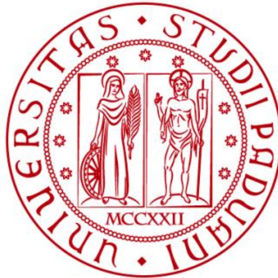
UNIVERSITY OF PADUA
Department of Biology



PhD School in Bioscience and Biotechnology
Curriculum: Biotechnology
XXVI cycle

APPLE AND STRAWBERRY MADS-BOX GENES AND THEIR FUNCTION IN PLANT DEVELOPMENTAL PATHWAYS

Director of the School: Ch.mo Prof. Giuseppe Zanotti

Coordinator of the curriculum: Ch.mo Prof. Fiorella Lo Schiavo

Supervisor at University of Padua: Ch.mo Prof. Fiorella Lo Schiavo

Supervisor at Edmund Mach Foundation: Dr. Azeddine Si Ammour

PhD Student: Giulia Valentina Miolli

ABSTRACT

The main role of MADS-box transcription factors in plant developmental processes has been well described in the model plant *Arabidopsis thaliana*. However, little is known about their function in crops of important agricultural and commercial value. Our study aims to investigate their role in two agronomical relevant *Rosaceae* crops: apple (*Malus* x *domestica* Borkh.) and strawberry (*Fragaria vesca*).

Expression studies using qPCR and RNA seq have identified two apple Dormancy Associated MADS-box (*DAM*) genes. They group with the StMADS11 clade, and were named *MdDAM1* and *MdDAM2,* the last one discovered *ex novo.* Real time expression studies in dormant buds collected during the chilling period and chromatin immunoprecipitation (ChIP) analyses confirmed that the genes are downregulated by exposure to cold and *MdDAM1* is epigenetically repressed, as it has been demonstrated for *Arabidopsis FLC* and peach DAM genes.

In parallel we worked on strawberry MADS-box genes of known function involved in flower development. We chose three MADS-box genes that are homologs of *Arabidopsis PISTILLATA* and A*GAMOUS* to perform gene expression and functional analysis using a RNA interference approach to obtain post-transcriptional gene silencing. The positive transgenic lines of each transformation were evaluated at the molecular and phenotypic level. Single gene mutants does not show altered flower phenotype, suggesting a different mechanism of flower development in strawberry, probably due to the peculiar flower structure.

ABSTRACT - ITALIAN

Il ruolo fondamentale svolto dai fattori di trascrizione MADS-box nei diversi processi di sviluppo delle piante è stato descritto in dettaglio nell'organismo modello *Arabidopsis thaliana*. Tuttavia la loro funzione in colture di maggior valore agricolo e commerciale rimane da indagare. La presente ricerca si propone di comprendere il loro ruolo in due colture agronomicamente importanti appartenenti alle Rosacee: melo (*Malus x domestica* Borkh.) e fragola (*Fragaria vesca*).

Studi dell'espressione genica attraverso Real time PCR e RNA-seq hanno permesso l'identificazione di due geni di melo appartenenti ai geni DAM (Dormancy Associated MADS-box). I due geni appartengono alla *clade* StMAD11 e sono stati denominati *MdDAM1* e *MdDAM2,* quest'ultimo scoperto *ex novo*. Analisi di espressione con Real time PCR in gemme dormienti raccolte durante il periodo invernale e studi di immunoprecipitazione di cromatina (ChIP) hanno confermato che i geni sono silenziati in seguito all'espozione al freddo. Inoltre si è provato che solo *MdDAM1* è epigeneticamente represso, come era stato in precedenza dimostrato in *Arabidopsis* per il gene *FLC* e in pesca per i geni *DAM*.

In parallelo si è lavorato su alcuni geni MADS-box di fragola, di cui era nota la funzione, coinvolti nello sviluppo del fiore. Tra questi geni ne sono stati scelti tre, i probabili omologhi di P*ISTILLATA* e *AGAMOUS* in *Arabidopsis*, per svolgere sia analisi di espressione, sia analisi funzionali che sfruttano l'approccio di RNA *interference* per ottenere silenziamento genico post-trascrizionale. Le linee transgeniche risultate positive sono state valutate a livello molecolare e fenotipico. Il silenziamento dei singoli geni non ha mostrato alterazioni nello sviluppo del fiore, suggerendo un diverso meccanismo coinvolto nello sviluppo del fiore in fragola, probabilmente a causa della sua particolare strutture.

Chapter I

General Introduction

I.1 MADS-box transcription factors in plants

Regulation of gene expression is a molecular mechanism found in all living organisms that allows to adjust appropriate gene activity in response to stimuli and during development (Yant, 2012). For instance, homeotic genes are transcription factors (TFs) regulating gene activity during body and organ formation in both animals and plants (Gehring et al., 1994; Meyerowitz, 2002). In plants, homeotic genes are represented by a class of transcription factors named MADS containing proteins or MADS-box genes. These genes are one of the most extensively studied transcription factors in plants (Smaczniak et al., 2012). MADS acronym derives from MINICHROMOSOME MAINTENANCE 1 (MCM1), AGAMOUS (AG), DEFICIENS (DEF), and serum response factor (SRF) genes that encode TFs that share a common highly conserved DNA-binding domain in yeast, Arabidopsis, *Antirrhinum*, and humans, respectively (Norman et al., 1988; Passmore et al., 1988; Sommer et al., 1990; Yanofsky et al., 1990). MADS-box genes have been identified only in eukaryotes (Gramzow et al., 2010). The MADS domain is composed of 55 to 60 amino acid residues that fold into N-terminal protruding extension, followed by amphipathic alpha-helix, and two antiparallel beta-strands (Gramzow et al., 2010). MADS-box transcription factors specifically recognize similar target DNA sequences named CArG boxes [$CC(A/T)_6GG$] (Taylor et al., 1989). Based on phylogenetic analyses, the members of the MADS-box family have been divided into two main classes (type I and II) originated after an ancient duplication of a common eukaryotic ancestor. In animal and fungi type I SRF-like proteins contain a conserved region, referred to as SAM, whereas type II MEF2-like (*MYOCYTE ENHANCER FACTOR 2*) proteins contain a conserved MEF2 domain (*Figure 1*) (Shore and Sharrocks, 1995). In plants, type I MADS domain proteins contain a SRF-like domain similar to the one found in animals and fungi whereas type II contain a *MEF2*-like domain followed by an Intervening (I) region, a Keratin-like (K) domain, and a conserved C-terminal (C) domain and are therefore named MIKC-type (*Figure 1*) (Alvarez-Buylla et al., 2000b). The I-region is composed of ~30 weakly conserved amino acids that contribute to dimerization. The K domain is composed of ~70 amino acids which folds into a coiled-coil structure that facilitates dimerization. The C domain has variable length and is involved in transcriptional activation or multimeric complexes formation (Ng and Yanofsky, 2001).

Plant type II genes have been further categorized into $MIKC^c$- and MIKC*-type based on structural features (Henschel et al., 2002). Members of the two different classes diverge from each other especially in the I-regions and to less extent in the K-boxes. MIKC*-type genes encode longer I-domains than $MIKC^C$-type, the former usually span from four to five exons, while the latter are rather encoded by one exon (Henschel et al.,

2002). The MIKC^c genes have been further classified into 12 clades based on phylogeny (Becker and Theissen, 2003). Type I genes have been categorized into M- and N-type based on the protein motifs (De Bodt et al., 2003) and also as Mα, Mβ, Mγ and Mδ, based on the phylogenetic relationships between MADS-box regions (Parenicova, 2003). The Mδ group, can also be referred as the MIKC* class.

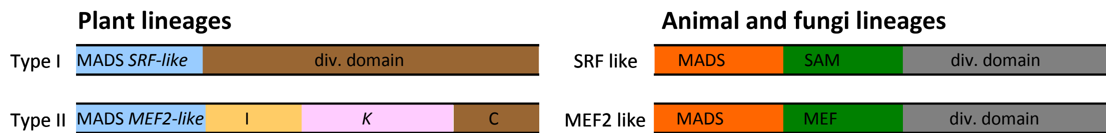


Figure 1 - Type I and type II MADS-box proteins

Plant type I MADS-box genes group with animal SRF-like genes. Plant type II MADS-box genes resemble animal MEF2 genes in their MADS domain. They contain the **Keratin-like (K)** domain, a conserved coiled-coil structure which facilitates dimerization. The MADS-box and K-domain are separated by a weakly conserved **Intervening (I)** domain, which contributes to dimerization. The conserved **C-terminal (C)** domain can contain transactivation domain or contributes to formation of multimeric MADS-box protein complexes.

Early phylogenetic studies of MADS-box lineages suggested that one MADS-box gene was present in the common ancestor of plants, animals, and fungi, and that a probable duplication occurred after animals diverged from plants (Theissen et al., 1996). The duplication gave rise to MEF2- and SRF-like lineages of MADS-box genes in animals. Even though the conclusions were convincing, these studies were conducted with a limited number of genes, especially of plant genes. Some years later Alvarez-Buylla et al. (2000b) extended the sample size to a wider range of genes from different organisms and their finding suggested that an ancestral duplication, occurred before the divergence of plants and animals, gave rise to two lineages. The authors named them type I and type II implying that the protein motifs that define each group were already fixed in the common ancestors of plants, fungi, and animals. They were able to associate most functionally characterized plant MADS-box genes to the animal MEF2-like lineage and identified them as type II. Moreover, they identified a clade of plant MADS-box genes that seemed to be closely related to SRF-like genes forming the group called type I. Thus, confirming the presence of the two lineages in plants, animals, fungi. At last it was also shown that only type II plant MADS-box genes contain the so called K-domain, suggesting that this domain evolved in plants only after the divergence from type I lineage.

Phylogenetic analyses have already shown that proliferation of MADS-box genes in flowering plants hold its origin in gene duplication (Theissen et al., 1996; Parenicova,

2003). MADS-box genes of type II involved in flower development duplicated to a great extent through several whole genome duplication (WGD) events (Causier et al., 2005). On the other hand, the duplications that gave rise to the type I MADS-box genes are generally associated with local duplication events (Nam et al., 2004). Following a WGD, the duplicated genes can have very different fates: loss, subfunctionalization, neofunctionalization, and a combination of the last two. Duplicated genes are not under selective pressure. In condition of single gene duplications or duplication involving only few genes, one copy of the gene accumulates deleterious mutations and its function is gradually lost over time. In contrast genes involved in networks, in signal transduction, and components of multimeric complexes are preferentially retained to maintain the necessary gene balance (Blanc and Wolfe, 2004; Seoighe and Gehring, 2004; Edger and Pires, 2009). Duplicated genes retained after WGD can evolve and partition existing function (subfunctionalization) or acquire new ones (neofunctionalization). The latter mechanism increases the biological complexity assuming a new evolutionary key role in a process (Airoldi and Davies, 2012). Usually the diversification of the function occurs through mutation of the regulatory regions, changing the spatial-temporal expression of the gene or modulating its activity. Evolution of the type II MADS-box genes belonging to class B and class C genes provides examples of all three post-duplication fates (Airoldi and Davies, 2012). For instance, the *PI/GLO* and paleo*AP3/DEF* are two B-function lineages that arose from a duplication that occurred about 280 million years ago, before the emergence of angiosperms (Purugganan et al., 1995; Kramer et al., 1998). A later duplication occurred in the paleoAP3/DEF and produced in eudicots two additional distinct lineages, *euAP3* and *TM6* (Kramer et al., 2006; Causier et al., 2010). The *TM6* lineage was lost in both *Arabidopsis* and *Antirrhinum* as synteny studies have demonstrated (Causier et al., 2010). A direct consequence of this loss is the dramatic phenotype observed in Arabidopsis *ap3* and *Antirrhinum glo* mutants demonstrating the role of MADS-box genes with B-function in petal and stamen development (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005). The *TM6* lineage genes have been maintained in other plant species, like tomato, tobacco and petunia, which contain *AP3* and *TM6* genes (Vandenbussche et al., 2004; de Martino et al., 2006). In contrast the *AP3* lineage has been lost in papaya that retains *TM6* only, suggesting that is possible to retain either or both of the *AP3/TM6* paralogs pair and still keep the possibility to produce flowers. Moreover, there is evidence of subfunctionalization in petunia and tomato (de Martino et al., 2006; Rijpkema et al., 2006).

