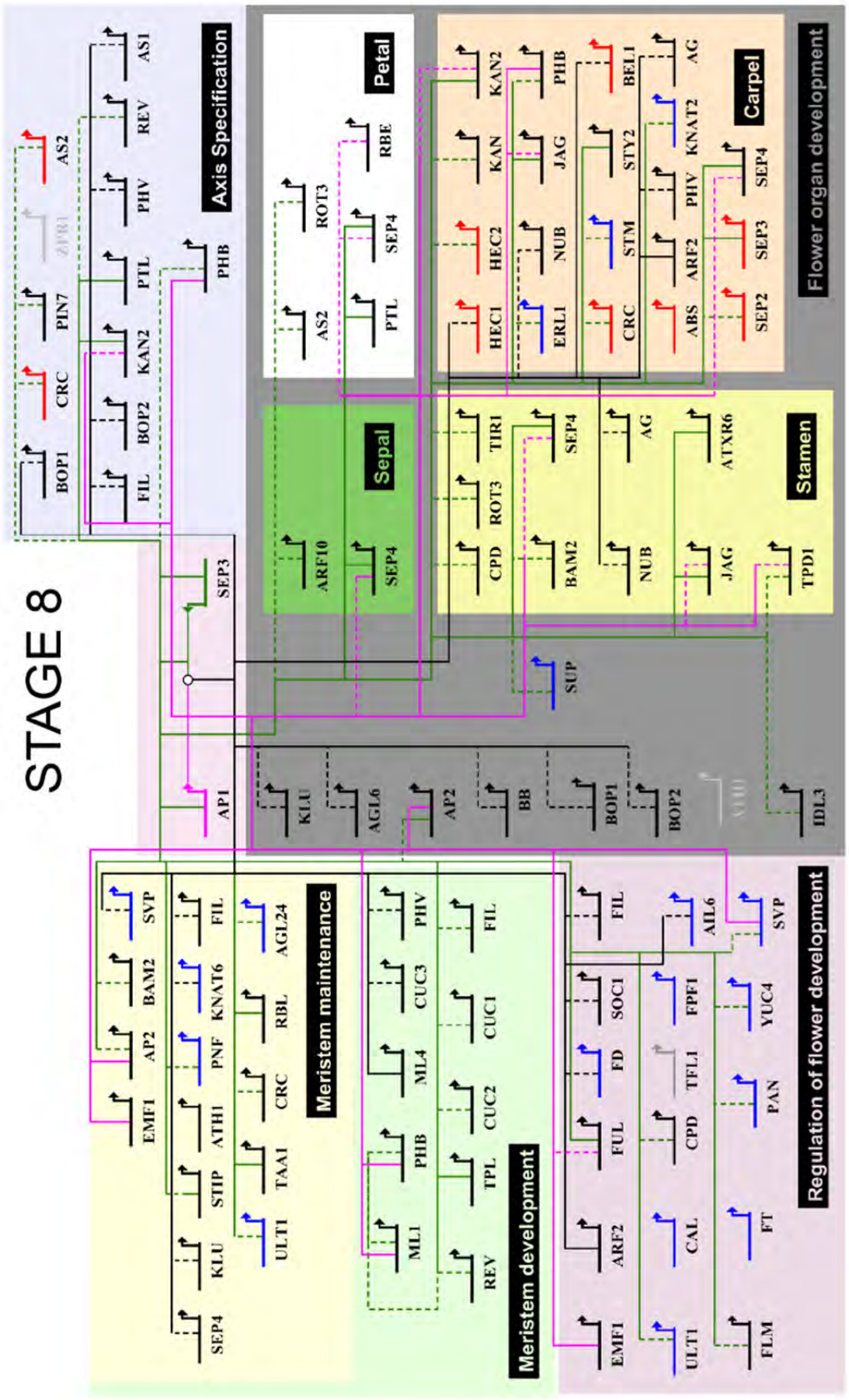
FPF1
AIL6
CAL
CPD
TFL1
SOC1
FLM
FT
PAN
YUC4
SVP
ULT1


STAGE 8
Axis Specification
BOP1
CRC
PIN7
ZIP1
AS2
FIL
BOP2
KAN2
PTL
PHV
REV
AS1
PHB
SEP3
AP1
Petal
AS2
ROT3
PTL
SEP4
RBE
Sepal
ARF10
SEP4
Flower organ development
Carpel
HEC1
HEC2
KAN
KAN2
ERL1
NUB
JAG
PHB
CRC
STM
STY2
BEL1
ABS
ARF2
PHV
KNAT2
AG
SEP2
SEP3
SEP4
Stamen
CPD
ROT3
TIR1
BAM2
SEP4
NUB
AG
JAG
ATXR6
TPD1
SUP
KLU
AGL6
AP2
BB
BOP1
BOP2
VHA
IDL3
Meristem maintenance
EMF1
AP2
BAM2
SVP
SEP4
KLU
STIP
ATH1
PNF
KNAT6
FIL
ULT1
TAA1
CRC
RBL
AGL24
Meristem development
ML1
PHB
ML4
CUC3
PHV
REV
TPL
CUC2
CUC1
FIL
Regulation of flower development
EMF1
ARF2
FUL
FD
SOC1
FIL
ULT1
CAL
CPD
TFL1
FPF1
AIL6
FLM
FT
PAN
YUC4
SVP

MATERIALS AND METHODS

Plant material. All plants were grown at 20 °C under long day condition (16 h light, 8 h dark). Plants for ChIP-seq and DNase-seq were grown on rock-wool, whereas plants for gene expression analysis were grown on soil.

Tissue collection. For DNase-seq and ChIP-seq experiments: *pAP1*:AP1-GR *ap1-1 cal-1* plants were dipped after bolting (2 cm to 5 cm height) in the DEX- induction solution (2 µM Dexamethasone, 0.01% (v/v) ethanol, and 0.01% Silwet L-77) daily. First induction was performed 8 hours after lights on and daily induction at 4 hr after lights on. Material was collected before DEX-induction, as well as at 2 days, 4 days and 8 days after the first treatment (8 h after lights on). Two biological samples were generated for each timepoint. For gene expression profiling experiments: Approximately 4 week-old *pAP1*:AP1-GR *ap1-1 cal-1* plants were used. For each sample, inflorescence tissue from ~25 plants was collected using jeweler's forceps as previously described (Wellmer *et al.*, 2006). Four biologically independent sets of samples were generated for each experiment. For induction, inflorescences were treated with a DEX-induction solution, or with an identical mock solution that lacked dexamethasone. Using plastic pipettes, the solutions were directly applied onto the inflorescences so that the cauliflower-like structures were completely drenched. As for the DNase-seq and ChIP-seq experiments, after the first induction, daily induction was performed 4h after lights on, and material was collected at the corresponding time-point 8hr after lights on. Material was collected immediately after solution application (0 days, mock), and at 2 days, 4 days and 8 days after the first treatment.

DNase-seq experiments. Nuclei isolation was performed according to (Zhang *et al.*, 2007) with minor modifications. Tissue was ground in liquid nitrogen. For each timepoint, 0.2 g of plant material was used. Ground material was resuspended in 2 ml of cold modified Honda buffer (HBM: 25 mM Tris, 0.44 M sucrose, 10 mM $MgCl_2$, 10 mM -mercaptoethanol, 2 mM spermine, and 0.1% Triton) and filtrated through a 55 m membrane. The membrane was washed with 1 ml HBM buffer. The filtrate was applied to a sucrose 2.5 M /40% Percoll gradient and centrifuged 30 min 2500 xg at 4 °C. Nuclei were collected in the interphase and washed with 10 ml cold HBB (HBM without spermine) and 10 ml cold HBC (HBB with 20% glycerol). Between each wash, nuclei

were centrifuged for 10 min 1000 xg at 4C. DNA digestion was performed according to (Hesselberth *et al.*, 2009) with minor modifications. Nuclei were resuspended in 2.5 ml buffer A (15 mM Tris-HCl (pH 8.0), 15 mM NaCl, 60 mM KCl, 1 mM EDTA (pH 8.0), 0.5 mM EGTA, 0.5 mM spermidine and 11% sucrose) and divided into 12 1.5ml tubes (aliquots of 200 l). To each aliquot, 200 l of 2x reaction buffer (Buffer A with 12 mM CaCl2, 150 mM NaCl) was added. Nuclei were mixed by inversion. DNase I was added (Roche Applied Science, Cat.no.04716728001) to attain final concentrations of 110U-90U-70U-50U-35U-20U-15U-10U-7.5U-5U-2.5U-0U. Samples were incubated for 10 min at 37 °C in a thermomixer. The DNase reaction was terminated by adding 400 l of stop buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% SDS, 100 mM EDTA (pH 8.0), 10 g/ml Ribonuclease A, 1 mM spermidine, 0.3 mM spermine) and incubating at RT for 15 min. To each sample, 10 l of 20 g/ml proteinase K was added. After O/N incubation at 55 °C, samples were centrifuged for 10 min at 13.000 xg. An aliquot of 10 l of each sample were run on a 1% agarose gel. Samples that were not completely digested were selected for library preparation (Figure S3E). DNA was precipitated by adding 0.9 volumes of isopropanol. The precipitated DNA was dried and left to resuspend in 100 l HPLC water O/N at 4 °C. DNA was purified with QIAGEN PCR purification kit (Cat.no.28104). Two biological replicates for each timepoint were sequenced on Illumina HighSeq2000.

ChIP-seq experiments. ChIP experiments were performed following a previously published protocol (Kaufmann *et al.*, 2010b) using an anti-GR antibody (Glucocorticoid Receptor alpha Polyclonal antibody (PA1-516, Thermo Scientific), to precipitate AP1-GR), or a peptide SEP3 antibody (Kaufmann *et al.*, 2009). 0.75 g of plant material were used for each biological replicate. ChIP experiments performed using pre-immuneserum were used as negative control for each timepoint. Two biological replicates for each experiment were sequenced on Illumina GAII or MySEQ.

DNase-seq and ChIP-seq data analysis. Basecalls was performed using CASAVA version 1.7 for AP1 4 and 8 days ChIP-seq experiments days, while CASAVA version 1.8 was used for all the other analysis. Sequence reads reported by the Illumina's CASAVA v1.8 pipeline as low quality reads were removed from further analysis. CASAVA v1.7 does this automatically. FASTQ files were mapped to the Arabidopsis

thaliana genome (TAIR10, http://www.arabidopsis.org/) using Bowtie (Langmead *et al.*, 2009) version 0.12.7, allowing up to 3 mismatches. Sequence reads mapped to mitochondrial and chloroplast chromosomes or mapping on multiple locations were removed. An overview of sequencing data is reported in Table S8. Reproducibility between biological replicates was assessed using the Pearson correlation coefficient (PCC) for the genome-wide reads distribution at each pair of replicates on a single nucleotide resolution, for this, we used the script 'correlation.awk' provided by (Bardet *et al.*, 2012), the results were: PCC>0.99 for DNase-seq experiments, and 0.80<PCC<0.977 for ChIP-seq experiments. Because of the high reproducibility of the data, FASTAQ files for replicates of the same experiment were combined. We used MACS 2.0.10 (Zhang *et al.*, 2008) with default parameters except --mfold which was set to '2,20') to identify significant BSs for ChIP-seq experiments and significant DNase I hypersensitive sites (DHSs) for DNase-seq experiments . We used a cut-off of FDR 0.01 and FDR 0.001 (--qvalue parameter in MACS) for DNase-seq and ChIP-seq experiments, respectively. Genomic regions were associated with genes if located 3 Kb upstream of the start of the gene up to 1 Kb downstream of the end of the gene using the function distance2Genes in the Bioconductor package CSAR (Muino *et al.*, 2011) for genes annotated in TAIR10.

Quantitative comparison of ChIP-seq and DNase-seq experiments. We followed the Bardet *et al.* (2012) protocol for the quantitative comparison. Namely, we created an aggregated list of ChIP-seq and DHSs peaks in a region ± 75 bp around the peak summit, and then scored each one of those regions by the highest mapped read count normalized by total number of mapped reads in the library. This score was subsequently scaled by the score in the corresponding control sample in the same region. Quantile normalization implemented in the preprocessCore R package (Bioconductor; http://www.bioconductor.org/) was then applied independently to all DNase-seq and to all ChIP-seq score values.

Changes in DHSs and putative TF BSs across the different time stages were quantified by means of (fold-change ratio). We classified regions as invariant when the fold-change was 2 for DNase-seq data, and 2 for ChIP-seq data. Otherwise the region was classified as being an increasing or decreasing region according to the sign of the log2.

The simultaneous analysis of dependence between chromatin accessibility changes, and TFs binding changes and of the influence of these factors on changes in gene expression

(Figure 2E, 4C) was done by the chi-square test in Genstat 15. DNA sequences and overlapping regions were extracted using BEDTools (Quinlan and Hall, 2010).

*Motif analysis and DNase I cleavage***.** For motif identification sequences of ChIP-seq peaks ±50 bp around the peak summits, were submitted to MEME-ChIP (Machanick and Bailey, 2011) after processed with RepeatMasker (http://repeatmasker.org); we used default parameters for MEME-ChIP except the motif site distribution ('-mod') parameter that was set to *any number of repetitions* (*anr*). Motif occurrences were found in TF BSs (located 3 Kb up to 1 Kb downstream of genes annotated in TAIR10) using FIMO (Grant *et al.*, 2011) at p-value < 1e-5, and the DNase I cuts ±100 bp around the motif matches at the same time stage were submitted to CENTIPEDE (Pique-Regi *et al.*, 2011) together with the proximity to the nearest TSS and the FIMO log-likelihood score ratio to infer TF binding by digital genomic footprinting. Then, each site was classified according to its posterior probability (pp) into 3 classes: footprint (pp 0.9), no footprint (pp 0.1), and unclear bound state (0.1 < pp < 0.9). For visualization of the average DNase I cleavage in Figure 5 in a window ±500 bp around the footprint, running-median smoothing was applied (width of median window equal to 5).
Information from The Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de/v3.0/) was used to identify overrepresented TF families. GO overrepresentation analysis was performed using the Cytoscape plugin BINGO (Maere *et al.*, 2005).

RNA preparation for microarray experiments. Total RNA was isolated from tissue samples using the Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. Quality of RNA samples was evaluated using a Bioanalyzer and a RNA Nano 6000 kit (Agilent). RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Microarray setup and experiments. Agilent microarrays were designed using the *e*Array software pipeline (https://earray.chem.agilent.com/earray/) and TAIR genome annotation v8, and contain probes corresponding to 28,327 annotated genes (see (Kaufmann *et al.*, 2010c). Microarrays were used following manufacturer's instructions. RNA samples were labelled with fluorescent dyes using the Quick Amp Labeling Kit (Agilent). Microarray hybridizations (65ºC, 16h) and washes were performed with Agilent reagents

and following standard protocols. Microarrays were scanned using an Agilent DNA Microarray Scanner, and data were acquired using Agilent's Feature Extraction Software. Four independent sets of biological samples were used for the experiments. The dyes used for labelling RNA from a given time point were switched in the replicate experiments to reduce dye-related artefacts. Samples were co-hybridized as follows: 0 days to 2 days, 2 days to 4 days, and 4 days to 8 days, resulting in a total of three hybridizations per set, and two biological replicate sets labelled with each dye polarity.

Gene expression microarray data analysis. Feature extraction software pre-processed data from the Agilent microarrays were imported into the Resolver gene expression data analysis system version 7.1 (Rosetta Biosoftware, Seattle, WA) and processed as described (Samach *et al.*, 2000). Resolver uses a platform-specific error model-based approach to stabilize the variance estimation to improve the specificity and sensitivity in differential gene expression detection (Weng *et al.*, 2006). The data from the four biological replicates of each condition were combined, resulting in an error-model weighted average of the four. The p-values for differential expression calculated by Resolver were adjusted for multi-hypothesis testing using the Benjamini & Hochberg procedure, as implemented in the Bioconductor *multtest* package in R (http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/multtest.html).
Genes for which the Benjamini & Hochberg-adjusted p-value was <0.05 in at least one of the comparisons (i.e., time-points), and that passed an absolute fold-change (FC) cut-off of 1.8, were considered as differentially expressed (**Table S4**). Genes that were detected as differentially expressed were subjected to cluster analysis using the k-means algorithm implemented in Resolver (partitioning into different numbers of clusters was tested, and k=6 was selected for producing the most consistent clusters (**Figure 4B**).

Isolation of RNA and real-time PCR analysis. Total RNA was extracted using Invitek Kit according to the manufacturer's protocol. DNase I digestion was performed on total RNA using DNase I from Invitrogen. RNA integrity was checked on 1% (w/v) agarose gels before and after DNase I treatment. Absence of genomic DNA was confirmed subsequently by qRT-PCR using primers, which amplify an intron sequence of the gene At5g65080 (Forward 5 -TTTTTTGCCCCCTTCGAATC-3 and reverse 5 -ATCTTCCGCCACCACATTGTAC-3). First-strand cDNA was synthesized from 4 µg

of total RNA using TaqMan kit (Roche) cDNA Synthesis Kit following the manufacturer's protocol. The efficiency of cDNA was estimated by qRT-PCR using two different primer sets annealing 5'- and 3'- ends of a control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH=At3g26650), respectively GAPDH3': fw 5'-TTGGTGACAACAGGTCAAGCA-3'; rev 5'-AAACTTGTCGCTCAATGCAATC-3' and GAPDH5': fw 5'-TCTCGATCTCAATTTCGCAAAA- 3' and rev 5'-CGAAACCGTTG ATTCCGATTC-3'.

Transcript levels of each gene were normalized to *ACTIN2* gene (5'- TCCCTCAG CACATTCCAGCAGAT-3' and reverse 5'-AACGATTCCTGGACCTGCCTCATC-3'). Large-scale qRT-PCR for 1.880 TFs was performed as described previously (Balazadeh *et al.*, 2008; Caldana *et al.*, 2007), using an ABI PRISM 7900HT sequence detection system (Applied Biosystems Applera, Darmstadt, Germany). Amplification products were visualized using SYBR Green (Applied Biosystems).

MIR396 constructs and GFP fusion reporter gene constructs. 35S:miR396a was generated by fusing 400 bp of MIR396a precursor to the 35S promoter in the pCHF3 binary plasmid (Jarvis *et al.*, 1998). ANT:miR396a was generated by replacing the 35S viral promoter in the previous vector with the ANT promoter (5.8 kb upstream regulatory sequences) (Wang *et al.*, 2008).

AtGRF2, *AtGRF5*, *AtGRF7* and *AtGRF8* genomic regions were amplified by PCR using the following primers:

AtGRF2	fw: 5'-AACATTTGGTTGGTAATGTCAGCGT-3'
	rev: 5'-GGTTGTGTAATGAAAGTAATCGCCA-3',
AtGRF5	fw: 5'-GTATGTTCAAATAATGTGAATCGTGG-3'
	rev: 5'-GCTACCTGAGAAAATAAATTTAAACT-3'
AtGRF7	fw: 5'-GAATCTTGTTCTTCAGAAAGATGAAC-3'
	rev: 5'-AACCTGGCTGCTTTCGTCGGAC-3'
AtGRF8	fw: 5'-GTTTGTTTGTTACATTGCCGTTT-3'
	rev: 5'-GCTTGAGCTTCTGCTGCA-3'.

The PCR fragments were cloned into the GATEWAY vector pCR8/GW/TOPO from Invitrogen and transferred via LR reaction into the destination vector pMDC107 (Curtis and Grossniklaus, 2003). Expression vectors were introduced into *Arabidopsis thaliana*

ecotype Col-0 by floral dip transformation (Clough and Bent, 1998). Transformant plants were select on MS medium with Hygromycin (10ug/ml).

Confocal Scanning Laser Microscopy (CSLM). GFP tagged protein localization was observed trough CSLM on Leica SPE DM5500 upright microscope using a ACS APO 40x/1.15 oil lens and using the LAS AF 1.8.2 software. FM4-64 dye was added to 0.1% agar at a concentration of 5 M and used as staining for cell membranes. GFP and FM4-64 dye were excited with the 488-nm line of an Argon ion laser. The GFP emission was detected at a bandwidth of 505-530 nm, while FM4-64 dye and chloroplast auto fluorescence were detected at a bandwidth of 650 nm. After acquisition optical slices were median filtered and three-dimensional projections were generated with LAS AF 1.8.2 software package.

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SUPPLEMENTAL DATA

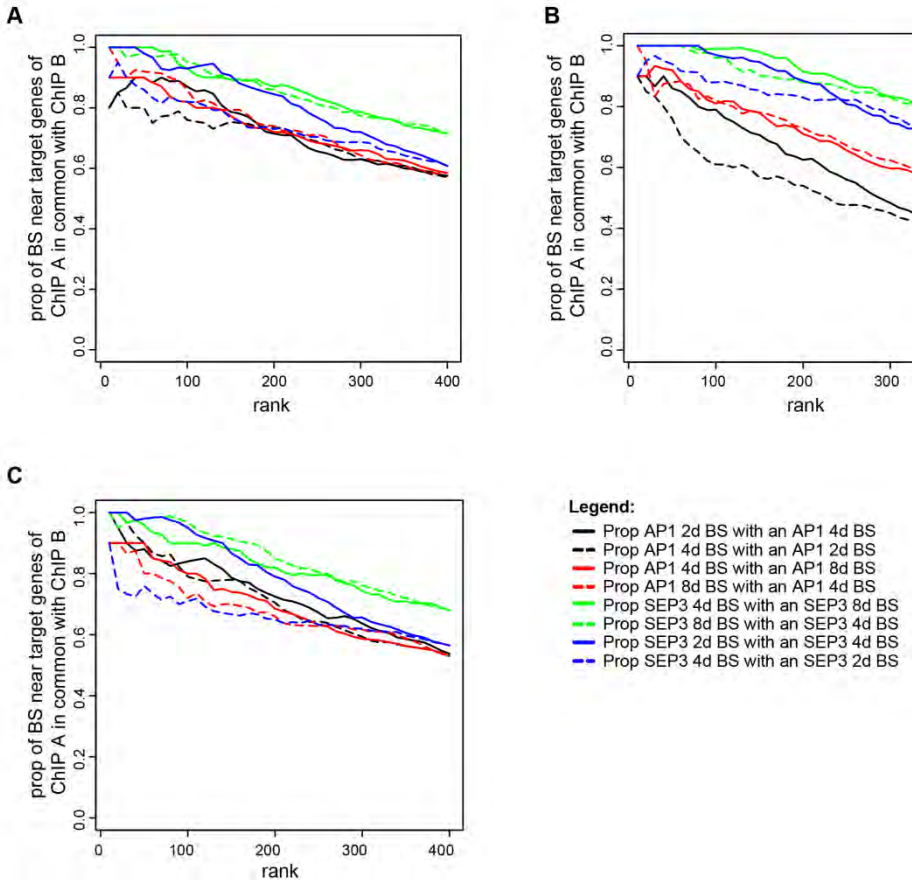


Figure S1. Proportion of overlapping AP1 or SEP3 BSs between different timepoints depending on their rank (1=highest rank) for pooled dataset and separate biological replicates. The figures were obtained in the same way like Figure 2A. We have performed the analysis for the same data as reported in the main manuscript (A) and for each replicate independently (B and C), only analyzing replicates 1 for each experiment (B) or only analyzing replicates 2 for each experiment (C). These figures shows that the rank-dependent pattern of overlap that we found is the same when combining the replicates or treating them independently.

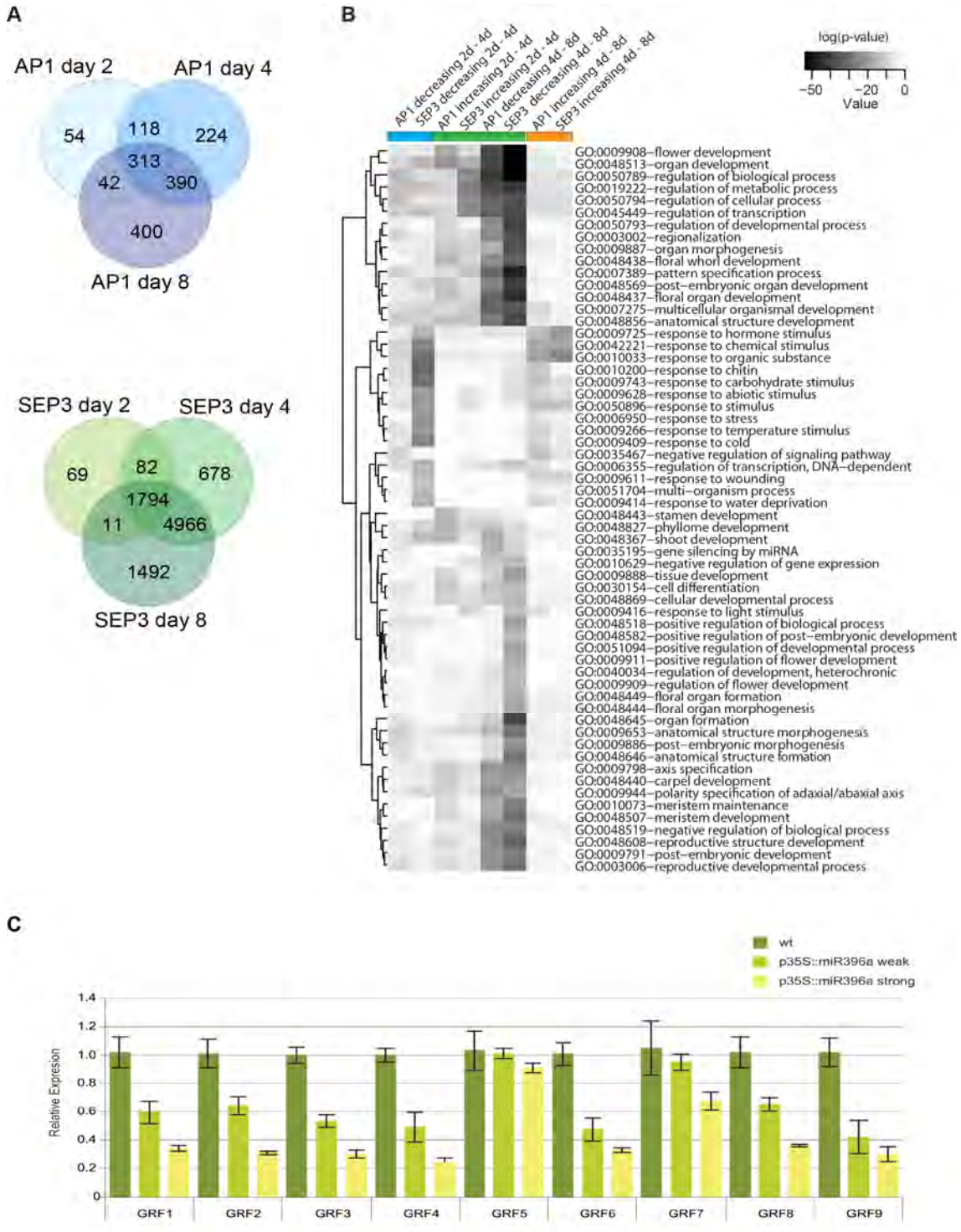


Figure S2. **MADS-domain TF binding dynamics. A.** Overview of AP1 and SEP3 ChIP-seq datasets from different time points. The number of target genes that were unique to, or shared across, the different time-points is indicated (Table S1). **B.** Gene ontology enrichment for increasing and decreasing AP1 and SEP3-bound genomic regions. The heat map includes all overrepresented categories with at least 5 genes and p value <0.0001. Parental categories with more that 90% overlap with the child category have been removed. **C.** AtGRF expression levels in plants overexpressing miR396a.

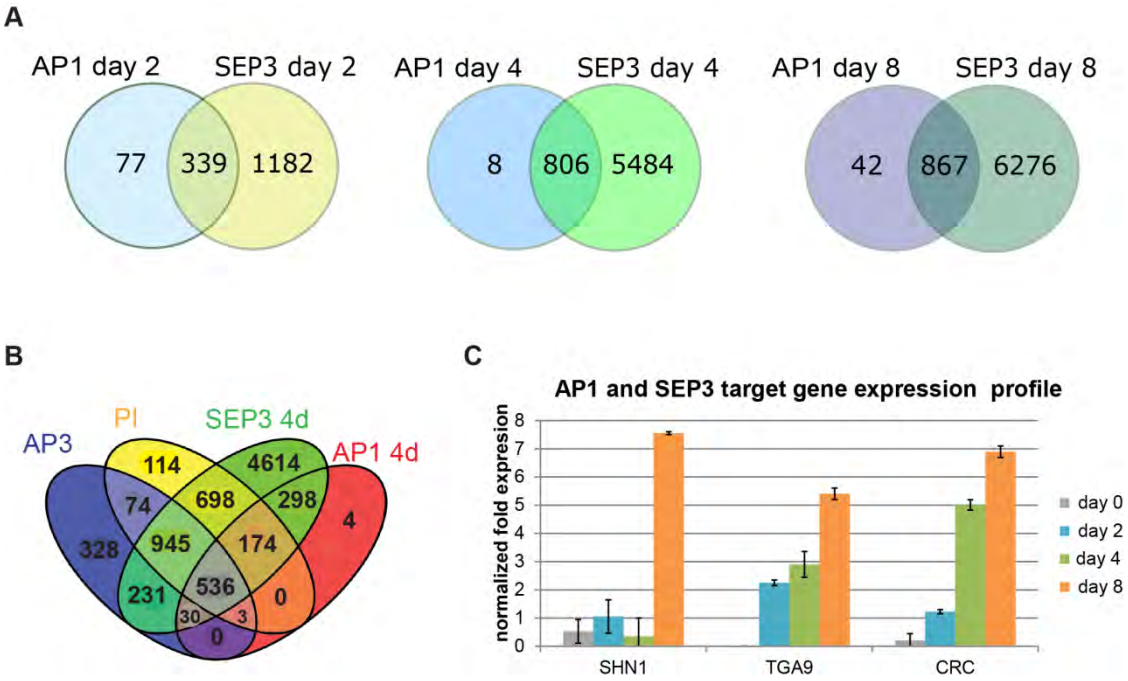
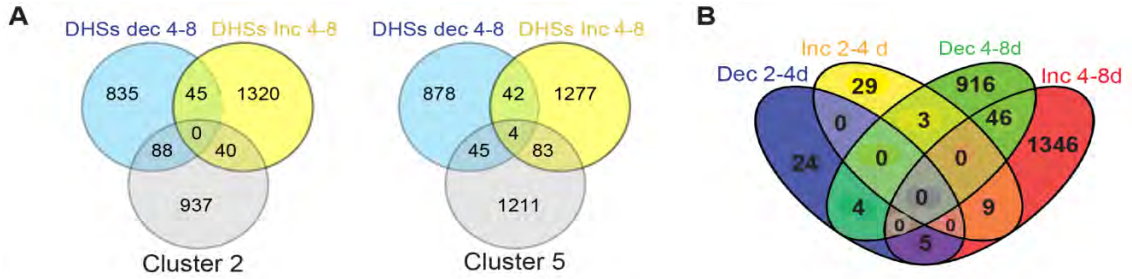
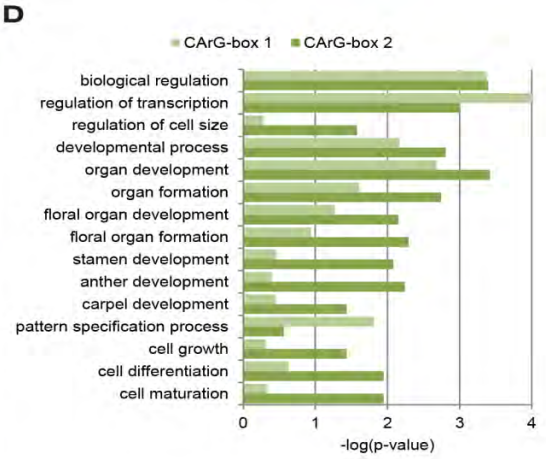


Figure S3. AP1 and SEP3 specific binding. A. Overview of AP1 and SEP3 ChIP-seq datasets from different time points. The number of BSs that were unique to, or shared across, the two TFs is indicated. **B**. Venn diagrams show overlap in potential direct target genes (genes with peak between 3 kb upstream of the start of the gene and 1 kb downstream of the end of the gene) between AP1, SEP3, AP3 and PI ChIP-seq datasets. **C.** qPCR results showing expression level at different time-points for selected target genes in Figure 3.



C

Consensus	Motif	Identified TFs (TOMTOM, only plants)
AP1 day 2		
GAGAAAGAGAGAGAG		MA0120.1 (id1)
TTACCAAAAAAGGAA		MA0082.1 (SQUAMOSA) MA0001.1 (AGL3) MA0005.1 (AG)
TTTTTTTGTTTGTCTCTTT		MA0120.1 (id1) MA0045.1 (HMG-I/Y) MA0082.1 (SQUAMOSA)
TGTCACGTCCCATC		MA0129.1 (TGA1A) MA0097.1 (bZIP911) MA0096.1 (bZIP910)
AP1 day 4		
CCAAAAAAGGAAAA		MA0082.1 (SQUAMOSA) MA0045.1 (HMG-I/Y) MA0001.1 (AGL3) MA0005.1 (AG)
TTTCTTCTTTTTTTCTCTCT		MA0045.1 (HMG-I/Y)
AAAGAGAGAAAAAAA		-
AP1 day 8		
TTTTTTTTTCCTTTTTTGG		MA0082.1 (SQUAMOSA)
TTCCATTTTTGGAAA		MA0082.1 (SQUAMOSA) MA0001.1 (AGL3) MA0005.1 (AG)
ATTGGACCACA		-
SEP3 day 2		
CCAAAAAAGGAA		MA0082.1 (SQUAMOSA) MA0001.1 (AGL3) MA0045.1 (HMG-I/Y) MA0005.1 (AG)
GGAAAAAAAAGAAGAGAAAG		MA0120.1 (id1)
TTTCCTAAAATGG		MA0001.1 (AGL3) MA0082.1 (SQUAMOSA) MA0005.1 (AG)
TTTTCTTGTTTTTGTTTCTT		MA0045.1 (HMG-I/Y) MA0082.1 (SQUAMOSA) MA0005.1 (AG)
SEP3 day 4		
TTACCAAAAAAGGAAAGAAA		MA0082.1 (SQUAMOSA) MA0005.1 (AG)
CTTCTTTTTCTTTCTTTTTT		-
TTTCCAAAAATGG		MA0001.1 (AGL3) MA0005.1 (AG) MA0082.1 (SQUAMOSA)
SEP3 day 8		
TTACCAAAAAAGGAAAGAA		MA0082.1 (SQUAMOSA) MA0005.1 (AG)
AAAAAAGAAAGAGAGAGAAA		-
TATTTCCAAAAATGG		MA0001.1 (AGL3) MA0005.1 (AG)



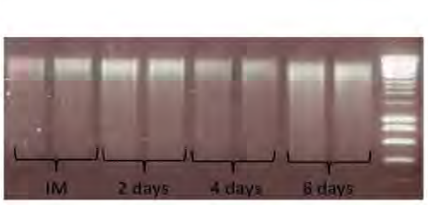


Figure S4. Chromatin accessibility and TF expression at different stages of flower development. A. Venn diagram showing the distribution in the expression cluster 2 and cluster 5 genes with increasing and decreasing DHSs between 4 days and 8 days. **B.** Venn diagram shows genes with increasing and decreasing DHSs between 2 days vs 4 days and 4 days vs 8 days. 46 genes have both increasing and decreasing DHSs from day 4 to day 8. **C.** Full list of motifs identified by MEME-ChIP in the AP1 and SEP3 peaks regions. Table shows consensus sequences and motifs based on position-specific probability matrices that were identified by MEME-ChIP, and TFs that potentially recognize those motifs identified by TOMTOM. **D.** Gene ontology enrichment for SEP3-bound genomic regions at day 8 with CArG-box motif 1 and CArG-box motif 2. The graph shows terminal overrepresented categories that belong to "biological regulation" and "developmental process". Only categories with at least 5 genes and p-value < 0.05 were considered. **E.** Gel showing partially DNase I-digested chromatin that was submitted for sequencing.

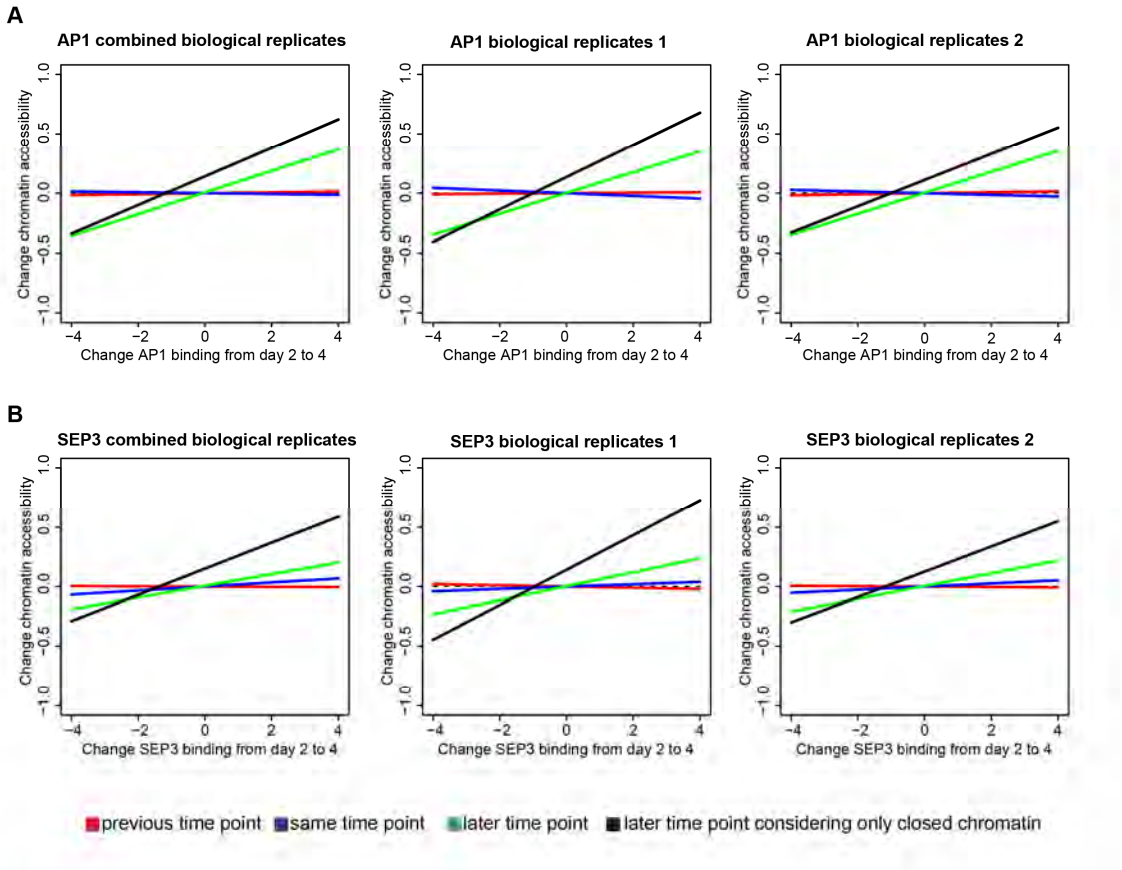


Figure S5. Change in MADS-DNA binding precedes change in chromatin accessibility. The figures were obtained in the same way as for Figure 6B. The analysis was repeated for each replicate independently and for the combined analysis for both AP1 (A) and SEP3 (B). The results and conclusions are similar in all cases.

DESCRIPTION OF ADDITIONAL TABLES

The following additional tables are available online.

http://genomebiology.com/2014/15/3/R41/additional

Table S1. ChIP-seq peak calling for AP1 and SEP3 ChIP-seq at different timepoints. The table shows peaks with FDR<0.001 and nearby genes for each dataset. Nearby genes are genes with the compared peaks 3kb upstream of the start of the gene and 1kb downstream of the end of the gene. In the overview table (sheet: overview) are summarised the total number of peaks for each dataset and the number of peaks nearby a gene for FDR < 0.001.

Table S2. ChIP-seq quantitative comparison between AP1 and SEP3 binding at different timepoints and between the two transcription factors at the same timepoint. The table shows the list of genomic regions that are increasing, decreasing or invariant between the two compared timepoints. Only regions with a significant peak (FDR < 0.001) in at least one of the two datasets compared are considered.

Table S3. Overrepresentation of TF families among significant (FDR < 0.001) potential direct target genes of AP1 or SEP3 at different timepoints. TF families that are overrepresented (p-value < 0.05) among either AP1 or SEP3 targets at least one timepoint are shown in the table.

Table S4. Genes identified as differentially expressed after AP1 activation. Microarray results show genes that are differentially expressed between IM and day 2, days 2 and 4 and days 4 and 8.

Table S5. DNase-seq peak calling for the different timepoints. The table shows DHSs with FDR<0.01 and nearby genes for each dataset. Nearby genes are genes with the compared peaks 3kb upstream of the start of the gene and 1kb downstream of the end of the gene. The total number of DHSs for each timepoint and the number of DHSs nearby a gene for FDR < 0.01 are summarised in the overview table.

Table S6. DHS quantitative comparison between different timepoints. The table shows the list of genomic regions that are increasing, decreasing or invariant between the two compared timepoints. Only regions with a significant DHS (FDR < 0.01) in at least one of the two datasets compared are considered.

Table S7. Number of genes in vicinity of different classes of BSs classified according to accessibility change. Numbers in yellow indicate cells in which significant deviations from independence are located.

Table S8. Overview of sequencing data. Total number of reads obtained with Illumina sequencing, aligned reads, and uniquely aligned reads for each sample.

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CHAPTER 4

Global changes in nucleosome occupancy during flower development

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ABSTRACT

Recent advances in genome-wide methods are revealing increasingly detailed profiles of the genomic distribution of nucleosomes, their modifications and their modifiers. The picture now emerging is one in which chromatin accessibility, governed by contributions from chromatin remodelers and chromatin modifiers, highly contributes to the regulation of transcription. MADS-domain proteins have been found to interact with chromatin modifiers and chromatin remodelers suggesting that transcriptional and epigenetic regulation may be interconnected. In this study we profile genome-wide nucleosome position at different stages of flower development. We found that nucleosome occupancy is dynamic during development and that changes in nucleosome occupancy happen prevalently in core promoter regions. Moreover, we observed low nucleosome occupancy in the proximity of transcription factor-bound regions, suggesting that transcription factors predominantly bind to nucleosome free regions.

INTRODUCTION

In the nucleus DNA is packed into chromatin and therefore, the chromatin landscape affects many DNA related processes, such as transcription. The fundamental building block of eukaryotic chromatin is the nucleosome. A nucleosome is constituted by 147 bp of DNA wrapped around a histone octamer, formed by two of each core histone H2A, H2B, H3 and H4. Dynamic post-transcriptional modifications of histones and histone tails, such as methylation, acetylation or ubiquitination, lead to a more or less compact chromatin structure that affects gene expression. Moreover, packaging of the DNA into nucleosomes restricts DNA accessibility for regulatory proteins but also provides an opportunity to regulate DNA-based processes through modulating nucleosome positions and local chromatin structure. In order to facilitate access to DNA and in order to alter nucleosome composition in chromosomal regions, cells have evolved a set of specialized proteins, the chromatin remodeling complexes (chromatin remodelers). Chromatin remodelers are ATP-driven protein complexes that can alter the chromatin structure by sliding histone octamers, modifying histone-DNA interactions or changing histone variants, leading to an increased or decreased accessibility of DNA elements to regulatory proteins. Although, chromatin remodeling complexes can be recruited to the chromatin via specific protein domains, for example the bromo-domain binds to acetylated histones and the chromo-domain to methylated histones, these complexes lack DNA-sequence specificity, as reflected in the pleiotropic effects observed in loss of function mutants (Kwon and Wagner, 2007). Thus, how can they act in specific gene regulation? Recent studies in *Arabidopsis* showed that transcription factors can interact with chromatin remodelers and histone modifiers (Efroni *et al.*, 2013; Smaczniak *et al.*, 2012; Vercruyssen *et al.*, 2014). For example, MADS-domain proteins interact with SWI/SNF family members, such as *BRAHMA* (*BRM*) and *SPLAYED* (*SYD*) (Smaczniak *et al.*, 2012; Wu *et al.*, 2012). *SYD* and *BRM* also play a role during floral organ development (Farrona *et al.*, 2004; Fritsch *et al.*, 2004; Noh and Amasino, 2003; Ogas *et al.*, 1999; Sang *et al.*, 2012; Wagner and Meyerowitz, 2002). Loss of *SYD* causes defects in many different developmental pathways such as developmental transitions, growth, patterning and stem cell maintenance (Wagner and Meyerowitz, 2002). *SYD* represses *LFY* before floral transition, and it activates B and C-class organ identity genes after the transition to flower (Wagner and Meyerowitz, 2002). Next to *SYD*, also *BRM* was found to play a role

in the activation of homeotic genes. In *brm* flowers lower expression of the C-class genes was observed. In agreement, loss of function of *BRM* causes homeotic floral organ conversions (Hurtado *et al.*, 2006). Besides BRM and SYD, members of the ISWI family, such as CHROMATIN REMODELING 11 (CHR11) and CHR17, were found in the same complex with the MADS-domain proteins (Smaczniak *et al.*, 2012). The defects in floral organ development observed in *chr11 chr17* double mutants (Li *et al.*, 2012a; Smaczniak *et al.*, 2012), suggest a role for these ISWI family remodelers in flower development. These findings reveal a new picture of gene regulation in which transcription factors and chromatin remodelers act in concert in the regulation of gene expression during development. Furthermore, changes in chromatin accessibility appear to follow DNA-binding of MADS-domain proteins (Pajoro *et al.*, 2014), suggesting that transcription factors may recruit chromatin remodelers to specific loci to shape the chromatin landscape and alter nucleosome distribution. Recent studies revealed that two MADS-domain protein interacting partners, CHR11 and CHR17, slide nucleosomes in the gene body and at cis-regulatory elements (Li *et al.*, 2014). Despite some recent advances, our knowledge about the dynamics in nucleosome distribution during flower development is still very limited. In this chapter, we used the micrococcal nuclease digestion followed by deep sequencing (MNase-seq) assay to investigate the genome-wide nucleosome distribution at different stages of flower development in *Arabidopsis*. We found that nucleosome occupancy is dynamic during flower development. Changes in nucleosome occupancy located predominantly nearby genes and are specific for the time intervals studied. Moreover, both up- and down-regulated genes are subject to changes in nucleosome occupancy and position. We then investigated the relationship between nucleosome occupancy, chromatin accessibility and transcription factor DNA-binding events. We observed that DNase I hypersensitivity sites (DHSs) and transcription factor DNA-binding events are mainly located in regions with low nucleosome occupancy.

RESULTS

Nucleosome occupancy profiles during flower development.

We studied nucleosome occupancy at genome-wide scale at different stages of flower development, making use of an inducible system to obtain homogenous material (Wellmer *et al.*, 2006). We performed MNase–seq experiments with three biological

replicates in inflorescence meristems (day 0), during floral meristem specification (day 2), floral organ specification (day 4), and floral organ differentiation (day 8) (**Figure 1**).

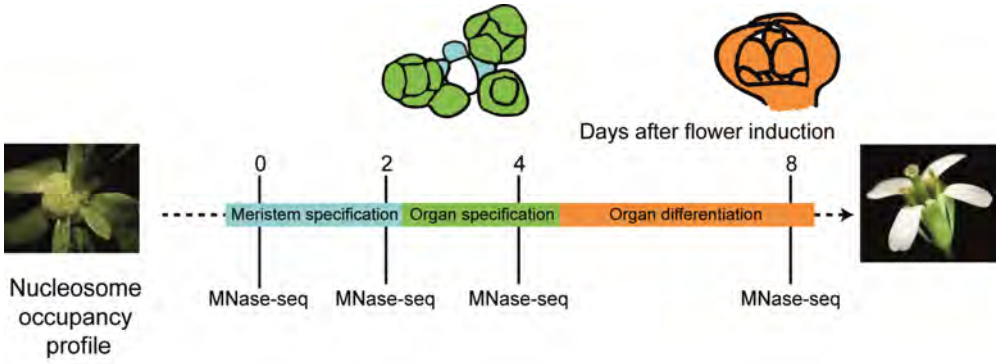


Figure 1. Experimental set up. A. Using a system for synchronized floral induction (*pAP1*:AP1:GR *ap1 cal*), we performed MNase-seq at different developmental stages: meristem specification (stage 2; 2 days after induction), organ specification (stage 4-5; 4 days after induction) and organ differentiation (stage 7-8; 8 days after induction). Around day 4, organ identity genes specify the floral whorls within the meristem, and sepal growth has been initiated. At day 8, sepals are largely differentiated, and the organs in the inner whorls are being formed. For illustrative purposes, images of wild type floral meristems of the respective stages (colour) are indicated above the graph.

We treated nuclear chromatin with MNase, which preferentially cleaves the linker DNA between nucleosomes, leaving nucleosomal DNA intact, and therefore generating a nucleosome footprint. We isolated and sequenced mononucleosomal DNA for each sample (**Figure 2**).

We sequenced our samples on Illumina HiSeq2000 and obtained between 65 and 144 million reads per sample (**Table S1**). Considering only uniquely aligned reads and after removing clonal reads, our final coverage was between 11 and 18 fold as average per sample (**Table S1**). We detected nucleosome occupancy in each sample using the DANPOS pipeline (Chen *et al.*, 2013a; Chen *et al.*, 2013b; Li *et al.*, 2012b) and identified around 200,000 nucleosomes (pv<0.001) at each time point. Considering a size of about 150 bp per nucleosome, approximately 22 % of the *Arabidopsis* genome appeared to be packed into nucleosomes (**Table 1**). To assess the reproducibility of our experiment we calculated the Pearson's correlation (PCC) between the three biological replicates, which revealed a good correlation (0.79 < PCC < 0.84) between the biological replicates at all time points (**Table S2**). Remarkably, we also observed a high correlation between samples from different time points (**Table S2**), which might be due to a largely stable nucleosome positioning throughout flower development.

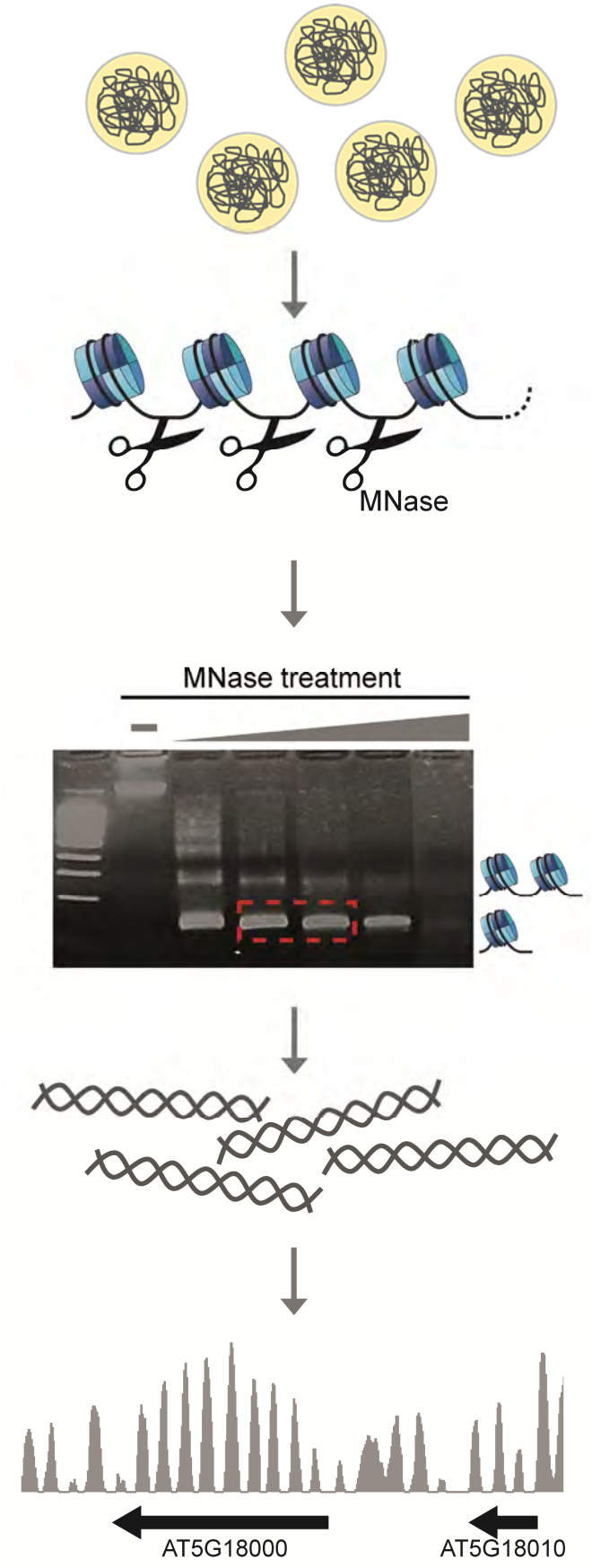


Figure 2. Experimental work flow. After nuclei isolation the chromatin is subject to MNase digestion. This enzyme preferentially cleaves linker DNA, leaving nucleosomal DNA intact. Chromatin is digested with different concentration of MNase, the mononucleosomal DNA fraction is purified from gel and sequenced, leading to identification of nucleosome occupancy along the genome.

Table 1. Number of nucleosomes assessed at the different stages of flower development.

Time point	Nucleosome number
Day 0	204,509
Day 2	210,866
Day 4	201,189
Day 8	203,983

Previous studies, conducted in yeast and human, indicate that nucleosome positions have a conserved pattern relative to the gene structure. When nucleosome density is plotted as a function of distance relative to the transcription start site (TSS), a -1 nucleosome is positioned close to the TSS from -307 bp to -111 bp, a +1 nucleosome is positioned from -5 bp to +144 bp and a 100-bp nucleosome free region (NFR) is found between the -111 and -5 position (Chen *et al.*, 2013a; Jiang and Pugh, 2009). Moreover, following the +1 nucleosome, multiple nucleosomes are well positioned along the gene body.

We analysed the nucleosome distribution relative to the TSS of all annotated *Arabidopsis* genes at different time points during flower development (**Figure 3A**).

We observed similar patterns in nucleosome occupancy flanking the TSS to the ones previously reported for yeast and human, with conserved nucleosomes at +1 and -1 and clear positioned nucleosomes in the gene body, while an NFR was observed in a region up to 100 bp upstream the TSS. This result confirms the quality of our data and suggests that nucleosome positions relative to the TSS are conserved in different organisms (**Figure 3A**).

Looking at nucleosome position around the TSS, we observed a clear nucleosome pattern in the gene body of *VERDANDI* (*VDD*), *SEPALLATA1* (*SEP1*) and *SHOOT MERISTEMLESS* (*STM*) (**Figure 3B**). At the same loci nucleosomes at +1 and -1 positions were identified. Remarkably, the nucleosome in position -1 at the *VDD* locus, as well as the nucleosome in position +1 at the *SEP1* locus, seem to be depleted when comparing the early time points to the latest time point, indicating dynamics in nucleosome occupancy during flower development.

To further verify the reproducibility of our experiment we looked at nucleosome occupancy around the TSS for each biological replicate (**Figure S1**). We observed similar patterns between the biological replicates at each the time points confirming the high reproducibility of our experiments.

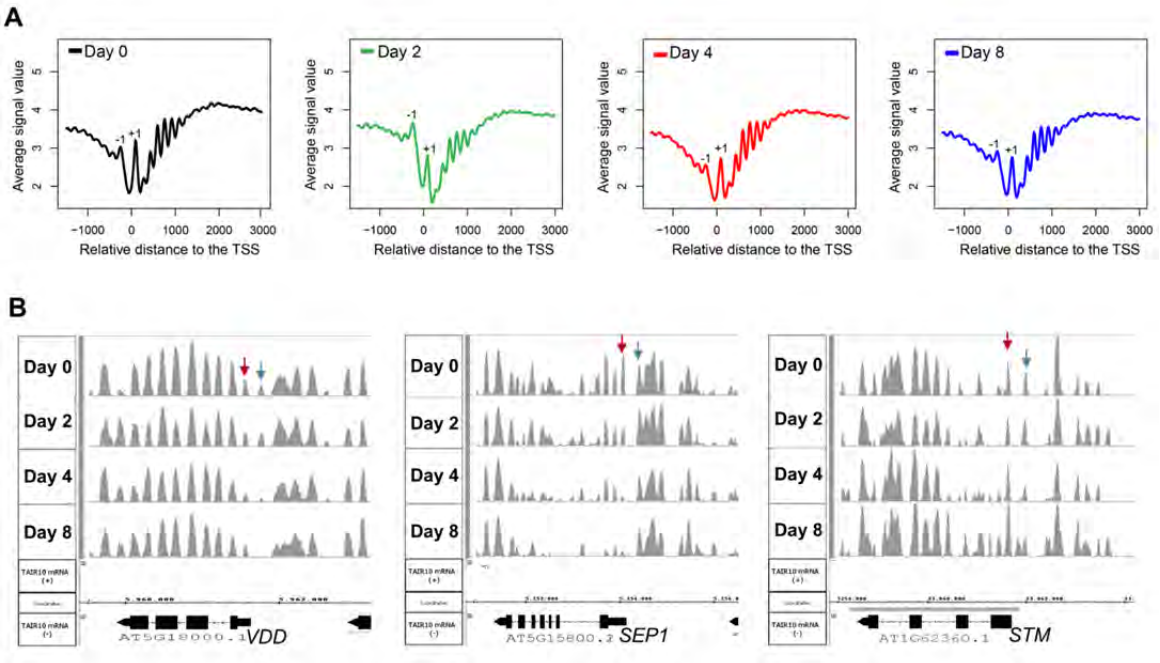


Figure 3. Nucleosome profiles during flower development. A. Nucleosome occupancy around the transcription start site (TSS) at the different time points. The average signal for all the annotated genes is plotted in the graphs. Conserved nucleosomes at position +1 and -1 were observed. An NFR was observed immediately upstream the TSS, and a clear nucleosome pattern was found in the gene body. **B.** Nucleosome occupancy profile for selected genes at different time points during flower development: *VERDANDI* (*VDD*), *SEPALLATA1* (*SEP1*) and *SHOOT MERISTEMLESS* (*STM*). Arrows indicate the conserved nucleosomes at position +1 (red) and -1 (blue).

Nucleosome occupancy dynamics during flower development.

During development, nucleosome compositions along the genome are rearranged. Thus, we studied changes in nucleosome occupancy during flower development. We identified regions where nucleosome occupancy changed between day 0-2, day 2-4 and day 4-8 using the DANPOS pipeline (Chen *et al.*, 2013a; Chen *et al.*, 2013b; Li *et al.*, 2012b). We observed changes in nucleosome occupancy between all-time points: around 10,000 regions (~5%) were dynamic (p-value < 1e-7) between 0 and 2 days and between 2 and 4 days, while only around 3,000 (~1.5%) nucleosomes changed (p-value < 1e-7) between 4 and 8 days (**Figure 4A**). We classified the changes as "nucleosome depleted regions" (Nucl.Dep), when the nucleosome occupancy was reduced compared to the previous time point, and "nucleosome gain regions" (Nucl.Gain), when the nucleosome occupancy was higher compared to the previous time point. We observed a prevalence of increased nucleosome occupancy between days 0-2 and days 4-8, and a prevalence in nucleosome

depleted regions between days 2-4 (**Figure 4A**). Most variations in occupancy occurred in the core promoter region (1 kb upstream of the TSS), although some changes were also observed in the gene body (**Figure 4B-D**). For example, dynamic nucleosomes were detected nearby key regulators of flower development such as *AGAMOUS* (*AG*) and *APETALA3* (*AP3*). At both loci nucleosome depleted regions as well as nucleosome gain regions were observed in the promoter as well as in the gene body.

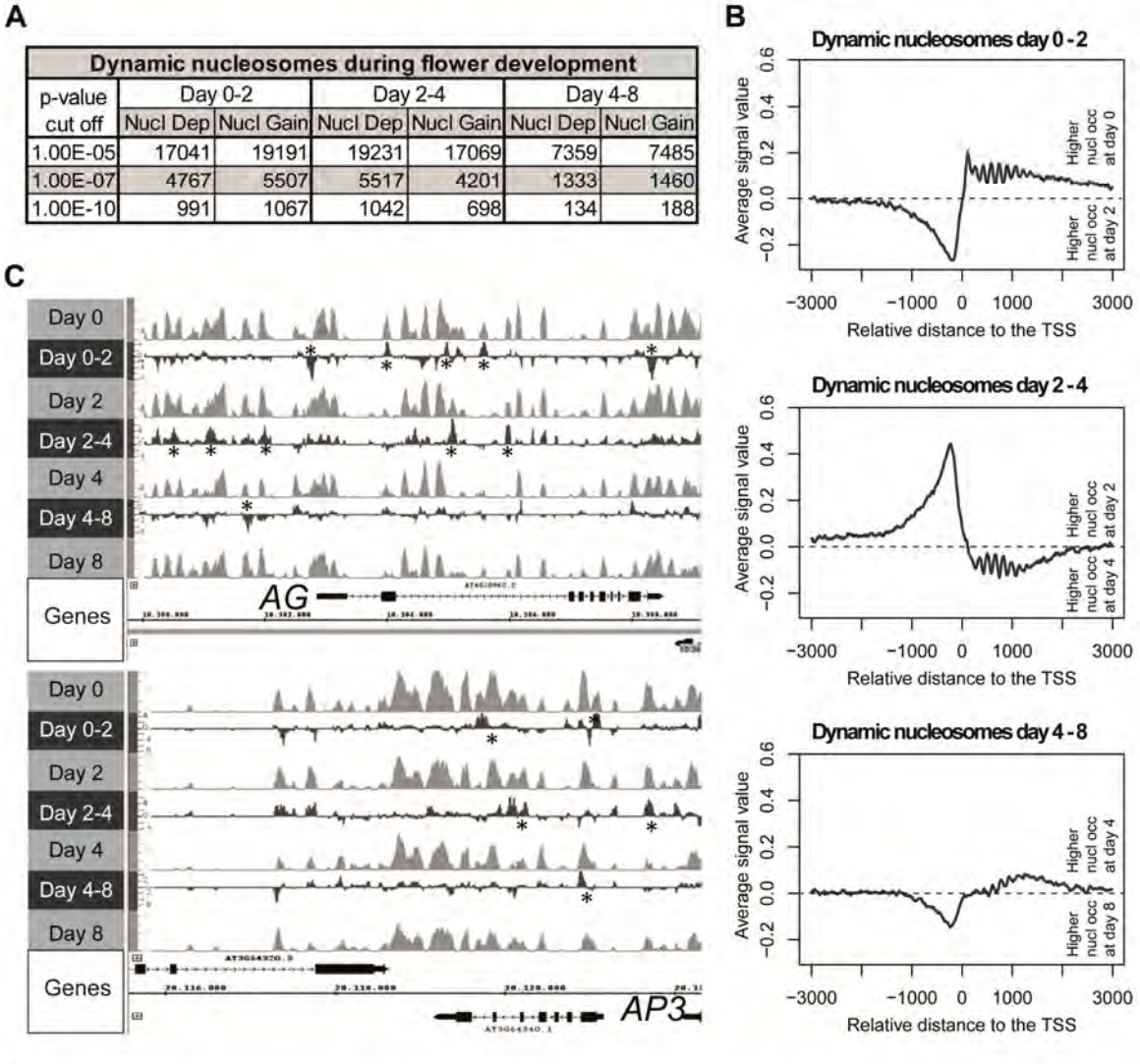
Next, we studied the biological functions of genes that showed dynamics in nucleosome occupancy. As expected, Gene Ontology (GO) analysis showed an overrepresentation of genes involved in flower development and reproduction in most of the time point comparisons (**Table 2**). Remarkably, some categories showed time point-specific enrichment, for example, 'regulation of cell cycle' was overrepresented in the day 0-2 and 2-4 comparisons, while differences in nucleosome occupancy were observed for genes involved in carpel and ovule development at the later time points.

Besides *AP3* and *AG*, we found also other known regulators of flower development among genes with dynamic nucleosomes. For example, between days 0 and 2 a nucleosome gain region was observed at the *WUSCHEL* (*WUS*) locus. An increased nucleosome occupancy was also detected around genes involved in the regulation of flowering time such as *FLOWERING LOCUS C* (*FLC*) and *MIR172* between days 0 and 2, *SCHLAFMUTZE* (*SMZ*) between days 2 and 4, and *FLOWERING LOCUS D* (*FD*), *AGAMOUS-LIKE 24* (*AGL24*), *CAULIFLOWER* (*CAL*) between day 4 and 8. In contrast, nucleosome depleted regions were detected at *SEP1* and *SEP2* loci. Interestingly, the chromatin remodeler *BRM* locus shows an increase in nucleosome occupancy in the core promoter between days 2 and 4 and another, nucleosome depleted region between days 4 and 8 in the gene body.

Remarkably, in each comparison between 70% and 80 % of the genes showed time point specific nucleosome occupied or depleted regions, and only a small portion of genes was affected throughout flower development (**Figure 5A**), among them we found the homeotic genes *AGAMOUS* (*AG*) and *SEP3*. Moreover, some genes showed nucleosome occupied as well as depleted regions in the same time interval at different genomic locations (**Figure 5B**), among them were *AG* (**Figure 4C**), *FLOWERING LOCUS M* (*FLM*) and *SMZ*.

A

Dynamic nucleosomes during flower development						
p-value cut off	Day 0-2		Day 2-4		Day 4-8	
	Nucl Dep	Nucl Gain	Nucl Dep	Nucl Gain	Nucl Dep	Nucl Gain
1.00E-05	17041	19191	19231	17069	7359	7485
1.00E-07	4767	5507	5517	4201	1333	1460
1.00E-10	991	1067	1042	698	134	188



D

Dynamic nucleosomes near by genes												
p-value cut off	Day 0-2				Day 2-4				Day 4-8			
	Nucl Dep	%	Nucl Gain	%	Nucl Dep	%	Nucl Gain	%	Nucl Dep	%	Nucl Gain	%
1.00E-05	13434	78.8	10148	52.9	11187	58.2	12045	70.6	5809	78.9	4850	64.8
1.00E-07	3598	75.5	2903	52.7	3238	58.7	2500	59.5	1064	79.8	926	63.4
1.00E-10	645	65.1	558	52.3	621	59.6	335	48.0	101	75.4	117	62.2

Figure 4. Changes in nucleosome occupancy during flower development. A. Number of all dynamic nucleosomes identified with DANPOS during flower development at three different p-value cut offs. **B.** Changes in nucleosome occupancy around the TSS, the largest changes were observed in the core promoter up to 1 kb upstream of the TSS. **C.** Nucleosome occupancy in grey and changes in occupancy in black for *AGAMOUS* (*AG*) and *APETALA3* (*AP3*). * significant changes in nucleosome occupancy at p-value < 1e-5. **D.** Number of dynamic nucleosomes nearby genes (1kb upstream the TSS and the gene body) for each time point. Percentages indicate portion of nucleosome nearby genes out of all dynamic nucleosomes identified with DANPOS.

Table 2. Gene ontology (GO) enrichment analysis.

GO	GO category description	NuclGain Day 0-2	NuclDep Day 0-2	NuclGain Day 2-4	NuclDep Day 2-4	NuclGain Day 4-8	NuclDep Day 4-8
GO-ID	description	0-2 loss	0-2 gain	2-4 loss	2-4 gain	4-8 loss	4-8 gain
48856	anatomical structure development	1.99E-03	1.40E-04	2.29E-09	2.85E-03	2.50E-01	8.37E-02
9653	anatomical structure morphogenesis	3.03E-01	1.01E-02	3.34E-07	4.52E-01	4.41E-01	1.24E-01
90066	regulation of anatomical structure size	6.29E-01	8.69E-02	3.31E-03	6.86E-01	6.63E-01	2.88E-01
48513	organ development	1.07E-03	3.40E-01	1.72E-03	3.09E-04	1.64E-01	8.55E-02
48437	floral organ development	2.41E-02	9.37E-01	6.45E-01	1.92E-03	7.40E-01	6.48E-02
48438	floral whorl development	1.00E-02	8.81E-01	7.59E-01	4.29E-03	5.37E-01	3.74E-02
9908	flower development	2.44E-02	7.33E-01	2.31E-01	4.41E-03	5.57E-01	1.30E-01
3	reproduction	1.73E-02	4.42E-06	3.24E-07	1.34E-01	3.54E-01	7.33E-02
3006	reproductive developmental process	5.04E-02	1.45E-04	2.48E-07	2.19E-02	4.78E-01	1.05E-01
22414	reproductive process	2.61E-02	6.10E-06	8.82E-07	9.29E-02	4.08E-01	8.55E-02
48608	reproductive structure development	4.71E-02	6.27E-04	1.09E-06	2.14E-02	5.39E-01	5.37E-02
48440	carpel development	7.50E-02	6.86E-01	4.96E-01	1.70E-03	8.54E-01	3.23E-03
48467	gynoecium development	7.49E-02	6.86E-01	5.78E-01	1.70E-03	8.73E-01	1.89E-03
48481	ovule development	3.08E-01	6.86E-01	5.79E-01	1.70E-03	1.00E+00	5.37E-02
10154	fruit development	1.47E-01	6.38E-04	6.83E-05	5.10E-01	4.08E-01	1.30E-01
48229	gametophyte development	7.52E-01	6.38E-04	6.79E-03	8.80E-01	6.09E-01	3.69E-01
9555	pollen development	7.64E-01	6.27E-04	5.63E-02	9.02E-01	4.67E-01	2.79E-01
9846	pollen germination	5.42E-01	2.68E-03	3.06E-02	9.65E-01	8.76E-01	7.97E-01
65007	biological regulation	9.18E-04	7.33E-01	3.87E-03	1.70E-03	1.64E-01	1.77E-01
7049	cell cycle	5.42E-01	8.22E-04	3.87E-03	6.86E-01	7.77E-01	2.83E-01
279	M phase	5.40E-01	2.30E-05	2.28E-03	8.58E-01	7.07E-01	1.40E-01
22403	cell cycle phase	6.63E-01	5.10E-04	1.12E-02	8.32E-01	8.00E-01	2.00E-01
22402	cell cycle process	6.44E-01	1.32E-03	2.41E-03	7.57E-01	8.67E-01	2.00E-01
48468	cell development	6.44E-01	2.45E-02	1.53E-03	5.99E-01	4.08E-01	4.16E-01
30154	cell differentiation	5.22E-01	4.64E-02	8.09E-04	5.34E-01	2.12E-01	3.62E-01
16049	cell growth	6.93E-01	8.34E-02	1.29E-03	6.58E-01	6.95E-01	2.46E-01
8361	regulation of cell size	6.29E-01	8.64E-02	3.18E-03	6.86E-01	6.56E-01	2.85E-01
40007	growth	7.64E-01	5.61E-02	1.11E-04	6.86E-01	6.56E-01	1.24E-01
904	cell morphogenesis involved in differentiation	7.97E-01	1.43E-02	1.53E-03	7.36E-01	9.37E-01	3.16E-01
48588	developmental cell growth	6.63E-01	8.69E-02	4.43E-03	8.06E-01	8.65E-01	6.25E-01
48589	developmental growth	6.63E-01	8.97E-02	1.94E-03	9.29E-01	7.68E-01	4.05E-01
32502	developmental process	2.33E-03	9.33E-05	2.32E-08	2.17E-02	2.12E-01	5.11E-02
90304	nucleic acid metabolic process	1.00E+00	2.42E-07	2.86E-09	1.00E+00	1.00E+00	4.40E-03
6306	DNA methylation	6.63E-01	3.69E-04	9.28E-02	8.39E-01	1.00E+00	5.72E-01
6304	DNA modification	6.96E-01	5.10E-04	1.02E-01	8.58E-01	1.00E+00	5.83E-01
6996	organelle organization	1.00E+00	3.24E-02	1.34E-06	9.02E-01	9.62E-01	5.21E-04
43687	post-translational protein modification	9.53E-01	1.12E-05	7.57E-03	1.95E-01	1.40E-01	1.32E-01
50789	regulation of biological process	2.80E-04	9.35E-01	2.04E-01	3.28E-03	1.72E-01	2.00E-01
65008	regulation of biological quality	7.64E-01	2.08E-01	2.21E-06	5.10E-01	4.64E-01	1.14E-01
9889	regulation of biosynthetic process	1.95E-07	1.00E+00	9.22E-01	2.86E-03	3.94E-01	7.39E-01
31326	regulation of cellular biosynthetic process	1.95E-07	1.00E+00	9.22E-01	2.86E-03	3.94E-01	7.39E-01
51128	regulation of cellular component organization	2.56E-01	1.38E-02	3.25E-03	7.96E-01	7.25E-01	2.61E-01
31323	regulation of cellular metabolic process	1.95E-07	1.00E+00	7.34E-01	1.70E-03	4.08E-01	5.58E-01
50794	regulation of cellular process	4.86E-05	9.48E-01	3.35E-01	1.16E-02	1.80E-01	2.79E-01
10468	regulation of gene expression	1.58E-06	1.00E+00	7.69E-01	1.70E-03	4.35E-01	6.57E-01
45449	regulation of transcription	1.95E-07	1.00E+00	9.18E-01	1.70E-03	3.96E-01	7.43E-01
16070	RNA metabolic process	1.00E+00	1.43E-02	4.99E-04	1.00E+00	1.00E+00	1.18E-01
48731	system development	1.08E-03	3.42E-01	1.78E-03	3.09E-04	1.64E-01	8.55E-02
9888	tissue development	1.51E-01	1.55E-01	1.01E-04	4.74E-01	1.80E-01	1.67E-01

For the analysis genes with changes in nucleosome occupancy at p-value < 1e-07 were used. Only GO categories with more than 10 genes and p-value < 0.005 in at least one of the comparisons are represented. In red are significant overrepresented categories and in blue are not significant ones.