The MADS-box genes with a C-function, which were shown to be involved in stamen and carpel development, provide another example of evolution through duplication events. A pair of genes referred as *AGAMOUS/FARINELLI* (*AG/FAR*[a]) and ancestral *PLENA/SHATTERPROOF* (*PLE/SHP*[a]) appeared through a WGD event that occurred 120 million ago (Airoldi and Davies, 2012). In *Antirrhinum* they evolved into *PLE*, which control both male and female organ development (Bradley et al., 1993), and *FAR*, which plays minor role in stamen development (Davies et al., 1999). In Arabidopsis they evolved into *AG*, controlling both male and female reproductive organs, and *SHP1*, *SHP2* that control seed pod shatter (Yanofsky et al., 1990; Angenent et al., 1993; Liljegren et al., 2000). *AG* and *SHP1/2* are the orthologs of FAR and PLE, respectively. The two *SHP* copies, *SHP1* and *SHP2*, arose from recent genome duplication in Brassicaceae that produced also a second copy of *AG* that has subsequently been lost. Similar events were observed also in monocots, such as rice and maize (Kramer et al., 2004; Yamaguchi et al., 2006). In contrast, type I MADS-box genes have been mainly duplicated by smaller scale and more recent duplications. The difficulty in finding orthologs of Arabidopsis type I genes in other plant species and the fact that the loci are confined to localized areas of two specific chromosomes support this hypothesis (Arora et al., 2007). As suggested by Walia et al. (2009), the most likely explanation for the lack of orthologs is that type I MADS-box genes are involved in the maintenance of species barrier and are therefore genus-specific. A direct consequence of duplication events that occurred in Arabidopsis is the presence of many copies of the same MADS-box genes leading to redundancy in biological function. A striking example is the redundant function of the four *SEP* genes acting throughout the whole process of flower development and A-function *APETALA1* and *CAULIFLOWER* (Irish and Sussex, 1990; Pelaz et al., 2000; Ditta et al., 2004). Moreover, studies with mutant combinations have demonstrated that MADS-box genes can have more than one function (pleiotropy) (Airoldi and Davies, 2012). For instance, the Arabidopsis *FRUITFUL* (*FUL*) gene that plays a specific role in carpel development showed an additive and pleiotropic effect by enhancing the production of leafy shoots meristem phenotype in the *ap1 cal ful* triple mutant (Ferrandiz et al., 2000). Another example is *FLOWERING LOCUS C* (*FLC*) that was originally described as a repressor of flowering in Arabidopsis in response to temperature and has been shown to have a pleiotropic effect on temperature dependent seed germination (Michaels and Amasino, 1999; Chiang et al., 2009).

I.2 Biological functions of MADS-box genes

Since the first study demonstrating the involvement of MADS-box transcription factors in floral organ identity in the model plants Arabidopsis and *Antirrhinum* was published, the role of this gene family in flowering plants has been widely investigated in different species (Coen and Meyerowitz, 1991). MADS-box genes were extensively studied in Arabidopsis revealing their role in different aspects of plant growth and development (*Table 1*). These include control of flowering time, meristem identity, floral organ identity, formation of dehiscence zone, fruit ripening, embryo development as well as development of vegetative organs such as root and leaf (*Table 1*) (Rounsley et al., 1995; Riechmann and Meyerowitz, 1997; Alvarez-Buylla et al., 2000a; Saedler et al., 2001; Moore et al., 2002). The MADS-box transcription factors involved in those processes have been widely studied in Arabidopsis and they all group with type II lineage but new insights are produced by the gradual characterization of type I genes.

The number of type I MADS-box genes in Arabidopsis is more important than type II genes, though their role has remained unclear until recently (reviewed by Masiero et al. 2011). The first Arabidopsis type I gene to be characterized was *AGL80/FEM1* that together with *DIANA* (*DIA/AGL61*) forms a protein dimer and control differentiation of the central cell during embryonic development (Portereiko et al., 2006). They are generally expressed during early stages of endosperm and embryo development and two subclasses of type I MADS-box genes are predominantly expressed in inflorescences and siliques (Day et al., 2008; Walia et al., 2009; Tiwari et al., 2010; Wuest et al., 2010). Many type I MADS-box genes are epigenetically repressed by PRC2-type polycomb group, for example *AGL23* that has a role in embryo sac development (Colombo et al., 2008) and PHE1, one of the first example of imprinting in plants, which has a parent-of-origin dependent expression (Kohler et al., 2005). The epigenetic regulation of many type I genes is supposed to contribute to post-zygotic compatibility and maintain species boundaries minimizing gene flow between species (Walia et al., 2009). Following the release of other plant genomes, type I MADS-box genes that group with the Arabidopsis one have been identified also in poplar, rice, and apple (Leseberg et al., 2006; Arora et al., 2007; Velasco et al., 2010).

As described previously in this chapter, there are two classes of type II MADS-box genes, named MIKC^c-type and MIKC*-type on the basis of structural features (Henschel et al., 2002). The MIKC^c genes have been further subdivided into 12 clades in Arabidopsis that correlate with gene function by Becker and Theissen (2003). The clades and their relative functions are summarized in *Table 1* and described in details as follows.

The AGAMOUS (AG) clade contains four genes in Arabidopsis: *AG, AGL11*, *SHATTERPROOF 1* and *2* (Yanofsky et al., 1990; Ma et al., 1991; Rounsley et al., 1995). *AG* was the first C-class floral homeotic MADS-box gene to be cloned. Like *PLENA*, a member of the same clade found in *Antirrhinum*, it is involved in stamen and carpel development and provides flower determinacy (Bradley et al., 1993). *SHP1* and *SHP2* encode redundant proteins required for the development of the siliques in Arabidopsis (Liljegren et al., 2000). In Antirrhinum the *FARINELLI* (*FAR*) gene is involved in male fertility (Davies et al., 1999).

The AGL2 clade is composed of four genes, *AGL2*, *AGL3*, *AGL4*, and *AGL9*. *AGL3* is expressed in all major plant organs, while the expression of the other three genes is restricted to the four floral organ primordia (Flanagan and Ma, 1994; Savidge et al., 1995; Mandel and Yanofsky, 1998). The analysis of the phenotype of triple mutant *agl2 agl4 agl9* and their redundant function prompted the author to rename them *SEPALLATA 1* (*SEP1*), *SEP2*, and *SEP*3 (Pelaz et al., 2000). They are classified as class E of floral homeotic genes (Theissen, 2001). A gene from the same clade was found also in tomato. *LeMADS-RIN* regulates late ripening in tomato fruit, showing a new function for this clade (Vrebalov et al., 2002). *AGL2*-like genes were also found in maize and rice, in rice they are involved in the determination of floral meristem at early stages of development of rice florets (Jeon et al., 2000). *AGL6* is expressed in all four classes of floral organ whereas *AGL13* expression is restricted to ovules (Rounsley et al., 1995; Mouradov et al., 1998).

The AGL6 clade is composed by two genes, *AGL6* and *AGL13* (Ma et al., 1991; Rounsley et al., 1995). They are closely related, however they show quite different expression pattern. *AGL6* is expressed in all four floral organs, while *AGL13* expression is restricted to ovules (Rounsley et al., 1995; Mouradov et al., 1998).

Only one gene forms the AGL12 clade and is conserved in monocotyledons. *AGL12* (also called *XAAANTAL1*, *XAL1*) is preferentially expressed in roots (Rounsley et al., 1995; Alvarez-Buylla et al., 2000a). The *AGL12* ortholog in rice is *OsMADS26* gene and is expressed mainly in leaves and inflorescences (Pelucchi et al., 2002).

AGL15 and *AGL18* are the two members from of the AGL15 clade (Rounsley et al., 1995; Alvarez-Buylla et al., 2000a). These genes are preferentially expressed during embryogenesis and seed development (Perry et al., 1996).

The AGL17 clade is formed of four genes *AGL16*, *AGL17*, *AGL21*, and *ARABIDOPSIS NITRATE REGULATED 1* (*ANR1*). *AGL16* plays a role in stomatal development and is regulated by miR824 (Alvarez-Buylla et al., 2000a; Kutter et al., 2007). *AGL17* is expressed in lateral root cap and the epidermis around the elongation zone, while *AGL21* is

predominantly expressed in lateral root primordia (Burgeff et al., 2002). *ANR1* controls lateral root elongation in response to nitrate (Zhang and Forde, 1998; Gan et al., 2005).

The DEF/GLO clade includes *DEFICIENS (DEF)* and *GLOBOSA* genes with B class function (Vernoux et al., 2011). *DEF* and *GLO* genes were identified in *Antirrhinum* and correspond to the *APETALA3* (*AP3*) and *PISTILLATA (PI)* in Arabidopsis, respectively (Goto and Meyerowitz, 1994; Jack et al., 1994). Their function in extant gymnosperm is to distinguish between male and female organs (Winter et al., 1999; 2002a). In angiosperms they specify petal identity (Winter et al., 2002b).

The FLC lineage includes five *MADS AFFECTING FLOWERING genes (MAF1 to MAF5),* and *FLOWERING LOCUS C* (*FLC*). *AGL27* was reported by two studies in the same year as *FLOWERING LOCUS M* (*FLM*) and *MADS AFFECTING FLOWERING 1* (*MAF1*), (Ratcliffe et al., 2001; Scortecci et al., 2001). In Arabidopsis*, AGL27* acts as inhibitor of flowering independently from photoperiod. *FLC* also act as repressor of flowering in response to both environmental and endogenous stimuli. *FLC* expression is repressed in the inflorescences by vernalization and the autonomous pathway and upregulated by *FRIGIDA* (*FRI*) (Michaels and Amasino, 1999).

The B_{sister} (GGM13) clade includes *ARABIDOPSIS BSISTER* gene (*ABS)* also called *TT16* or *AGL32*, and the *GORDITA* gene (*GOA*). The first member, *GGM13*, was found in gymnosperm *Gnetum gnemon*. They are mainly expressed in female reproductive organs, especially in ovules (Becker et al., 2000).

The SQUA clade contains the genes *APETALA 1* (*AP1*), *CAULIFLOWER* (*CAL*), and *FRUITFULL* (*FUL*) (Mandel et al., 1992; Kempin et al., 1995; Gu et al., 1998). The first SQUA-like gene identified was *SQUAMOSA* (*SQUA*) from *Antirrhinum* (Huijser et al., 1992). *AP1*, *CAL*, and *FUL* are usually expressed in inflorescence or floral meristem and classified as meristem identity genes (Theissen et al., 1996). Moreover, *AP1* determines sepal and petal development and is thus considered a class A floral organ identity gene (Mandel et al., 1992). Kempin et al. (1995) demonstrated that *AP1* and *CAL* have redundant function.