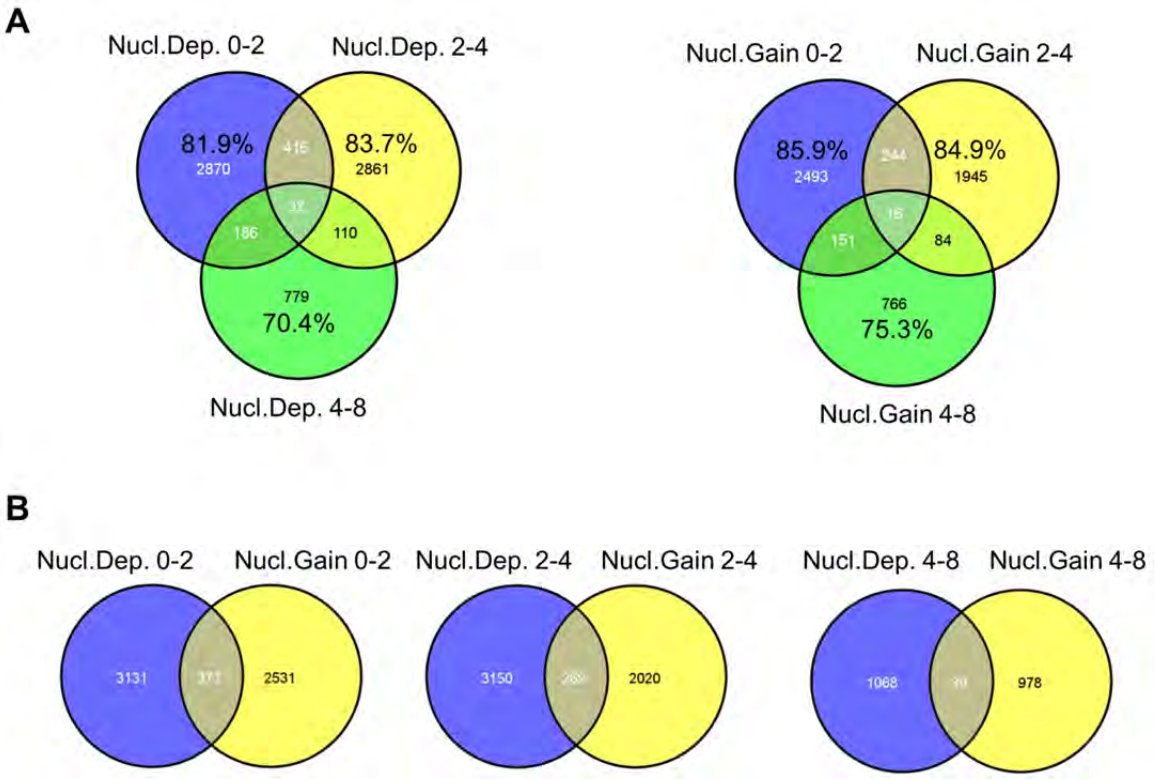


Figure 5. Genes with dynamic nucleosomes in promoter regions (1 kb upstream) and gene body. A. In the Venn diagrams are indicated genes that showed significant (p-value < 1e-7) nucleosome gain and nucleosome depleted regions at different time points. Most of changes in nucleosomes are time-point specific. **B.** In the Venn diagrams are indicated genes that showed significant (p-value < 1e-7) increase of nucleosome occupancy and nucleosome depletion at each time point comparison. Few genes showed both gain and loss in nucleosome occupancy.

Changes in nucleosome occupancy and gene expression.

Flower development is a process associated with extensive changes in gene expression (Kaufmann *et al.*, 2010; Wellmer *et al.*, 2006). In a previous study we profiled changes in gene expression between the same stages of flower development analysed in this chapter (Pajoro *et al.*, 2014; Chapter 3). Using a customized microarray, (Pajoro *et al.*, 2014) we assessed changes in gene expression during various time intervals from inflorescence meristem to floral meristem specification (day 0-2), from floral meristem specification to floral organ specification (day 2-4) and from floral organ specification to floral organ differentiation (day 4-8) (Pajoro *et al.*, 2014; **Figure 1**). We found 2,000-4,000 genes differentially expressed in the three comparisons, with a prevalence of up-regulated genes between day 0-2 and day 4-8 and a prevalence of down-regulated between day 2-4 (**Figure 6A**).

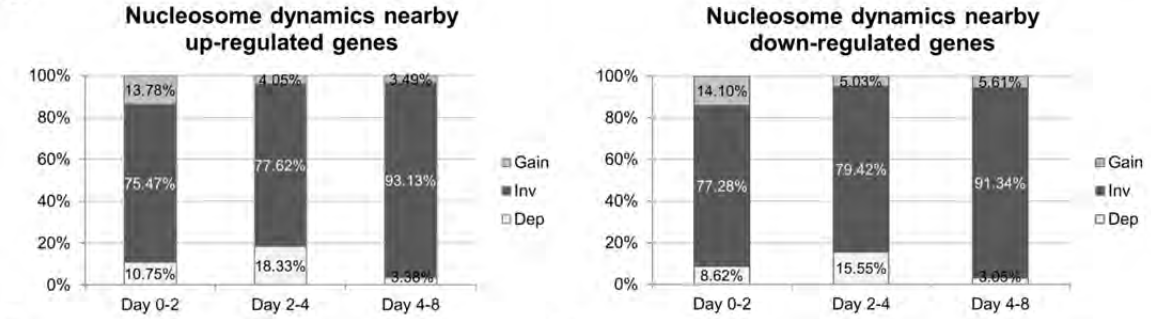
A

Number of genes with changes in expression			
	Day 0-2	Day 2-4	Day 4-8
Up-regulated	1851	791	2576
Down-regulated	1241	1273	1247
Total	3092	2064	3823

B

Number of genes with dynamic nucleosomes						
	Day 0-2		Day 2-4		Day 4-8	
p-value cut off	Nucl Dep	Nucl Gain	Nucl Dep	Nucl Gain	Nucl Dep	Nucl Gain
1.00E-05	10349	8508	9785	7975	5239	4891
1.00E-07	3504	2904	3419	2289	1107	1017
1.00E-10	635	598	701	336	120	135

C



D

Nucleosome position shift			
	Day 0-2	Day 2-4	Day 4-8
total	3555	3185	1590
promoter & gene body	2381 (67%)	1905 (60%)	1189 (75%)
gene body	1723 (48%)	1413 (44%)	945 (59%)
promoter	658 (19%)	492 (15%)	492 (15%)

E

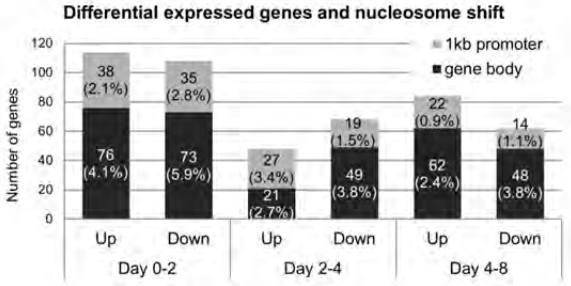


Figure 6. Changes in nucleosome occupancy and gene expression. A. Number of genes that were found up or down-regulated (fold change 1.8) in each comparison **B.** Number of genes that show a significant change in nucleosome occupancy at p-value 1e-05, 1e-07 and 1e-10 in a region 1 kb upstream of the gene and within the gene. **C.** Graphs show percentage of up- and down-regulated genes for which nucleosome depleted or occupied regions were observed at p-value cut-off 1e-07. Similar results were obtained selecting a different cut-off, see Figure S2. Between day 0-2 and day 4-8 a higher percentage of up- and down-regulated genes showed nucleosome occupied regions than nucleosome depleted regions while the opposite scenario was observed between day 2-4 where a higher percentage of up- and down-regulated genes showed nucleosome depleted regions. **D.** Number of nucleosomes that change in position at p-value 0.01. We retrieved dynamic nucleosome position shifts by selecting nucleosomes that moved between 50 bp to 90 bp between the two time points. Between 60 % and 75 % of the significant nucleosome position shift events happened nearby a gene (1 kb upstream the TSS and gene body) and between 44 % and 56 %, depending on the time points compared, are located in the gene body. **E.** Nucleosome position shift events located in the promoter (1 kb upstream the TSS) and in the gene body of differentially expressed genes. In the graph, the numbers of up- and down-regulated genes are shown for which a nucleosome changed in position either in the gene body or in the promoter between the two time points. Percentage indicates the fraction of differentially expressed genes with nucleosomes changes.

Since transcriptional regulation is associated with rearrangements of chromatin structure that include histone modifications and changes in nucleosome variants (Van Lijsebettens and Grasser, 2014), we investigated nucleosome dynamics nearby differentially expressed genes during flower development. First, we estimated the number of genes with dynamic nucleosomes in the promoter (1 kb upstream the TSS) and in the gene body for different p-value cut offs (**Figure 6B**). Then, we investigated whether there is a correlation between change in gene expression and nucleosome dynamics of differentially expressed genes (**Figure 6C**). We found that only a minority of differentially expressed genes associate with dynamic nucleosomes (p-value < 1e-07) and that nucleosome depleted regions as well as nucleosome gain regions are present nearby both up- and down-regulated genes. Though, we observed a prevalence of nucleosome gain regions in the day 0-2 interval and a prevalence of nucleosome depleted regions in the 2-4 interval, while no clear difference was observed for the day 4-8 comparison (**Figure 6C**).

Besides change in occupancy, due to nucleosome eviction or deposition, nucleosomes can also change in position, due to nucleosome sliding. Therefore, we determined nucleosome position shifts at the different time points. We found between 1,500 and 3,500 nucleosomes that shift from 50 bp to 90 bp (p-value < 0.01) for each time interval, the majority is located nearby genes with a prevalence in gene bodies (**Figure 6D**).

Less than 10% of differentially expressed genes were subjected to nucleosome sliding. The majority of changes were in the gene body except for up-regulated genes between 2 and 4 days, where we observed more changes in the promoter than in the gene body (**Figure 6E**).

In conclusion, we could not observe a clear correlation between changes in gene expression and changes in nucleosome occupancy or position.

Nucleosome occupancy around DNase I hypersensitivity sites.

Cis-regulatory DNA elements such as promoters, enhancers and insulators can be detected by their characteristic sensitivity to the endonuclease DNase I digestion. In previous experiments we identified DNase I hypersensitivity sites (DHSs) at the same stages of flower development studied in this chapter (Pajoro *et al.*, 2014; Chapter 3). By analyzing nucleosome occupancy around the previously identified DHSs, we found that the DHS summits are located in regions with low nucleosome occupancy (**Figure 7**).

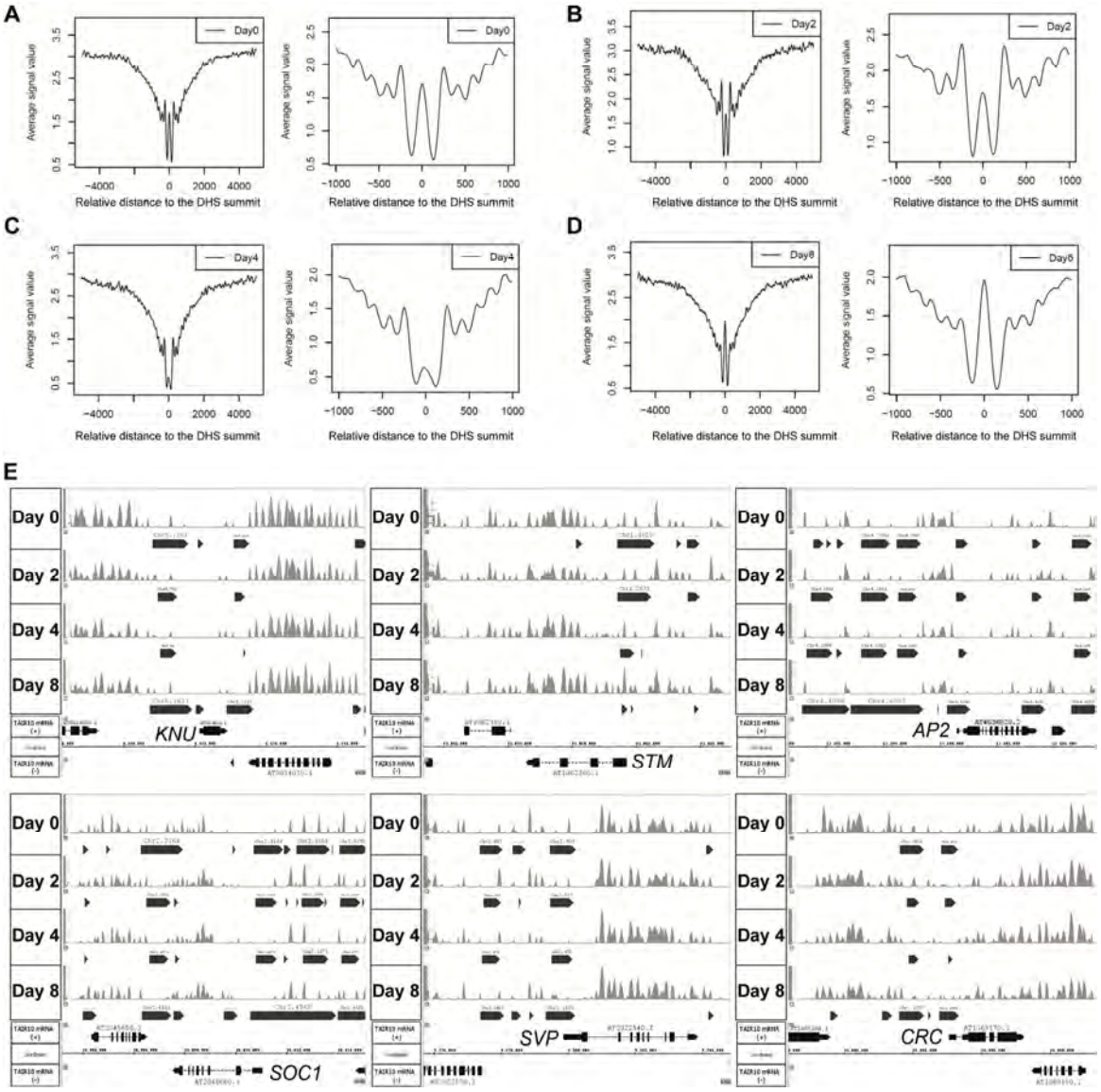


Figure 7. Nucleosome occupancy and DNase I high sensitivity sites (DHSs) during flower development.
A. Nucleosome occupancy profile (average signal value) around the summit of all DHS identified at day 0.
B. Nucleosome occupancy profile (average signal value) around the summit of all DHS identified at day 2.
C. Nucleosome occupancy profile (average signal value) around the summit of all DHS identified at day 4.
D. Nucleosome occupancy profile (average signal value) around the summit of all DHS identified at day 8.
E. Nucleosome occupancy profile and DHSs profiles for selected genes at the different time points. Black blocks represent DHSs, while in grey are represented nucleosomes. Most of the DHSs are located in nucleosome free regions.

Interestingly, the MNase-seq signal was higher at the DHS summit than in the immediate surrounding regions (**Figure 7A-D**). This distinctive profile could be caused by the presence of a nucleosome in the DHS region, as can be observed for *KNU*, *SOC1* and *STM* loci (**Figure 7E**). Similar profiles were observed in the ISWI mutants. ISWI family chromatin remodelers, CHR11 and CHR17, play a role in sliding nucleosomes along the

DNA. In *chr11 chr17* an increased nucleosome occupancy in the middle of DHSs was observed (Li *et al.*, 2014).

MNase preferentially cleaves the linker DNA between nucleosomes, leaving nucleosomal DNA intact, and generating a nucleosome footprint. However when subnucleosome-sized fragments (<80 bp) are selected, MNase digestion can be used, similar to DNase I digestion, to generate the footprints of DNA-binding proteins (Carone *et al.*, 2014). Therefore, the increased signal observed at the DHSs summit could be produced by the presence of other DNA-binding proteins, such as transcription factors that bound the DNA, protecting it from the digestion. Although we specifically selected for mononucleosomal-size fragments, we cannot exclude the presence of a fraction of subnucleosome-sized fragments in our samples that leads to the characteristic footprint profile of DNA-binding proteins.

Nucleosome occupancy around MADS-domain protein binding sites.

Recent studies revealed that MADS-domain proteins interact with chromatin modellers and modifiers (Smaczniak *et al.*, 2012; Wu *et al.*, 2012). We previously observed that changes in chromatin accessibility follow changes in MADS-domain DNA-binding (Pajoro *et al.*, 2014). Taken together, these results suggest a role for MADS-domain proteins in shaping the chromatin landscape during plant development. Therefore we investigated the nucleosome occupancy and dynamics around MADS-domain protein binding events.

In proximity of genes with a known role in flower development, AP1 and SEP3 binding sites co-localize with low nucleosome occupied regions as well as with regions where high nucleosome occupancy was detected. For example, AP1 and SEP3 binding events at *SOC1* and *SEP3* loci are located in nucleosome-free regions, while at *PI, SEP4* and *AG* loci co-localize with nucleosome occupied region (**Figure 8**).

We then analysed the genome-wide nucleosome occupancy levels around the summit of AP1 and SEP3 bound regions (**Figure 9**). A ‘valley’ is present around the summit of both AP1 and SEP3 bound regions at all the studied time points (**Figure 9A-B**). Interestingly, a region with higher nucleosome occupancy was present in the centre of the valley for AP1 bound regions (**Figure 9A**), which could represent either the binding of the TF as well as the presence of a nucleosome.

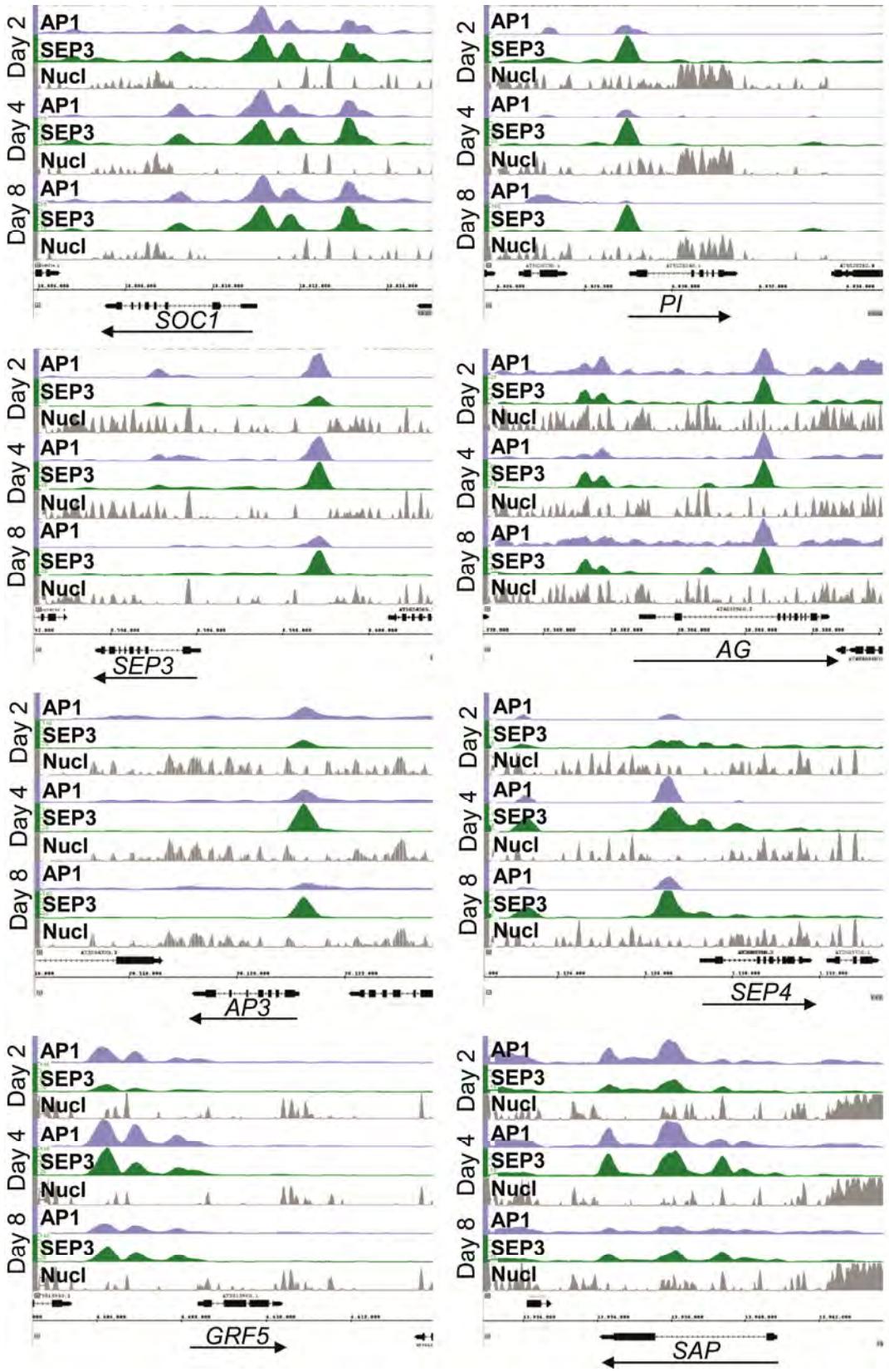


Figure 8. Nucleosome occupancy and AP1 and SEP3 DNA-binding profile during flower development for selected target genes. Nucleosome occupancy (grey), AP1 binding (purple) and SEP3 binding (green) profiles for selected genes. Most of the binding events are in regions with low nucleosome occupancy (on the left), although some nucleosomes were detected in regions bound by AP1 and SEP3 (on the right).

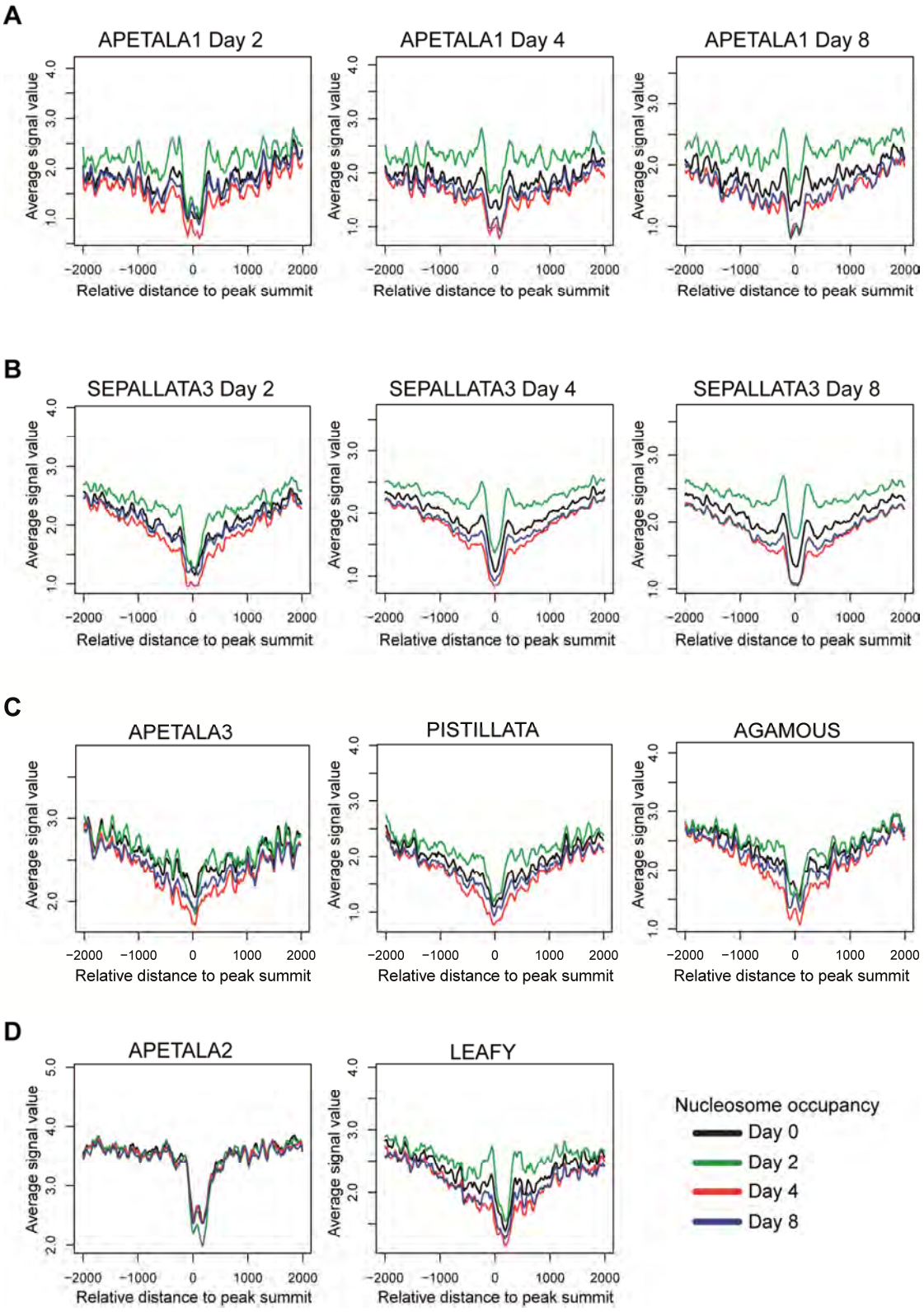


Figure 9. Nucleosome occupancy (as average MNase signal value) at TFs DNA-binding events during flower development. A. Nucleosome occupancy around AP1 bound region summits at day 2, day 4 and day 8. **B.** Nucleosome occupancy around SEP3 bound region summits at day 2, day 4 and day 8. **C.** Nucleosome occupancy around AP3, PI and AG bound region summits **D.** Nucleosome occupancy around AP2 and LFY bound region summits.

Next, we looked at nucleosome occupancy around regions bound by other MADS-domain proteins such as AG (O'Maoileidigh *et al.*, 2013), AP3 and PI (Wuest *et al.*, 2012) (**Figure 9C**) and members of other transcription factor families such as AP2 (Yant *et al.*, 2010) and LFY (Winter *et al.*, 2011) (**Figure 9D**). For all the transcription factors a valley was present around the centre of the peak summit, suggesting that transcription factors generally bind to nucleosome-depleted regions.

Finally, we analysed nucleosome dynamics at AP1 and SEP3 bound loci. Remarkably, within regions bound by AP1 and SEP3 at day 4 we detected respectively 83 (0.43%) and 768 (4%) nucleosome-depleted regions between 2 and 4 days. For example, we observed nucleosome-depleted regions within AP1 and SEP3 binding sites located nearby *AG* and *AP3* loci at day 4 (**Figure 8**).

DISCUSSION

Nucleosome occupancy and dynamics during flower development.

Transcription factors regulate gene expression and act in a complex chromatin environment. The structural unit of chromatin is the nucleosome. To gain a better understanding in the relationship between gene regulation and chromatin landscape during flower development, we studied nucleosome occupancy at different stages of flower development. We developed the MNase-seq technique to detect nucleosome occupancy and nucleosome dynamics in *Arabidopsis*. We observed that only a small portion of the genome (~22%) is stably occupied by nucleosomes during flower development. Similar findings in human nucleosome occupancy studies (Carone *et al.*, 2014), suggest a conservation between different organisms.

Moreover, as previously observed in other organisms, such as human and yeast (Chen *et al.*, 2013a; Jiang and Pugh, 2009), also in Arabidopsis flowers, nucleosome position is strongly conserved around the transcription starting site.

Nucleosome turnover during gene transcription is a rapid mechanism that cannot be captured in a day-time frame (Kristjuhan and Svejstrup, 2004; Lee *et al.*, 2004). In our study, the majority of dynamic nucleosomes are located in the core promoter region. Thus, the changes we observed may reflect more permanent alterations in chromatin structure that happen mostly at cis-regulatory elements.

Only a small portion (~5%) of nucleosomes is dynamic during flower development, indicating that we are dealing with a fairly stable nucleosome composition over a 0-8 days interval. Nevertheless, the changes that we observed in nucleosome occupancy happen nearby genes with a known role in processes involved in flower development, such as floral whorl development or carpel, ovule and pollen development, suggesting that the changes are relevant for the action of the genes involved in these processes.

Changes in genes expression do not correlate with nucleosome dynamics.

In yeast, gene repression is associated with gain in nucleosome occupancy at corresponding loci while nucleosome depletion is observed nearby by up-regulated genes (Huebert *et al.*, 2012). In our experiments, we observed nucleosome-depleted regions as well as nucleosome gain regions nearby both up- and down-regulated genes, indicated that there is no clear correlation between change in expression and nucleosome remodeling at corresponding loci. The lack of correlation between changes in gene expression and nucleosome occupancy could be attributed to the time intervals used in the study. Changes in nucleosome occupancy due to transcription may only be observed in short time intervals. In our experiment nucleosome dynamics may be due to changes at specific cis-regulatory elements instead of chromatin remodeling related to gene transcription. In agreement of this hypothesis most of the nucleosome dynamic regions were located in the promoters.

Cis-regulatory elements are prevalently located in nucleosome free regions.

Cis-regulatory elements, defined as DHSs or regions bound by a transcription factor, appear to be generally nucleosome-depleted.

Interestingly, we detected a higher level of occupancy at the summit of cis-regulatory regions compared to the immediate surrounding regions. This could indicate either the presence of a nucleosome or a DNA-binding protein complex that protects the DNA from MNase digestion, resulting in an higher signal at the summit of the region.

MNase preferentially cleaves the linker DNA between nucleosomes, leaving nucleosomal DNA intact, and generating a nucleosome footprint. However, recent studies (Carone *et al.*, 2014; Henikoff *et al.*, 2011; Kent *et al.*, 2011) showed that when subnucleosome-sized fragments are selected, MNase digestion can be used to generate the footprints of

DNA-binding proteins. To recognize the presence of a nucleosome in cis-regulatory elements ChIP-seq experiments using antibody targeting histones could be performed.

At early stages of flower development, during the meristem and organ specification phases, chromatin is subjected predominantly to local rearrangements, which is reflected in the higher dynamics in nucleosome occupancy observed between days 0-2 and 2-4. In contrast, at later stage of development, during organ differentiation, nucleosomes appear to be more static, when the highest dynamics in DHS is observed (Pajoro *et al.*, 2014; chapter 3). Taken together these results suggest that chromatin is initially subject to rearrangements at nucleosome level followed by larger changes later on at the cis-regulatory elements.

In conclusion, the fact that we couldn't detect high nucleosome occupancy prior transcription factor binding, indicate that MADS-domain transcription factors bind mostly to nucleosome free regions. Thus, MADS-domain transcription factors do not directly promote nucleosome eviction, as an active pioneer factor does (Drouin, 2014; Zaret and Carroll, 2011). Alternatively, active pioneer action of MADS-domain protein is a fast mechanism that can't be detected at our experimental condition. Moreover the lack of tissue-specificity in our current experiment could mask pioneering action at a specific locus.

Our previous studies showed that chromatin accessibility as cis-regulatory elements (identified by DHSs) increases upon MADS-domain transcription factor binding (Pajoro *et al.*, 2014; chapter 3) and that MADS-domain proteins interact with chromatin remodelers (Smaczniak *et al.*, 2012), both characteristic features of "pioneer factors" (Drouin, 2014; Zaret and Carroll, 2011). Taken together these observations suggest that MADS-domain proteins, upon binding to free nucleosome loci, affect chromatin accessibility at their binding sites by interaction with chromatin remodelers, therefore MADS-domain proteins may act as "pioneer factors".

MATERIALS and METHODS

Tissue collection. Approximately 4 week-old *pAP1*:AP1-GR *ap1-1 cal-1* plants were used. *pAP1*:AP1-GR *ap1-1 cal-1* plants were dipped after bolting (2 cm to 5 cm height) in DEX- induction solution (2 M Dexamethasone, 0.01% (v/v) ethanol, and 0.01% Silwet

L-77) daily. First induction was performed 8 hours after switching on the lights and subsequent daily induction at 4 hr after start of the light period. Material was collected just before DEX-induction, as well as at 2 days, 4 days and 8 days after the first treatment. Three biological samples were generated for each time point.

MNase-seq experiments. Nuclei isolation was performed according to (Zhang *et al.*, 2007) with minor modifications. Tissue was grinded in liquid nitrogen. For each time point, 0.2 g of plant material was used. Grinded material was resuspended in 2 ml of cold modified Honda buffer (HBM: 25 mM Tris, 0.44 M sucrose, 10 mM $MgCl_2$, 10 mM -mercaptoethanol, 2 mM spermine, and 0.1% Triton) and filtrated through a 55 m membrane. The membrane was washed with 1 ml HBM buffer. The filtrate was applied to a sucrose 2.5 M /40% Percoll gradient and centrifuged 30 min 2500 xg at 4°C. Nuclei were collected in the interphase and washed with 10 ml cold HBB (HBM without spermine) and 10 ml cold HBC (HBB with 20% glycerol). Between each wash, nuclei were centrifuged for 10 min 1000 xg at 4C. DNA digestion was performed according to (Li *et al.*, 2012b) with minor modifications. Nuclei were resuspended in 5 ml Wash Buffer (1mM Tris-HCl ph7.5, 5mM $MgCl_2$, 60mM KCl, 0.5mM DTT, 15mM NaCl, 300mM sucrose) and centrifuged 5 min 2500 xg at 4°C. 3. Pellet was washed with 5ml cold Reaction Buffer (1mM Tris-HCl ph7.5, 1mM $CaCl_2$, 60mM KCl, 0.5mM DTT, 15mM NaCl, 300mM sucrose) and centrifuged 5 min 2500 xg at 4°C. Nuclei were resuspended in 2 ml Reaction Buffer and divided into 10 1.5 ml tubes (aliquots of 200 l). To each aliquot 0-0.2-0.4-0.6-0.8-1-1.5-2-3-5 U of S7 nuclease was added (Roche Applied Science, Catalog number 10107921001). Samples were incubated for 10 min at 37°C in a thermomixer gently shaking (500rpm). The reaction was terminated by adding 23 l of EDTA and incubating at RT for 5 min. To each aliquot, 350 l Lysis Solution (1% SDS, 50mM Tris-HCl ph8, 20mM EDTA, 10mg/ml RNaseA) was added and samples were mixed by inversion. After adding 10 l of 20 g/ml proteinase K, samples were incubated O/N incubation at 37°C. Samples were centrifuged for 10 min at 13.000 xg at RT. Supernatant was transferred to a new tube and 10 l of each aliquot were run on a 1% agarose gel. Aliquots with manly (80%) mononucleosomal DNA were selected. DNA from selected aliquots was extract with chloroform-isoamylalcohol and precipitated by adding 0.9 volumes of isopropanol. The precipitated DNA was dried and left to resuspend in 100 l HPLC water O/N at 4°C. 50 l were load on 2% agarose gel and

mononucleosomal DNA (bands at 150 bp) was excised and purified with QIAGEN gel purification kit. Libraries were prepared using TruSeq DNA sample preparation kit from Illumina following the manufacturer instructions. Three biological replicates for each time point were sequenced on Illumina HighSeq2000 in a 50 bp single-end run.