The *StMADS11* lineage comprises genes found in a wide range of plant species suggesting their ancestral function in vegetative organs such as timing of vegetative development and the transition to reproductive stage (Becker and Theissen, 2003). In Arabidopsis, two genes belong to this clade, *AGL24* and *SHORT VEGETATIVE PHASE* (*SVP*). *AGL24* is strongly expressed in apical meristem and leaf primordial. The expression can be detected also in floral meristem and later in stamen and carpel (Yu et al., 2002). RNA interference of *AGL24* results in dosage dependent late flowering, while constitutive expression leads to early flowering (Yu et al., 2002). Thus, *AGL24* acts within the flowering

pathway as floral promoter (Michaels et al., 2003). *SVP* is expressed in young leaves and throughout shoot apical meristem. During flower development its expression is abolished prior to the formation of sepals (Hartmann et al., 2000). Since *svp* mutant plants are early flowering and show a weaker response to vernalization, *SVP* is considered as a dosage dependent repressor of flowering (Becker and Theissen, 2003; Lee et al., 2007). A *StMADS11* gene was found in tomato and named *JOINTLESS* (Mao et al., 2000). The *jointless* mutants showed defects in the development of abscission zones that influences flower and fruit abscission. The *JOINTLESS* mutants bear 'stemless' tomato fruits that is a desired agronomical trait. Six genes of this subfamily were recently found also in peach (*Prunus persica*) and called *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*) (Jimenez et al., 2009).

The TM3 (SOC1) clade is composed of *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), *AGL14*, *AGL19*, *AGL42, AGL71, and AGL72*. *AGL14* and *AGL19* are expressed in roots (Rounsley et al., 1995; Alvarez-Buylla et al., 2000a). *SOC1* is preferentially expressed in the apical meristem and is regulated by the duration of the daylength (Onouchi et al., 2000; Samach et al., 2000). *SOC1* is involved in the photoperiod flowering pathway, as it is a direct target of *CONSTANS* (*CO*), however it receives input also from the autonomous and vernalization flowering pathway as it is repressed by *FLC* (Borner et al., 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000).

Recently, member of MICK-type genes have been identified also in other plant species thanks to the increasing availability of whole genome sequence, and the development of more powerful sequencing and bioinformatics tools.

Table 1 - MIKC^c-type MADS-box genes functions in development of *Arabidopsis thaliana* and other plants

Clade	Gene name	Arabidopsis Gene ID	Functions in Arabidopsis (References)	Functions in other plant species (References)
AGAMOUS (AG)	*AGAMOUS (AG)*	At4g18960	Homeotic C-class gene, carpel and stamen specification (Yanofsky et al., 1990).	Subfunctionalization of homeotic C-function; fruit development (Causier et al., 2005; Airoldi et al., 2010; 2012)
	SHATTERPROOF1,2 (SHP1, 2)	At3g58780 At2g42830	Carpel, ovule and fruit development; dehiscence; periodic lateral root formation (Liljegren et al., 2000; Moreno-Risueno et al., 2010).	
	SEEDSTICK (STK)	At4g09960	Carpel, ovule and fruit development; periodic lateral root formation (Pinyopich et al., 2003; Moreno-Risueno et al., 2010)	
AGL2	*SEPALLATA1-4 (SEP1-4)*	At5g15800, At2g03710, At1g24260, At3g02310	Homeotic E-class gene; sepal, petal, stamen and carpel specification (Mandel and Yanofsky, 1998; Pelaz et al., 2000; Ditta et al., 2004)	Inflorescence meristem identity in Gerbera; tomato fruit ripening (Vrebalov et al., 2002; Uimari et al., 2004)
AGL6	*AGAMOUS-LIKE 6 (AGL6)*	At2g45650	Transition to flowering (activator); lateral organ development (Koo et al., 2010; Yoo et al., 2011)	None
AGL12	*XAANTAL1 (XAL1)*	At1g71692	Root development cell cycle regulation; transition to flowering (activator) (Tapia-López et al., 2008)	None
AGL15	*AGAMOUS-LIKE 15 (AGL15)*	At5g13790	Embryogenesis, transition to flowering (repressor) with AGL18; sepal and petal longevity; fruit maturation (Heck et al., 1995; Fernandez et al., 2000; Harding et al., 2003)	None
AGL17	*AGAMOUS-LIKE 18 (AGL18)*	At5g57390	Transition to flowering (repressor) with AGL15 (Adamczyk et al., 2007)	None
	AGAMOUS-LIKE 16 (AGL16)	At3g57230	Stomatal development and distribution (Kutter et al., 2007)	
	AGAMOUS-LIKE 17 (AGL17)	At2g22630	Transition to flowering (activator) (Han et al., 2008)	
	ARABIDOPSIS NITRATE REGULATED 1 (ANR1)	At2g14210	Root development; nutrient response (Zhang and Forde, 1998)	
B$_{SISTER}$ (GGM13)	*ARABIDOPSIS BSISTER (ABS)*	At5g23260	Seed pigmentation and endothelium development (Nesi et al., 2002; Kaufmann et al., 2005; de Folter et al., 2006)	None
	GORDITA (GOA)	At1g31140	Fruit development (Prasad et al., 2010)	
DEF/GLO	*APETALA 3 (AP3)*	At3g54340	Homeotic B-class gene; petal and stamen specification (Jack et al., 1992)	Tepal specification in orchids; specification of various petaloid organs (Mondragon-Palomino and Theissen, 2008; Chang et al., 2010)
	PISTILLATA (PI)	At5g20240	Homeotic B-class gene; petal and stamen specification (Goto and Meyerowitz, 1994)	
FLC	*FLOWERING LOCUS C (FLC)*	At5g10140	Transition to flowering (repressor); germination, juvenile-to-adult transition; initiation of flowering; flower organ development (Michaels and Amasino, 1999; Chiang et al., 2009; Deng et al., 2011)	Role in floral bud dormancy; perennial life cycle of *A. alpina* (Du et al., 2008; Wang et al., 2009b; Zhang et al., 2009)
	MADS AFFECTING FLOWERING 1-4 (MAF1-4)	At1g77080, At5g65050, At5g65060, At5g65070	Transition to flowering (repressors) (Ratcliffe et al., 2001; 2003)	
SQUA	*MADS AFFECTING FLOWERING 5 (MAF5)*	At5g65080	Transition to flowering (activator) (Ratcliffe et al., 2003)	Axillary bud formation (potato); roles in fruit development; sepal size and floral abscission in tomato, variable roles in floral transition (Rosin et al., 2003; Calonje et al., 2004; Elo et al., 2007; Nakano et al., 2012)
	APETALA1 (AP1)	At1g69120	Meristem identity specification; homeotic A-class gene (Mandel et al., 1992; Weigel et al., 1992; Ferrandiz et al., 2000)	
	CAULIFLOWER (CAL)	At1g26310	Meristem identity specification (Kempin et al., 1995; Ferrandiz et al., 2000)	
	FRUITFULL (FUL)	At5g60910	Meristem identity specification; annual life cycle regulator with *SOC1*, fruit development; cauline leaf growth (Gu et al., 1998; Ferrandiz et al., 2000; Ferrándiz et al., 2000; Melzer et al., 2008)	
STMADS11	*AGAMOUS-LIKE 24 (AGL24)*	At4g24540	Transition to flowering (activator) (Michaels et al., 2003)	Floral bud dormancy in *Prunus* and *Pyrus*; flower abscission in tomato; role in prophyll development in Antirrhinum; inflated calyx syndrome in *Physalis* (Mao et al., 2000; Masiero et al., 2004; He and Saedler, 2005; Li et al., 2009; Saito et al., 2013)
	SHORT VEGETATIVE PHASE (SVP)	At2g22540	Transition to flowering (repressor) (Hartmann et al., 2000)	
TM3/SOC1	*AGAMOUS-LIKE 19 (AGL19)*	At4g22950	Transition to flowering (activator) (Schonrock et al., 2006)	
	AGAMOUS-LIKE 42 (AGL42) (FOREVER YOUNG FLOWER, FYF)	At5g62165	Transition to flowering (activator); flower organ senescence and abscission; root development (Nawy et al., 2005; Chen et al., 2011; Dorca-Fornell et al., 2011)	
	AGAMOUS-LIKE 71, 72 (AGL71, 72)	At5g51870, At5g51860	Transition to flowering (activators) with AGL42 (Dorca-Fornell et al., 2011)	
	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)	At2g45660	Transition to flowering (activator); periodic lateral root formation (Lee et al., 2000; Moreno-Risueno et al., 2010)	

I.3 Role of MADS-box genes in vernalization and dormancy

The strict regulation of flowering time is essential for successful reproduction in angiosperms. This mechanism enables completion of seed development in optimal environmental conditions in response to internal and external stimuli. Genetic and phenotypic analyses in Arabidopsis allowed unraveling the mechanisms and pathways that regulate the switch from vegetative growth to reproductive state. The flowering in response to seasonal changes is controlled by environmental parameters such as vernalization, photoperiod, and temperature that act in coordination with endogenous and developmental signals: the autonomous, gibberellin, and age-dependent pathways (reviewed by Wellmer and Riechmann, 2010). A schematic diagram of the genetic pathways that regulate flowering time in Arabidopsis is represented in *Figure 2*. The photoperiod pathway is mediated by the zinc-finger transcription factor *CONSTANS* (*CO*), that activates the expression the flowering activator *FLOWERING LOCUS T* (*FT*) under long day growth conditions (An et al., 2004). *CO* function is antagonised by repressing *FT* through diverse mechanisms to prevent flowering in adverse season condition (Yant et al., 2009). Moreover, *FLC* represses *SOC1* and *FD*, a bZIP transcription factor, which normally promote flowering (Abe et al., 2005; Michaels et al., 2005; Helliwell et al., 2006; Lee and Lee, 2010). *FD* interacts with FT to upregulate *SOC1* that is positively regulated by both the gibberellin and the age-related pathway (Lee et al., 2000).

The vernalization pathway is activated when the plant is exposed to cold temperatures. Many plants require prolonged exposure to cold during winter to become able to initiate flowering in the following spring. This process has been deeply investigated in the model plant Arabidopsis through the identification of many components of the vernalization regulatory network; however, the mechanism by which the plant senses the temperature still remains unclear (Andres and Coupland, 2012). Flowering is repressed during winter until the plant is exposed to low winter temperatures. Exposure to cold for long periods gradually abolishes the repression and flowering is activated. In Arabidopsis and numerous plants chilling requirements varies among accessions and cultivars (reviewed by Amasino, 2010).