MNase-seq data analysis. Basecalls was performed using CASAVA version 1.8. to obtain FASTQ files, later sequenced reads were mapped to the *Arabidopsis thaliana* TAIR10 genome using Bowtie (Langmead *et al.*, 2009) version 0.12.7, allowing up to 2 mismatches. Low quality reads and duplicate reads were removed with samtools view (with –q parameter = 1) and samtools rmdup (Li *et al.*, 2009). Only reads mapping in a single position in the genome were kept. An overview of sequencing data is reported in Table S1. Reproducibility between biological replicates was assessed using the Pearson correlation coefficient (PCC) for the genome-wide reads distribution at each pair of replicates after comparison to the control using 10 bp window, an overview is reported in Table S2. To identify nucleosome positions, we used DANPOS 2.1.4 (Chen *et al.*, 2013b) with default parameters. We used a cut-off of p-value 1e-05, 1e-07 and 1e-10 (testcut parameter in DANPOS) to identify region differentially occupied by nucleosomes.
WigAnalysis function from DANTOOLS 0.2.1 (Chen *et al.*, 2013b) was used to retrieve nucleosome occupancy profiles around the transcription starting site (TSS) or the DHS and transcription factor binding summits.
Genomic regions were associated with genes if located 1 kb upstream of the start of the gene to the end of the gene using the function distance2Genes in the Bioconductor package CSAR (Muino *et al.*, 2011) for genes annotated in TAIR10.

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SUPPLEMENTAL DATA

Table S1. Number of reads obtained for each sample. 3 biological replicates for each time point were sequenced on HiSeq2000. In the table are reported the total number of reads obtained from the sequencing, the number of reads that mapped to the *Arabidopsis* TAIR 10 genome and the number of reads left after removing duplicate reads with relative percentages.

SAMPLE	Total number of reads obtained from Hiseq2000 sequencing	Number of reads aligned in *A thaliana* genome (TAIR10) with good quality mapping	% mapped reads	Number of uniquely aligned reads (duplicates removed)	% of unique reads	coverage
genomic DNA 1	113,112,346	106,186,577	93.88%	43,812,510	39.87%	16.27
genomic DNA 2	101,314,431	94,862,768	93.63%	39,335,438	40.11%	14.61
day 0 bio repl 1	98,826,702	88,821,598	89.88%	39,530,869	41.35%	14.68
day 0 bio repl 2	73,418,463	66,253,016	90.24%	32,913,743	46.36%	12.22
day 0 bio repl 3	102,812,591	92,858,851	90.32%	41,313,239	41.49%	15.34
day 2 bio repl 1	143,910,691	130,142,957	90.43%	48,574,217	34.80%	18.04
day 2 bio repl 2	98,436,129	88,747,865	90.16%	38,598,933	40.49%	14.33
day 2 bio repl 3	129,241,787	116,500,377	90.14%	46,111,197	36.98%	17.12
day 4 bio repl 1	144,502,872	130,478,570	90.29%	47,440,960	44.57%	17.62
day 4 bio repl 2	96,846,069	87,756,796	90.61%	39,277,069	41.89%	14.59
day 4 bio repl 3	83,092,100	74,676,877	89.87%	34,175,273	42.68%	12.69
day 8 bio repl 1	65,406,707	59,269,945	90.62%	30,063,679	47.41%	11.16
day 8 bio repl 2	96,208,223	86,846,256	90.27%	37,662,724	40.42%	13.99
day 8 bio repl 3	91,391,677	82,882,745	90.69%	37,342,272	42.16%	13.87
TOT	**1,438,520,788**	**1,306,285,198**	**90.81%**	**556,152,123**	**42.58%**	**206.54**

Table S2. Pearson correlation between the samples.

		day 0			day 2			day 4			day 8		
		Repl 1	Repl 2	Repl 3	Repl 1	Repl 2	Repl 3	Repl 1	Repl 2	Repl 3	Repl 1	Repl 2	Repl 3
day 0	Repl 1	1	0.8	0.82	0.79	0.81	0.81	0.8	0.78	0.78	0.77	0.79	0.78
	Repl 2		1	0.79	0.74	0.77	0.76	0.78	0.78	0.79	0.78	0.77	0.78
	Repl 3			1	0.78	0.78	0.79	0.82	0.8	0.79	0.78	0.79	0.79
day 2	Repl 1				1	0.83	0.84	0.81	0.8	0.75	0.74	0.82	0.8
	Repl 2					1	0.84	0.79	0.79	0.76	0.75	0.81	0.79
	Repl 3						1	0.82	0.8	0.76	0.75	0.81	0.8
day 4	Repl 1							1	0.83	0.81	0.78	0.82	0.82
	Repl 2								1	0.81	0.79	0.82	0.82
	Repl 3									1	0.8	0.79	0.8
day 8	Repl 1										1	0.79	0.79
	Repl 2											1	0.82
	Repl 3												1

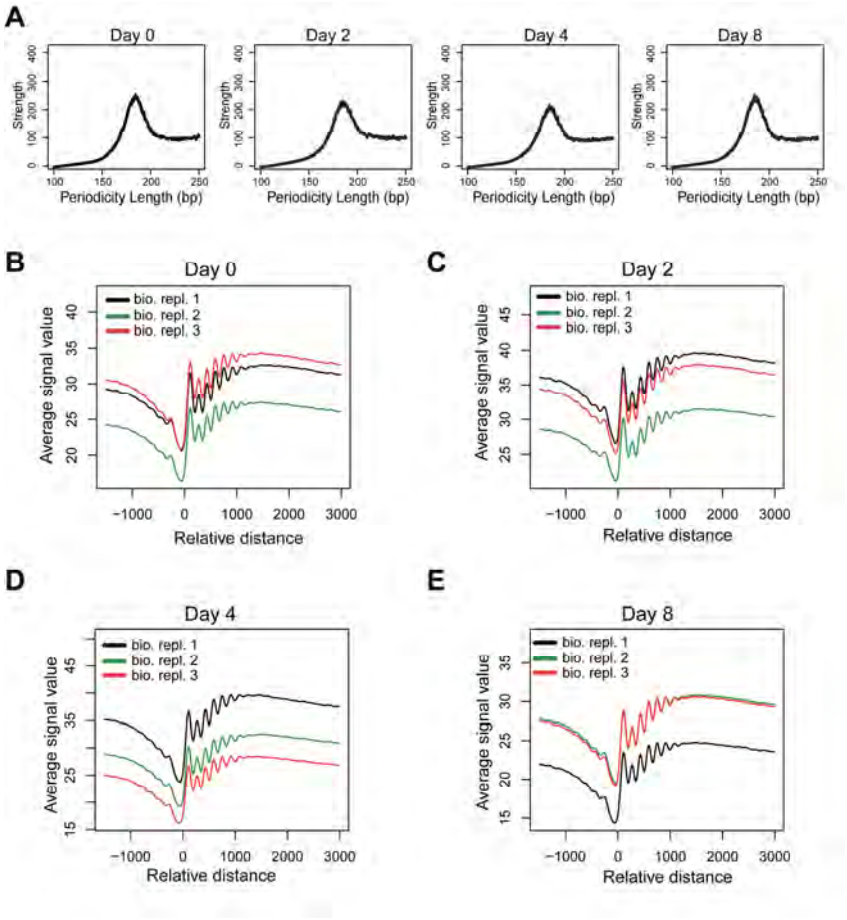

Figure S1. Nucleosome distribution in *Arabidopsis thaliana* **A**. Signal periodicity at the different time points. The signal periodicity represents the distance between two summits. For all time points we observed an average of 180 bp. **B**. Nucleosome occupancy profile for three biological replicates at day 0 before normalisation and comparison with the control. **C.** Nucleosome occupancy profile for three biological replicates at day 2 before normalisation and comparison with the control. **D.** Nucleosome occupancy profile for three biological replicates at day 4 before normalisation and comparison with the control. **E.** Nucleosome occupancy profile for three biological replicates at day 8 before normalisation and comparison with the control.

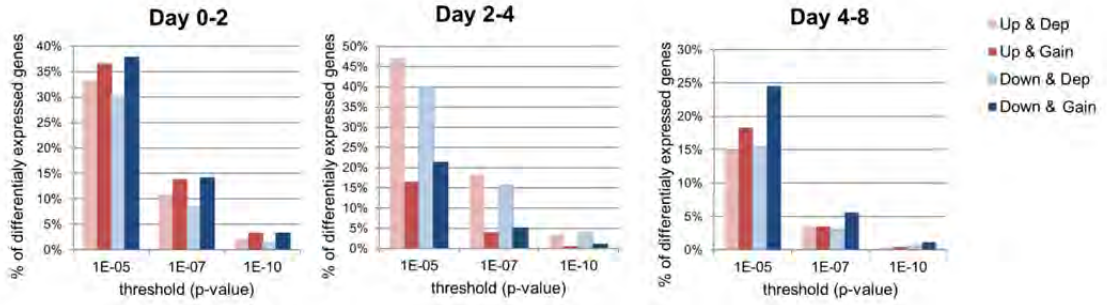

Figure S2. Changes in nucleosome occupancy and gene expression. Graphs show percentage of up- and down-regulated genes for which nucleosome depleted or gain regions were observed at p-value cut-off 1e-05 and 1e-10. Up, up-regulated genes; down, down-regulated genes; Dep, nucleosome depletion events; Occ, increased nucleosome occupancy.

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CHAPTER 5

The role of *STERILE APETALA* during early stages of flower development in *Arabidopsis thaliana*

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ABSTRACT

Flower development is a key process for successful plant reproduction, which is tightly regulated at the genetic level. The backbone of the gene regulatory network controlling flower development has been unveiled in the past decades with genes that belong to the MADS-domain transcription factor family. Recent genome wide studies revealed a much higher complexity of the interconnections between the genes in the network than previously assumed. The genome wide approaches also allowed to identify many new genes with a putative role in flower development. In a previous study we investigated the dynamics in regulation of target genes of the MADS-domain transcription factors controlling flower development. In this chapter, we focus on the further functional characterisation of one of these target genes that has been found to be part of the network: *STERILE APETALA* (*SAP*). *SAP* is a target of many MADS-domain proteins including APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), AGAMOUS (AG) and SEPALLATA3 (SEP3). Expression analysis revealed that *SAP* is strongly expressed in inflorescence meristems and at early stages of flower development. Loss of function of *SAP* causes strong aberrations in flowers, such as reduction in petal and stamen numbers. Preliminary results indicate that SAP interacts with proteins of the SCF ubiquitin ligase complex, suggesting that SAP could act in the ubiquitination pathway.

INTRODUCTION

Flower development is a key step for successful plant reproduction. During vegetative growth the apical meristem produces leaves, while during the reproductive phase it produces flowers. This developmental change requires changes in gene expression by the action of key regulators. In the past decades key regulators of floral organ specification have been identified by genetic studies (Bowman *et al.*, 1989), leading to the inference of an elegant model, known as ABC model (Haughn and Somerville, 1988). The ABC model has later been extended to the ABCDE model (for review see (Causier *et al.*, 2010; Pajoro *et al.*, 2014a)). In this model, transcription factors of the MADS-domain family act in a combinatorial manner to define floral organ identity: A and E class genes promote sepal identity; A, B and E class genes determine petal identity; B, C and E class genes specify stamen identity; and the combination of C and E class gene functions confers carpel identity, while D and E class genes are responsible for ovule identity. The master regulators representing the Arabidopsis class A function comprise APETALA1 (AP1) and the non-MADS transcription factor APETALA2 (AP2) (Bowman *et al.*, 1993; Mandel *et al.*, 1992). The genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) have been identified to act as class B genes (Goto and Meyerowitz, 1994; Jack *et al.*, 1992), while *AGAMOUS* (*AG*) is the class C gene in Arabidopsis (Yanofsky *et al.*, 1990). Three D class genes *SEEDSTICK* (*STK*), and *SHATTERPROOF 1* and *2* (*SHP1, SHP2*) are redundantly involved in ovule development (Pinyopich *et al.*, 2003). Four E class genes *SEPALLATA1-4* (*SEP1-4*) act redundantly and are required for the formation of all floral organs (Ditta *et al.*, 2004; Pelaz *et al.*, 2000; Pelaz *et al.*, 2001). Once these key regulators of floral organ identity were identified, the next question was how these transcription factors interact with each other in order to determine floral organ identity in a combinatorial manner. Yeast two hybrid and protein-immunoprecipitation experiments revealed interactions among these transcription factors forming dimers and higher-order complexes (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001). These key regulators specify the identity and further differentiation of the organs to their final size, shape and function by controlling the expression of a large set of target genes. Target genes were identified by expression profiling and chromatin immunoprecipiation (ChIP), which provided information on the direct transcriptional regulation of downstream targets during early stages of flower development (Kaufmann *et al.*, 2009; Kaufmann *et al.*, 2010b;

O'Maoileidigh *et al.*, 2013; Wuest *et al.*, 2012). These approaches revealed that many transcription factors could act as transcriptional activators or repressors depending on the tissue type, stage of development as well as endogenous and exogenous signals. Furthermore, using an inducible system for synchronised flower development global changes in gene expression with high temporal resolution could be studied (Kaufmann *et al.*, 2010b; Wellmer *et al.*, 2006). These studies showed that the expression of genes is highly dynamic during flower initiation and floral organ development. To correlate dynamic changes in expression with changes in TF DNA-binding, we recently determined the dynamic binding profiles of two master regulators of flower development: AP1 and SEP3 (Pajoro *et al.*, 2014b; Chapter 3). The ChIP-seq experiments at different stages of flower development revealed the *STERILE APETALA* (*SAP*) gene as dynamically bound by AP1 and SEP3 (Pajoro *et al.*, 2014b). We found an increase in AP1 binding at the *SAP* locus from day 2 to day 4 after *AP1* induction and a decrease in both AP1 and SEP3 binding from day 4 to day 8. Other MADS-domain proteins, such as AG (O'Maoileidigh *et al.*, 2013), PI and AP3 (Wuest *et al.*, 2012) bind to the *SAP* locus at stage 4 of flower development. The *sap* mutant has previously been described to be strongly affected in flower and ovule development (Byzova *et al.*, 1999). The *sap* mutant was identified in a transposon tagging screen. Mutant plants are bushy and sterile due to defects in megasporogenesis. *Sap* flowers lack petals and late arising flowers resemble an *ap2* mutant with conversions of sepals into carpelloid structures (Byzova *et al.*, 1999).

Based on this previous study, *SAP* appears to be another important player in establishing the body plan of the flower. Therefore, we decided to investigate the role of *SAP* at early stages of flower development in more detail and aim to understand its regulation by ABC-class genes and its biological function.

RESULTS

The SAP locus

In previous experiments (Pajoro *et al.*, 2014b), Chapter 3) we identified genomic regions in the *SAP* locus bound by AP1 and SEP3 (**Figure 1**). Since the published mutant line (Byzova *et al.*, 1999) was no longer available we searched for new mutant lines. We selected three lines that had T-DNA insertions in the intron and exons: *sap-1* (SALK-

129750), *sap-2* (SALK-023467) and *sap-3* (FLAG-359F07), respectively (**Figure 1**). The *sap-1* and *sap-2* T-DNA insertions are located in the 1st intron, and *sap-3* has a T-DNA insertion in the 3' UTR.

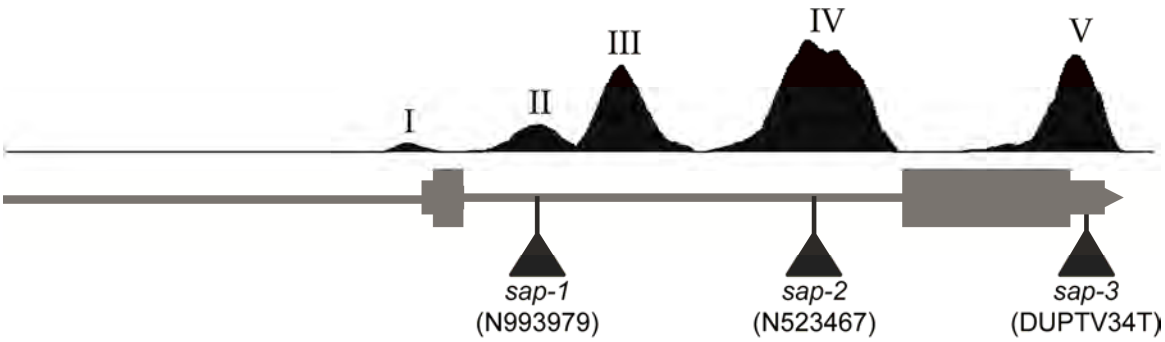

Figure 1. Schematic representation of the SAP locus. Regulatory regions numbered from I to V have been identified through AP1 and SEP3 DNA binding profiles, in the figure the SEP3 binding profile at day 4 is reported as example. The regulatory regions II and IV were found to be preferentially bound at day 4 by both AP1 and SEP3. AG binds to the regulatory region III and AP3 and PI bind to the regulatory region IV. T-DNA insertions in corresponding mutant lines are indicated by a triangle. Dashed line is the promoter region, blocks are exons with coding region indicated by thicker rectangles, solid line is an intron.

The sap mutant phenotype.

The phenotype of all three homozygous T-DNA insertion lines was studied and compared to wild-type plants. All insertion lines were smaller and bushier compared to wild-type. Mutants were sterile with serrated leaves and reduced internode length (**Figure 2A-D**). In *sap* plants severe defects in flower development were observed (**Figure 2E-L**). Mutant flowers are smaller with a reduction in organ numbers (**Figure 2H**), aberrant stamens and longer style compared to wild-type (**Figure 2G**). A significant difference in petal numbers between the mutant lines was observed: strong reduction in petal number or completely apetala flowers were found in the *sap*-3 line as described by Byzova et al (1999), while the reduction in petal number was less in the *sap*-1 and *sap*-2 lines (**Figure 2H-L**). Since *sap-1* and *sap-2* mutant alleles were alike only *sap-2* was considered further. *sap* mutant plants show an extended longevity, most likely because these plants are sterile and continue to form flowers. 11 weeks old *sap* plants were bushy and stronger defects in the flowers were observed (**Figure 3A**). The late arising flowers showed a more severe phenotype than early formed flowers with a conversion of sepals into carpelloid structures and the formation of ectopic ovules (**Figure 3B-E**).

In conclusion, the phenotypes observed in the T-DNA insertion lines are in agreement with the phenotype previously reported by Byzova and colleagues (Byzova *et al.*, 1999), although the phenotype of late arising flowers is likely not specific for the *sap* mutant but due to the sterility of the plants.

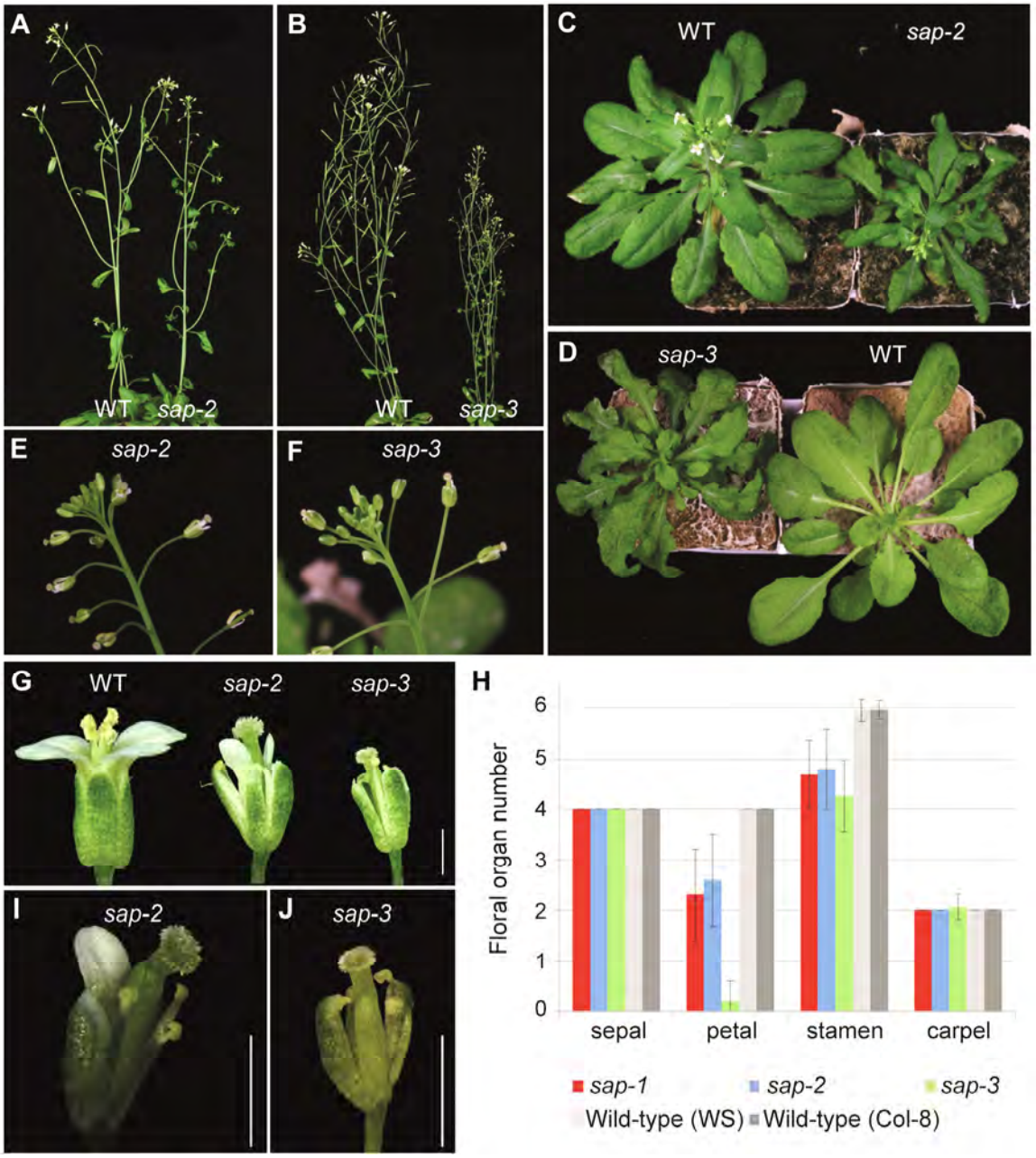

Figure 2. *sterile apetala* mutant phenotype. Since *sap-1* and *sap-2* mutant alleles were alike only *sap-2* flowers are shown in the figure. **A.** *sap-2* mutant plant compared to wild-type. Mutant plant is smaller than wild-type. All mutant plants are sterile. **B**. *sap-3* mutant plant compared to wild-type. *sap-3* is smaller and more bushy than wild-type due to the reduction in internode length. All mutant plants are sterile. **C**. *sap-2* mutant plant compared to wild-type. *sap-2* has a smaller rosette and leaves are serrated. **D**. *sap-3* mutant plant compared to wild-type. *sap-3* has a smaller rosette and leaves are serrated. **E**. *sap-2* inflorescence. Siliques fail to elongate due to sterility. **F.** *sap-3* inflorescence. Absence of petals can be observed. **G.** Phenotypes observed in flowers of *sap-1*, *sap-2* and *sap-3*. *sap* flowers are smaller than wild–type flowers. *sap*-1 and *sap*-2 mutant flowers are very similar, while a stronger mutant phenotype is

observed in the *sap*-3 mutant flower. In all mutant alleles the anther filaments do not fully elongate and anthers fail to release pollen. Mutant gynoecium has a longer style compared to wild type. Moreover, *sap* flowers show a reduction in petal and stamen numbers. Bar = 1mm. **H.** Histogram shows the average number of flower organs in the different *sap* mutant alleles compared to wild-type (n = 40). **I.** *sap-2* flower, one sepal has been removed to reveal inside organs. Reduction in petal numbers and aberrations in the anthers can be observed. Bar = 1mm. **J**. *sap-3* flower. No petals are present, stamens show severe defects in the anthers and no pollen seems to be formed. The gynoecium has a longer style. Bar = 1mm.

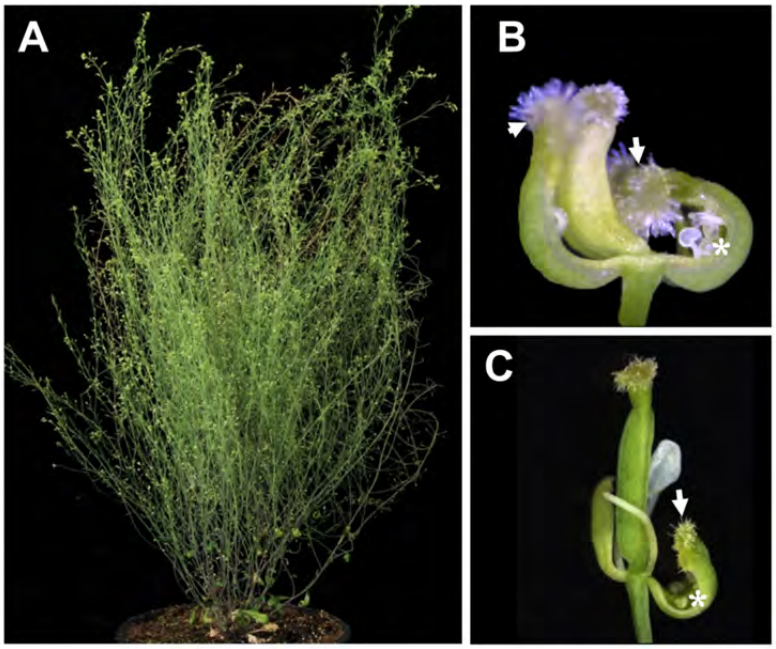

Figure 3. ***sap* phenotype in old plants A.** 11 weeks old *sap* mutant plant with a bushy appearance and it continues to grow while no seeds are produced. **B.** *sap-2* late arising flower. Flower shows a stronger phenotype with conversions of sepals into carpelloid structures with ectopic ovule formation **C.** *sap-3* late arising flower with sepals converted into carpelloid structures and ectopic ovule formation. Arrows indicate ectopic stigmatic tissue. * indicates ectopic ovule formation.

SAP expression in mutant lines and wild-type.

To confirm the reduction in *SAP* expression in the mutant lines we assessed the presence of *SAP* transcripts by real-time PCR (**Figure 4A-B**). We designed two different sets of primers: one pair of primers was designed to amplify a region spanning the 1st and 2nd exon to assess the absence of transcript in the *sap-1* and *sap-2* lines, the other couple of primers was designed at the 3' UTR of the gene, to assess absence of the transcript in the *sap-3* line. No transcript was detected in *sap-1* (not shown) and *sap-2* insertion lines (**Figure 4A)**. We also observed a strong reduction in *SAP* expression in *sap-3* plants using primers designed between the two exons, indicating that the T-DNA insertion in the 3' UTR causes instability of the mRNA and no transcript was detected with the pair of primers in the 3' UTR (**Figure 4B**).

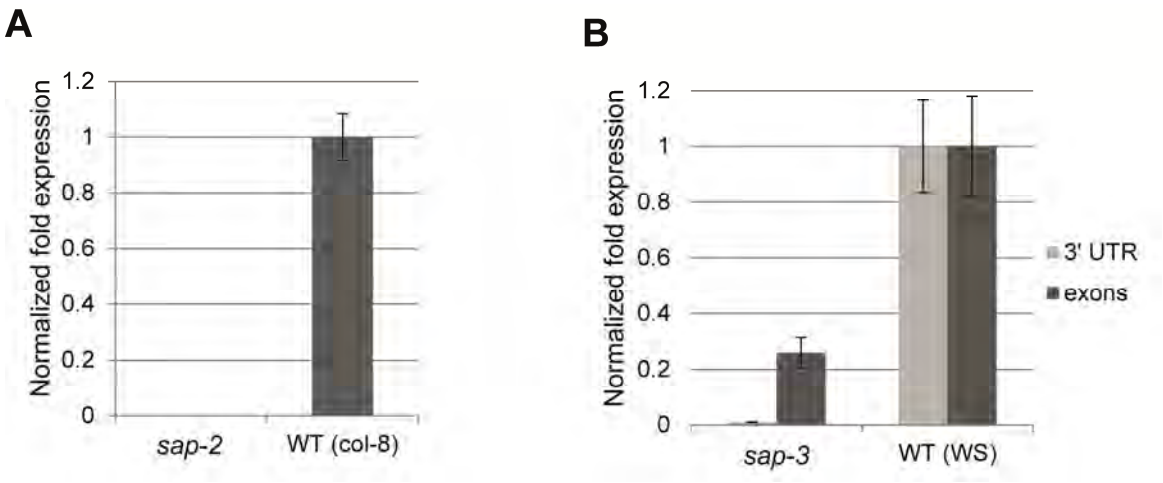

Figure 4. *SAP* expression in wild-type and mutant lines. A. Reduction of *SAP* expression in the *sap-2* mutant line. We could not detect any expression in mutant inflorescences using primers designed between the two exons, indicating that the presence of the T-DNA insertion abolished *SAP* expression completely. Bars indicate standard error calculated based on 3 biological replicates. **B.** Reduction of SAP expression in the *sap-3* mutant line. We could not detect any expression in mutant inflorescences using primers designed in the 3'UTR, while we observed a strong reduction in expression using primers designed between the two exons. These results indicate that the T-DNA insertion in the 3'ÚTR causes instability of the mRNA. Bars indicate standard error calculated based on 3 biological replicates.

Previous studies reported that *SAP* is strongly expressed in the inflorescence meristem, young flower buds and ovules (Byzova *et al.*, 1999). In a developmental time-series (0-8 days after AP1 induction) we found *SAP* expression to be down-regulated during floral organ development (Pajoro *et al.*, 2014b) (**Figure 5A**). Moreover, we found that *SAP* is higher expressed in *ap1* than in wild-type inflorescences (**Figure 5B**), suggesting that *AP1* negatively regulates *SAP*.

To study *SAP* expression in more detail, we generated a reporter construct for the *SAP* locus. We fused the *SAP* genomic region, consisting of 2 kb promoter region and the transcribed region of *SAP* (gSAP) to the green fluorescent protein (GFP). Ten *gSAP:GFP* T1 lines were studied, and two representative lines were analysed in more detail by confocal microscopy. Confocal imaging of *gSAP:GFP* showed nuclear localization of the GFP signal (**Figure 5C-H**). GFP signal was detected in the inflorescence meristem (**Figure 5C**) and in flower meristems until stage 5 (**Figure 5C-D**), while no GFP signal was observed in floral organs at later stages of flower development (**Figure 5D**). *SAP* seems to be most strongly expressed in the meristems. This result is in agreement with

previous RNA *in situ* hybridization studies, which reported *SAP* expression in inflorescence meristems and in flower buds from stage 1 to 6 (Byzova *et al.*, 1999).

To better characterize the upstream regulation of *SAP* we introduced the reporter gene *gSAP:GFP* in *ap1* and *ap1 cal* mutants. We found that *SAP* is expressed more broadly and higher in *ap1* than in wild-type flowers from stage 2 to stage 6 (compare **Figure 5C and F**). *SAP* ectopic expression was detected in sepalloid-leaves in *ap1* flowers while no expression was detectable in wild-type sepals (**Figure 5D, F-G**). This result is in agreement with the qPCR analysis that showed higher *SAP* expression in *ap1* mutant inflorescences than in wild-type inflorescences (**Figure 5B**). The findings suggest that AP1 may bind to the *SAP* locus to repress *SAP* expression in sepals. In contrast to later floral stages, *SAP* expression in the inflorescence meristem of *ap1* appears to be the same as in wild-type flowers, which is in line with the lack of expression of *AP1* in the inflorescence meristem (Mandel *et al.*, 1992; Urbanus *et al.*, 2009). *SAP* expression in the centre of the flower meristem of stage 2-6 floral buds, where *AP1* is not expressed is most likely regulated by other MADS-domain TFs. Indeed, *SAP* is also bound by other transcription factors that regulate flower development and are expressed in the flower meristem, such as AP3, PI, AG and SEP3 (Kaufmann *et al.*, 2009; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014b; Wuest *et al.*, 2012).

Next, we analyzed *SAP* expression profile in an *ap1 cal* mutant background and in the synchronized system for flower development (Wellmer *et al.*, 2006). As expected, we observed strong expression in *ap1 cal* (**Figure 5H**) since the inflorescences of *ap1 cal* mutant plants are composed of indeterminate propagation of inflorescence meristems that fail to develop proper floral organs.

Furthermore, we analysed the expression profile of *SAP* in the synchronised system for flower development, pAP1:AP1:GR *ap1 cal* (**Figure 5I-K**). We studied the expression profile before induction of flower development (**Figure 5I**), 1 day after induction (**Figure 5J**) and 4 days after induction (**Figure 5K**). We observed that 4 days after induction, *SAP* expression is confined to the floral meristem which explains the lower level of expression detected in the microarray experiments (**Figure 5A**).

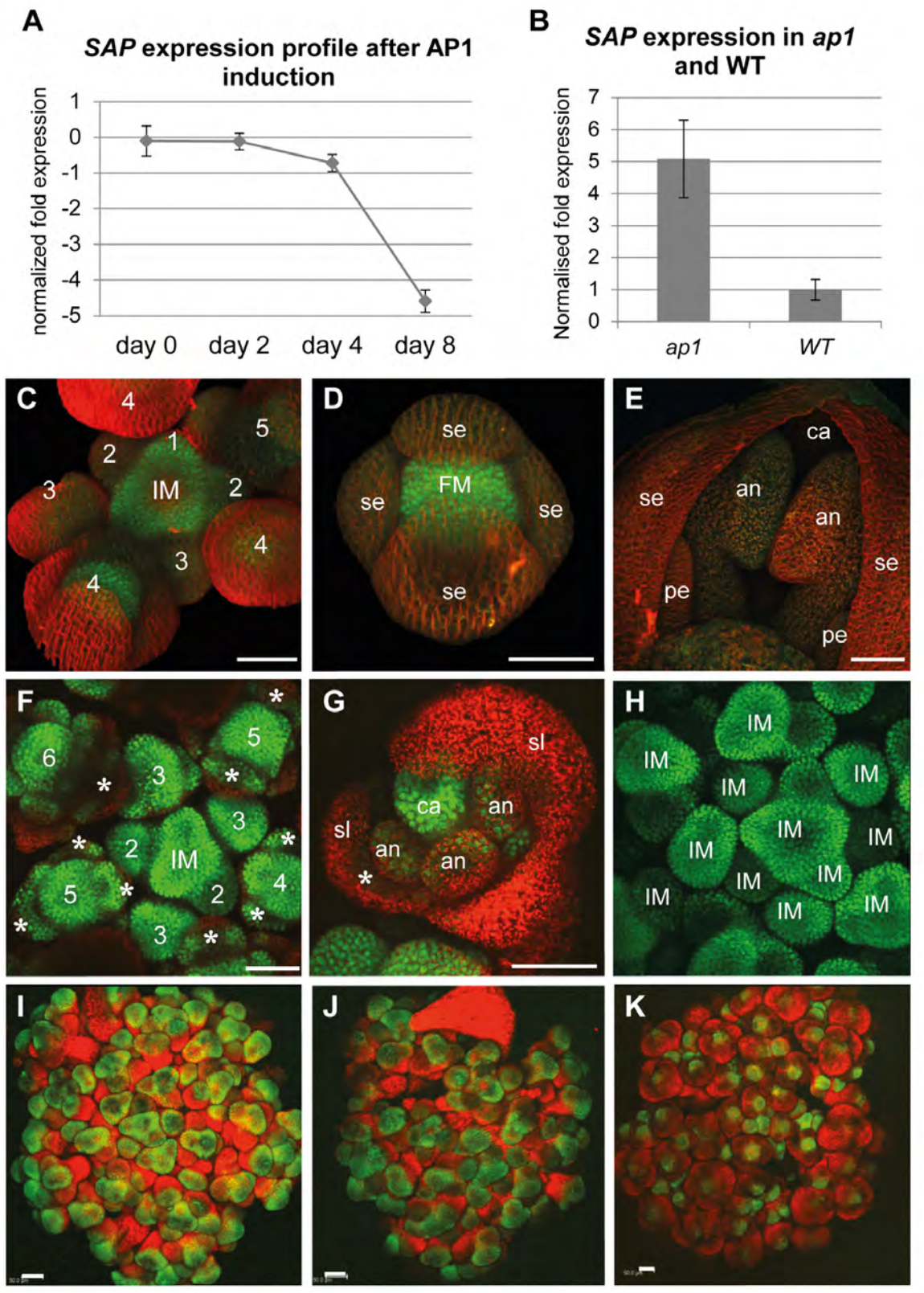

Figure 5. **SAP expression profile at early stages of flower development in wild type, *ap1* and *ap1 cal* background. A.** *SAP* expression profile in the synchronised flower system at 0-8 days after AP1 induction. RT-qPCR was performed with 3 biological replicates for plants treated with DEX or mock **B**. *SAP* expression in *ap1* and wild-type inflorescences. Bars indicate SE between three biological replicates. **C.** *gSAP:GFP* expression in inflorescence meristem (IM) and flower buds until stage 5 in a wild-type plant (Col-0). **D.**

gSAP:GFP expression in a flower at stage 4: expression is observed only in the floral meristematic region, no expression was detected in sepals. **E.** *gSAP:GFP* expression in floral stage 8, no expression was detected at this stage. **F.** *SAP* expression in *ap1* mutant background. *SAP* is more broadly and higher expressed in *ap1* flowers. *GFP* expression can be observed in the sepal-leaf structures in *ap1* flowers between stage 3 and 6 (*). **G.** *SAP* expression in an *ap1* mutant flower at stage 6. GFP signal was detected in the abaxial site of the sepaloid-leaves and in stamen and carpel primordia. **H.** *gSAP:GFP* expression in *ap1 cal* mutant background. **I.** *gSAP:GFP* expression profile in *ap1 cal* pAP1:AP1:GR mutant background before induction. **J**. *gSAP:GFP* expression profile in *ap1 cal* pAP1:AP1:GR mutant background 1 day after induction. *GFP* expression seems to decrease. **K**. *gSAP:GFP* expression profile in *ap1 cal* pAP1:AP1:GR mutant background 4 days after induction. *GFP* expression was detected in meristematic tissues and not in developing sepals. Numbers indicate stage of flower development according to (Smyth *et al.*, 1990). IM, inflorescence meristem; FM, flower meristem; se, sepal; sl, sepaloid-leaf ca, carpel; pe, petal; an, anther. Bars=50um.