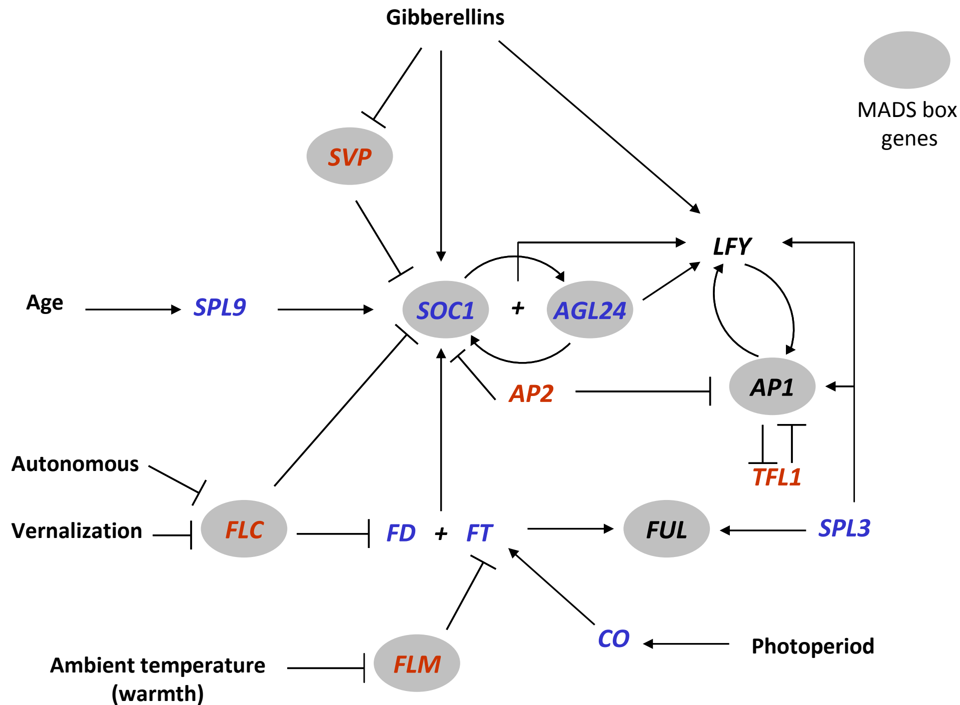


Figure 2 – Schematic diagram of the genetic pathways that regulate flowering time in Arabidopsis

Different pathways respond to various external (photoperiod, vernalization, ambient temperature) and internal (autonomous, age, gibberellins) cues to regulate the floral transition through a complex genetic network. Inductive signals have first to overcome the activity of several repressors of flowering (genes indicated in red), for activators (genes indicated in blue) to eventually turn on the meristem identity genes (*AP1* and *LFY*). Long days (photoperiod) result in upregulation of *FT* by *CO* (a zinc finger and CCT-domain transcription factor). *FT* is upregulated by warm temperatures by repression of *FLM*. Repressors of *FT* are *FLC*, which integrate autonomous and vernalization pathways; *AP2* transcription factor; and *SVP*. *FD* (a bZIP transcription factor) and *FT* coordinately upregulate the MADS-box genes *SOC1*, *AP1* and *FUL*. *SOC1* forms a positive feedback loop with *AGL24*. *AP1* and *LFY* are also upregulated by members of the SPL family of transcription factors (Wang et al., 2009a).

The key regulator of this pathway is the MADS-box gene *FLC* (Michaels and Amasino, 1999). Its expression is promoted by *FRIGIDA* (*FRI*) (Johanson et al., 2000). FLC form a multimeric complex with SVP (Lee et al., 2007; Li et al., 2008; Deng et al., 2011). *SVP* represses the expression of *FT* and other genes that initiate floral transition while it directly activates the expression of flowering repressors (*Figure 2*) (Searle et al., 2006; Li et al., 2008; Jang et al., 2009). *FLC* regulatory mechanism has been investigated in detail and it has been shown that the gene is epigenetically regulated (Figure 3). Before cold exposure, FLC chromatin is in active state, whereby active histone marks, e.g. histone H3 Lys 4 (H3K4), histone H3 Lys 36 (H3K36) methylation, and histone H3 acetylation, are present (He et al., 2003; Kim et al., 2005; Zhao et al., 2005). Cold temperatures induce

quantitative accumulation of Polycomb-based silencing complexes (PRC2) and repressive histone modifications at the *FLC* locus (Dennis and Peacock, 2007; Kim et al., 2009; Heo and Sung, 2011). In particular H3K27 and H3K9 methylation accumulate during the vernalization response (Bastow et al., 2004; Sung and Amasino, 2004). The dosage-dependent nature of vernalization is reflected in the progressive enrichment of H3K27me3 (tri-methylation of Lysine 27 on histone 3) at the nucleation region with the increasing chilling hours (Angel et al., 2011).

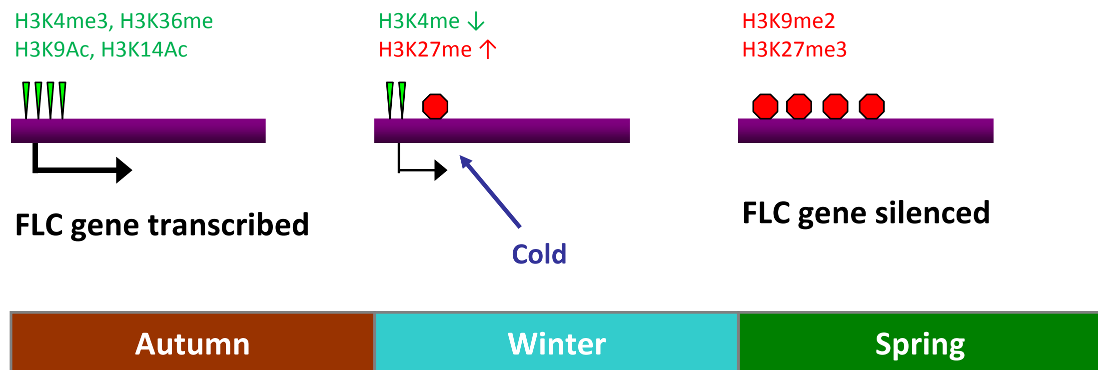


Figure 3 – Epigenetic regulation of *FLC* in Arabidopsis

Histone modifications at *FLC* locus are correlated to exposition to prolonged cold. Before cold exposure *FLC* is actively transcribed due to histone marks at the chromatin level. During vernalization, repressive marks are accumulated by Polycomb-based silencing complexes. In spring, *FLC* is repressed due to the presence of histone marks coding for closed chromatin.

Vernalization response evolved differently in other grass plants. For instance, in cereals, the interaction between photoperiod pathway and vernalization is mediated by an unrelated gene *VERNALIZATION 2* (*VRN2*) (Lin et al., 2005; Wang et al., 2009b). Under long day conditions *VRN2* represses at least one of the cereals *FT* gene and its transcription is repressed after vernalization by the MADS-box transcription factor *VRN1* that is, in contrast, expressed in response to cold (Yan et al., 2004; Dubcovsky et al., 2006).

The vernalization mechanism through a *FLC* ortholog has been identified also in other Brassicaceae (Kuittinen et al., 2008; Wang et al., 2009b; Aikawa et al., 2010). For example, *Arabis alpina* is a diploid perennial able to flower only if it is exposed to vernalization (Wang et al., 2009b). However, *A. alpina* plants show an age-dependent response to vernalization due to *TERMINAL FLOWER 1* (*TFL1*) gene (Wang et al., 2011). This feature is typical of perennials and is not shown by annual Arabidopsis plants.

Another characteristic of the life cycle of perennials is seasonal flowering, in *A. alpina* this aspect is regulated by *PERPETUAL FLOWERING 1* (*PEP1*), a MADS-box transcription factor that is the ortholog of Arabidopsis *FLC*. Similarly to *FLC*, *PEP1* expression is differentially regulated by vernalization and correlates with changes in histone methylation (Wang et al., 2009b). Moreover, plants that are homozygous for *pep1* mutation are able to flower without vernalization, as *flc* mutant do, but they also do not return to vegetative growth and flower continuously.

Unlike annual plants, such as Arabidopsis and cereals, perennial plants and trees flower multiple times during their lifespan and live for many years. As consequence, their life cycle switch continuously between periods of flowering and vegetative growth. The period of vegetative growth for the apical buds is indicated as *dormancy*, and divided into *paradormancy* in summer, *endodormancy* in autumn, and *ecodormancy* in winter. In spring dormancy is released, the buds break and flowers start to form. In the perennial plant poplar *FLC* homologues were not found, the alternate periods of vegetative and reproductive growth are conferred by different *FT*-like genes. *FT1* mRNA increase in response to cold temperature and promotes reproduction, in contrast, *FT2* mRNA is induced by long days and warm temperature and promotes vegetative growth in spring and summer (Hsu et al., 2011). In silver birch tree (*Betula pendula*) *BpMADS4*, member of the *SQUA* family, plays a role in the initiation of inflorescence development and transition from vegetative to reproductive phase (Elo et al., 2007). In peach tree (*Prunus persica*) tandem duplication of *StMADS11* clade originated six genes associated with bud dormancy called *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*) (Jimenez et al., 2009; Li et al., 2009). These genes have been demonstrated to be epigenetically regulated in response to cold similarly to Arabidopsis *FLC* (Leida et al., 2012). Recently, three *DAM* genes were isolated in Japanese pear (*Pyrus pyrifolia*) by Saito el al. (2013).

I.4 Role of MADS-box genes in flower development

In 1991, the first genes involved in flower development were cloned in Arabidopsis and in *Antirrhinum* and the ABC model was proposed to explain the function of transcription factors in flower development (Coen and Meyerowitz). The A-class *AP1* and *APETALA 2* (*AP2*) genes specify sepals in the first whorl. The B-class genes *AP3* and *PI* together with A-class genes specify petals and together with C-class genes specify stamens. The C-class gene *AG* specifies carpels in the fourth whorl. Except for *AP2*, which is member of the AP2/ETHYLENE RESPONSE FACTOR (ERF) transcription factor family (Okamuro et al.,

1997), all the other genes encode MIKC^{c}-type MADS-box transcription factors (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994; Jofuku et al., 1994; Parenicova, 2003). The ABC genetic model was based on the observations made on Arabidopsis mutants isolated by forward genetic approaches. The *apetala2* mutant flowers contain carpels instead of sepals and stamens instead of petals (*Figure 4B*). *apetala3* and *pistillata* mutants show homeotic transformation of petals into sepals, and stamens into carpels (*Figure 4C*)**,** while *agamous* mutant flowers develop petals in the position normally occupied by stamens and sepals in the position of carpels (*Figure 4D*) (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005).

Further studies led to the identification of E-function *SEPALLATA* (*SEP1-4*) genes, which are required throughout the whole process, and in particular *SEP4* is required to specify sepals identity (Pelaz et al., 2000; Ditta et al., 2004). The *sep1 sep2 sep3* triple mutant of Arabidopsis develops only sepals whereas the quadruple mutant shows the conversion of all floral organs into a leaf-like structure (*Figure 4E*) (Krizek and Fletcher, 2005). The fifth class of floral homeotic genes specify for D-function and confers ovule identity (Angenent et al., 1995; Colombo et al., 1995). The Arabidopsis homologs are *AG*, *SEEDSTICK* (*STK*), and *SHATTERPROOF 1* and *2* (S*HP1* and *SHP2*) (Pinyopich et al., 2003). In *stk shp1 shp2* triple mutant ovules are sometimes transformed into leaf-like or carpel-like structures (Pinyopich et al., 2003).