Molecular function of SAP.

Finally, we were interested to study the molecular function of SAP. Due to the nuclear localisation of SAP:GFP (**Figure 5**), we hypothesised that SAP could act as DNA-binding factor. To identify putative direct targets of SAP, we performed chromatin immunoprecipitation (ChIP) using the *gSAP:GFP* line. Based on the strong petal phenotype observed in the *sap* mutant plants, we decided to look for SAP binding to genomic loci involved in petal development, such as *AP3* and *SEP3*. Next to these genes we examined binding events at the *AG* locus, since *AG* was previously found to be ectopically expressed in the *sap* mutant (Byzova *et al.*, 1999). We performed ChIP-qPCR using specific primers for the *AP3*, *SEP3* and *AG* loci. We could not observe any enrichment for the *AP3* and *AG* loci, while for the *SEP3* locus a low enrichment (P-value=0.1) was observed in sample 1 (**Figure 6**). This result could be due to the fact that SAP is not binding to DNA, which is in agreement with the absence of a predicted DNA-binding domain in the SAP protein. Alternatively, we selected the wrong genomic regions for our ChIP-qPCR.

As SAP did not show significant DNA-binding to the examined loci, we had a closer look at the predicted domain organization of the protein. SAP contains a WD40 repeat-like-containing domain (www.ebi.ac.uk/interpro), which is typically involved in protein-protein interactions (Smith, 2008; Smith *et al.*, 1999). Proteins containing WD40 repeats are known to serve as platforms for the assembly of protein complexes or mediators of transient interplay among other proteins. Thus, SAP could act as part of a protein complex; to investigate this hypothesis we performed a protein pull-down experiment to identify proteins that interact with SAP (Smaczniak *et al.*, 2012).

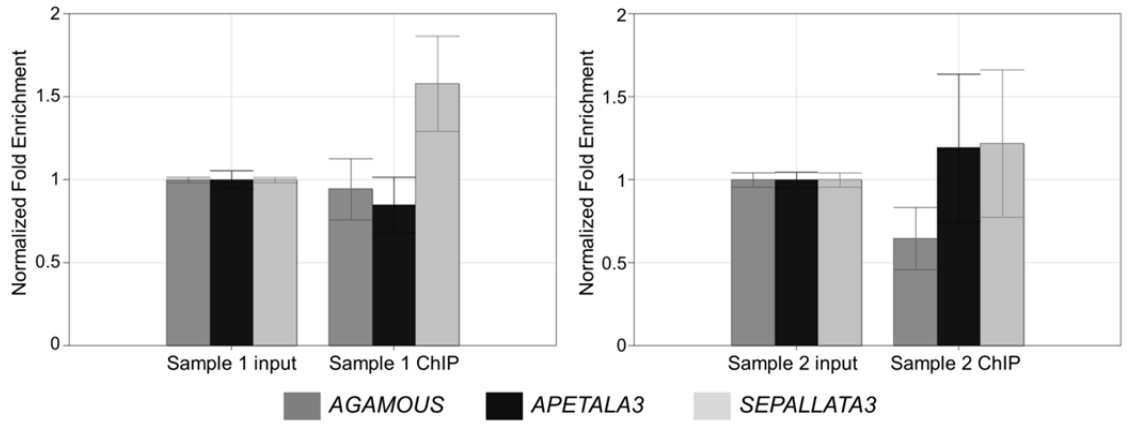

Figure 6. qPCR on selected loci after chromatin immunoprecipitation (ChIP). ChIP experiments were performed with gSAP:GFP inflorescences using anti-GFP antibody. Sample 1 inflorescences were fixed with FAA, while Sample 2 inflorescences were fixed with FAA and DMA. Enrichment was tested for *AG*, *AP3* and *SEP3* loci. *RESPONSE REGULATOR 6* (*ARR6*) and *HEAT SHOCK FACTOR 1* (*HSF1*) loci were used as negative control, since no enrichment is expected at these loci. No enrichment was found for AG and AP3 loci in both samples, while a low enrichment was observed for the SEP3 locus in sample 1. Bars indicate SD between two technical replicates.

The results of the LC-MS-based complex isolation using the gSAP:GFP transgenic plants revealed ARABIDOPSIS SKP1-HOMOLOGUE 1 (ASK1) and ARABIDOPSIS SKP1-HOMOLOGUE 2 (ASK2) as significantly enriched compared to the control sample (**Figure 7** and **Table 1**).

SKP1 is part of the Skp, Cullin, F-box protein complex (SCF-complex), an E3 ubiquitin ligase (Porat *et al.*, 1998; Wang *et al.*, 2006). SCF complexes act at the post-transcriptional level by ubiquitinating proteins designated for degradation via the 26S proteasome. SCF-complexes are typically composed by four units: Cul1, Rbx1, Skp1 and a member of the F-box protein family. Rbx1, which contains the RING domain, and Cul1 form a catalytic core of the complex, a variable F-box protein subunit binds the target protein, and Skp1 serves as bridge between the F-box protein and Cul1 (Shabek and Zheng, 2014; Zheng *et al.*, 2002).

F-box proteins that function in SCF-complexes are characterized by an amino-terminal F-box motif that binds to Skp1 and by a C-terminal protein-protein interaction domain, such as Leucine-rich repeats (LRR), Kelch-repeats or Tryptophan-Asparagine-rich repeats (WD40), that binds substrate proteins to the complex. Remarkably, an F-box motif is predicted at the N-terminus of SAP (http://www.ebi.ac.uk/interpro/protein/Q9FKH1), suggesting that SAP may play the role of an F-box protein in such an SCF complex. Interestingly, CUL1 was also found between the top enriched proteins, although not

significant at FDR 0.01. The lower level of CUL1 enrichment can be explained by the indirect interaction between the F-box protein and the cullin.

Table 1. Results of LC-MS-based complex isolation of gSAP:GFP inflorescence meristems.

ATG	Protein	Log10 Ratio	Peptide number		Description
			All	Unique	
AT5G35770	SAP *	5.58	29	29	
AT1G75950	ASK1 *	4.97	9	5	SKP1 is core component of the SCF family of E3 ubiquitin ligases
AT5G42190	ASK2 *	4.88	11	7	SKP1 is core component of the SCF family of E3 ubiquitin ligases
	GFP *	3.82	13	13	
AT1G79930	HSP70-14 *	2.39	32	4	High molecular weight heat shock protein 70
	Q93YS7	2.29	11	11	Putative WD-repeat membrane protein
AT2G04550	IBR5 *	2.15	4	4	Protein-tyrosine-phosphatase, IBR5 promotes auxin responses
AT5G62390	BAG7	2.07	10	10	BAG family molecular chaperone regulators
AT4G02570	CUL1	1.81	4	4	Encodes a cullin that is a component of SCF ubiquitin ligase complexes
AT5G64040	PSAN	1.78	3	3	Photosystem I subunit
AT3G22330	RH53	1.68	5	5	DEAD-box ATP-dependent RNA helicase
AT4G14713	TIFY4	1.58	3	3	Plant-specific putative DNA-binding protein
AT5G25980	TGG2	1.47	17	14	Myrosinase, involved in glucosinoloate metabolism
AT2G29550	TUB7	1.45	22	3	Beta-ubulin

(*) indicates proteins significantly enriched at FDR0.01.

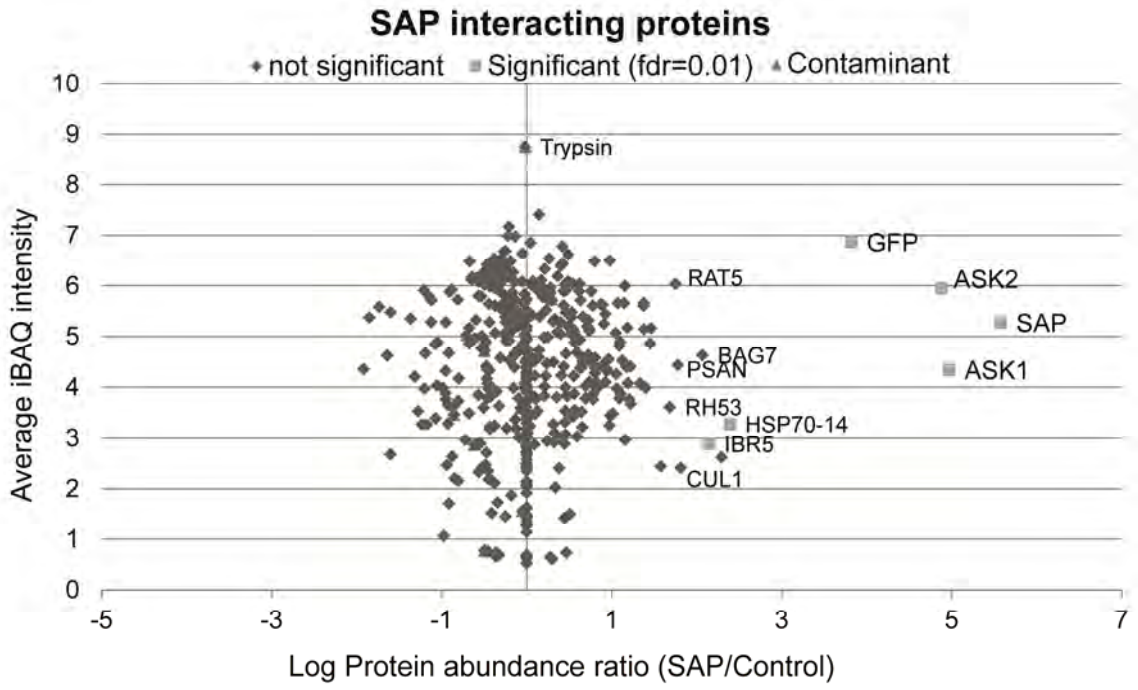

Figure 7. Protein interaction profile. Scatterplot of gSAP:GFP IP. Protein abundance ratios between sample and control plotted against the iBAQ intensities for a particular protein. In squares are significant enriched proteins at FDR 0.01.

DISCUSSION

The *SAP* genomic locus is bound by many key regulators of flower development, such as AP1, AP3, PI, AG and SEP3. Moreover, loss of function of *SAP* leads to severe aberrations in floral organs, such as the lack of petals and the conversion of sepals into carpelloid organs. Taken together these results indicate a fundamental role of *SAP* during early stages of flower development. We found that *SAP* is strongly expressed in meristematic tissues, while no expression was detected in floral organs. The characterisation of *SAP* expression in the *ap1* mutant indicates that *AP1* negatively regulates *SAP* expression in sepals. Moreover, the severe defects in petal and stamen development in the *sap* flowers, as well as previously reported binding of PI, AP3 and AG to the *SAP* locus suggest that *SAP* acts down-stream the homeotic genes in the regulation of flower organ development. However, more studies are needed to further characterize the *cis*-regulatory regions in the *SAP* locus and the regulation of *SAP* by MADS-domain transcription factors. Next, the result of our pull-down experiment prompted us to speculate about a mechanism for SAP: SAP may act at the post-transcriptional level by being part of a SCF-complex together with ASK1/2 and CUL1 (**Figure 8**). The *ask-1* mutant shows severe defects in flower development such as reduction in petal number and defects in petal and stamen growth (Zhao *et al.*, 1999). Similar to *SAP*, *ASK1* is expressed in meristems and floral organ primordia (Porat *et al.*, 1998). The similarities between the loss of function mutants and the expression profiles of *SAP* and *ASK1* suggest a genetic interaction between the two genes.

In conclusion, SAP together with ASK1/2 and CUL could form an E3 ubiquitin ligase SCF^{SAP} complex, and target proteins involved in organ development for ubiquitination. However, further experiments are needed to identify the proteins ubiquitinated by the SCF^{SAP} complex and whether SAP can act as an F-box protein.

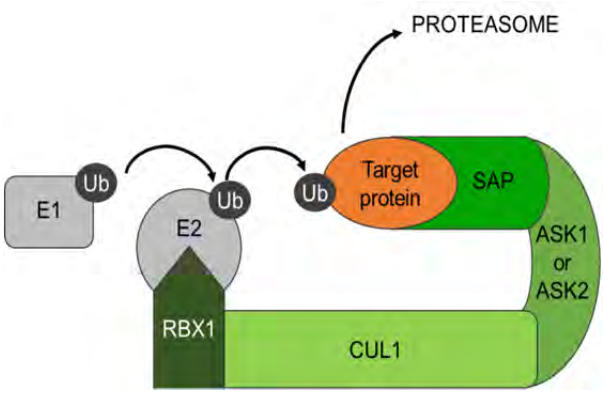

Figure 8. Model of SAP mode of action. Ubiquitin (Ub) is activated by the ubiquitin-activating enzyme (E1) and then transferred to the ubiquitin-conjugating enzyme (E2). The E2 interacts with the ubiquitin-ligase (E3), here the SCF complex (green), through a RING-protein RBX1. SAP may be part of an SCF complex composed by the SPK1 homologs ASK1 or ASK2 and CUL1. SAP, as F-box protein recognizes target proteins for ubiquitination. The ubiquitin is transferred from the E2 to the target protein, which is then degraded via the proteasome.

MATERIALS and METHODS

Plant material. All plants were grown at 20 °C under long day condition (16 h light, 8 h dark) on rock wool. Seeds of knock-out lines SALK_129750 (N663979) and SALK_023467 (N523467) were obtained from the Nottingham Arabidopsis Stock Center (NASC). Seeds of knock-out line FLAG_359F07 (DUPTV34T3) were obtained from INRA.

Isolation of RNA and real-time PCR analysis. Total RNA was extracted using the Invitek Kit according to the manufacturer's protocol. DNase I digestion was performed on total RNA using DBase I from Ambion. RNA integrity was checked on 1% (w/v) agarose gel after DNase I treatment. First-strand cDNA was synthesized from 1 µg of total RNA using TaqMan kit (Roche) cDNA Synthesis Kit following the manufacturer's protocol. For *SAP* expression 2 pairs of primers were used, one designed between the first and second exon (forward: 5'-GGAGAAGTTGACGCCATTGTTGC-3' reverse 5'-ACTCTGAGCCGTTGATGAAGCTGA-3') and the second one designed in the 3'UTR region exon (forward: 5'-CAGGAGGGTGAGATTTACAAGG-3' reverse 5'-TGAGAG AGCCCTAAACACAAGG-3').

GFP fusion reporter gene constructs. SAP genomic region was amplified by PCR using the following primers: forward: 5'-GAAAATGTACAGTCTTACGAGACCA-3' reverse: 5'- CAGTGCACCGAAATCCCATA-3'. The PCR fragment was cloned into the GATEWAY vector pCR8/GW/TOPO from Invitrogen and transferred via LR reaction into the pGREEN destination vector pGBGWG-AM884387 (Curtis and Grossniklaus, 2003), Zhong et al., 2008). Expression vectors were introduced into Arabidopsis thaliana ecotype Col-0 by floral dip transformation (Clough and Bent, 1998). Transformant plants were selected on plates containing ½ Murashige-Skoog (MS) medium pH 6 with 10 g/ml phosphinothricin (ppt) and 0.7% Agar.

Confocal Scanning Laser Microscopy (CSLM). GFP tagged protein localization was observed trough CSLM on Leica SPE DM5500 upright microscope using an ACS APO 40x/1.15 oil lens and using the LAS AF 1.8.2 software. FM4-64 dye was added to 0.1% agar at a concentration of 5 M and used as staining of cell membranes. GFP and FM4-64 dye were excited with the 488-nm line of an Argon ion laser. The GFP emission was detected at a bandwidth of 505-530 nm, while FM4-64 dye and chloroplast auto-

fluorescence were detected at a bandwidth of 650 nm. After acquisition, optical slices were median filtered and three-dimensional projections were generated with LAS AF 1.8.2 software package.

Chromatin immunoprecipitation (ChIP). ChIP was performed according to (Kaufmann *et al.*, 2010a; Smaczniak *et al.*, 2012b). Inflorescences from gSAP:GFP were collected on ice and fixed on ice with 1% FAA for 30 min or with 10 mM DMA for 30 min followed by fixation with 1% FAA for 30 min. After the ChIP, DNA fragments were amplified using the following primers and the enrichment was calculated using the non-targets HSF1 and ARR6.

Gene ID	**Alias**	**Forward primer 5'→ 3'**	**Reverse primer 5'→ 3'**
AT4G17750	HSF1	gctatccacaggttagataaaggag	gagaaagattgtgtgagaatgaaa
AT5G62920	ARR6	gccaccatggtttcacatcatatc	cctttgcaagaagatactctgagc
AT1G24260	SEP3	aaacccagacgtgacttgtttgacg	tgagaatcggacggctttgagg
AT3G54340	AP3	caattgatttaagcagtgtc	ggaaagtattgcctaatccatgaaag
AT4G18960	AG	ctacgagcagcttatgccacca	gagtaatggtgattgttaggttgc

LC-MS-based complex isolation. Immunoprecipitation was performed using GFP antibody coupled to magnetic beads using inflorescence from gSAP:GFP *ap1 cal* plants. Experiment was performed according to Smaczniak *et al*., (Smaczniak *et al.*, 2012a) on "crude extract" for 3 biological replicates for gSAP:GFP *ap1 cal* and *ap1 cal* as control.

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CHAPTER 6

The role of the homeobox gene *WOX12* during flower development in *Arabidopsis thaliana*

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ABSTRACT

The *WUSCHEL*-related homeobox (*WOX*) genes play key roles in many developmental processes, from embryo formation to root and flower development. Flower development is a key step in plant reproduction, which is tightly controlled by . the master regulators of flower development. These master regulators have been identified and they are predominantly represented by transcription factors belonging to the MADS-domain family. Recently, MADS-domain protein DNA-binding profiles showed that many of the WOX genes are direct targets of MADS-domain proteins, among them is *WOX12*, which was also reported to be differentially expressed during flower development. In this chapter we focus on the functional characterisation of *WOX12* and its role in Arabidopsis flower development. *WOX12* acts downstream *APETALA1 (AP1)* and ectopic expression of *WOX12* leads to reduction of *AGAMOUS (AG)* expression, suggesting a role for *WOX12* in regulating the antagonistic interplay between the homeotic genes *AP1* and *AG*. The phenotypes obtained by down-regulation and overexpression of *WOX12* are consistent with a proposed role of this gene in defining the border between *AP1* and *AG* gene expression domains, exactly where *WOX12* is expressed in the flower meristem.

INTRODUCTION

Flower development is a fundamental process in the plant life cycle since the correct formation of flower organs is a prerequisite to produce seeds. In Arabidopsis, flowers are composed of four different types of organs arranged in concentric whorls: four sepals, four petals, six stamens and two fused carpels. In the 90s an elegant model, called ABC model, was proposed to explain the identity determination of these organs (Coen and Meyerowitz, 1991). According to the ABC model, the expression of A-class genes, such as *APETALA1* (*AP1*) and *APETALA2* (*AP2*), determines sepal identity in whorl 1, while the expression of C-class genes, such as *AGAMOUS* (*AG*), determines carpel identity in whorl 4. B-class genes, represented by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), specify the petals together with A-class genes in whorl 2 and the stamens in whorl 3 when expressed in combination with C-class genes (Bowman *et al.*, 1989). The genes mention above represent the master regulators of flower development since they are necessary and sufficient to determine proper flower organ formation.

In the past decades a number of genes that act downstream of these master regulators have been identified. However, only recently more global information became available about the complexity of the gene regulatory network that determines flower organ formation by genome-wide studies. DNA-binding profile experiments and transcriptome analysis revealed hundreds to thousands of direct target for each master regulator. Among the newly identified targets an overrepresentation of homeobox transcription factors was observed (Kaufmann *et al.*, 2009; Kaufmann *et al.*, 2010b; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014; Wellmer *et al.*, 2006; Wuest *et al.*, 2012).

In Arabidopsis, the homeobox transcription factor family consists of 110 genes that can be further subdivided into 14 subfamilies based on sequence similarity and unique domain architecture (Mukherjee *et al.*, 2009). The homeobox transcription factors are distinguishable by the presence of a typical DNA-binding domain of 60 amino acids, known as homeodomain, which is characterized by a helix-loop-helix-turn-helix structure (Kamiya *et al.*, 2003). Homeobox transcription factors are key regulators involved in the determination of cell fate and cell differentiation in both plants and animals. Many members of the *WUSCHEL*-related homeobox (WOX) subfamily (van der Graaff *et al.*, 2009), which forms a plant specific subgroup of the homeobox transcription factor family, were found as direct targets of the flower master regulators (Kaufmann *et al.*,

2009; Kaufmann *et al.*, 2010b; O'Maoileidigh *et al.*, 2013; Pajoro *et al.*, 2014; Wellmer *et al.*, 2006; Wuest *et al.*, 2012). The WOX subfamily is named after the founder gene called *WUSCHEL* (*WUS*). The WOX subfamily differs from the other homeobox members by the presence of a WUS-box motif, the presence of one or two extra residues between helix 1 and 2, and four or five extra residues between helix 2 and 3 (Mukherjee *et al.*, 2009). The WOX subfamily includes 15 members, which differ in their expression pattern and their role in development (van der Graaff *et al.*, 2009). Although WOX genes are mostly characterised for their functions during embryogenesis, many of the subfamily members play a role in flower development. Indeed, *WUS, WOX2, WOX5, WOX8* and *WOX9* are expressed during early embryogenesis and their specific expression profiles reflect their role in developmental processes such as apical-basal axis formation and the establishment of shoot and root meristems (Breuninger *et al.*, 2008; Haecker *et al.*, 2004; Lau *et al.*, 2010; Mayer *et al.*, 1998)

A role during flower development has been shown for *WUS, WOX1, WOX3, WOX6, WOX9, WOX13* and *WOX14*. *WUS* is essential to maintain stem cell homeostasis in the shoot apical meristem (SAM) and floral meristem (FM). It is part of the CLAVATA (CLV)-WUS feedback loop mechanism that maintains the stem cell niche in the meristem but also controls cell division activity by restricting the expression of *WUS* to the organising centre (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). *WUS* has besides its function as repressor of stem cell differentiation (Schoof *et al.*, 2000), a role in floral patterning. It plays a key role in flower development by acting as activator of *AG* in the floral meristem (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). Ectopic expression of *WUS* leads to the formation of carpels and stamens in whorls 1 and 2 as a consequence of the expansion of *AG* expression in these whorls (Ikeda *et al.*, 2009; Lenhard *et al.*, 2001). At later stages of flower development, the flower becomes determinate by the loss of *WUS* expression, which is caused by the AG target *KNUCKLES (KNU)* that acts as an repressor of *WUS* (Sun *et al.*, 2009).

WOX1 and *WOX3*, the latter also called *PRESSED FLOWER* (*PRS*), are expressed at early stages of flower development (Matsumoto and Okada, 2001; Zhang *et al.*, 2011). *PRS* is required for sepal development (Matsumoto and Okada, 2001) and acts redundantly with *WOX1* in sepal and petal growth. Flowers of *wox1 prs* mutants have narrow sepals and petals (Vandenbussche *et al.*, 2009). The phenotype observed in *wox1 prs* double mutants suggests a role for *WOX1* and *PRS* in polarity determination during

sepal and petal development. In line with this, *WOX1* and *WOX3* have also been found to determine abaxial/adaxial fate in leaves (Nakata *et al.*, 2012).

WOX6, also called *PRETTY FEW SEEDS 2* (*PFS2*), is preferentially expressed in ovules, where it is needed for proper integument development. In *pfs2* mutants, the integuments are abnormal and most of the embryo sacs show impaired development resulting in a reduced number of seeds (Park *et al.*, 2005). *pfs2* mutants also have defects in flowers having ragged and wavy petal margins. Ectopic expression of *PSF2* leads to strong abnormalities in the flower, such as the formation of carpelloid stamens and reduction in petal size (Park *et al.*, 2005). The defects observed in flowers ectopically expressing *PSF2* are due to a reduction of *AG* expression in the floral primordia (Park *et al.*, 2005). The expression profile of *PSF2* and the phenotypes observed by ectopic expression of *PFS2* suggest that, alike *WUS* and *PRS*/*WOX3, PFS2* acts in the regulation of cell differentiation in the floral meristem, possibly in part through the regulation of *AG*.

WOX9, also called *STIMPY* (*STIP*), is expressed in meristematic tissues, in flowers at early stages of development and in ovules (Wu *et al.*, 2005). *STIP* plays an important role in meristem growth and maintenance. Loss of function of *STIP* leads to an arrest in meristem growth (Skylar *et al.*, 2010; Skylar *et al.*, 2011; Wu *et al.*, 2005), while overexpression of *STIP* in a *clavata3* mutant background results in a proliferation of extra inflorescence meristems leading to an inflorescence apex that resembles an *ap1 cal* mutant (Wu *et al.*, 2005).

WOX13 and *WOX14* play a role during floral transition, while later in development, *WOX13* is important for replum development and *WOX14* is required for proper anther differentiation (Deveaux *et al.*, 2008; Romera-Branchat *et al.*, 2012). *WOX13* is expressed in inflorescence meristems, floral meristems and in floral buds before and after anthesis, and at lower levels in siliques, yet *WOX13* loss-of-function causes defects in replum formation only. In *wox13* mutants, repla are narrower and valve margins are wider and more lignified than in wild-type (Romera-Branchat *et al.*, 2012). Ectopic expression of *WOX13* causes aberrations in all flower organs (Romera-Branchat *et al.*, 2012). *WOX12* together with its close homolog *WOX11* are involved in *de novo* root organogenesis when explants are grown in *in vitro* culture (Liu *et al.*, 2014), however a role of these *WOX* genes in flower development has never been reported.

Here we show that *WOX12* is also involved in flower development. Previously, it was reported that *WOX12* is directly activated by AP1 during the initiation of the floral

meristem (Kaufmann *et al.*, 2010b). In more recent studies we found that *WOX12* is differentially expressed throughout flower development and it is bound by both AP1 and SEPALLATA3 (SEP3) at different stages of flower development (Pajoro *et al.*, 2014; chapter 3). In this chapter we show that *WOX12* plays a role in the determination of floral organ identities. We found that *WOX12* acts downstream of *AP1* in petal identity specification and ectopic expression of *WOX12* causes reduction in *AG* expression and subsequent modifications of third whorl organ identities.

RESULTS

The WOX subfamily.

The *WUSCHEL*-related homeobox (*WOX*) genes are specifically expressed in plants and form a large subgroup of the homodomain (HD)-containing transcription factor gene family (Mukherjee *et al.*, 2009). The WOX subfamily includes 15 members that differ in their expression patterns and play different roles during Arabidopsis development (**Table 1**). The WOX subfamily can be subdivided into 3 clades based on phylogenetic analysis: the WUS clade, the ancient clade and the intermediate clade. The WUS clade includes *WUS*, *WOX1*, *WOX2*, *WOX3*, *WOX4*, *WOX5*, *WOX6* and, *WOX7*; the intermediate clade includes *WOX8*, *WOX9*, *WOX11* and *WOX12*, while the ancient clade includes *WOX10*, *WOX13* and *WOX14* (**Figure 1A**).

In agreement with their role in flower development, WOX genes are target of transcription factors (TFs) involved in flower development such as AG, AP1, AP2, AP3, PI and SEP3 (Kaufmann *et al.*, 2009; Kaufmann *et al.*, 2010b; O'Maoileidigh *et al.*, 2013; Wuest *et al.*, 2012; Yant *et al.*, 2010), while proteins involved in the regulation of flowering time, such as SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (SOC1), FLOWERING LOCUS M (FLM), FLOWERING LOCUS C (FLC) and SCHNARCHZAPFEN (SNZ) do not bind to these WOX genes (Deng *et al.*, 2011; Immink *et al.*, 2012; Pose *et al.*, 2013) (**Table 2**). For example, *WOX1* and *WOX3*, are bound by TFs with a known role in sepal and petal initiation and development, such as AP1, AP2, AP3, PI and SEP3. This finding is in agreement with the role of *WOX1* and *WOX3* in sepal and petal development (Vandenbussche *et al.*, 2009). There are still some family members that are not bound by any of the studied TFs, which is in agreement with

Table 1. ***Arabidopsis thaliana*** **WOX subfamily.**

LOCUS	GENE NAME	CLADE	EXPRESSION DOMAIN	FUNCTION	REFERENCES
AT2G17950	WUS	WUS clade	SAM, floral meristem, ovule and anther	regulation of cell fates, ovule and anther development	Brand et al., 2002; Laux et al., 1996; Mayer et al., 1998; Gross-Hardt et al., 2002; Deyhle et al., 2007; Ikeda et al., 2009
AT3G18010	WOX1	WUS clade	lateral organ primordia, leaves and flowers	lateral organ formation abaxial/adaxial polarity establishment in leaves	Haecker et al., 2004; Vandenbussche et al., 2009; Zhang et al 2011; Nakata et al., 2012
AT5G59340	WOX2	WUS clade	female gametophyte, embryo apical domain	embryo patterning	Haecker et al., 2004; Wu et al., 2007; Breuninger et al., 2008
AT2G28610	WOX3 (PRS)	WUS clade	lateral organ primordia, leaves	lateral organ formation abaxial/adaxial polarity establishment in leaves	Matsumoto and Okada, 2001; Haecker et al., 2004; Vandenbussche et al., 2009; Nakata et al., 2012
AT1G46480	WOX4	WUS clade	cambium	cambium activity	Ji et al., 2010; Hirakawa et al., 2010; Suer et al., 2011
AT3G11260	WOX5	WUS clade	root QC, pollen	stem cell maintenance pollen tube elongation	Haecker et al., 2004; Dorantes-Acosta et al 2006
AT2G01500	WOX6 (PSF2)	WUS clade	Ovule, SAM, leaves primordia, inflorescence meristem, floral meristem, anthers, petals and carpels	ovule patterning and differentiation, petal and leaf morphology	Park et al., 2005
AT5G05770	WOX7	WUS clade	unknown	unknown	
AT5G45980	WOX8 (STPL)	Intermediate clade	female gametophyte and embryo basal domain	embryo patterning	Haecker et al., 2004; Wu et al., 2007; Breuninger et al., 2008
AT2G33880	WOX9 (STIP)	Intermediate clade	female gametophyte and embryo basal domain inflorescence meristem, floral meristem, ovules	embryo patterning	Haecker et al., 2004; Wu et al., 2007
AT1G20710	WOX10	Ancient clade	unknown	unknown	
AT3G03660	WOX11	Intermediate clade	procambium and xylem parenchyma cells	root organogenesis	Liu et al., 2014
AT5G17810	WOX12	Intermediate clade	procambium and xylem parenchyma cells	root organogenesis	Liu et al., 2014
AT4G35550	WOX13	Ancient clade	root, leaves, gynoecium, embryo siliques	delays flowering promote replum formation	Deveaux et al., 2008; Romera-Branchat et al., 2013;
AT1G20700	WOX14	Ancient clade	root, vascular tissue stamens	promotes flowering, stamen development, root growth,	Deveaux et al., 2008

the broad functions played by the WOX subfamily genes. Next, we investigated the binding of AP1 and SEP3 to WOX loci at different stages of flower development (Pajoro *et al.*, 2014). We performed ChIP-seq experiments at different stages of flower development using an inducible system for synchronised flower initiation (pAP1:AP1:GR in *ap1 cal*). We identified AP1 and SEP3 target genes during meristem specification (day 2), floral organ specification (day 4) and organ differentiation (day 8). Stage-specific binding events were observed for most of the family members (**Figure 1B**). For example, *WOX1* and *WOX12* were bound by both TFs at days 4 and 8, while *WOX2* was only bound by SEP3 at day 8. Remarkably, only 3 genes were found to be bound at all stages: *WOX3* was bound at all-time points by AP1, while *WOX9* and *WOX13* were bound at all-time points by SEP3. Moreover, due to the higher resolution obtained in the stage specific

ChIP-seq experiments, not previously reported binding events could be identified, such as the binding of SEP3 to *WUS*, *WOX2*, *WOX9*, *WOX12* and *WOX13* (**Table 2** and **Figure 1B**).

We investigated the changes in expression of the WOX subfamily genes during early stages of flowers development (**Figure 1C**). We profiled gene expression in the inducible system pAP1:AP1:GR *ap1 cal* before treatment (day 0) and 2, 4 and 8 days after DEX or mock treatments. *WOX6* and *WOX10* expression decreases from day 2 to day 4 and increases from day 4 to day 8. *WOX8* and *WOX12* expression increases between day 2 and day 4. *WOX1* expression increases at a later stage of development (**Figure 1C**).

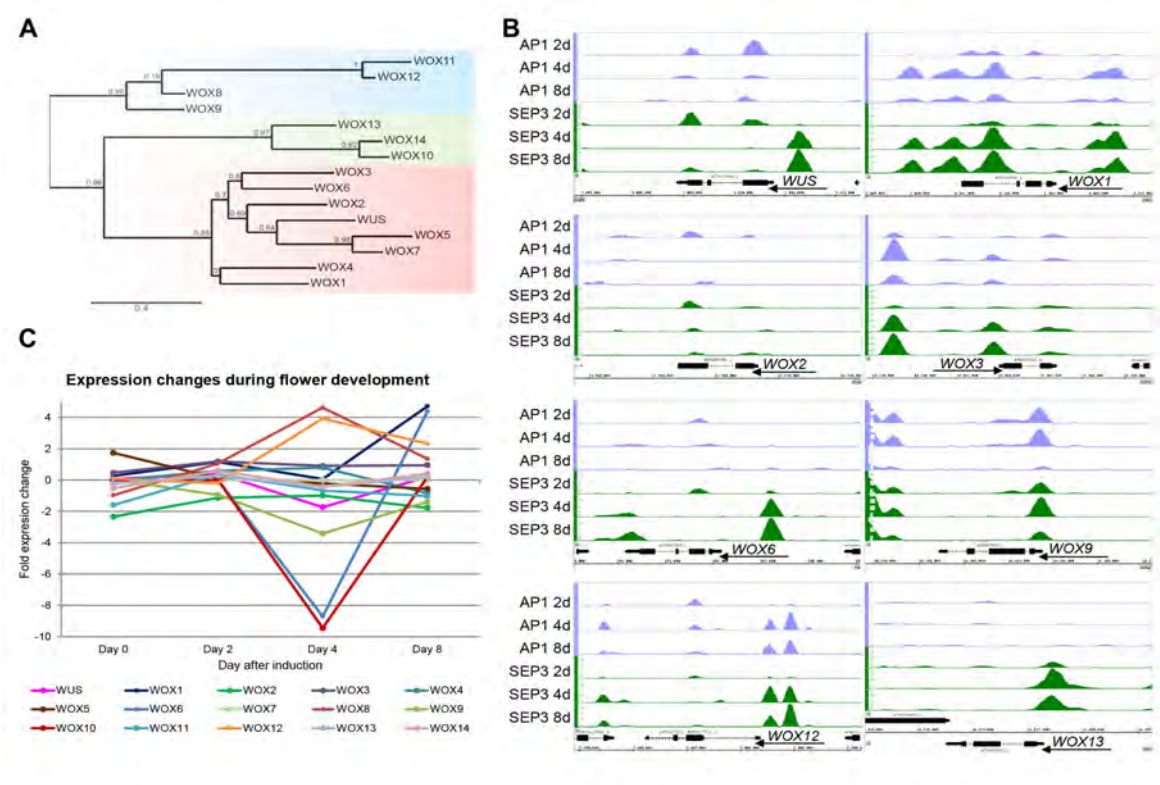

Figure 1. The WOX subfamily. A. Phylogenetic tree showing the WOX subfamily genes. The analysis was performed on the Phylogeny.fr platform (Dereeper *et al.*, 2008) using entire coding sequences of all WOX subfamily members. Sequences were aligned with MUSCLE (Edgar, 2004) and the phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (Guindon *et al.*, 2010). The WOX subfamily can be subdivided into 3 clades: the WUS clade (red), the ancient clade (green) and the intermediate clade (blue). **B.** AP1 and SEP3 DNA binding profiles in *WOX* loci. In purple are the AP1 binding profiles and in green, the SEP3 binding profiles. From the top to the bottom binding events are shown at day 2, day 4 and day 8 for each transcription factor (Pajoro *et al.*, 2014). **C.** Changes in expression of WOX subfamily genes during flower development. Fold change in expression, as assessed by qPCR, represents the difference in expression between plants treated with DEX compared to plants treated with mock solution at each time point.

Table 2. TFs binding to WOX loci.

		WUS	*WOX1*	*WOX2*	*WOX3*	*WOX4*	*WOX5*	*WOX6*	*WOX7*	*WOX8*	*WOX9*	*WOX10*	*WOX11*	*WOX12*	*WOX13*	*WOX14*
AG																
AP1	2h															
	day 2															
	day 4															
	day 8															
AP2																
AP3																
LFY																
PI																
SEP3	inflo															
	day 2															
	day 4															
	day 8															
SVP																
FLC																
FLM																
SOC1																
SNZ																

Dark cells in the table indicate significant binding events in WOX loci reported for AG (O'Maoileidigh *et al.*, 2013), AP1 (Kaufmann *et al.*, 2010b) (Pajoro *et al.*, 2014), AP2 (Yant *et al.*, 2010), AP3 (Wuest *et al.*, 2012), FLC (Deng *et al.*, 2011), FLM (Pose *et al.*, 2013), LFY (Moyroud *et al.*, 2011), PI (Wuest *et al.*, 2012), SNZ (Mathieu *et al.*, 2009), SEP3 (Kaufmann *et al.*, 2009), (Pajoro *et al.*, 2014), SVP (Gregis *et al.*, 2013), SOC1 (Immink *et al.*, 2012).

WOX12 expression profile during flower development.

WOX12 is bound and activated by AP1 during the first stages of floral meristem and floral organ development (Kaufmann *et al.*, 2010b). To study the expression profile of *WOX12* during flower development in more detail, we generated a reporter construct expressing the WOX12 protein C-terminally fused to the reporter GREEN FLUORESCENT PROTEIN (GFP). This reporter construct, gWOX12:GFP, consists of a 5kb region upstream the ATG and the *WOX12* genomic region up to the stop codon. Confocal microscopy analysis showed that gWOX12:GFP is initially expressed in the IM at the border of newly arising flower buds, while no expression was detected in the floral meristem (FM) during the first stages of flower development (up to stage 4; Smyth et al 1990). GFP signal was detected again in the central dome of flower buds at stage 4-5 of development (**Figure 2A-B**). In flowers from stage 5, gWOX12:GFP is expressed in the central domain in a ring-like shape at the position where the stamen primordia will arise and no expression was observed in the developing sepals nor at the place where petal primordia emerge (**Figure 2B**). In flowers at stage 11 the expression was detected in anthers and in the carpels (**Figure 2C-E**). Expression in petals is only observed in flowers at stage 9, before petal elongation (**Figure 2C-D**). At anthesis (stage 12), gWOX12:GFP is expressed only in the carpels where it is predominantly expressed in the valve margins (**Figure 2F**).

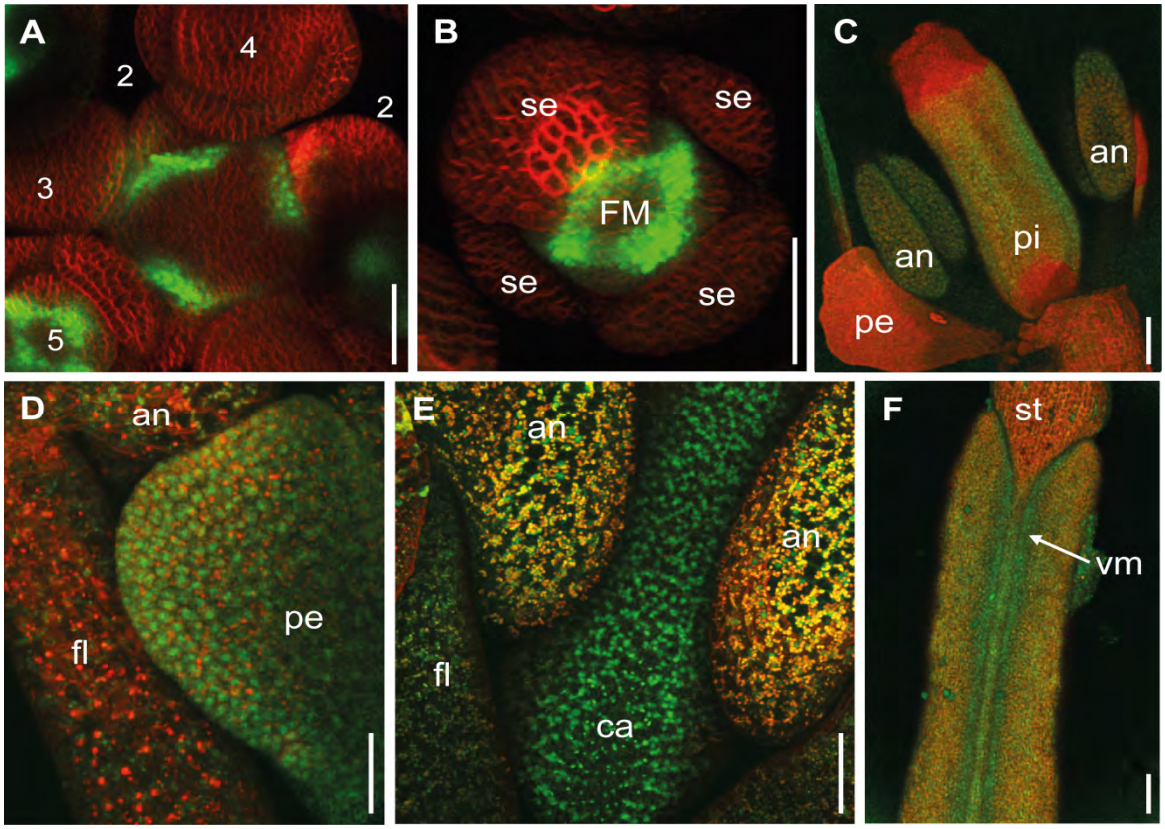

Figure 2. *WOX12* expression profile during flower development. A. gWOX12:GFP expression in an inflorescence with young floral buds arising. GFP expression was detected at the border between inflorescence meristem (IM) and newly formed flowers. **B.** gWOX12:GFP expression in a flower bud at stage 5. GFP expression is detected in a ring shape in the centre of the flower buds. No expression is detected in sepals. **C.** gWOX12:GFP expression in a developing pistil and anthers in a flower at stage 11. **D.** gWOX12:GFP expression in a petal in a stage 9 flower. **E.** stage 10 flower where GFP expression is detectable in the pistil and in the anthers. **F.** gWOX12:GFP expression in a silique of a stage 12 flower. GFP expression is mainly detectable in the valve margins. Numbers indicate flower stages according to Smyth et al. (1990), se=sepal, pe=petal, , an=anther, fl=filament, pi=pistil, st=stigma, vm=valve margin. Bars=100um

Regulation of WOX12 by MADS-domain transcription factors.

Both AP1 and SEP3 bind to the *WOX12* locus at different stages of flower development (Pajoro *et al.*, 2014, chapter 3) and *WOX12* is differentially expressed upon induction by AP1 (see Figure 1), suggesting that *WOX12* acts downstream of *AP1* in the regulation of flower development. To get insights into the regulation of *WOX12* by AP1, we analysed the gWOX12:GFP expression profile in various homeotic mutant backgrounds (**Figures 3A-E**). The GFP expression profiles in wild-type and *ap1-10* plants were similar at the periphery of the IM where the newly arising buds appear, while broader expression was

observed at stage 5. In wild-type plants, *WOX12* is expressed in a ring-like shape at the position where the stamens will emerge, however, in *ap1*, WOX12 seems to be broader expressed in this area. This difference in expression is consistent with the absence of petal primordia in *ap1*. The similar expression profile observed in the IM indicates that other genes are involved in *WOX12* regulation (**Figure 3 A-B**). A candidate is *CALIFLOWER* (*CAL*), which is acting redundantly with *AP1* in the regulation of flower initiation. Therefore, we analysed the *WOX12* expression profile in the *ap1 cal* mutant. An *ap1 cal* inflorescence consists of a massive production of inflorescence meristems and no flowers are produced (**Figure 3C**). At early stages of flower development, gWOX12:GFP was expressed at the border of the inflorescence meristems in the *ap1 cal* mutant, similar to the pattern observed in wild-type inflorescence meristems. This suggests that *WOX12* is expressed in the IM at positions where new FMs arise. Alternatively, *WOX12* is expressed exactly at the border of any two meristems, independent whether it is an IM or a FM. In contrast to wild-type, no expression was detectable as a ring-like shape in the FM, which can be linked to the fact that flower development is arrested in the *ap1 cal* mutants (**Figure 3C**). Next, we analysed the gWOX12:GFP expression profile in the C-class mutant *ag*. According to the ABC model the knock-out of C-class genes causes an expansion of the A-class gene expression domain (Gustafson-Brown *et al.*, 1994). *ag* flowers are composed of only sepals and petals and no stamens or carpels are present. However, gWOX12:GFP expression was similar between wild-type and *ag* plants. Remarkably, as in wild-type, gWOX12:GFP is expressed in a ring-like shape in the central domain of *ag* flowers at stage 5. This result suggests that the expression in the central domain is not determined by the identity of the emerging organs, but is position-specific. Finally, we studied the gWOX12:GFP expression profile in a *ful* mutant background (**Figure 3E**). In this mutant background the GFP signal was the same as in wild-type plants (**Figure 3A and E**). In conclusion, the expression in the IM was not affected in the floral homeotic mutants, which is not surprising because *AP1* and *AG* are not expressed in the IM, while *FUL* and *CAL* act redundantly with other genes. The difference observed in stage 5 flowers may be caused by the difference in flower morphology between wild-type and homeotic mutants, although the expression in a ring-shape fashion appears to be independent of organ identity.

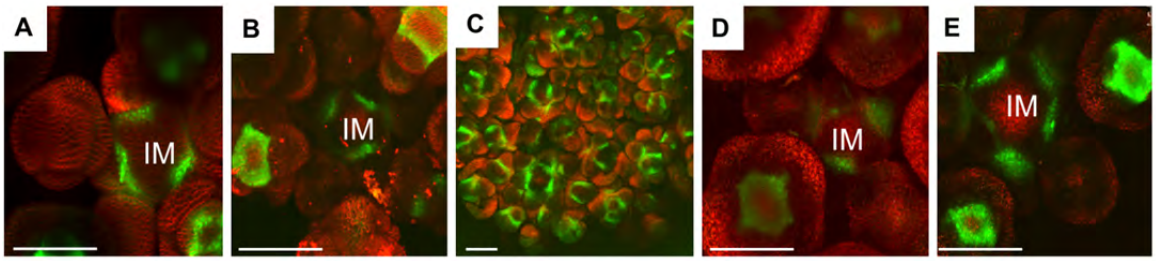

Figure 3. gWOX12:GFP expression profile in wild-type and homeotic mutants at early stages of flower development. A. gWOX12:GFP expression profile in wild-type. **B.** gWOX12:GFP expression profile in *ap1.* **C.** gWOX12:GFP expression profile in *ap1 cal*. **D.** gWOX12:GFP expression profile in *ag.* **E.** gWOX12:GFP expression profile in *ful.* Bars=100 µm.

The characterisation of WOX12 upstream regulatory elements.

Our previous DNaseI-seq and ChIP-seq experiments suggest the presence of two cis-regulatory regions in the *WOX12* promoter (Pajoro *et al.*, 2014; chapter 3) (**Figure 1B; Figure 4A**). To further characterise the role of these two putative regions, we cloned the *WOX12* genomic locus (including coding sequence) in front of the GFP reporter using different promoter lengths: a long promoter of 5 kb that includes both cis-regulatory regions; a short promoter of 3 kb that includes only the cis-regulatory region "a" and a minimal promoter of 0.5 kb where both cis-regulatory elements have been deleted (**Figure 4A**). Arabidopsis plants were stably transformed with these constructs and the level of GFP expression in inflorescences of these plants were measured by qPCR (**Figure 4B**). GFP expression levels obtained with the long promoter and the short promoter were similar, while the level of GFP expression was highly reduced when using the minimal promoter. Next, we characterised the expression pattern at early stages of flower development. The long promoter (5 kb) gave GFP expression in the IM at the border between the IM and stage 2 flowers (see also Figure 2), no expression was detected in a floral meristem (FM) during the first stages of flower development (from stage 2 to stage 4), while GFP signal was detected again in a floral meristem of flower buds at stage 4-5 in a ring-like shape and no expression was observed in the developing sepals (**Figure 4C**). The GFP expression profile was broader in plants expressing the short promoter (3 kb). In those plants, additional GFP signal was often detectable in stage 2 flowers and in sepal primordia of stage 3-5 flowers (**Figure 4D**). The ectopic expression observed in the sepals after deletion of the distal cis-regulatory region suggests

that TFs bind to this region to repress *WOX12* expression in sepals. In plants expressing WOX12:GFP under the control of the basal promoter (500 bp) no GFP signal was detected (**Figure 4E**).

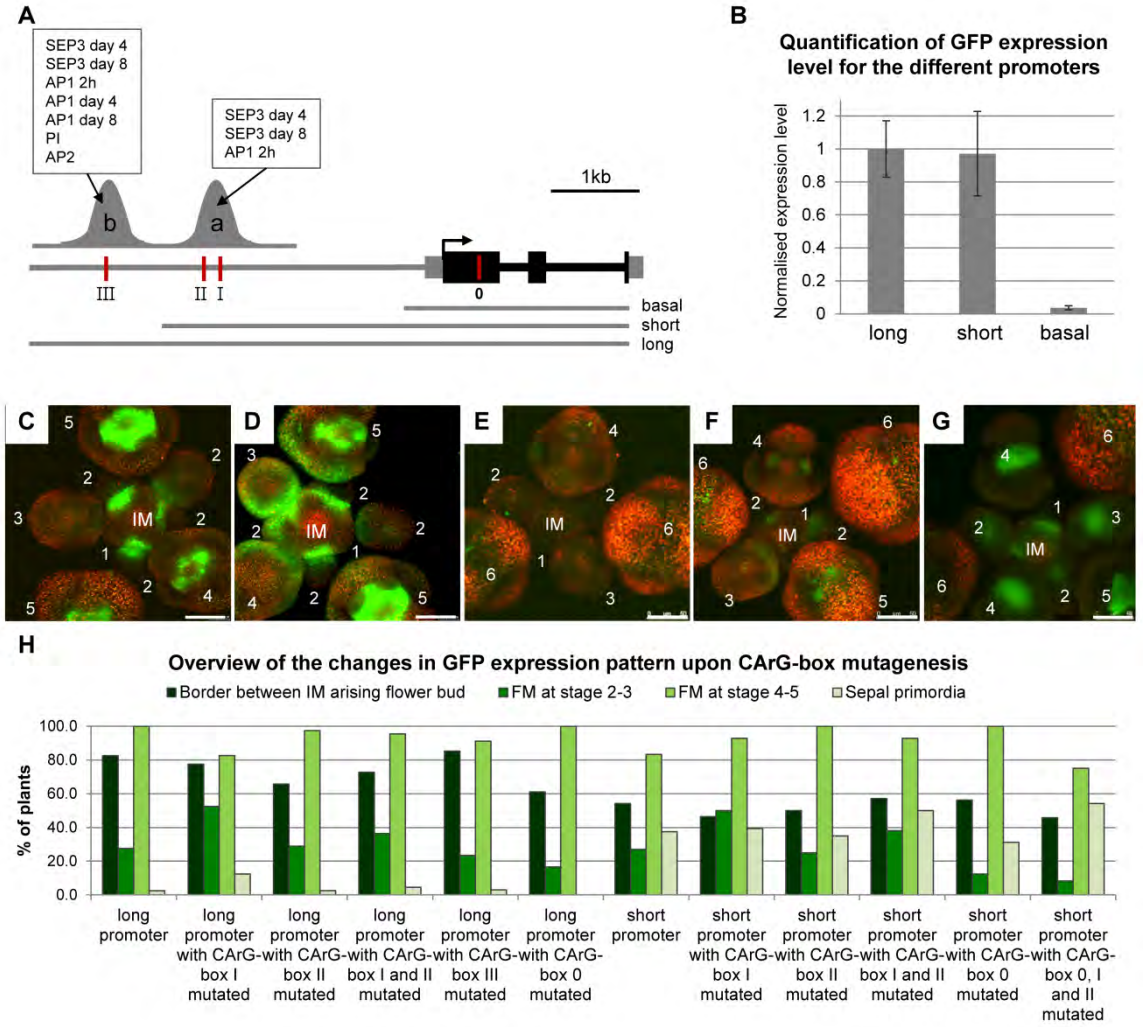

Figure 4. **Regulation of *WOX12* expression**. **A.** Schematic representation of the *WOX12* locus. Black boxes represent coding regions, grey boxes represent UTRs, black lines depict introns and the dashed line represents the promoter region. Black arrow indicates the coding start site (CSS). Peaks "a" and "b" represent regions containing cis-regulatory regions identified in DNAse I accessibility profile experiments (see chapter 3). The first cis-regulatory region is located at 2.5 kb upstream the CSS while the second cis-regulatory element is located at 3.5 kb upstream the CSS. Above each regulatory region the transcription factors are indicated that have been reported to bind to that region. SEP3 binds both cis-regulatory regions. AP1 binds to the most upstream cis-regulatory region (b) in inflorescence meristems and in flowers at stage 4 and 8 of development. Binding of AP1 to elements in region (a) is significant only in inflorescence meristem (AP1IM). The distal region (b) is also bound by PI and AP2. Red lines indicate CArG-box like elements. **B.** GFP expression levels measured by qPCR. No quantitative differences in GFP expression level was observed between long and short promoters. GFP expression is significantly lower in plants with the basal promoter compared to the long and short promoters. GFP expression was normalised against a housekeeping gene (AT4G34270, *TIP41*). Values are reported as means and error bars represent SE between 8 biological replicates for the basal promoter and 19 for the short and long

promoters. **C.** Confocal image of a representative expression pattern observed in plants expressing the *WOX12* locus including a long promoter region (5 kb region upstream of the CSS) fused to GFP. **D.** The same as **C,** but here the short promoter region (3 kb region upstream of the CSS) was used. Ectopic GFP signal was observed in arising sepals of flowers at stages 3-5 of development. **E.** The same as **C** and **D**, but here a minimal promoter region (500 bp region upstream of the CSS) was used in the reporter construct. No GFP signal was detected in those plants. **F.** Confocal image of a representative GFP expression pattern observed in plants expressing the *WOX12* locus including the short promoter region upon mutagenesis of the CArG-boxes 0, I and II. Substantially lower GFP signals were detected at the border between inflorescence meristem and newly formed flowers, and in floral meristems of flowers at stage 4 of development when compared to the intact short promoter shown in D. **G.** Confocal image of a representative GFP expression pattern observed in plants expressing the *WOX12* upon mutagenesis of the CArG-box I. Higher GFP signals were detected in floral meristems of flowers at stage 2 and 3 of development when compared to the intact long and short promoters shown in C and D, respectively. **H.** Overview of the changes in GFP expression pattern upon CArG-box mutagenesis. The graph shows percentages of T1 plants that expressed GFP at the border between IM and newly arising buds, in FMs of flowers at stage 2-3 of development, in FMs of flowers at stage 4-5 of development, and in sepal primordia. GFP expression patterns were characterised for 20 independent transgenic plants from each T1 generation. Here, we considered the number of plants showing a particular spatial expression distribution, we did not include information about the level of expression.

We then looked at transcription factors that bind to the *WOX12* locus based on available data. APETALA2 (AP2) and the MADS-domain TFs AP1, SEP3 and PISTILLATA (PI) bind to the cis-regulatory elements in the *WOX12* promoter region (**Figure 4A**) (Kaufmann *et al.*, 2009; Kaufmann *et al.*, 2010b; Pajoro *et al.*, 2014; Wuest *et al.*, 2012; Yant *et al.*, 2010). Both cis-regulatory elements are bound by AP1 in inflorescence meristems of the *ap1 cal* mutant and by SEP3 in flowers at stage 4 and 8 of development (Pajoro *et al.*, 2014). The cis-regulatory element "b" is also bound by AP2 and PI in inflorescences, and by AP1 in flowers of stage 4 and stage 8 of development.

To characterise the role of MADS-domain proteins in the regulation of *WOX12* expression, we mutated the CArG-box like sequences present in the cis-regulatory elements. MADS-domain proteins bind to specific consensus sequences in the DNA called CArG-box. A CArG-box consist of a stretch of 10 nucleotides containing a core of six to eight "A/T" nucleotides flanked by either two or one "C" and "G", respectively. One CArG-box-like sequence was identified in the cis-regulatory region "b" (named CArG-box III, **Table 6)** and two CArG-box-like sequences in the cis-regulatory region "a" (named CArG-box I and CArG-box II, **Table 6**). An additional CArG-box-like sequence was present in the first exon in a region bound by AP1 and SEP3 at stage 2 of flower development (named CArG-box 0). We mutated single and multiple CArG-boxes

in both long and short promoters (**Figure 4G**). Although in most cases the GFP expression patterns were not affected by the mutation of the CArG-boxes, we observed a higher frequency of plants expressing GFP in floral meristems (FM) of flowers at stage 2-3 of development (**Figure 4G**) upon mutation of the CArG-box I (**Figure 4H**). Furthermore, we detected lower GFP expression at the borders between the IM and newly arising buds and in the FM at stage 4-5 when mutating the CArG-box 0, I and II in the short promoter (compare **Figures 4D and F**).

The observation that mutation of CArG-boxes have only minor effects on spatial GFP expression patterns could have several explanations. An option is that the MADS-domain proteins bind to another CArG-box-like sequence after the elimination of the bound CArG-boxes by mutagenesis. Recently, Mendes and colleagues showed that mutation of a CArG-box bound by SEP3 and STK in the *VERDANDI* (*VDD*) promoter leads to binding of a nearby CArG-box that was not previously bound (Mendes *et al.*, 2013). Therefore, we searched for other CArG-box-like sequences in the *WOX12* locus that could functionally replace the mutated CArG-boxes and we identified four of these alternative sites (see **Table 6** in material and methods section). To test whether these CArG-boxes could be bound by either AP1 or SEP3, we performed Electrophoretic Mobility Shift Assay (EMSA) experiments. No *in vitro* binding of AP1 and SEP3 homodimers or heterodimers to the DNA probes were observed suggesting that these alternative elements could not function as replacement for the mutated CArG-boxes (**Figure 5A**).

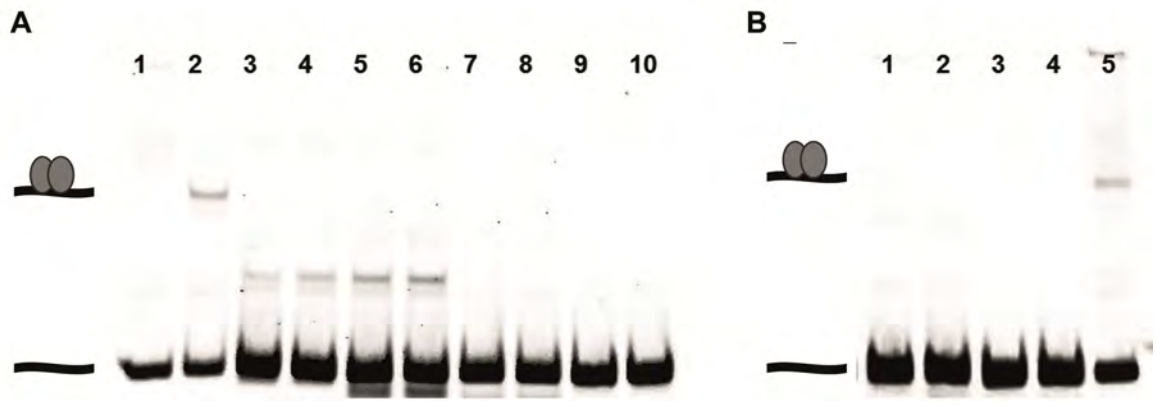

Figure 5. EMSA experiment with AP1 and SEP3 binding to CArG-boxes present in the WOX12 locus. A. Analysis of binding of SEP3 homodimer to the alternative CArG-box-like sequences present in the WOX12 locus. Lanes 1-2: positive control, lanes 3-4: CArG box A, lanes 5-6: CArG-box B, lanes 7-8: CArG box C, lanes 9-10: CArG-box D. Lanes 1-3-5-7-9 represent the negative control where the DNA was incubate

without proteins. Lanes 2-4-6-8-10 show the DNA that was incubated with SEP3. The band observed in lane 2 represents the binding of a homodimer. The bands observed in lane 3 to 6 represent background binding since they are also present when the DNA is incubated without MADS –domain proteins. The same results were obtained when incubating the DNA with the heterodimer SEP3-AP1 (results not shown). **B.** Analysis of binding of the SEP3 homodimer to DNA fragments containing the CArG-boxes 0, I, II and III (lanes 1-4, respectively). The band observed in lane 5 represents the binding of the SEP3 homodimer to a control DNA fragment (positive control), no SEP3 binding was observed for any of the other CArG-boxes. The same results were obtained when incubating the DNA with the heterodimer SEP3-AP1 or the SEP3-AP1-AP3-PI complex (results not shown).

This result suggests that the MADS-domain protein binding observed in previous ChIP experiments may not be direct. Indeed MADS-domain proteins are found to be part of bigger complexes, specifically AP1 interacts *in vivo* with other TFs such as KNAT3, BLH1, BLR, ARF2 and SPL8 (Smaczniak *et al.*, 2012). Therefore, we tested the binding of AP1 and SEP3 to the CArG-box 0, I, II and III by EMSA (**Figure 5B**). None of the CArG-boxes was bound by either AP1 or SEP3 homodimers, neither by an AP1-SEP3 complex indicating that AP1 and SEP3 do not bind directly to the proposed cis-regulatory elements, despite the ChIP peaks reported previously (Pajoro *et al.*, 2014).

Loss of function of WOX12 affects flower development.

To investigate the role of *WOX12* during flower development, we characterised the loss of function mutant allele *wox12-1* (SALK_087882) (Liu *et al.*, 2014). Next, we designed an artificial microRNA (amiRNA) (Schwab *et al.*, 2006), which specifically targets the *WOX12* locus (amiR-wox12). The WOX12ami was designed to target a region in the first intron of the gene and was expressed under the control of the constitutive Cauliflower Mosaic Virus 35S promoter (p35S) (**Figure 6A**). We confirmed a reduction in *WOX12* expression in inflorescences of the *wox12-1* and the amiR-wox12 lines by qPCR analysis. We tested *WOX12* expression levels using primers designed to amplify two different regions of the *WOX12* locus. The first couple of primers amplifies a region in the first exon, which is the same region targeted by the amiRNA. The second couple of primers was designed at the end of the gene, flanking the second intron, which includes the T-DNA insertion (**Figure 6A**). Expression analysis using these two primer sets revealed that approximately 20 % residual expression is left, but that these mRNAs most likely lack the third exon, encoding the C-terminal 5 amino acid residues (**Figure 6B**). Furthermore, we

could observe a substantial reduction in *WOX12* expression in several lines expressing the WOX12ami transcript (**Figure 6C**).

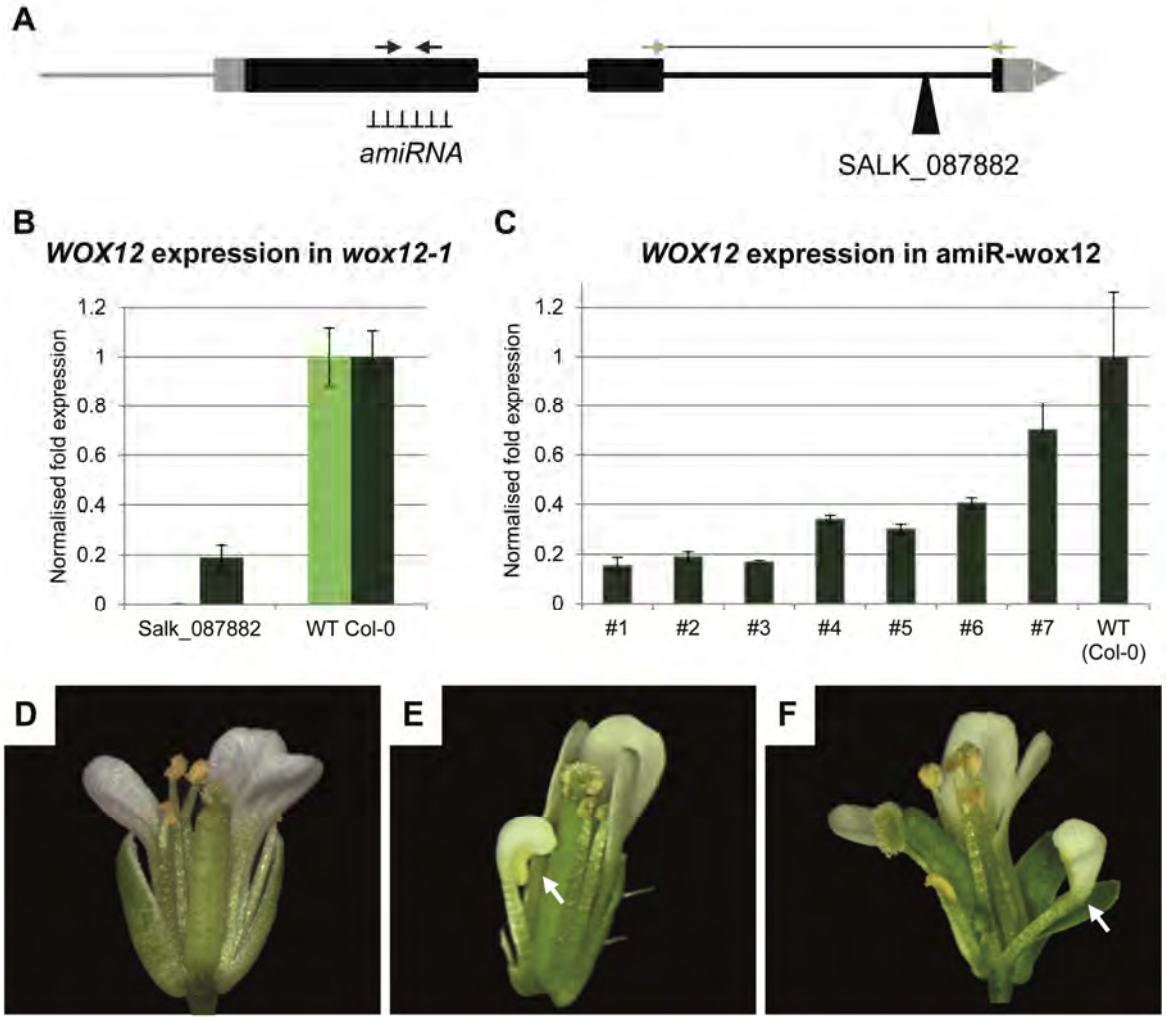

Figure 6. Knockdown of *WOX12* causes aberrations in flowers. A. Schematic representation of the WOX12 locus. Dark grey blocks represent coding regions while dark lines represent introns, light grey blocks represent 5'and 3' UTR regions and the light grey line represents the promoter region. The artificial microRNA (ami) was designed to target a *WOX12* specific sequence in the first exon. The *wox12-1* (SALK_087882) line has a T-DNA insertion in the second intron. **B.** *WOX12* expression in *wox12-1* inflorescences. No transcript was detectable using primers flanking the second intron (black), while a reduction in expression was detectable using primers in the first exon (grey). *WOX12* expression was tested in 6 plants homozygous for the T-DNA insertion. Bars indicate SE between two replicates for wild-type and 6 biological replicates for SALK_087882. **C.** *WOX12* expression in inflorescences of amiR-wox12 plants. *WOX12* expression was assessed in 7 independent lines. *WOX12* was significantly lower in 6 lines overexpressing the amiR-wox12, although some differences in reduction levels were observed between the lines. Bars indicate SD between two technical replicates for each T1 line. **D.** Wild-type *Arabidopsis thaliana* Col-0 flower with one sepal and one petal removed. **E.** amiR-wox12flower where the formation of a stamenoid petal can be observed (arrow). Sepal removed to allow the visualisation of the inner organs. **F.** *ap1-3* flower. Formation of a stamenoid petal can be observed (arrow). Sepal removed to reveal the inner organs.

Next, we investigated the flower morphology by microscopic analysis. Arabidopsis flowers have a canonical body plan with 4 sepals, 4 petals, 6 stamens and 2 fused carpels forming one pistil (**Figure 6D**). We could observe defects in flowers of both *wox12-1* and amiR-wox12 mutant lines, such as reduction in stamen number and the formation of stamenoid petals (**Figure 6D-E and Table 4**). These stamenoid petals are positioned in whorl 2 and replaces normal petals. These aberrations are rare (**Table 4**) and when they occur, only one petal in a flower is converted into a chimeric organ with stamenoid tissues. We observed a higher frequency of defected flowers in the amiR-wox12 line compared to *wox12-1*, probably due to the presence of a partially functional protein in *wox12-1* (**Table 4**). Furthermore, in *wox12-1* flowers we observed a significant reduction in stamen numbers compared to wild-type. In some mutant flowers only 4 or 5 stamens are produced, suggesting a role for *WOX12* in stamen development or the initiation of whorl 3 primordia (**Table 4**). *WOX12* is indeed expressed in the floral meristem in flowers at stage 5 when stamens are determined (**Figure 2B**). We also observed chimeric stamenoid petal organ formation (**Figure 6E**), which resembles a phenotype observed in the weak *ap1* mutant allele *ap1-3* (**Figure 6F**), indicating a role for *WOX12* downstream *AP1* in the regulation of flower development.

Table 4. Identity and numbers of floral organs in wild-type, *ap1-3* and *WOX12* mutant lines.

		First whorl				Second whorl		Third whorl			Fourth whorl	N° flowers observed
		sepal	leaf-sepal	petaloid sepals	carpelloid sepal	petal	stamenoid petal	stamen	filament	petaloid stamen	carpel	
WT Col-0		4 ± 0	0	0	0	4 ± 0	0	5.94 ± 0.24	0	0	2 ± 0	200
Salk_087882		4 ± 0	0	0	0	3.99 ± 0.08	0.01 ± 0.08*	5.78 ± 0.45*	0	0	2 ± 0	170
amiR-wox12	#1	4 ± 0	0	0	0	4 ± 0	0	5.83 ± 0.50	0	0	2 ± 0	40
	#2	4.03 ± 0.16"	0	0	0	3.93 ± 0.35"	0.10 ± 0.30*	5.6 ± 0.71*	0	0	2 ± 0	40
	#3	4 ± 0	0	0	0	3.98 ± 0.16	0.03 ± 0.16"	5.73 ± 0.60*	0	0	2 ± 0	40
	#4	4.03 ± 0.16"	0	0	0	3.95 ± 0.22"	0.05 ± 0.22"	5.33 ± 0.76*	0	0	2 ± 0	40
	#5	4.05 ± 0.32"	0	0	0	4 ± 0	0	5.68 ± 0.47*	0	0	2 ± 0	40
	#6	4 ± 0	0	0	0	4 ± 0.23	0.03 ± 0.16"	5.55 ± 0.60*	0	0	2 ± 0	40
	#7	4 ± 0	0	0	0	4 ± 0	0	5.73 ± 0.53*	0	0	2 ± 0	26
ap1-3		0	2.57 ± 0.89*	0.23 ± 0.45*	0.10 ± 0.36*	3.76 ± 0.57*	0.18 ± 0.52*	5.99 ± 0.10	0	0	2.03 ± 0.3	100

Flowers from 2 months old plants were analysed at anthesis. A reduction in stamen numbers compared to wild-type was observed in both *wox12* mutant lines. In amiR-wox12 flowers, and at a lower frequency in *wox12-1* flowers, formation of stamenoid-petal organs were observed. A similar phenotype is also observed in *ap1-3* flowers. (*) indicates significant differences according to unpaired t-Test at p-value <0.001. (“) indicates significant differences according to unpaired t-Test at p-value <0.05.