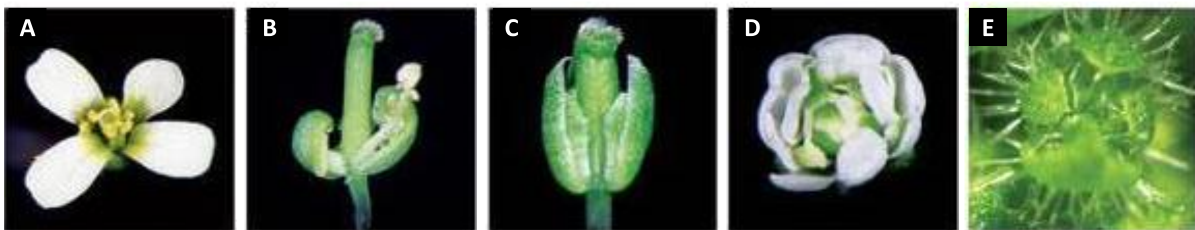


Figure 4 - Arabidopsis mutant of floral homeotic genes

The wild-type flower possesses the four floral organs: sepals, petals, stamens, and carpel (A). An *apetala2* flower lacks A-function that determines sepals and petals (B); a *pistillata* flower lacks B-function that determines stamens and carpels (C); an *agamous* flower lacks C-function that determines the formation of the reproductive organs (D). A quadruple mutant for SEPALLATA genes (*sep1 sep2 sep3 sep4*) consist of repeated whorls of leaf-like organs (E). From Krizek and Fletcher, 2005.

On the basis of the discovery of the new E- and D-function genes, a more complex and complete ABC(D)E model for flower development was proposed in replacement to the previous ABC model (Krizek and Fletcher, 2005). According to this model (*Figure 5*), the

development of each of the four flower whorls is regulated by four homeotic genes belonging to the A, B, C, E class in coordination with genes of the D class devoted to ovules development (Krizek and Fletcher, 2005). Theissen and Saedler (2001) proposed the floral quartet model to explain the molecular interactions between MADS domain proteins. Two interacting MADS box protein dimers (homo- or hetero-dimers) bind to two CArG boxes that are present in close proximity within a target DNA sequence (Melzer et al., 2010). In Arabidopsis the interactions between MADS domain proteins involved in floral organ development have been disclosed (*Figure 5*) (Honma and Goto, 2001; Favaro et al., 2003). Interactions between SEP protein and B-sister (Bs) class of MADS domain protein were also detected. ARABIDOPSIS BSISTER (ABS)-SEP and STK-SHP quartet was found within the ovule during endothelium development (*Figure 5*) (Kaufmann et al., 2005; de Folter et al., 2006). The presence of E-class SEP protein in all the quaternary complexes confirmed their role in binding all components of protein dimerization during the whole pattern of flower formation (Immink et al., 2009).

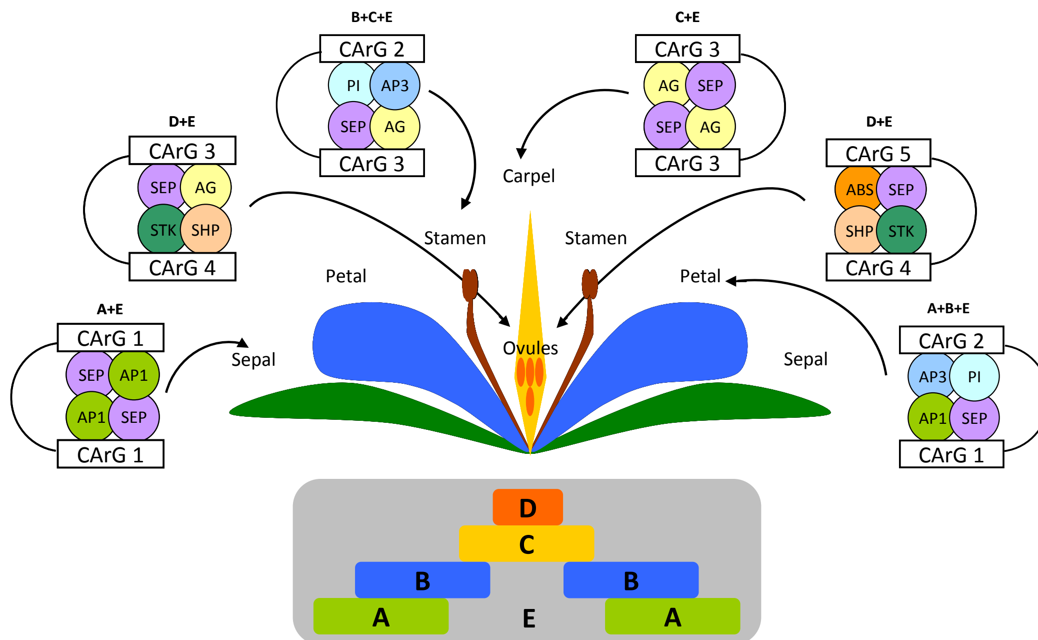


Figure 5 - Schematic representation of ABC(D)E model for flower development and floral quartet model
The formation of the four different floral organs is determined by the expression of A-, B-, C- and E- function floral homeotic genes. Ovule development is determined by D-function genes. The MADS box genes involved in the flowering pattern act as quaternary complexes to specify the identity of the floral organs.

I.5 Agricultural relevance of dormancy in Rosaceae and apple

Rosaceae family includes various economically and agronomically relevant perennial crops. *Fragaria spp.* (strawberry), *Malus spp.* (apple), *Prunus spp*., among them *Prunus armeniaca* (apricot), *Prunus dulcis* (almond), *Prunus serotina* (black cherry), *Prunus persica* (peach), *Prunus avium* (sweet cherry), *Prunus cerasus* (sour cherry), and *Pyrus spp*. (pear) all belong to Rosaceae. Our work focuses mainly on apple and strawberry.

The world apple production reached more than 67 million tons in year 2012, with Asia being the first producer with 55% of the entire production, followed by Europe with 17%, and USA with 6% (sources: FAO and USDA). Italy, ranking as the 6th apple producer worldwide, is the biggest producer in Europe and with and average production of more than 2 millions tonnes provides around 20% of the EU-28 apple production. Trentino-South Tyrol is the region that covers 70% of Italian production and 15% of the production in Europe (sources: Assomela, CSO - *Italy's Fresh Produce Service Centre;* data referred to year 2012).

The first apple cultivation dated to 2000 b.C. in ancient Anatolia and north Mesopotamia, around 500 b.C. it was cultivated in the region of the Persian empire and it is supposed that Alexander the great imported them in ancient Greek around 300 b.C. Later, during the ages of the Roman Empire, apple cultivation spread to north Western Europe (Ferree and Warrington, 2003). Nowadays, domesticated apple is an interspecific hybrid named *Malus x domestica* Borkh (Pratt, 1988).

Like other fruit trees apple requires a long juvenile period of up to 8 years to acquire competency to flower (Zimmerman, 1972; Goldschmidt and Samach, 2004). Interestingly, ectopic expression of the silver birch FT-like MADS-box gene *BpMADS4* in apple shortens considerably the juvenile period and enable constitutive flowering (Flachowsky et al., 2011). This study indicates the existence of molecular mechanisms repressing flower formation. Thanks to the availability of the apple genome it is possible to investigate molecular mechanisms regulating dormancy and flowering (Velasco et al., 2010).

Another fundamental requirement for apple tree to flower is the break of buds endodormancy in response to cold temperatures. Even though different apple varieties have different chilling requirements (*Table 2*), without a prolonged exposure to cold, apple trees are not able to flower and consequently to produce fruits. The accumulation of chilling hours necessary to break bud dormancy is hard to reach in warm zones, such as the Mediterranean Basin and South America. For this reason, farmers turned to an

extensive use of chemical compounds in order to induce bud break. Hydrogen cyanamide (CH_2N_2) is the principal ingredient of Dormex®, a plant fertilizer applied by nebulization and used to stimulate uniform bud break of fruit trees. The substance is classified in the European Union as "toxic" if swallowed, "harmful" in contact with skin, "irritating" to eyes and skin, and capable of producing sensitization after skin contact. The U.S. Environmental Protection Agency (EPA) places both the active ingredient (hydrogen cyanamide) and the product (Dormex®), which contains 50% hydrogen cyanamide, into the acute toxicity category I (danger). Adverse health effects from contact with hydrogen cyanamide include severe irritation and ulceration of the eyes, skin, and respiratory tract (Hathaway et al., 1996). The substance can also induce vomiting, parasympathetic hyperactivity, dyspnea, hypotension, and confusion when exposure coincides with alcohol use. Several cases of illness due to CH_2N_2 exposure were reported in South Italy and US (CDC, 2001; Calvert et al., 2004; CDC, 2005). An aware use of toxic compounds in agriculture, for example the correct use of personal protective equipments, might reduce the outbreak of intoxication cases. More beneficial will be the possibility to completely avoid chemicals that are toxic for human health introducing genetically modified varieties able to flower in warm climate.

To determine the end of bud dormancy and predict initiation of flowering, different phenological models have been proposed. From the first empirical and linear regression models that linked phenophases with low temperatures, the modern models evolved towards hourly algorithms-basis (Landsberg, 1974; White, 1979; Floyd and Braddock, 1984; Reicosky et al., 1989; Kajfez-Bogataj and Bergant, 1998; Bergant et al., 2001). In the recent models, estimating both chilling and temperature forcing, bud development is split into two stages. The first phase is where “chilling units” CU are accumulated. CU are defined as the chilling hours accumulated to break bud dormancy when the “requirement” value is reached. The second stage, starting after the fulfillment of CU requirement, is where “growing degree days/hours” (GDD or GDH) are accumulated and it leads to bud opening (Rea and Eccel, 2006). Several phenological flowering models were described, among them Bidabè, “Utah” and Anderson make use of “chilling and forcing” algorithms with different parameterizations of CU and GDH summarization (Bidabé, 1967; Ashcroft et al., 1977; Anderson et al., 1986). In 2006 a more general model was proposed and named “Progressive Utah” (Rea and Eccel, 2006). According this model, GDH is a function of temperature and photoperiodic effect:

$$GDH(k) = \sum_{i=r}^{k} f_f(T_h(i)) f_a(GDH(i-1)) f_p(i);$$

where $f_f(T_h(i))$ is the “Utah” model forcing function, $f_p(i)$ is the photoperiod function for day i, and $f_a(GDH(i-1)$ is the function of actual GDH accumulation. The general form for this function is:

$$f(GDH(I-1)) = \left[\frac{GDH(I-1)}{GDH_{in.flow.}}\right]^a ;$$

where $GDH_{in.flow.}$ is the requirement for initial flowering phase.

That was converted into the general equation of the “Progressive Utah” model:

$$GDH(k) = GDH(K-1) + \sum_{h=1}^{24} \max\left\{0, (T_h(k) - T_b)\left[1 + \left(\frac{GDH(k-1)}{GDH_{in.flow}}\right)^2\right]\right\}.$$

The “Progressive Utah” model allows to determine both the CU required to break dormancy and the day of flowering initiation with an error of ~2 days (Rea and Eccel, 2006; Eccel et al., 2009). In our study this model was used to calculate the CU requirement of ‘Golden Delicious’ in correlation to meteorological data of our region.

Table 2 – Chilling hours requirement in different apple varieties

Chilling requirement	Apple variety	Chilling Hours
High and intermediate chilling requirement	'Golden Delicious'	1000
	'Red Delicious'	800
	'Jonagold'	700-800
	'Gala'	< 600
	'Granny Smith'	500-600
Low chilling requirement	'Pink Lady'	200-400
	'Fuji'	200-400
	'Ein Shemer'	100-400
	'Anna'	100-300
	'Dorsett Golden'	< 100

I.6 Economical importance of strawberry

The first attempt to cultivate strawberry started in the sixteenth century in France and England, at that time *F. vesca*, *F. moschata*, and *F. Viridis* were identified and propagated asexually by cutting off the runners. In the 1600's, *F. virginiana* was introduced in Europe from eastern North America giving later rise to the modern cultivated strawberry. In the 1750's the garden strawberry was initially a crossing *Fragaria virginiana* x *Fragaria chiloensis*, the latter imported from Chile (Darrow, 1966). Nowadays, the result of an interspecific breeding *Fragaria x ananassa* is the most commercialized cultivar and preferred to the woodland strawberry *Fragaria vesca* (Davis et al., 2007; Shulaev et al., 2008). Nevertheless, the diploid *F. vesca* is preferred for structural and functional genomic studies due to its intrinsic features (Folta and Davis, 2006). *F. vesca* is diploid (2n = 2x = 14) and has one of the smallest genome size among cultivated plants (Folta and Davis, 2006; Shulaev et al., 2008; 2010). Furthermore, *F. vesca* plants are compact enough to be grown in large scale in laboratory and greenhouse, easy to propagate vegetatively, self-compatible, with short generation time from seed to seed, and produce many seeds per plants (Darrow, 1966). Another advantage of strawberry is the availability of varieties with different flowering competences. Most of the cultivars are "*junebearing"*, that is, the fruit can be harvested once each growing season, typically in June, but everbearing varieties are also present (i.e. 'Rügen', 'Baron Solemacher', 'Hawaii-4'), whose fruit can be harvested possibly during the whole year (Albani et al., 2004; Heide and Sønsteby, 2007). The *Fragaria spp.* berry is an aggregate accessory fruit: the fleshy edible part is derived from the receptacle and not from ovaries which actually develop into many achenes (the "true" fruit). Recently, two studies have investigated how hormonal signals produced in the endosperm and seed coat coordinate seed, ovary wall, and receptacle fruit development (Kang et al., 2013; Merchante et al., 2013).

I.7 Aim of the thesis

In flowering plants, MADS-box genes are deputed to the regulation of developmental processes. The best studied plant MADS-box transcription factors are the homeotic floral genes APETALA (AP), AGAMOUS (AG), and PISTILLATA (PI) which determine the identity of the four flower organs following the ABC model (Coen and Meyerowitz,

1991). MADS-box proteins also control flowering time through different pathways, among them vernalization in which *FLC* plays a key role (Michaels and Amasino, 1999). The role of the above mentioned MADS-box genes has been widely studied in the model plant *Arabidopsis thaliana*, however, poorly investigated in major crop plants and fruit trees. Our work will focus on MADS-box genes specific of two representative Rosaceae species of important agricultural and commercial value worldwide, apple (*Malus x domestica* Borkh.) and wild strawberry (*Fragaria vesca*).

A great variety of apple cultivars is available on the market. However, fruit trees remain in a long juvenile phase for 3 up to 7 years before they acquire the competence to switch to the reproductive phase. Moreover, buds endodormancy is broken only after a prolonged exposure to cold temperatures in winter. To induce and enhance bud break and flowering initiation in regions where chilling requirement could not be fulfilled, toxic compounds, such as hydrogen cyanamide, are exploited. Our study aims to characterize the gene(s) involved in dormancy in the apple cv. 'Golden Delicious', taking into account what is known for other Rosaceae species such as peach and pear (Li et al., 2009; Leida et al., 2012; Saito et al., 2013). The activity of the genes will be investigated through gene expression studies in buds, while Chromatin Immunoprecipitation (ChIP) and ChIP seq analyses will be used to investigate epigenetic regulation at the histone level. The final goal is to obtain a "molecular mark" for dormancy break to be used for production of apple varieties with the desired chilling requirements. This in order to avoid the use of toxic compounds in agriculture and to produce apple trees meeting the progressive global warming.

The woodland strawberry present many convenient physical and genetic features to study flower development. Among them, the diploid genome, the short seed-to-seed period, the availability of tools for genetic manipulation, and the conserved Rosaceae flower structure. Moreover, the fruit develops from the receptacle similarly to apple. In *F. vesca* we conducted a phylogenetic analysis of MADS-box genes and selected three *AGAMOUS*- and *PISTILLATA*-like genes to perform gene expression analyses. The first step is to produce knock-down mutants of the three flowering genes using RNA interference approach. A novel protocol for strawberry transformation that exploits a gateway based RNA silencing vector will be used to transform diploid strawberry cv. 'Rügen'. The phenotypic analysis of silenced mutant will help to elucidate the role of B- and C- function genes in strawberry. The data will be then compared to Arabidopsis and other Rosaceae species to eventually propose a model for flower development in strawberry with possible extension to Rosaceae family.

I.8 References Chapter I

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

Adamczyk, B.J., Lehti-Shiu, M.D., and Fernandez, D.E. (2007). The MADS domain factors AGL15 and AGL18 act redundantly as repressors of the floral transition in Arabidopsis. The Plant Journal **50,** 1007-1019.

Aikawa, S., Kobayashi, M.J., Satake, A., Shimizu, K.K., and Kudoh, H. (2010). Robust control of the seasonal expression of the Arabidopsis FLC gene in a fluctuating environment. Proc Natl Acad Sci U S A **107,** 11632-11637.

Airoldi, C.A., and Davies, B. (2012). Gene duplication and the evolution of plant MADS-box transcription factors. J Genet Genomics **39,** 157-165.

Airoldi, C.A., Bergonzi, S., and Davies, B. (2010). Single amino acid change alters the ability to specify male or female organ identity. Proceedings of the National Academy of Sciences **107,** 18898-18902.

Albani, M.C., Battey, N.H., and Wilkinson, M.J. (2004). The development of ISSR-derived SCAR markers around the SEASONAL FLOWERING LOCUS (SFL) in Fragaria vesca. Theoretical and Applied Genetics **109,** 571-579.

Alvarez-Buylla, E.R., Liljegren, S.J., Pelaz, S., Gold, S.E., Burgeff, C., Ditta, G.S., Vergara-Silva, F., and Yanofsky, M.F. (2000a). MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. Plant J **24,** 457-466.

Alvarez-Buylla, E.R., Pelaz, S., Liljegren, S.J., Gold, S.E., Burgeff, C., Ditta, G.S., Ribas de Pouplana, L., Martinez-Castilla, L., and Yanofsky, M.F. (2000b). An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. Proc Natl Acad Sci U S A **97,** 5328-5333.

Amasino, R. (2010). Seasonal and developmental timing of flowering. Plant J **61,** 1001-1013.

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

Anderson, J.L., Richardson, E.A., and Kesner, C.D. (1986). Validation of chill unit and flower bud phenology models for “Montmorency” sour cherry. . Acta Hortic **184,** 71-78.

Andres, F., and Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. Nat Rev Genet **13,** 627-639.

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

Angenent, G.C., Franken, J., Busscher, M., Colombo, L., and van Tunen, A.J. (1993). Petal and stamen formation in petunia is regulated by the homeotic gene fbp1. Plant J **4,** 101-112.

Angenent, G.C., Franken, J., Busscher, M., van Dijken, A., van Went, J.L., Dons, H.J., and van Tunen, A.J. (1995). A novel class of MADS box genes is involved in ovule development in petunia. Plant Cell **7,** 1569-1582.

Arora, R., Agarwal, P., Ray, S., Singh, A.K., Singh, V.P., Tyagi, A.K., and Kapoor, S. (2007). MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics **8,** 242.

Ashcroft, L.G., Richardson, E.A., and Schuyler, D.S. (1977). A statistical method of determining chill unit and growing degree hour requirements for deciduous fruit trees. . HortScience **12,** 347–348.

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

Becker, A., and Theissen, G. (2003). The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol **29,** 464-489.

Becker, A., Winter, K.U., Meyer, B., Saedler, H., and Theissen, G. (2000). MADS-Box gene diversity in seed plants 300 million years ago. Mol Biol Evol **17,** 1425-1434.

Bergant, K., Črepinšek, Z., and Kajfez-Bogataj, L. (2001). Flowering prediction of pear tree (Pyrus communis L.), apple tree (Malus domestica Borkh) and plum tree (Prunus domestica L.)-similarities and differences. . Research Reports, Biotechnical Faculty University of Ljubljana **77**.

Bidabé, B. (1967). Action de la température sur l'évolution des bourgeons de pommier et comparaison de méthodes de contrôle de l'époque de floraison. Ann Physiol vég **9,** 65-86.

Blanc, G., and Wolfe, K.H. (2004). Functional divergence of duplicated genes formed by polyploidy during Arabidopsis evolution. Plant Cell **16,** 1679-1691.

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

Bradley, D., Carpenter, R., Sommer, H., Hartley, N., and Coen, E. (1993). Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. Cell **72,** 85-95.

Burgeff, C., Liljegren, S.J., Tapia-Lopez, R., Yanofsky, M.F., and Alvarez-Buylla, E.R. (2002). MADS-box gene expression in lateral primordia, meristems and differentiated tissues of Arabidopsis thaliana roots. Planta **214,** 365-372.

Calonje, M., Cubas, P., Martínez-Zapater, J.M., and Carmona, M.J. (2004). Floral Meristem Identity Genes Are Expressed during Tendril Development in Grapevine. Plant Physiology **135,** 1491-1501.

Calvert, G.M., Plate, D.K., and Das, R. (2004). Acute occupational pesticide-related illness in the US, 1998--1999: surveillance findings from the SENSOR-Pesticides program. . Am J Ind Med **45,** 14-23.

Causier, B., Castillo, R., Xue, Y., Schwarz-Sommer, Z., and Davies, B. (2010). Tracing the evolution of the floral homeotic B- and C-function genes through genome synteny. Mol Biol Evol **27,** 2651-2664.

Causier, B., Castillo, R., Zhou, J., Ingram, R., Xue, Y., Schwarz-Sommer, Z., and Davies, B. (2005). Evolution in action: following function in duplicated floral homeotic genes. Curr Biol **15,** 1508-1512.

CDC. (2001). Pesticide-related illnesses associated with the use of a plant growth regulator--Italy, 2001. MMWR Morb Mortal Wkly Rep **50,** 845-847.

CDC. (2005). Update: hydrogen cyanamide-related illnesses--Italy, 2002-2004. MMWR Morb Mortal Wkly Rep **54,** 405-408.

Chang, Y.-Y., Kao, N.-H., Li, J.-Y., Hsu, W.-H., Liang, Y.-L., Wu, J.-W., and Yang, C.-H. (2010). Characterization of the Possible Roles for B Class MADS Box Genes in Regulation of Perianth Formation in Orchid. Plant Physiology **152,** 837-853.

Chen, M.-K., Hsu, W.-H., Lee, P.-F., Thiruvengadam, M., Chen, H.-I., and Yang, C.-H. (2011). The MADS box gene, FOREVER YOUNG FLOWER, acts as a repressor controlling floral organ senescence and abscission in Arabidopsis. The Plant Journal **68,** 168-185.

Chiang, G.C., Barua, D., Kramer, E.M., Amasino, R.M., and Donohue, K. (2009). Major flowering time gene, flowering locus C, regulates seed germination in Arabidopsis thaliana. Proc Natl Acad Sci U S A **106,** 11661-11666.