Ectopic expression of WOX12 leads to reduction in AGAMOUS expression.

We analysed the effects of *WOX12* overexpression during *Arabidopsis* development with a main focus on flower development. qRT-PCR analysis showed that most of the obtained lines overexpress *WOX12* in comparison with wild-type plants (**Figure S1**). The most prominent phenotypes observed in flowers of plants overexpressing *WOX12* (*WOX12-OE*) were a reduction in stamen numbers and the formation of petaloid-stamens in whorl 3 (**Figure 7A-B and Table 5**). More rarely, the formation of an extra sepal is observed, resulting in two fused sepals (**Table 5**). The conversion of stamens into stamen-petal mosaic structures was previously reported in the weak *ag* mutant allele, *ag-4* (Chen and Meyerowitz, 1999). *AG* is a key regulator of male and female reproductive organ development and the loss of *AG* function results in flowers forming only sepals and petals (Bowman *et al.*, 1989). Therefore, we studied the expression of *AG* in *WOX12-OE* inflorescences by qPCR. We indeed found that *AG* is less expressed in *WOX12-OE* inflorescences than in wild-type inflorescences (**Figure 7C**). Taken together these results suggest that *WOX12* play a role in the repression of *AG*.

Table 5. Identity and numbers of defects observed in flowers of plants overexpressing *WOX12*.

	First whorl	Second whorl	Third whorl			Fourth whorl	N° flowers observed
	sepal	petal	stamen	filament	petaloid-stamen	carpel	
WT Col-0	4 ± 0	4 ± 0	5.94 ± 0.24	0	0	2 ± 0	200
p35S:WOX12 #1	4 ± 0	4 ± 0	5.7 ± 0.66*	0	0.05 ± 0.22*	2 ± 0	20
#2	4 ± 0	4 ± 0	5.85 ± 0.37	0.05 ± 0.22*	0.05 ± 0.22*	2 ± 0	20
#3	4 ± 0	4 ± 0	5.9 ± 0.31	0	0.05 ± 0.22*	2 ± 0	20
#4	4 ± 0	4 ± 0	5.8 ± 0.52"	0	0	2 ± 0	20
#5	4.05 ± 0.22"	4.05 ± 0.22"	5.75 ± 0.55"	0	0.05 ± 0.22*	2 ± 0	20
#6	4 ± 0	4 ± 0	5.55 ± 0.51*	0	0.05 ± 0.22*	2 ± 0	20
#7	4 ± 0	4 ± 0	5.7 ± 0.66*	0	0	2 ± 0	20
#8	4 ± 0	4 ± 0	5.7 ± 0.66*	0	0.05 ± 0.22*	2 ± 0	20
#9	4.05 ± 0.22"	4.05 ± 0.22"	5.7 ± 0.66*	0	0.05 ± 0.22*	2 ± 0	20
#10	4 ± 0	4 ± 0	5.7 ± 0.66*	0.1 ± 0.31*	0	2 ± 0	20
#11	4 ± 0	4 ± 0	5.65 ± 0.67*	0.05 ± 0.22*	0	2 ± 0	20
#12	4.05 ± 0.22"	4 ± 0	5.75 ± 0.55"	0	0.1 ± 0.31*	2 ± 0	20
#13	4 ± 0	4 ± 0	5.9 ± 0.31	0	0	2 ± 0	20
#14	4 ± 0	4 ± 0	5.8 ± 0.52"	0	0.05 ± 0.22*	2 ± 0	20
#15	4.05 ± 0.22"	4 ± 0	5.8 ± 0.52"	0	0	2 ± 0	20
#16	4 ± 0	4 ± 0	5.75 ± 0.55"	0	0	2 ± 0	20
#17	4 ± 0	4 ± 0	5.85 ± 0.37	0	0	2 ± 0	20
#18	4 ± 0	4 ± 0	5.55 ± 0.51*	0.05 ± 0.22*	0.05 ± 0.22*	2 ± 0	20
#19	4 ± 0	4 ± 0	5.65 ± 0.67*	0	0	2 ± 0	20
#20	4 ± 0	4 ± 0	5.5 ± 0.76*	0	0	2 ± 0	20
#21	4 ± 0	4 ± 0	5.7 ± 0.66*	0	0	2 ± 0	20
#22	4 ± 0	4 ± 0	5.65 ± 0.67*	0	0.1 ± 0.31*	2 ± 0	20
#23	4 ± 0	4 ± 0	5.85 ± 0.37	0	0.05 ± 0.22*	2 ± 0	20
#24	4 ± 0	4 ± 0	5.95 ± 0.22	0	0	2 ± 0	20

Flowers from 2 months old plants were observed at anthesis. 20 flowers from 24 independent T1 plants overexpressing WOX12 were observed. (*) indicates significant differences according to unpaired t-Test at p-value <0.001. (") indicates significant differences according to unpaired t-Test at p-value <0.05.

Figure 7. *WOX12* overexpression causes aberrations in flowers. A. Wild-type *Arabidopsis* flower, composed of 4 sepals, 4 petals, 6 stamens and 2 fused carpels arranged in 4 concentric whorls. **B.** Flower from a plant overexpressing *WOX12*. A petaloid-stamen can be observed (arrowhead). One sepal was removed to reveal the inner flower organs. **C.** *AGAMOUS* expression is lower in *WOX12OE* plants compared to wild-type. Bars indicate SE between 5 biological replicates.

DISCUSSION

WOX12 is a member of the homeobox gene family and it is expressed in the IM at the border of newly formed floral meristems. Furthermore, *WOX12* becomes activated in the FM from stage 4 onwards at the position where the stamen primordia will emerge. Our mutant analyses point to a function of *WOX12* in floral organ specification, which is in line with its expression in the FM. The expression at the border of newly arising floral meristems could not be associated with any phenotypic aberration in the overexpression or knock-down lines, suggesting that other genes are needed or act redundantly with *WOX12* at this stage of development.

Regulation of WOX12 expression during flower development.

The characterisation of cis-regulatory regions in the *WOX12* locus shows that the most upstream cis-regulatory element (‘b’, figure 4A) is needed to repress *WOX12* in the sepal primordia. However, how and which TFs regulate *WOX12* remains unclear. Our results suggest that the MADS-domain protein binding to the *WOX12* locus is indirect or needs co-factors that are not present in our *in vitro* DNA-binding assays. MADS-domain proteins are able to interact with other transcription factors, especially AP1 was found to interact with other proteins (Smaczniak *et al.*, 2012). Among the AP1 interacting proteins are SPL8 and BLR. BLR is expressed in IM and floral meristems and later on in carpels. A consensus for BLR binding is present in the *WOX12* locus suggesting that BRL could mediate the AP1 binding to the *WOX12* locus and act as a repressor of *WOX12*. *SPL8* is expressed in sepals and an SBP binding sites is present in the *WOX12* promoter region,

thus a complex SEP3-AP1-SPL8 could also be responsible for the suppression of *WOX12* expression in the sepal primordia. Alternatively, *AP2* may regulate *WOX12* expression in the sepal primordia, since binding to the most upstream cis regulatory region was reported (Yant *et al.*, 2010). Further experiments, such as yeast-one-hybrid screens, are needed to identify regulators of *WOX12.*

WOX12 acts downstream AP1 and is involved in the regulation of AG in flower development.

Previous work has shown that the *WOX12* locus is bound by the AP1 protein and *WOX12* is activated by AP1 during the first stages of flower meristem development (Kaufmann *et al.*, 2010b). This gene is dynamically expressed throughout flower development, suggesting a role of this gene during floral organ formation. We found that *WOX12* down-regulation leads to defects in floral organ identity specification with the formation of stamenoid-petals. The phenotype observed upon down-regulation of *WOX12* resembles a weak *ap1* mutant confirming that *WOX12* acts downstream *AP1* in the regulation of proper floral organ formation and spatial patterning of organ identities (**Figure 8**). Notably, we found that ectopic expression of *WOX12* leads to an opposite homeotic effect: it causes the formation of petaloid stamens in the third whorl. A similar phenotype was observed in the weak *ag* mutant allele, *ag-4* (Chen and Meyerowitz, 1999; Sieburth *et al.*, 1995). In agreement, we observed a reduction in *AG* expression in inflorescences ectopically expressing *WOX12*. Taken together these results suggest that *WOX12* may play a role in the spatial regulation of *AG*. According to the ABC model, the A class genes, such as *AP1* and *AP2* act antagonistically with the C-class gene *AG* (Drews *et al.*, 1991). Thus, the *ap1-3* phenotype is correlated with an expansion of the *AG* expression domain to the second whorl, resulting in the formation of stamenoid-petals. Similarly, the phenotype observed in the *wox12* flowers could be explained by an expansion of the *AG* expression domain to whorl 2. In an opposite manner, the phenotype observed upon ectopic expression of *WOX12* suggests an expansion of the A-class gene expression to the third whorl, with the consequent formation of petaloid-stamens.

Remarkably, the phenotypes were very mild and only observed in a few flowers per inflorescence. For the knock-down line it is possible that the remaining expression or the expression of the C-terminally truncated protein is still partly functional and may be

enough for proper petal development in most flowers. Alternatively, other genes are (partly) redundant with *WOX12*. Overexpression of *WOX12* reduces *AG* expression, but this may be insufficient or happen too late during organ formation to fully transform stamens into petals. Furthermore, the regulation of *AG* expression is complex, many transcription factors as well as chromatin remodelers have been shown to play a role in the regulation of *AG* expression (Kaufmann *et al.*, 2010a). Therefore the repression of *AG* by WOX12 may be overcome by other regulators.

It also remains unclear whether *WOX12* regulates *AG* directly or indirectly through the activation of an *AG* repressor (**Figure 8**). WOX12 contains a WUS-box, which is a repressive protein domain (Ikeda *et al.*, 2009) and therefore *WOX12* may act directly as a repressor. Also other WOX family members such as *WOX6* have been found to repress *AG*. Ectopic expression of *WOX6* leads to strong abnormalities in the flower, such as the formation of carpelloid stamens (Park *et al.*, 2005).

The characterisation of the spatial and temporal expression of *AG* in flowers differentially expressing *WOX12* will give a better understanding of the role of *WOX12*. In conclusion our results suggest that, like other *WOX* genes, *WOX12* may act in the specification of organ identity through regulation of *AG*. Since the A class homeotic genes and *AG* control each other antagonistically, we hypothesise that *WOX12* is involved in establishing the border between class A and C expression domains.

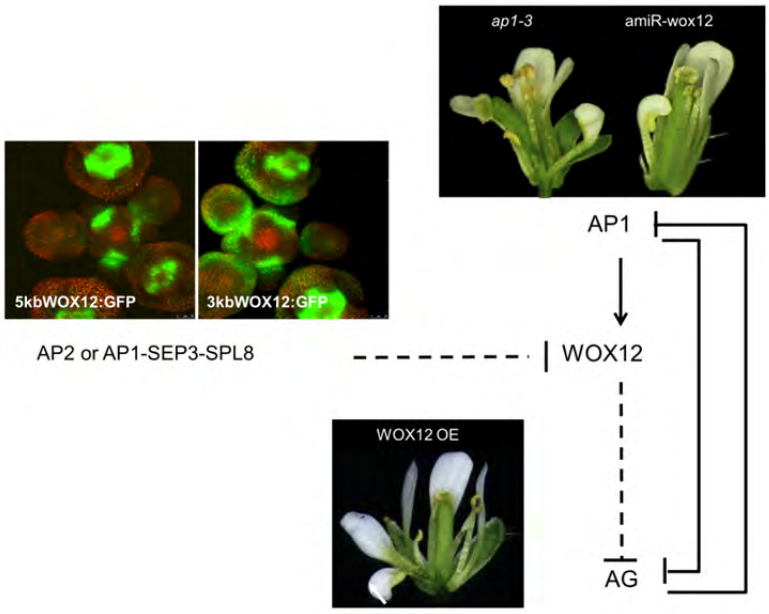

Figure 8. Model of WOX12 regulation. *WOX12* acts downstream *AP1* during flower development and represses *AG* expression.
Binding of the most upstream cis-regulatory element in the *WOX12* promoter leads to repression of *WOX12* expression in sepal primordia.

MATERIALS AND METHODS

Plant material. All plants were grown at 20 °C under long day condition (16 h light, 8 h dark) on rock wool. Seeds of knock-out lines SALK_087882 was obtained from the Nottingham Arabidopsis Stock Center (NASC). Plants were genotyped for the presence of the T-DNA insertion using the following primers: fw: 5'-AATGTCTGCAGAAATTAACCTCAAG-3' rev: 5'- TCGATCGAGAATGGTATAAAGTACC-3'; T-DNA: 5'-ATTTTGCCGATTTCGGAAC-3'.

Isolation of RNA and real-time PCR analysis. Total RNA was extracted using the Invitek Kit according to the manufacturer's protocol. DNase I digestion was performed on total RNA using DBase I from Ambion. RNA integrity was checked on 1% (w/v) agarose gel after DNase I treatment. Absence of genomic DNA was confirmed subsequently by qRT-PCR using primers, which amplify an intron sequence of the gene At5g65080 (Forward 5 -TTTTTTGCCCCCTTCGAATC-3 and reverse 5 -ATCTTCCGCCACCACATTGTAC-3). First-strand cDNA was synthesized from 1 µg of total RNA using TaqMan kit (Roche) cDNA Synthesis Kit following the manufacturer's protocol. The efficiency of cDNA was estimated by qRT-PCR using two different primer sets annealing 5'- and 3'- ends of a control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (At3g26650), respectively (GAPDH3': forward 5'-TTGGTGACAACAGGTCAAGCA-3' and reverse 5'-AAACTTGTCGCTCAATGCA ATC-3') (GAPDH5': forward 5'-TCTCGATCTCAATTTCGCAAAA-3' and reverse 5'-CGAA ACCGTTGATTCCGATTC-3'). Primers used to detect WOX subfamily gene expression at different stages of flower development are listed in Table S1**.**

To test *WOX12* expression level in the SALK_087882 line and in the amiR-wox12 lines two pairs of primers were used, one designed in the first exon (forward: 5'-CTTGCAGCAACAACGAGATT-3' reverse 5'- AAGAGAGGCCGAGGAAAAAG-3') and the second one designed between the second exon and the 3'UTR region (forward: 5'-GATGAGTTTGGTTTCTTGATGC-3' reverse 5'-GTTCCCACATAAAACAGCCAG-3').

GFP fusion reporter lines. WOX12 genomic region was amplified by PCR using the following primers: forward: 5'-CAGCTTTTAATGGGGCCTCTTTACTG-3' for the 5 kb promoter, forward: 5'- CAATTTATCTGTTTAGTTTAACAAAAC-3' for the 3 kb promoter, forward: 5'-GGTGTTTTCAC AATTGCAT-3' for the 500 bp promoter and reverse: 5'-TGTCTGTCTCGGTACCTGTCGA-3'. The PCR fragment was cloned into the GATEWAY vector pCR8/GW/TOPO from Invitrogen. The CArG-box sequences were mutate via site-direct mutagenesis: the plasmid were amplified using mutated primers (see table S1) that introduced the desired changes; the amplified, containing the desired mutation, was circularized in a ligation reaction with T4 DNA Ligase. The constructs were transferred via LR reaction into the

destination vector pARC384 (Curtis and Grossniklaus, 2003). Expression vectors were introduced into Arabidopsis thaliana ecotype Col-0 by floral dip transformation (Clough and Bent, 1998). Transformant plants were selected on plates containing ½ Murashige-Skoog (MS) medium pH 6 with 10ug/ml hygromicin and 0.7% Agar.

Confocal Scanning Laser Microscopy (CSLM). GFP tagged protein localization was observed trough CSLM on Leica SPE DM5500 upright microscope using an ACS APO 40x/1.15 oil lens and using the LAS AF 1.8.2 software. FM4-64 dye was added to 0.1% agar at a concentration of 5uM and used as staining of cell membranes, alternatively chloroplast auto-fluorescence was used as back-ground. GFP and FM4-64 dye were excited with the 488-nm line of an Argon ion laser. The GFP emission was detected at a bandwidth of 505-530 nm, while FM4-64 dye and chloroplast auto-fluorescence were detected at a bandwidth of 650 nm. After acquisition, optical slices were median filtered and three-dimensional projections were generated with LAS AF 1.8.2 software package.

Protein DNA-binding assay. EMSA experiments were performed as described in (Smaczniak *et al.*, 2012). Complementary oligos were aligned to generate the probes (**Table 6**). The probes were cloned into pGEM-T vector and amplified with vector-specific florescent labelled primers. Proteins were produced in vitro using TNT SP6 High-Yield Protein Expression System from Promega. Detection was performed using Odyssey scanner.

Table 6. Probes used in the EMSA experiment.

sequence 5' → 3'	description
TGATTGAAATCTTTTTTTTGTTAATTATATA	CArG-box A
TATATAAAATCTTTTTTTTGTTAATTATATA	CArG-box B
TCCTAGACGTCTTATATATGAATCTAATAAA	CArG-box C
AGTGGTAGGTCTAATTTATGAGTATCCGATA	CArG-box D
GCTAATTACTTCCTTAATGGATCGTCGTCA	CArG-box 0
TAGAACGTAACATAAAAAAGGAAGAAAAAA	CArG-box I
CGTCCCAAAAGCAATTTTGGGATTGTGGTT	CArG-box II
AAAGAGAGTGGCTTAAAGGTAACTTTTGTG	CArG-box III

WOX12 knock-down lines. The artificial microRNA was generated as described in Schwab *et al.*, 2006. An online tool was used to designed specific oligos to generate the ami construct (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi).

oligo I: 5'-GATAATTAGCTGATAAGTTGCTCTCTCTCTTTTGTATTCC-3',

oligo II: 5'- GAGAGCAACTTATCAGCTAATTATCAAAGAGAATCAATGA-3',

oligo III: 5'- GAGAACAACTTATCACCTAATTTTCACAGGTCGTGATATG-3',

oligo VI: 5'- GAAAATTAGGTGATAAGTTGTTCTCTACATATATATTCCT-3'.

The final PCR product was then ligate into the GATEWAY vector pCR8/GW/TOPO from Invitrogen and transferred via LR reaction into the destination vector pB7WG2 (Curtis and Grossniklaus, 2003). Expression vector was introduced into Arabidopsis thaliana ecotype Col-0 by floral dip transformation (Clough and Bent, 1998). Transformant plants were selected on plates containing ½ Murashige-Skoog (MS) medium pH 6 with 10ug/ml ppt and 0.7% Agar.

WOX12 overexpression lines. WOX12 coding sequence was amplified using the following primers: forward: 5'-CACCATGAATCAAGAAGGTGCTTCACATAG-3' reverse 5'- TCATGT CTGTCTCGGTACCAG-3'. The PCR product was then ligate into the GATEWAY vector pENTRY TOPO from Invitrogen and transferred via LR reaction into the destination vector pB7WG2 (Curtis and Grossniklaus, 2003). Expression vector was introduced into Arabidopsis thaliana ecotype Col-0 by floral dip transformation (Clough and Bent, 1998). Transformant plants were selected on plates containing ½ Murashige-Skoog (MS) medium pH 6 with 10ug/ml ppt and 0.7% Agar.

Phylogenetic analysis. The analysis was performed on the Phylogeny.fr platform (Dereeper *et al.*, 2008) using the entire coding sequences of all WOX subfamily members as input data. Sequences were aligned with MUSCLE v3.7, with MUSCLE default settings (Edgar, 2004). After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks v0.91b (Castresana, 2000) using the following parameters: minimum length of a block after gap cleaning: 10; no gap positions were allowed in the final alignment; all segments with contiguous non conserved positions bigger than 8 were rejected; minimum number of sequences for a flank position: 85%. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program v3.0 (Guindon *et al.*, 2010). The HKY85 substitution model was selected assuming an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn v198.3 (Chevenet *et al.*, 2006).

SUPLEMENTAL DATA

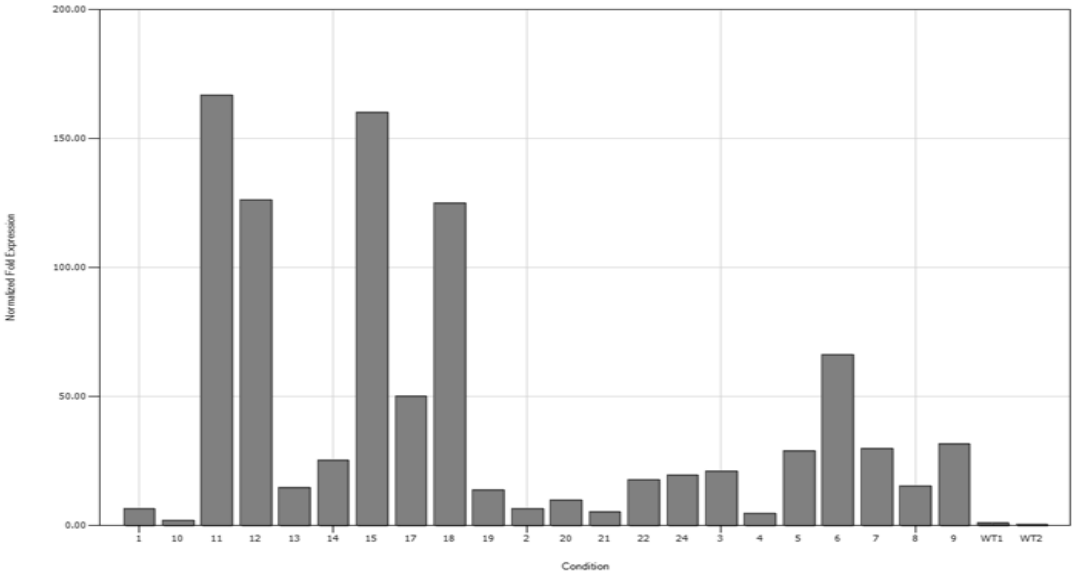

Figure S1. WOX12 expression in OE lines. Level of WOX12 expression in inflorescences of 24 T1 plants was measured by qPCR. All plants showed higher expression level than wild-type (on the right).

Table S1. Primers used in the study

WOX family gene expression primers			
gene	**atg code**	**forward primer**	**reverse primer**
WUS	AT2G17950	TCCCAGCTTCAATAACGGGAAT	GCCATTAGAAGCATTAACAACACC
WOX1	AT3G18010	GCGACACGCAACCAGAGAAACCTT	AACGAGCATTGTGCTCCACCCGTA
WOX2	AT5G59340	GCTAGGCAACGCCAAAAGC	TGTGGAGGAGGCGATTGAAG
WOX3	AT2G28610	TCAGGGAACTGGAGTAGGAGAAGC	TCTTCAGCTCCACTTTTGGTGCAG
WOX4	AT1G46480	TGAGAGAACCAATGGTGGAGAAGG	TCAAATCCCCAGCTCCTACATGTC
WOX5	AT3G11260	GGCAGAAACGTCGTAAAATCTCCA	TCCTCTTGACAATCTTCTTCGCTT
WOX6	AT2G01500	CGACCACAGCCACAGCATGAATTA	TTTACCAACCTCTGATGCCCTCTG
WOX7	AT5G05770	TGCCGGAAAATCTCCACCGTCAAG	TCGGCGAGGCTTAGAAAGATCTGT
WOX8	AT5G45980	TCCTTTTCCTCAGATCGGATACCA	TTTGAATCTCCTCTCTAGGTGGGT
WOX9	AT2G33880	TCAGGATGTGAAGTGGAGAGGAGT	GGAGGATTCACCATCCCGGAGTTA
WOX10	AT1G20710	GCAACCTCCGACAACGACAATTAC	TCCCTAAATCAGGACTCGGGAACA
WOX11	AT3G03660	TTCAAAACCGGCGGTCAAGGT	GCTAGAAGTGTTGGTGGTTGCGT
WOX12	AT5G17810	CGAACAAGAAGGGTTTATGACGGT	TCAGTGGGAAGAGGAAGACCAGAG
WOX13	AT4G35550	ACGGCACCATTTGTGAGCGTCT	GCACGATGCCCACCTTTTCTCTTA
WOX14	AT1G20700	TCTCATCAGGAGTGAGGCCAATGG	TCCACCGATGCCTAGTCGATATCC
WOX12 mutagenesis primers			
Primer	**mutation**	**sequence 5'--> 3'**	
PDS5419	CArG-box I	TTTTTTCTTCGTTTTTTATCTTACGTTCTATTG	
PDS5420	CArG-box I	GGACAAGGAGCTAGAGAAGTTATAAAAGAC	
PDS5421	CArG-box II	AACCACAATCCGAAAATTCCTTTTGGGACG	
PDS5422	CArG-box II	TTGGATGATGGGAAGTGGATAGAGATCGTA	
PDS5423	CArG-box III	CACAAAAGTTACGTTTAACCCACTCTCTTTG	
PDS5424	CArG-box III	AATCCTCGTACAGTTTCTGCCTGATC	
PDS6415	CArG-box 0	AGCTAATTACTTTCTCAACGGCTCGTCGTCATC	
PDS6416	CArG-box 0	TAAGTTGCTACAACTTCCAAACCCCAAATCAATCTCG	

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CHAPTER 7

Concluding remarks and perspectives

During the life cycle, a plant undergoes a series of developmental changes. Each developmental change is controlled at the level of gene expression by the combined action of chromatin regulators and transcription factors (TFs). Using flower development as a model system, we studied the dynamics of TF DNA binding and chromatin accessibility.

After the initiation of the reproductive phase, a group of undifferentiated cells at the flank of the inflorescence meristem forms the flower meristem. The flower meristem gives rise to the four organ types that constitute the mature flower: the sepals, the petals, the stamens and the carpels (Smyth *et al.*, 1990). MADS-domain TFs play a key role in floral organ specification, and a particular combination of these TFs specifies each organ type (Bowman *et al.*, 1989). MADS-domain TFs can act as activators as well as repressors of transcription, for example AP1 acts predominantly as repressor during flower initiation (Kaufmann *et al.*, 2010b) and as activator at later stages of flower development (Pajoro *et al.*, 2014b; chapter 3). Moreover, we found that MADS-domain proteins, such as APETALA1 (AP1) and SEPALLATA3 (SEP3), select their binding sites, and thereby their target genes, in a partly stage-specific fashion.

Next, we addressed the question of how stage-specific binding is realised at the molecular level in a chromatin context. We characterized changes in chromatin accessibility in relation to MADS-domain protein occupancy and gene expression. Our results provide insights into the mechanisms by which MADS-domain TFs exert their master regulatory functions in meristem and organ differentiation in plants (chapter 3 and chapter 4).

Throughout our study we also identified new players in the floral gene regulatory network and characterized downstream regulators of floral organ development, such as the GRF family members (chapter 3), *STERILE APETALA* (*SAP*, chapter 5) and *WUSCHEL RELATED HOMEOBOX 12* (*WOX12*, chapter 6).

Dynamics of gene regulatory network underling flower development.

The introduction of next-generation sequencing and genome-wide approaches has changed our view on gene regulation and gene regulatory networks (GRNs). We moved from linear genetic interactions towards global highly connected gene networks (chapter 2, Kaufmann *et al.*, 2010a; Pajoro *et al.*, 2014a). Many genome-wide expression profiles have become available as well as protein-DNA binding profile data that rapidly increased

our knowledge of transcriptional regulation and network wiring. Developmental processes are characterised by continuous changes in cell fate, growth and direction, which has to be preceded by dynamics in regulatory circuits. Therefore, a main question of my research was how GRNs change during flower development. Protein-DNA binding experiments were mostly performed with mixed tissues and cannot reveal the dynamics of the GRNs. Therefore, to address this question we characterized protein-DNA binding dynamics of the two MADS-domain proteins, AP1 and SEP3, in the synchronised floral induction system, *ap1 cal* pAP1:AP1:GR (Wellmer *et al.*, 2006). Although many MADS-domain TF-bound regions are occupied by these factors throughout flower development, we observed stage-specific binding events. Binding site dynamics reflect regulatory dynamics of genes with stage-specific functions in flower development, such as floral meristem patterning and organ growth (chapter 3).

In our time-series experiment, many genes show no quantitative change in AP1 or SEP3 binding but they are differentially expressed throughout flower development, indicating that either single TF binding alone per se is not sufficient to explain changes in gene expression or other TFs determine gene expression changes. Alternatively, there is a delay in the regulatory response. It is possible that promoter binding by MADS-domain TFs is a prerequisite for regulatory response, but that additional factors are needed to promote gene expression. MADS-domain TFs are indeed part of protein complexes that include chromatin remodelers as well as other TFs (Smaczniak *et al.*, 2012; Wu *et al.*, 2012). In this scenario, the analysis of additional protein-DNA binding profiles in a stage-specific manner will give a better understanding of the regulation of gene expression and the dynamics of the GRN structure underling flower development.

Characterisation of new players in the control of flower development.

The isolation of developing flowers of specific stages increased the sensitivity of our ChIP-seq experiments, thereby enabling us to identify novel target genes with a role in flower development, such as the GRF family genes (chapter 3), *SAP* (chapter 5) and members of the WOX family (chapter 6).

The *GRF* TF family genes act redundantly in flower development in such a way that down-regulation of multiple members of the family is required to reveal defects in floral organ formation. Downregulation of all the family members leads to reduction in floral

organ numbers and homeotic conversion of floral organs, such as the formation of petaloid stamens. Although they mostly act redundantly during floral organ development, some *GRFs* show differences in their expression profiles. For example, *GRF2* is expressed prevalently at early stages of development, while *GRF8* is predominantly expressed at later stages of development. The dynamics in gene expression coincides with changes in binding behaviour of MADS-domain TFs. SEP3 binds to all the *GRF* loci, while AP1 only to a subset of the family members, reflecting the more organ-specific function and more confined expression profile of *AP1* compared to *SEP3*. For example, SEP3 but not AP1 binds to the *GRF8* locus. Moreover, while SEP3 binding to the *GRF2* locus is stronger at early stages of flower development, binding to the *GRF8* locus is stronger at later stages of flower development.

In summary, our results indicate that different, apparently redundantly acting GRF family members are regulated in different ways, and that the phenotype that was observed in the miRNA-directed knockdown lines probably reflects the overlapping function of these family members in floral meristem patterning and in floral organ differentiation.

Next to the GRF family, other families of TFs were found to be overrepresented among MADS-domain protein target genes, such as the WOX family (chapter 6). The role of some WOX genes in flower development has been previously reported, for example, *WUS* plays a key role in the regulation of *AG* expression in the floral meristem (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001), while *WOX1*, *WOX3* and *WOX6* play a role in petal growth (Park *et al.*, 2005; Vandenbussche *et al.*, 2009). In this thesis, we focused on the characterisation of *WOX12*. *WOX12* was initially identified as target gene of AP1 at the early stages of floral meristem formation (Kaufmann *et al.*, 2010b). AP1 binding to the *WOX12* locus and the change in expression upon AP1 induction, suggest a role of *WOX12* in flower development. Next, in ChIP-seq experiments conducted at different stages of flower development we observed binding of both AP1 and SEP3 during floral whorl specification (day 4) and during floral organ growth (day 8), indicating a role of *WOX12* at later stages of flower development (Pajoro *et al.*, 2014b). Finally, binding of homeotic proteins APETALA2 (AP2) and PISTILLATA (PI) to the *WOX12* locus was also reported (Wuest *et al.*, 2012; Yant *et al.*, 2010), highlighting a likely role in flower development.

The characterization of *WOX12* overexpression plants and knock-down mutant lines indicates that the main role of *WOX12* consists in the determination of the boundary

between whorls 2 and 3. *WOX12* is indeed expressed in a ring-like fashion in the flower meristem at stage 4 and 5. Moreover, *WOX12* downregulation leads to defects in floral organ identity specification with the formation of stamenoid petals, while ectopic expression of *WOX12* leads to an opposite effect: it causes the formation of petaloid stamens in the third whorl. Finally, the reduction of *AG* expression in inflorescences ectopically expressing *WOX12*, suggest that *WOX12* plays a role in the spatial regulation of *AG*.

WOX12 is also expressed in flowers at other developmental stages, such as at stage 1 and 2 in the flower meristem, at stage 9 in petals, at stage 10 and 11 in anthers and carpels and at stage 12 and 13 in the valve margins of the silique. We did not observe any aberrant phenotype in the knock-down lines in these tissues, suggesting possible redundancy with other genes. The diverse expression profile of *WOX12* at different floral developmental stages point towards a coordinated control by MADS-domain TFs and other transcriptional regulators to establish cell-type specific gene expression patterns. In agreement with this hypothesis, *WOX12* expression was not abolished in any of the homeotic mutants we analysed neither upon mutagenesis of the cis-regulatory elements that are putatively responsible for MADS-domain protein binding.

The regulation of *WOX12* is also an example of tissue specific and temporal mode of action of MADS-domain proteins. While AP1 activates *WOX12* at flower meristem initiation (Kaufmann *et al.*, 2010b), the lack of *WOX12* expression in sepal primordia at stage 3-5, and the ectopic *WOX12* expression observed in sepals upon AP1-bound region deletion indicate a repressive action of AP1 in sepal at the later developmental stage. This tissue- and stage-specific regulation may be achieved via the interaction with different TFs in each tissue and developmental stage.

Among dynamically bound target genes we did not only found TFs but also genes that possibly act at the post-transcriptional level such as *SAP* (Byzova et al, 1999). *SAP* was found to be preferentially bound by AP1 at stage 4 and *SAP* expression decreased after AP1 induction. Moreover, we observed higher level of *SAP* expression in *ap1* inflorescences than in wild-type and the *in planta* expression profile showed ectopic expression of *SAP* in *ap1* leaf-like sepals, indicating a repressive action of *AP1* at the *SAP* locus (chapter 5). A role for *SAP* in flower development was previously reported: phenotypical characterisation of the loss of function mutant indicates a role of *SAP* in petal and ovule development, however the mechanism of action remained unclear

(Byzova *et al.*, 1999). Our results suggest that SAP acts at the post-transcriptional level by being part of an SCF-complex together with ASK1/2 and CUL1. However, further experiments are needed to identify the proteins ubiquitinated by the SCF^{SAP} complex and to elucidate the exact role in this complex. Loss of function of *SAP* does not exclusively affect flower development; *sap* plants appear bushy and have curly leaves, indicating a broader role of *SAP* in plant development.

In conclusion, advances in genome-wide studies resulted in the confirmation of previously known genetic interactions at the molecular level, but also identified many novel regulatory interactions. The characterisation of new factors and interactions can reveal new regulatory mechanism of flower development.

Profiling chromatin landscape during flower development.

Recent studies of TF DNA-binding profiles and gene expression analyses have shown that there is only a weak correlation between binding of a TF and changes in expression of its target genes (O'Maoileidigh *et al.*, 2014), indicating that single TF binding events are not the only cause of gene regulation. Data from the animal field show that developmental control of gene expression is tightly linked with dynamic changes in chromatin accessibility. With that in mind, we aimed to understand how changes in the chromatin accessibility landscape during flower development can reflect changes in gene expression.

We characterised changes at cis-regulatory elements, via DNase I-seq, and profiled nucleosome occupancy through MNase-seq, at different stages of flower development. We observed a number of quantitative changes in chromatin accessibility at cis-regulatory elements, mostly in the transition from meristematic stages to floral organ differentiation. An opposite scenario was observed for changes in nucleosome occupancy: nucleosomes appear to be more dynamic during meristematic stages than comparing to floral organ differentiation phase. These observations lead us to the hypothesis that cell programming initiate with changes in nucleosome occupancy and result in different DNA accessibility at cis-regulatory elements between meristematic and differentiated cells.

Remarkably, changes in nucleosome occupancy involve mainly single nucleosomes and are prevalently localised in promoter regions. Similar findings have been reported recently in mouse and human cell studies. During the transition from pluripotent to

somatic cell identities, changes in nucleosome occupancy affect single nucleosomes and co-localize with binding sites of pluripotency and reprogramming proteins (West *et al.*, 2014).

The highest nucleosome dynamics, which was observed at promoter regions close to the transcription start site (TSS) could be caused either by chromatin remodelling at cis-regulatory elements upon binding of TFs (Li *et al.*, 2012) or to changes in chromatin structure during gene transcription (Lee *et al.*, 2004). The characterisation of plant chromatin states revealed that also histone marks typically associated with active transcription, such as H3K4me2, H3K4me3 and H3K36me3 as well as the histone variant H2A.Z peak upstream the TSS (Sequeira-Mendes *et al.*, 2014).