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

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C., and van Tunen, A.J. (1995). The petunia MADS box gene FBP11 determines ovule identity. Plant Cell **7,** 1859-1868.

Colombo, M., Masiero, S., Vanzulli, S., Lardelli, P., Kater, M.M., and Colombo, L. (2008). AGL23, a type I MADS-box gene that controls female gametophyte and embryo development in Arabidopsis. Plant J **54,** 1037-1048.

Darrow, G.M. (1966). The strawberry: history, breeding and physiology. .

Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H., and Schwarz-Sommer, Z. (1999). PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development. EMBO J **18,** 4023-4034.

Davis, T.M., Denoyes-Rothan, B., and Lerceteau-Köhler, E. (2007). Strawberry. In Fruits and Nuts, C. Kole, ed (Springer Berlin Heidelberg), pp. 189-205.

Day, R.C., Herridge, R.P., Ambrose, B.A., and Macknight, R.C. (2008). Transcriptome analysis of proliferating Arabidopsis endosperm reveals biological implications for the control of syncytial division, cytokinin signaling, and gene expression regulation. Plant Physiol **148,** 1964-1984.

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

de Folter, S., Shchennikova, A.V., Franken, J., Busscher, M., Baskar, R., Grossniklaus, U., Angenent, G.C., and Immink, R.G. (2006). A Bsister MADS-box gene involved in ovule and seed development in petunia and Arabidopsis. Plant J **47,** 934-946.

de Martino, G., Pan, I., Emmanuel, E., Levy, A., and Irish, V.F. (2006). Functional analyses of two tomato APETALA3 genes demonstrate diversification in their roles in regulating floral development. Plant Cell **18,** 1833-1845.

Deng, W., Ying, H., Helliwell, C.A., Taylor, J.M., Peacock, W.J., and Dennis, E.S. (2011). FLOWERING LOCUS C (FLC) regulates development pathways throughout the life cycle of Arabidopsis. Proc Natl Acad Sci U S A **108,** 6680-6685.

Dennis, E.S., and Peacock, W.J. (2007). Epigenetic regulation of flowering. Current Opinion in Plant Biology **10,** 520-527.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., and Yanofsky, M.F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Curr Biol **14,** 1935-1940.

Dorca-Fornell, C., Gregis, V., Grandi, V., Coupland, G., Colombo, L., and Kater, M.M. (2011). The Arabidopsis SOC1-like genes AGL42, AGL71 and AGL72 promote flowering in the shoot apical and axillary meristems. The Plant Journal **67,** 1006-1017.

Du, X., Xiao, Q., Zhao, R., Wu, F., Xu, Q., Chong, K., and Meng, Z. (2008). TrMADS3, a new MADS-box gene, from a perennial species Taihangia rupestris (Rosaceae) is upregulated by cold and experiences seasonal fluctuation in expression level. Dev Genes Evol **218,** 281-292.

Dubcovsky, J., Loukoianov, A., Fu, D., Valarik, M., Sanchez, A., and Yan, L. (2006). Effect of photoperiod on the regulation of wheat vernalization genes VRN1 and VRN2. Plant Mol Biol **60,** 469-480.

Eccel, E., Rea, R., Caffarra, A., and Crisci, A. (2009). Risk of spring frost to apple production under future climate scenarios: the role of phenological acclimation. Int J Biometeorol **53,** 273-286.

Edger, P.P., and Pires, J.C. (2009). Gene and genome duplications: the impact of dosage-sensitivity on the fate of nuclear genes. Chromosome Res **17,** 699-717.

Elo, A., Lemmetyinen, J., Novak, A., Keinonen, K., Porali, I., Hassinen, M., and Sopanen, T. (2007). BpMADS4 has a central role in inflorescence initiation in silver birch (Betula pendula). Physiol Plant **131,** 149-158.

Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M.F., Kater, M.M., and Colombo, L. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. Plant Cell **15,** 2603-2611.

Fernandez, D.E., Heck, G.R., Perry, S.E., Patterson, S.E., Bleecker, A.B., and Fang, S.-C. (2000). The Embryo MADS Domain Factor AGL15 Acts Postembryonically: Inhibition of Perianth Senescence and Abscission via Constitutive Expression. The Plant Cell Online **12,** 183-197.

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

Ferrándiz, C., Liljegren, S.J., and Yanofsky, M.F. (2000). Negative Regulation of the SHATTERPROOF Genes by FRUITFULL During Arabidopsis Fruit Development. Science **289,** 436-438.

Ferree, D.C., and Warrington, I. (2003). Apples: Botany, Production and Uses.

Flachowsky, H., Le Roux, P.M., Peil, A., Patocchi, A., Richter, K., and Hanke, M.V. (2011). Application of a high-speed breeding technology to apple (Malus x domestica) based on transgenic early flowering plants and marker-assisted selection. New Phytol **192,** 364-377.

Flanagan, C.A., and Ma, H. (1994). Spatially and temporally regulated expression of the MADS-box gene AGL2 in wild-type and mutant arabidopsis flowers. Plant Mol Biol **26,** 581-595.

Floyd, R.B., and Braddock, R.D. (1984). A simple method for fitting average diurnal temperature curves. Agricultural and Forest Meteorology **32,** 107-119.

Folta, K.M., and Davis, T.M. (2006). Strawberry Genes and Genomics. Critical Reviews in Plant Sciences **25,** 399-415.

Gan, Y., Filleur, S., Rahman, A., Gotensparre, S., and Forde, B.G. (2005). Nutritional regulation of ANR1 and other root-expressed MADS-box genes in Arabidopsis thaliana. Planta **222,** 730-742.

Gehring, W.J., Affolter, M., and Burglin, T. (1994). Homeodomain proteins. Annu Rev Biochem **63,** 487-526.

Goldschmidt, E.E., and Samach, A. (2004). Aspects of flowering in fruit trees. Acta Horticulturae **653,** 23-27.

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

Gramzow, L., Ritz, M.S., and Theißen, G. (2010). On the origin of MADS-domain transcription factors. Trends in genetics : TIG **26,** 149-153.

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

Guak, S., and Neilsen, D. (2013). Chill unit models for predicting dormancy completion of floral buds in apple and sweet cherry. Hortic. Environ. Biotechnol. **54,** 29-36.

Han, P., García-Ponce, B., Fonseca-Salazar, G., Alvarez-Buylla, E.R., and Yu, H. (2008). AGAMOUS-LIKE 17, a novel flowering promoter, acts in a FT-independent photoperiod pathway. The Plant Journal **55,** 253-265.

Harding, E.W., Tang, W., Nichols, K.W., Fernandez, D.E., and Perry, S.E. (2003). Expression and Maintenance of Embryogenic Potential Is Enhanced through Constitutive Expression of AGAMOUS-Like 15. Plant Physiology **133,** 653-663.

Hartmann, U., Hohmann, S., Nettesheim, K., Wisman, E., Saedler, H., and Huijser, P. (2000). Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. Plant J **21,** 351-360.

Hathaway, G.J., Proctor, N.H., and Hughes, J.P. (1996). Hathaway GJ, Proctor NH, Hughes JP. Proctor and Hughes' chemical hazards of the workplace. .

He, C., and Saedler, H. (2005). Heterotopic expression of MPF2 is the key to the evolution of the Chinese lantern of Physalis, a morphological novelty in Solanaceae. Proceedings of the National Academy of Sciences of the United States of America **102,** 5779-5784.

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

Heck, G.R., Perry, S.E., Nichols, K.W., and Fernandez, D.E. (1995). AGL15, a MADS domain protein expressed in developing embryos. The Plant Cell Online **7,** 1271-1282.

Heide, O.M., and Sønsteby, A. (2007). Interactions of temperature and photoperiod in the control of flowering of latitudinal and altitudinal populations of wild strawberry (Fragaria vesca). Physiologia Plantarum **130,** 280-289.

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

Henschel, K., Kofuji, R., Hasebe, M., Saedler, H., Munster, T., and Theissen, G. (2002). Two ancient classes of MIKC-type MADS-box genes are present in the moss Physcomitrella patens. Mol Biol Evol **19,** 801-814.

Heo, J.B., and Sung, S. (2011). Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. Science **331,** 76-79.

Honma, T., and Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409,** 525-529.

Hsu, C.Y., Adams, J.P., Kim, H., No, K., Ma, C., Strauss, S.H., Drnevich, J., Vandervelde, L., Ellis, J.D., Rice, B.M., Wickett, N., Gunter, L.E., Tuskan, G.A., Brunner, A.M., Page, G.P., Barakat, A., Carlson, J.E., DePamphilis, C.W., Luthe, D.S., and Yuceer, C. (2011). FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. Proc Natl Acad Sci U S A **108,** 10756-10761.

Huijser, P., Klein, J., Lonnig, W.E., Meijer, H., Saedler, H., and Sommer, H. (1992). Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in Antirrhinum majus. EMBO J **11,** 1239-1249.

Immink, R.G., Tonaco, I.A., de Folter, S., Shchennikova, A., van Dijk, A.D., Busscher-Lange, J., Borst, J.W., and Angenent, G.C. (2009). SEPALLATA3: the 'glue' for MADS box transcription factor complex formation. Genome Biol **10,** R24.

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

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

Jack, T., Fox, G.L., and Meyerowitz, E.M. (1994). Arabidopsis homeotic gene APETALA3 ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell **76,** 703-716.

Jang, S., Torti, S., and Coupland, G. (2009). Genetic and spatial interactions betweenFT,TSFandSVPduring the early stages of floral induction in Arabidopsis. The Plant Journal **60,** 614-625.

Jeon, J.S., Jang, S., Lee, S., Nam, J., Kim, C., Lee, S.H., Chung, Y.Y., Kim, S.R., Lee, Y.H., Cho, Y.G., and An, G. (2000). leafy hull sterile1 is a homeotic mutation in a rice MADS box gene affecting rice flower development. Plant Cell **12,** 871-884.

Jimenez, S., Lawton-Rauh, A.L., Reighard, G.L., Abbott, A.G., and Bielenberg, D.G. (2009). Phylogenetic analysis and molecular evolution of the dormancy associated MADS-box genes from peach. BMC Plant Biol **9,** 81.

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

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

Kajfez-Bogataj, L., and Bergant, K. (1998). Prediction of blossoming of apple tree (Malus domestica Borkh) by phenological models. . Research Reports Biotechnical Faculty University of Ljubljana **7**.

Kang, C., Darwish, O., Geretz, A., Shahan, R., Alkharouf, N., and Liu, Z. (2013). Genome-scale transcriptomic insights into early-stage fruit development in woodland strawberry Fragaria vesca. Plant Cell **25,** 1960-1978.

Kaufmann, K., Anfang, N., Saedler, H., and Theissen, G. (2005). Mutant analysis, protein-protein interactions and subcellular localization of the Arabidopsis Bsister (ABS) protein. Molecular Genetics and Genomics **274,** 103-118.

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

Kim, D.H., Doyle, M.R., Sung, S., and Amasino, R.M. (2009). Vernalization: winter and the timing of flowering in plants. Annu Rev Cell Dev Biol **25,** 277-299.

Kim, S.Y., He, Y., Jacob, Y., Noh, Y.S., Michaels, S., and Amasino, R. (2005). Establishment of the vernalization-responsive, winter-annual habit in Arabidopsis requires a putative histone H3 methyl transferase. Plant Cell **17,** 3301-3310.