While changes in DNA hypersensitivity sites (DHSs) globally correlate with changes in gene expression, no clear correlation was observed between changes in nucleosome occupancy and gene expression, with both up-and down-regulated genes showing gain as well as loss in nucleosome occupancy. Therefore, if changes at DHSs can reflect the establishment of multiple new cell types during flower differentiation, and be linked with the regulation of gene expression, changes in nucleosome occupancy may be hallmark of reprogramming.

In conclusion, our findings suggest that there are multiple mechanisms by which developmental changes in gene expression are controlled, and that developmental changes in gene expression are partly manifested in changes in chromatin structure in plants.

MADS-domain TFs may act as pioneer factors.

A result of the combined analysis of MADS-domain TF binding dynamics and chromatin accessibility is that MADS-domain proteins bind preferentially to nucleosome depleted-regions and that their binding to DNA is followed by opening of the surrounding chromatin (**Figure 1**).

While chapter 3 (Pajoro *et al.*, 2014b) is the first report proposing such a mode of action for a plant TF, a similar mode of action has been previously described for animal TFs that trigger reprogramming of cell fate, such as FoxA, Oct4, Sox2, Klf4 and c-Myc (Drouin, 2014; Zaret and Carroll, 2011). These TFs have been defined as “pioneer factors”.

Pioneer factors can play a passive or active role in gene activation (Drouin, 2014; Zaret and Carroll, 2011). In their passive role, binding of a pioneer factor to a cis-regulatory element reduces the number of additional factors required for efficient transcriptional response (Carroll *et al.*, 2005). This passive mode is supported by the observation that binding event of a single TF often does not cause change in gene expression and that multiple proteins are often required for transcriptional competence. On the other hand, the active role of pioneer factors is accomplished through alteration of the chromatin structure that facilitates binding of other TFs (Cirillo and Zaret, 1999). For example, *in vitro* studies showed that FoxA binds the target sites in highly compacted chromatin and thereby, increasing chromatin accessibility (Cirillo *et al.*, 2002). The FoxA C-terminal domain binds to histones and is necessary for chromatin opening *in vitro* (Cirillo *et al.*, 2002). *In vivo* nucleosome occupancy studies showed that Foxa2 is required for nucleosome depletion during embryonic stem cell differentiation (Li *et al.*, 2012). Moreover, it was shown that nucleosome depletion during embryonic stem cell differentiation involves SWI/SNF and INO80 chromatin remodelling complexes (Li *et al.*, 2012).

In a similar manner we observed an increase in chromatin accessibility upon binding of MADS-domain protein *in vivo*, suggesting that MADS-domain proteins trigger changes in chromatin accessibility. The characterisation of nucleosome occupancy around MADS-domain protein binding sites shows that, differently from FoxA, MADS-domain proteins bind predominantly to nucleosome depleted regions, indicating that MADS-domain proteins may not be able to displace nucleosomes and suggesting that chromatin remodelling proteins may be required for changes in chromatin accessibility. In agreement with this hypothesis, previous results have shown that floral homeotic MADS-domain proteins form larger complexes together with ATP-dependent nucleosome remodelers and with histone-modifying enzymes *in planta* (Smaczniak *et al.*, 2012; Wu *et al.*, 2012).

Alternatively, active pioneer action of MADS-domain protein is a fast mechanism that cannot be detected with our experimental conditions and temporal resolution. Moreover, the lack of cell-type specificity in our current experiment could mask pioneering action at a specific locus. It should be noted that, due to the fact that mixed cell types were used in the nucleosome profiling, the cell-type specific changes in nucleosome occupancy may be underestimated. In addition to the measurements of nucleosome density, most

measurements (such as gene expression, MADS-domain protein binding levels, etc.) in this study were conducted with mixed cell types. Although we were dealing with a highly synchronised system, the comparisons between time points could not capture differences in individual cell types. Cell type-specific measurements will be necessary to better elucidate the MADS-domain protein mode of action.

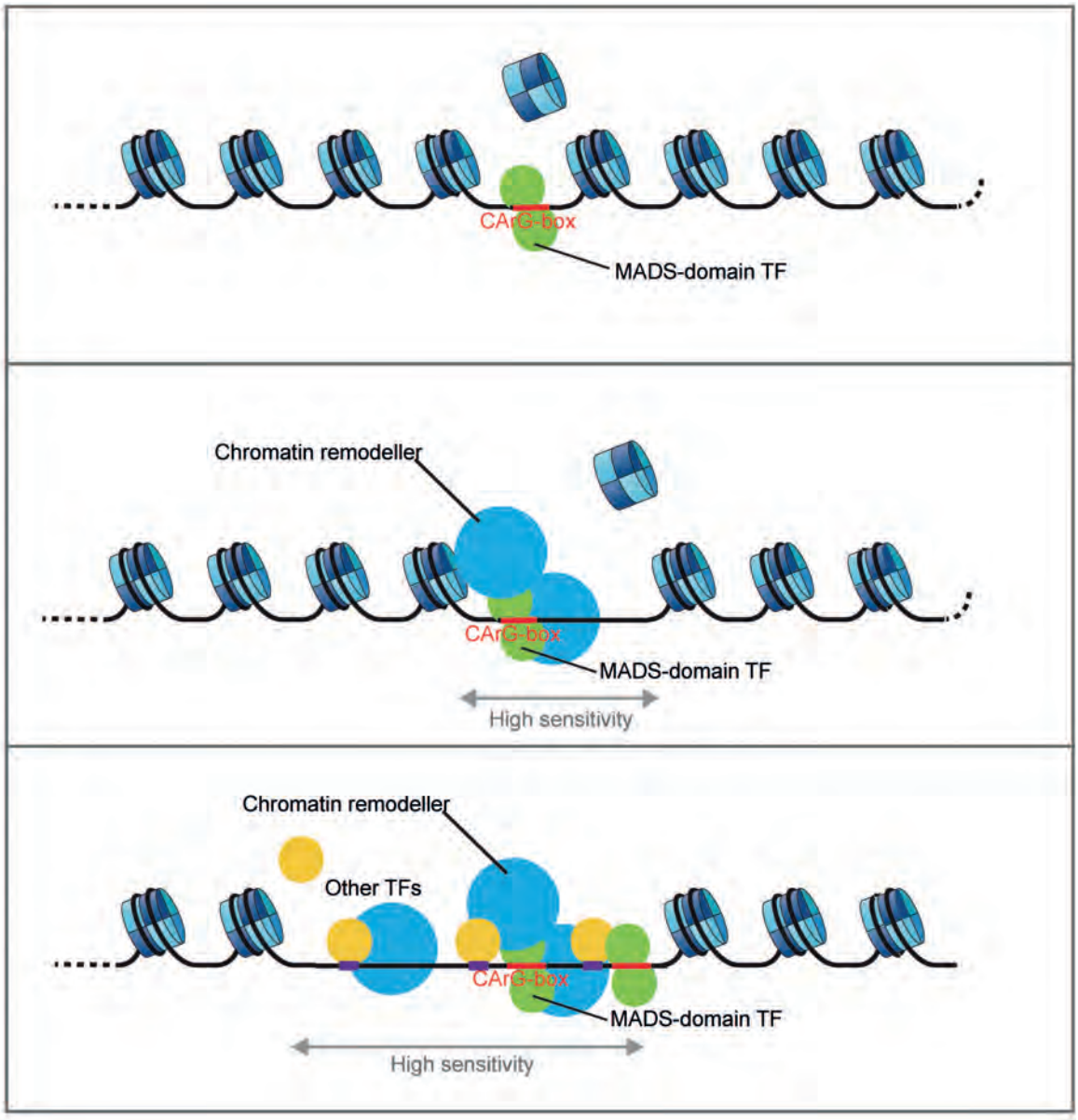

Figure 1. MADS-domain TFs mode of action. MADS domain proteins (green) bind to their target sites, the CArG-box sequences (red), prevalently in nucleosome depleted regions. MADS-domain TFs interact with chromatin remodellers (blue) to modify the chromatin landscape at their binding site surroundings. Chromatin becomes more accessible at MADS-domain TF bound regions and other TFs (yellow) may also bind to the DNA.

Given the important roles of MADS-domain proteins as master regulators of developmental switches and floral organ specification, their pioneer behaviour is an intriguing mode of action. But how do these proteins select regulatory regions at different stages of development? Based on the different properties of CArG-boxes that we found in SEP3- and AP1-bound regions (chapter 3), we propose that different MADS-domain protein dimers have different affinities for specific 'types' of CArG-boxes.

Another layer of specificity may be achieved via the modulation of higher-order complex formation. Developmental stage-specific change in chromatin conformation can lead to the formation of a specific higher-order complex that can drive gene activation. For example, a change in chromatin conformation of the *TFL1* locus is associated with gene repression (Liu *et al.*, 2013). New techniques, such as chromatin capture (Stadhouders *et al.*, 2013) and ChIA-Pet (Zhang *et al.*, 2012), can be used to describe cis-regulatory element interactions and characterise the proteins involved in gene regulation.

Perspectives

Information on epigenetic regulation of gene expression remains poorly represented in the current GRN models. Another complexity concerns spatial and temporal determinants. We know that genes can have different functions, depending on the tissue and stage at which they are expressed. For example, AP1 acts as a repressor of flowering time genes during flower initiation, while at later stages it activates genes involved in organ formation. Information about tissue and stage-specific gene expression (Jiao and Meyerowitz, 2010; Wellmer *et al.*, 2006) and protein-DNA binding (Gregis *et al.*, 2013; Pajoro *et al.*, 2014b) generates a better understanding of gene function and network dynamics. Most likely, more tissue and stage-specific information about protein-DNA binding will become available in the near future and will allow reconstruction of tissue and stage-specific GRNs. To achieve this resolution, novel technologies such as the INTACT system (Deal and Henikoff, 2011) and single cell approaches (Shapiro *et al.*, 2013) will be important.

Combined analysis of TF DNA-binding profiles and gene expression revealed a weak correlation between binding of a TF and changes in expression of its target genes (O'Maoileidigh *et al.*, 2014). An explanation could be that multiple TF binding events or co-factors are needed for gene regulation. In such a scenario only a specific combination

of TFs binding will trigger changes in expression. Sequential ChIP analysis (Oh *et al.*, 2012) or binding assays in mutant backgrounds (Kaufmann *et al.*, 2009) could be used to identify TF co-occupancy and obtain a better insight into the regulation of gene expression. Another explanation could be that a single binding event to a cis-regulatory element is not sufficient to drive expression and a conformational change of the DNA or chromatin. For instance, conformational changes are triggered by the binding of STK to multiple sites in the promoter of its target gene *VERDANDI* (Mendes *et al.*, 2013) and conformation change in gene looping of *TFL1*, *FT* and *FLC* locus regulates gene expression (Cao *et al.*, 2014; Jegu *et al.*, 2014; Liu *et al.*, 2013).

In conclusion, advances in genome-wide studies resulted in the confirmation of previously known genetic interactions at the molecular level, but also identified many novel regulatory interactions. We are just at the beginning of the genome-wide characterisation of GRNs and new factors and interactions are undoubtedly waiting to be discovered. A challenge for the near future will be to unravel the spatial and temporal regulation of the genes in the current networks.

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SUMMARY

During the life cycle, a plant undergoes a series of developmental phase changes. The first phase change is the transition from the initial juvenile vegetative stage into the adult vegetative phase. During the juvenile phase plants produce leaves and axillary buds, whereas during the adult phase the initiation of reproductive structures occurs. The next developmental change is the switch from vegetative to reproductive growth, when the shoot apical meristem acquires the identity of an inflorescence meristem that will then produce floral meristems. *Arabidopsis* floral meristems produce four concentric whorls of floral organs: sepals, petals, stamens and carpels. Each developmental change is controlled by coordinated network of regulators, known as gene regulatory networks (GRNs), which determine the transcription of a specific set of genes. The aim of the study presented in this thesis was to understand the dynamics of GRNs during floral organ development in Arabidopsis and correlate the binding of key regulatory MADS domain transcription factors with the accessibility of the chromatin in a genome-wide context.

In **chapter 1 and 2** we reviewed the current knowledge on the regulation of transcription in the model plant *Arabidopsis thaliana*. In chapter 1 we mainly focus on how the view of the GRN underling flower development has changed during the last decades, while in chapter 2 we more broadly revised the mechanisms that control developmental switches in plants. The recent introduction of next-generation sequencing and genome-wide approaches has changed our view on gene regulation and GRNs. We moved from linear genetic interactions towards global highly connected gene networks. The high numbers of interactions that were detected in protein-DNA binding profiles revealed a much higher network complexity than previously anticipated and demonstrated that master regulators of development not only control another layer of regulators, but also genes encoding structural proteins, enzymes and signalling proteins. Moreover, most transcription factors bind to their own locus, highlighting that auto-regulatory loops are a common mechanism of regulation.

The discovery of interactions between transcriptional master regulators with epigenetic factors provides new insights into general transcriptional regulatory mechanisms. Switches of developmental programmes and cell fates in complex organisms are

controlled at the level of gene expression by the combined action of chromatin regulators and transcription factors.

Although many master regulators of meristem and organ identities have been identified, it is still not well understood how they act at the molecular level and how they can switch an entire developmental program in which thousands of genes are involved. Using flower development as a model system, in **chapters 3 and 4** we investigated general concepts of transcription regulation by analysing the dynamics of protein-DNA binding, chromatin accessibility and gene expression.

Using an inducible system for synchronised flower formation, we characterised DNA-binding profiles of two MADS-domain transcription factors, APETALA1 (AP1) and SEPALLATA3 (SEP3), at three stages of flower development. Our study revealed that these MADS-domain proteins, select their binding sites, and thereby their target genes, in a partly stage-specific fashion. By combining the information from DNA-binding and gene expression data, we proposed models of stage-specific GRNs in flower development. Since developmental control of gene expression is tightly linked with dynamic changes in chromatin accessibility, we identified DNase I hypersensitive sites (DHSs, chapter 3) and we characterised nucleosome occupancy (chapter 4) at different stages of flower development. We observed dynamics in chromatin landscape manifested in increasing and decreasing DHSs as well as in changes in nucleosome occupancy and position.

Next, we addressed the question how MADS-domain protein stage-specific binding is achieved at the molecular level in a chromatin context. In the nucleus the DNA is wrapped around histone octamers to form nucleosomes, which are then packed into highly dense structures, and hence transcription factor binding sites may not be easily accessible. A result of the combined analysis of MADS-domain binding and chromatin dynamics is that MADS-domain proteins bind prevalently to nucleosome depleted regions, and that binding of AP1 and SEP3 to DNA precedes opening of the chromatin, which suggests that these MADS-domain transcription factors may act as so-called “pioneer factors”.

The isolation and analysis of developing flowers of specific stages increased the specificity of our genome-wide experiments, enabling the identification of novel actors in the GRN that regulates flower development. In this thesis we characterised the role of

some novel regulators in more detail: in **chapter 3** we focussed on the *GROWTH REGULATING FACTOR* (*GRF*) family genes; in **chapter 5** we investigated the action of *STERILE APETALA* (*SAP*); and in **chapter 6** we elucidated the regulation and the role of a member of the *WUSCHEL*-related homeobox (*WOX*) family, *WOX12*. GRF family genes are dynamically bound by AP1 and SEP3 at the different stages of flower development. All family members are bound by SEP3, while only a subset of the genes is bound by AP1. The defects in floral organs observed upon down-regulation of these genes highlight their role down-stream of MADS-domain transcription factors. In addition to AP1 and SEP3, *SAP* is also a target of other MADS-domain proteins, such as APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG). *SAP* is strongly expressed in meristems and loss of function of *SAP* causes strong aberrations in flowers, such as a reduction in petal and stamen numbers. We found that SAP interacts with proteins of the SCF ubiquitin ligase complex, suggesting that SAP could act in the ubiquitination pathway.

WOX12 down-regulation leads to defects in floral organ identity specification with the formation of stamenoid-petals, while ectopic expression of *WOX12* leads to an opposite effect: it causes the formation of petaloid-stamens in the third whorl. *WOX12* acts downstream of AP1. Ectopic expression of *WOX12* leads to reduction of *AG* expression, suggesting a role for *WOX12* in the antagonistic interplay between the homeotic genes *AP1* and *AG*.

In **chapter 7** we discuss the findings of this thesis. Taken together, the work performed in this thesis increased our knowledge on the GRN that regulates flower development and on the mode of action of MADS-domain transcription factors. We hypothesise that MADS-domain proteins may act as pioneer factors, proteins that access and remodel condensed chromatin. However, differently from other pioneer factors, MADS-domain transcription factors do not actively deplete nucleosomes, but instead they interact with chromatin remodelers to shape chromatin landscape. Given the important roles of MADS-domain proteins as master regulators of developmental switches, their pioneer behaviour represents an intriguing mode of action.

SAMENVATTING

Planten ondergaan een serie aan ontwikkelingsfases tijdens hun levenscyclus. De eerste faseverandering is de overgang van het initiële juveniele vegetatieve stadium naar het volwassen vegetatieve stadium. Tijdens het juveniele stadium produceert de plant bladeren en okselknoppen, terwijl in het volwassen stadium reproductieve groei wordt geïnitieerd. De daaropvolgende ontwikkelingsovergang is de faseverandering van vegetatieve naar reproductieve groei. Tijdens deze overgang verwerft het scheut apicale meristeem (SAM) de identiteit van bloeiwijze-meristeem welke bloemen produceert. De bloem meristemen in Arabidopsis produceren bloemorganen in vier concentrische ringen: kelkbladeren, kroonbladeren, meeldraden en vruchtbladeren. Elke ontwikkelingsfase wordt gecontroleerd door een gecoördineerd netwerk van regulatoren, bekend als gen regulatie netwerken (GRN) die de expressie van een specifieke set genen beïnvloeden. In dit proefschrift vergroten we de kennis van de dynamiek van het GRN dat de ontwikkeling van bloemorganen in Arabidopsis reguleert. Daarnaast correleren we de DNA-binding van MADS-domein eiwitten aan de toegankelijkheid van het chromatine in een genoom-wijde context.

In **hoofdstuk 1 en 2** geven we een overzicht van de huidige kennis van transcriptionele regulatie in de modelplant *Arabidopsis thaliana*. In hoofdstuk 1 besteden we vooral aandacht aan hoe het beeld van GRN tijdens bloemontwikkeling is veranderd tijdens de laatste decennia. Hoofdstuk 2 geeft een breder overzicht van de mechanismen die ontwikkelingsovergangen reguleren in planten. De recente introductie van het nieuwe-generatie sequencen en de genoomwijde aanpak heeft onze blik op genregulatie en GRN veranderd. Lineaire genomische netwerken zijn overgegaan in veel bredere netwerken met een groot aantal interacties. Het grote aantal aan interacties die we detecteren met eiwit-DNA bindingsprofielen toont aan dat de complexiteit van netwerken veel hoger is dan voorheen geanticipeerd was. Deze bindingsprofielen laten zien dat sleutel regulatoren van ontwikkeling niet alleen andere lagen van regulatoren controleren, maar ook genen die coderen voor structurele eiwitten, enzymen en signaaleiwitten. Ook binden de meeste transcriptiefactoren hun eigen locus; dit onderstreept dat auto-regulatie een veelvoorkomend fenomeen is.

De ontdekking dat transcriptionele sleutel regulatoren interacties kunnen aangaan met epigenetische factoren geeft nieuwe inzichten in algemene trancriptionele mechanismen. Veranderingen van ontwikkelingsprogramma's en celidentiteit in complexe organismen zijn gereguleerd op het niveau van genexpressie via het gecombineerde werk van chromatine regulatoren en transcriptiefactoren.

Hoewel de meeste sleutel regulatoren van meristeem- en orgaanidentiteit zijn geïdentificeerd, is nog niet bekend hoe deze op moleculair niveau werken en hoe deze sleutel regulatoren in staat zijn volledige ontwikkelingsprogramma's, waarin duizenden genen betrokken zijn, te veranderen. Door het gebruik van bloemontwikkeling als model systeem, in **hoofdstuk 3 en 4**, wordt transcriptionele regulatie onderzocht middels de analyse van dynamische eiwit-DNA binding, chromatine toegankelijkheid en genexpressie.

Het gebruik van een induceerbaar mechanisme voor bloemformatie maakte het mogelijk de DNA-bindingsprofielen van twee MADS-domein transcriptiefactoren, APETALA1 (AP1) en SEPALLATA3 (SEP3), te karakteriseren tijdens drie stadia van bloem ontwikkeling. Onze studie laat zien dat deze MADS-domein eiwitten voor een deel hun DNA bindingsplaatsen en daarbij de genen die ze reguleren specifiek kiezen voor een bepaald ontwikkelingsstadium. Door data van DNA-binding en genexpressie te combineren hebben we een model ontwikkeld van stadium-specifieke GRN tijdens bloemontwikkeling. Omdat stadium-specifieke genexpressie sterk verbonden is met dynamische veranderingen in chromatine toegankelijkheid hebben we DNase I hypersensitieve plekken (DHP, hoofdstuk 3) en nucleosoom bezetting (hoofdstuk 4) onderzocht tijdens verschillende stadia van bloemontwikkeling. We hebben aangetoond dat dynamiek in het chromatine landschap zich manifesteert als verhogingen en verlagingen van DHP en ook veranderingen in nucleosoom bezetting en positie.

In een volgende stap hebben we op moleculair niveau bestudeerd hoe de status van chromatine van invloed is op hoe MADS-domein eiwitten stadium-specifieke binding bereiken. DNA in de celkern is gewonden rond nucleosomen bestaande uit een achttal histonen, welke vervolgens nog compacter zijn georganiseerd. Door deze compacte organisatie zijn bindingsplaatsen van transcriptiefactoren slecht toegankelijk. Resultaten van de gecombineerde analyse van MADS-domein DNA-binding en chromatine dynamiek laten zien dat MADS-eiwitten bij voorkeur binden aan regio's met weinig

nucleosomen en dat de DNA binding van AP1 en SEP3 vooraf gaat aan opening van het chromatine. Dit suggereert dan deze MADS-domein eiwitten acteren als zogenaamde “pionier factoren”.

Doordat we specifieke stadia van bloemontwikkeling hebben gebruikt voor de analyse is de specificiteit van ons genoom-wijde experimenten vergroot. Deze specificiteit maakt het mogelijk om nieuwe factoren in het GRN van bloemontwikkeling te identificeren. In dit proefschrift karakteriseren we de rol van aan drietal nieuwe regulatoren in meer detail: in **hoofdstuk 3** richten we ons op GROWTH REGULATING FACTOR (GRF) family genen; in **hoofdstuk 5** bestuderen we de actie van STERILE APETALA (SAP); en in **hoofdstuk 6** verduidelijken we de regulatie en de rol van een lid van de WUSCHEL-gerelateerde homeobox (WOX) familie, WOX12. GRF familie genen zijn dynamisch gebonden door AP1 en SEP3 op verschillende stadia van bloem ontwikkeling. Alle (WOX)-familieleden zijn gebonden door SEP3, terwijl maar een deel van de genen is gebonden door AP1. Het belang van deze doelgenen van MADS-domein eiwitten wordt onderschreven doordat verminderde expressie van de GRF genen resulteert in bloemorgaan defecten. *SAP* is naast een doelgen van AP1 en SEP3 ook een doelgen van andere MADS-domein eiwitten zoals APETALA3 (AP3), PISTILLATA (PI) en AGAMOUS (AG). *SAP* komt sterk tot expressie in het meristeem. Functionele mutanten van *SAP* zorgen voor sterke afwijkende bloemen, zoals bloemen met een verminderd aantal kroonbladeren en meeldraden. We hebben aangetoond dat SAP interacties aangaat met eiwitten van het SCF ubiquitine ligase complex, suggererend dat SAP een functie heeft in eiwit afbraak.

Een verminderde *WOX12* expressie leid tot defecten in de specificatie van bloemorgaan identiteit met als resultaat de formatie van meeldraad-achtige kroonbladeren. Daarentegen leidt ectopische expressie van *WOX12* tot een tegenovergesteld effect: de formatie van kroonbladeren-achtige meeldraden. *WOX12* wordt gereguleerd door AP1 en ectopische expressie van *WOX12* leidt tot reductie van *AG* expressie, dit suggereert een rol voor WOX12 in het antagonistische samenspel tussen de homeotische genen *AP1* en *AG*.

In **hoofdstuk 7** worden de bevindingen van het proefschrift besproken. Samenvattend, het werk uitgevoerd voor dit proefschrift vergroot onze kennis van het GRN dat bloemformatie reguleert en de werkingswijze van MADS-domein transcriptiefactoren. We komen met de hypothese dat MADS-domein eiwitten een functie

hebben als pionier factoren; eiwitten die DNA in dicht chromatine kunnen binden en de toegankelijkheid ervan kunnen modeleren. Maar anders dan andere pionier factoren worden nucleosomen niet actief verminderd door MADS-domein transcriptie factoren. Ze gaan daarentegen interacties aan met zogenaamde “chromatin remodelers” om het chromatine landschap te modeleren. Het belang van MADS-domein eiwitten als sleutel regulatoren van ontwikkelingsprocessen en hun rol als pionier factoren geeft hun intrigerende manier van werken aan.

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Alice Pajoro was born on June 4th , 1984, in Milano, Italy. After finishing the high school at "Liceo A. Volta" in Milan in 2003, she started her study in Plant Biotechnology at Universitá degli Studi di Milano. For her BSc thesis research project in 2006, Alice joined the laboratory of Plant Genetics, and worked on the characterization of fused leaves mutant (*fdl*) in *Zea mays*. in the group of Prof G. Consonni and Prof G. Gavazzi.

In 2007, during her MSc in Agricultural Biotechnology, she visited the University of Valencia as exchange student (Erasmus fellowship). In 2008 she joined Prof. Martin Kater's group at Milan University to work on rice. Working with Dr L. Dreni she contributed to unravel the role of *OsMADS13* and *OsMADS21* in rice ovule and kernel development. Soon after obtaining her MSc degree in Plant Food and Environmental Biotechnology with *cum laude* in 2009, Alice joined the Laboratory of Plant Developmental Systems at Wageningen University. Her PhD project was supported by the Marie Curie fellowship via the SYSFLO network, where Alice was under the mentorship of Prof G.C. Angenent and Dr K. Kaufmann. Her research on the regulation of gene transcription during flower development in *Arabidopsis thaliana* resulted in the publication of this thesis.

In 2014, Alice joined the group of Prof R. Immink, where she studies the role of ambient temperature in the regulation of flowering time in *Arabidopsis thaliana*.

List of Publications:

Kaufmann K, **Pajoro A**, Angenent GC (2010) *"Regulation of transcription in plants: mechanisms controlling developmental switches."* **Nature Reviews Genetics** 11, 830–842

Dreni L, Erreni S, Caporali E, **Pajoro A**, Pilatone A, Yun D, Zhang D, Kater MM (2011) *"Functional analysis of all AGAMOUS subfamily members in rice reveals their roles in reproductive organ identity determination and meristem determinacy."* **Plant Cell** 23, 2850-2863

Pajoro A, Madrigal P, Muiño JM, Matus JT, Jin J, Mecchia MA, Debernardi JM, Palatnik JF, Balazadeh S, Arif M, Ó'Maoiléidigh DS, Wellmer F, Krajewski P, Riechmann JL, Angenent GC, Kaufmann K (2014) *"Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development."* **Genome Biology** 15:R41

Pajoro A*, Biewers S*, Dougali E*, Leal Valentim F*, Mendes MA*, Porri A*, Coupland G, Van de Peer Y, van Dijk ADJ, Colombo L, Davies B, Angenent GC (2014) *"The (r)evolution of gene regulatory networks controlling Arabidopsis plant reproduction, a two decades history."* **Journal of Experimental Botany** 65 (17): 4731-4745

Mourik H, Muiño JM, **Pajoro A**, Angenent GC, Kaufmann K (2015) *"Characterization of In Vivo DNA-Binding Events of Plant Transcription Factors by ChIP-seq: Experimental Protocol and Computational Analysis"* Plant Functional Genomics: Methods and Protocols, **Methods in Molecular Biology**, vol. 1284

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Alice Pajoro
Date: 12 March 2015
Group: Bioscience & Molecular Biology
University: Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Systems Biology Applied to Flowering	May 20, 2010
► **Writing or rewriting a project proposal**	
Transcriptional networks underlying Arabidopsis floral development	Jan 2010
► **Writing a review or book chapter**	
"Regulation of transcription in plants: mechanisms controlling developmental switches" Nature Reviews Genetics, vol 11 (Dec. 2010, p. 830	Dec 2010
"The (r)evolution of gene regulatory networks controlling Arabidopsis plant reproduction: a two-decade history" Journal of Experimental Botany, vol. 65 (June 2014), p. 4731-4745	Jun 2014
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*9.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day Wageningen, NL	May 20, 2011
EPS PhD student day Amsterdam, NL	Nov 30, 2012
EPS theme symposia	
EPS theme 1 symposium "Developmental Biology of Plants" Wageningen, NL	Jan 28, 2010
► EPS theme 1 symposium "Developmental Biology of Plants" Leiden, NL	Jan 20, 2011
EPS theme 1 symposium "Developmental Biology of Plants" Wageningen, NL	Jan 19, 2012
EPS theme 1 symposium "Developmental Biology of Plants" Leiden, NL	Jan 17, 2013
EPS theme 4 symposium "Genome Biology" Wageningen, NL	Dec 03, 2014
EPS theme 1 symposium "Developmental Biology of Plants" Leiden, NL	Jan 08, 2015
► **NWO Lunteren days and other National Platforms**	
ALW Meeting Experimental Plant Sciences Lunteren, NL	Apr 19-20, 2010
ALW Meeting Experimental Plant Sciences Lunteren, NL	Apr 04-05, 2011
ALW Meeting Experimental Plant Sciences Lunteren, NL	Apr 03-04, 2012
10th Dutch Chromatin Meeting, Amsterdam, NL	Oct 24, 2012
ALW Meeting Experimental Plant Sciences Lunteren, NL	Apr 22-23, 2013
ALW Meeting Experimental Plant Sciences Lunteren, NL	Apr 14-15, 2014
► **Seminars (series), workshops and symposia**	
Inv. Seminar Daniel Schubert "Dynamic control of histone methylation and plant cell fate by Polycomb-group proteins"	May 11, 2010
Inv. Seminar Peter Cook "Transcription factories as organizers of the genome: the role of fixed polymerases"	Oct 27, 2010
Inv. Seminar Kirsten Bomblies "Genetic incompatibility and the plant immune system"	Nov 19, 2010
Inv. Seminar Megan Adriankaja "Systems Biology of Yield"	Feb 03, 2011
Inv. Seminar Isabella Nougalli Tonaco " The use of chemical genomics to study light signaling and growth processes in Arabidopsis."	Apr 15, 2011
Inv. Seminar Javier Palatnik 'Biogenesis and function of plant microRNAs'	Aug 25, 2011
Illumina Seminar Series	Oct 07, 2011
Inv. Seminar Rainer Melzer "Minor groove - major impact? Towards analysing the DNA-binding specificity of SEPALLATA3."	Apr 23, 2012
Inv. Seminar Doris Wagner "Auxin mediated organogenesis in Arabidopsis"	Jul 10, 2012
Inv. Seminar Ortrun Mittelsten Sheid "Genetics and epigenetics: a complex relationship"	Nov 19, 2014
► **Seminar plus**	
Fabio Fornara (invited lecturer theme 1 day 2010)	Jan 28, 2010
► **International symposia and congresses**	
2nd European Joint Retreat for PhD students in Plant Sciences, Koln, GE	Apr 14-17, 2010
1st SYSFLO meeting, Palermo, IT	May 27-28, 2010
3rd International PhD School "Plant Development", Retzbach, GE	Oct 06-08, 2010
2nd SYSFLO meeting, Berlin, GE	Feb 08-09, 2011
Mid Term SYSFLO Review Meeting, Matarea, IT	Jun 13-14, 2011
International Workshop Molecular Mechanisms Controlling Flower Development, Maratea, IT	Jun 14-17, 2011
3rd SYSFLO Network Meeting, Grenoble, FR	Feb 02, 2012
3rd EMBO Conference on Plant Molecular Biology "Plant Development & Enviromental Interactions"	May 27-30, 2012
5th International PhD School in Plant development, Siena, IT	Sep 25-28, 2012
Final SYSFLO Network meeting, Norwich, UK	Jan 31, 2013
Workshop on Molecular Mechanisms Controlling Flower Development, Presqu'ile de Giens, FR	Jun 08-12, 2013
3rd European Workshop on Plant Chromatin, Madrid, SP	Aug 29-30, 2013

► **Presentations**	
Talk: 1st SYSFLO project report: "Dynamics of target-gene regulation by floral MADS-box transcription factors" Palermo, IT	May 28, 2010
Talk "Investigation of MADS-box transcription factors dynamics in target-gene regulation drive to the identification of new actors in flower development", Leiden, NL	Jan 20, 2011
Talk: 2nd SYSFLO Project report Berlin, GE	Feb 09, 2011
Talk: Mid Term SYSFLO Project report, Maratea, IT	Jun 14, 2011
Talk: 3rd SYSFLO Project report, Grenoble, FR	Feb 02, 2012
Talk: "Dynamic target-gene reguation by MADS-box transcription factors during flower development" Siena,	Sep 26, 2012
Talk: "Dynamics of chromatin accessibility and gene regulation by MADS-box transcription factors in flower development" Amsterdam, NL	Oct 24, 2012
Talk: Final SYSFLO Project report, Norwich, UK	Jan 31, 2013
Talk: "Dynamics of gene regulation in flower development" Potsdam, GE	Apr 17, 2013
Talk: "Dynamics of gene-regulatory networks controlling flower development" Lunteren, NL	Apr 23, 2013
Talk: Flash Poster Presetation, Presqu'ile de Giens, FR	Jun 10, 2013
Talk: "Dynamics of chromatin accessibility during flower development from a transcription factor point of view" Madrid, SP	Aug 30, 2013
Talk: "Dynamics of chromatin strucure and gene regulation by MADS-domain transcription factors" Golm,	Dec 11, 2014
Poster: "Dynamics of target-gene regulation by floral MADS-box transcription factors" Koln, GE	Apr 14-17, 2010
Poster: "Dynamics of target-gene regulation by floral MADS-box transcription factors" Maratea, IT	Jun 13-14, 2011
Poster: "A dynamic view of TF regulation during flower development" Matera, IT	May 27-30, 2012
Best Poster Award EPS "Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development" Lunteren, NL	Apr 22-23, 2013
Poster "Dynamics of chromatin accessibility and gene regulation by MADS-domain transcription factors in flower development" Presqu'ile de Giens, FR	Jun 08-12, 2013
► **IAB interview**	
Meeting with a membe of the International Advisory Board of EPS	Nov 14, 2012
► **Excursions**	
Subtotal Scientific Exposure	*33.5 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS PhD course "Bioinformatics, A user's approach", Wageningen, NL	Aug 30-Sep 03, 2010
European Networking Summer School Plant Epigenetic, Gatasleben, GE	Sep 20-24, 2010
EMBL Pratical Course Next Generation Sequencing Data Analysis, Heidelberg, GE	Oct 12-13, 2010
ESR Training Course on Gene Regulation Analysis Tools and IP / Commercialization, Wolfenbuttel, GE	Nov 25-26, 2010
EPS PhD course "Transcription Factors", Leiden,NL	May 09-11, 2011
► **Journal club**	
Participated in literature descussion group	2009-2013
► **Individual research training**	
TF platform - MPI Golm	Nov 07-18, 2011
Subtotal In-Depth Studies	*11.1 credits**

4) Personal development	*date*
► **Skill training courses**	
PhD competence assessment	Jan 19 & Feb 16, 2010
Mini Symposium: "How to write a world-class paper", Wageningen, NL	Oct 26, 2010
SYSFLO ESR Training Course "Getting published and Completing your PhD", Leeds, UK	Sep 07th-09, 2011
EPS PhD course"Project & Time Management", Wageningen, NL	Jan 10, 24-Feb 21, 2012
SYSFLO ESR training course "Grant writing", Grenoble, FR	Feb 03, 2012
EPS PhD course "Scientific writing"	Mar-May 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**58.5**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	

** A credit represents a normative study load of 28 hours of study.*

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Thesis layout and cover design by the author
On the cover: DNA-binding profiles of APETALA1 (front) and SEPALLATA 3 (back) along the chromosome 3 (spine) of Arabidpsis.

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