Kohler, C., Page, D.R., Gagliardini, V., and Grossniklaus, U. (2005). The Arabidopsis thaliana MEDEA Polycomb group protein controls expression of PHERES1 by parental imprinting. Nat Genet **37,** 28-30.

Koo, S.C., Bracko, O., Park, M.S., Schwab, R., Chun, H.J., Park, K.M., Seo, J.S., Grbic, V., Balasubramanian, S., Schmid, M., Godard, F., Yun, D.-J., Lee, S.Y., Cho, M.J., Weigel, D., and Kim, M.C. (2010). Control of lateral organ development and flowering time by the Arabidopsis thaliana MADS-box Gene AGAMOUS-LIKE6. The Plant Journal **62,** 807-816.

Kramer, E.M., Dorit, R.L., and Irish, V.F. (1998). Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. Genetics **149,** 765-783.

Kramer, E.M., Jaramillo, M.A., and Di Stilio, V.S. (2004). Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. Genetics **166,** 1011-1023.

Kramer, E.M., Su, H.J., Wu, C.C., and Hu, J.M. (2006). A simplified explanation for the frameshift mutation that created a novel C-terminal motif in the APETALA3 gene lineage. BMC Evol Biol **6,** 30.

Krizek, B.A., and Fletcher, J.C. (2005). Molecular mechanisms of flower development: an armchair guide. Nat Rev Genet **6,** 688-698.

Kuittinen, H., Niittyvuopio, A., Rinne, P., and Savolainen, O. (2008). Natural variation in Arabidopsis lyrata vernalization requirement conferred by a FRIGIDA indel polymorphism. Mol Biol Evol **25,** 319-329.

Kutter, C., Schob, H., Stadler, M., Meins, F., Jr., and Si-Ammour, A. (2007). MicroRNA-mediated regulation of stomatal development in Arabidopsis. Plant Cell **19,** 2417-2429.

Landsberg, J.J. (1974). Apple fruit bud development and growth: analysis and an empirical model. Ann Bot **38,** 1013-1023.

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

Lee, J., and Lee, I. (2010). Regulation and function of SOC1, a flowering pathway integrator. J Exp Bot **61,** 2247-2254.

Lee, J.H., Yoo, S.J., Park, S.H., Hwang, I., Lee, J.S., and Ahn, J.H. (2007). Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. Genes Dev **21,** 397-402.

Leida, C., Conesa, A., Llacer, G., Badenes, M.L., and Rios, G. (2012). Histone modifications and expression of DAM6 gene in peach are modulated during bud dormancy release in a cultivar-dependent manner. New Phytol **193,** 67-80.

Leseberg, C.H., Li, A., Kang, H., Duvall, M., and Mao, L. (2006). Genome-wide analysis of the MADS-box gene family in Populus trichocarpa. Gene **378,** 84-94.

Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C.A., Ito, T., Meyerowitz, E., and Yu, H. (2008). A repressor complex governs the integration of flowering signals in Arabidopsis. Dev Cell **15,** 110-120.

Li, Z., Reighard, G.L., Abbott, A.G., and Bielenberg, D.G. (2009). Dormancy-associated MADS genes from the EVG locus of peach [Prunus persica (L.) Batsch] have distinct seasonal and photoperiodic expression patterns. J Exp Bot **60,** 3521-3530.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L., and Yanofsky, M.F. (2000). SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. Nature **404,** 766-770.

Lin, S.I., Wang, J.G., Poon, S.Y., Su, C.L., Wang, S.S., and Chiou, T.J. (2005). Differential regulation of FLOWERING LOCUS C expression by vernalization in cabbage and Arabidopsis. Plant Physiol **137,** 1037-1048.

Ma, H., Yanofsky, M.F., and Meyerowitz, E.M. (1991). AGL1-AGL6, an Arabidopsis gene family with similarity to floral homeotic and transcription factor genes. Genes Dev **5,** 484-495.

Mandel, M.A., and Yanofsky, M.F. (1998). The Arabidopsis AGL9 MADS box gene is expressed in young flower primordia. Sex Plant Reprod. **11,** 22-28.

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

Mao, L., Begum, D., Chuang, H.W., Budiman, M.A., Szymkowiak, E.J., Irish, E.E., and Wing, R.A. (2000). JOINTLESS is a MADS-box gene controlling tomato flower abscission zone development. Nature **406,** 910-913.

Masiero, S., Colombo, L., Grini, P.E., Schnittger, A., and Kater, M.M. (2011). The emerging importance of type I MADS box transcription factors for plant reproduction. Plant Cell **23,** 865-872.

Masiero, S., Li, M.-A., Will, I., Hartmann, U., Saedler, H., Huijser, P., Schwarz-Sommer, Z., and Sommer, H. (2004). INCOMPOSITA: a MADS-box gene controlling prophyll development and floral meristem identity in Antirrhinum. Development **131,** 5981-5990.

Melzer, R., Wang, Y.-Q., and Theißen, G. (2010). The naked and the dead: The ABCs of gymnosperm reproduction and the origin of the angiosperm flower. Seminars in Cell & Developmental Biology **21,** 118-128.

Melzer, S., Lens, F., Gennen, J., Vanneste, S., Rohde, A., and Beeckman, T. (2008). Flowering-time genes modulate meristem determinacy and growth form in Arabidopsis thaliana. Nat Genet **40,** 1489-1492.

Merchante, C., Vallarino, J.G., Osorio, S., Araguez, I., Villarreal, N., Ariza, M.T., Martinez, G.A., Medina-Escobar, N., Civello, M.P., Fernie, A.R., Botella, M.A., and Valpuesta, V. (2013). Ethylene is involved in strawberry fruit ripening in an organ-specific manner. J Exp Bot **64,** 4421-4439.

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

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

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

Michaels, S.D., Ditta, G., Gustafson-Brown, C., Pelaz, S., Yanofsky, M., and Amasino, R.M. (2003). AGL24 acts as a promoter of flowering in Arabidopsis and is positively regulated by vernalization. Plant J **33,** 867-874.

Mondragon-Palomino, M., and Theissen, G. (2008). MADS about the evolution of orchid flowers. Trends Plant Sci **13,** 51-59.

Moore, S., Vrebalov, J., Payton, P., and Giovannoni, J. (2002). Use of genomics tools to isolate key ripening genes and analyse fruit maturation in tomato. J Exp Bot **53,** 2023-2030.

Moreno-Risueno, M.A., Van Norman, J.M., Moreno, A., Zhang, J., Ahnert, S.E., and Benfey, P.N. (2010). Oscillating Gene Expression Determines Competence for Periodic Arabidopsis Root Branching. Science **329,** 1306-1311.

Mouradov, A., Glassick, T.V., Hamdorf, B.A., Murphy, L.C., Marla, S.S., Yang, Y., and Teasdale, R.D. (1998). Family of MADS-Box genes expressed early in male and female reproductive structures of monterey pine. Plant Physiol **117,** 55-62.

Nakano, T., Kimbara, J., Fujisawa, M., Kitagawa, M., Ihashi, N., Maeda, H., Kasumi, T., and Ito, Y. (2012). MACROCALYX and JOINTLESS Interact in the Transcriptional Regulation of Tomato Fruit Abscission Zone Development. Plant Physiology **158,** 439-450.

Nam, J., Kim, J., Lee, S., An, G., Ma, H., and Nei, M. (2004). Type I MADS-box genes have experienced faster birth-and-death evolution than type II MADS-box genes in angiosperms. Proc Natl Acad Sci U S A **101,** 1910-1915.

Nawy, T., Lee, J.-Y., Colinas, J., Wang, J.Y., Thongrod, S.C., Malamy, J.E., Birnbaum, K., and Benfey, P.N. (2005). Transcriptional Profile of the Arabidopsis Root Quiescent Center. The Plant Cell Online **17,** 1908-1925.

Nesi, N., Debeaujon, I., Jond, C., Stewart, A.J., Jenkins, G.I., Caboche, M., and Lepiniec, L. (2002). The TRANSPARENT TESTA16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. Plant Cell **14,** 2463-2479.

Ng, M., and Yanofsky, M.F. (2001). Function and evolution of the plant MADS-box gene family. Nat Rev Genet **2,** 186-195.

Norman, C., Runswick, M., Pollock, R., and Treisman, R. (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell **55,** 989-1003.

Okamuro, J.K., Caster, B., Villarroel, R., Van Montagu, M., and Jofuku, K.D. (1997). The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. Proc Natl Acad Sci U S A **94,** 7076-7081.

Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K., and Coupland, G. (2000). Mutagenesis of plants overexpressing CONSTANS demonstrates novel interactions among Arabidopsis flowering-time genes. Plant Cell **12,** 885-900.

Parenicova, L. (2003). Molecular and Phylogenetic Analyses of the Complete MADS-Box Transcription Factor Family in Arabidopsis: New Openings to the MADS World. The Plant Cell Online **15,** 1538-1551.

Passmore, S., Maine, G.T., Elble, R., Christ, C., and Tye, B.K. (1988). Saccharomyces cerevisiae protein involved in plasmid maintenance is necessary for mating of MAT alpha cells. J Mol Biol **204,** 593-606.

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

Pelucchi, N., Fornara, F., Favalli, C., Masiero, S., Lago, C., Pè, E., Colombo, L., and Kater, M.M. (2002). Comparative analysis of rice MADS-box genes expressed during flower development. Sex Plant Reprod. **14,** 113-122.

Perry, S.E., Nichols, K.W., and Fernandez, D.E. (1996). The MADS domain protein AGL15 localizes to the nucleus during early stages of seed development. Plant Cell **8,** 1977-1989.

Pinyopich, A., Ditta, G.S., Savidge, B., Liljegren, S.J., Baumann, E., Wisman, E., and Yanofsky, M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature **424,** 85-88.

Portereiko, M.F., Lloyd, A., Steffen, J.G., Punwani, J.A., Otsuga, D., and Drews, G.N. (2006). AGL80 is required for central cell and endosperm development in Arabidopsis. Plant Cell **18,** 1862-1872.

Prasad, K., Zhang, X., Tobón, E., and Ambrose, B.A. (2010). The Arabidopsis B-sister MADS-box protein, GORDITA, represses fruit growth and contributes to integument development. The Plant Journal **62,** 203-214.

Pratt. (1988). Apple Flower and Fruit: Morphology and anatomy. Hort Rev **10**.

Purugganan, M.D., Rounsley, S.D., Schmidt, R.J., and Yanofsky, M.F. (1995). Molecular evolution of flower development: diversification of the plant MADS-box regulatory gene family. Genetics **140,** 345-356.

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

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J., and Riechmann, J.L. (2003). Analysis of the Arabidopsis MADS AFFECTING FLOWERING Gene Family: MAF2 Prevents Vernalization by Short Periods of Cold. The Plant Cell Online **15,** 1159-1169.

Rea, R., and Eccel, E. (2006). Phenological models for blooming of apple in a mountainous region. Int J Biometeorol **51,** 1-16.

Reicosky, D.C., Winkelman, L.J., Baker, J.M., and Baker, D.G. (1989). Accuracy of hourly air temperatures calculated from daily minima and maxima. Agricultural and Forest Meteorology **46,** 193-209.

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

Rijpkema, A.S., Royaert, S., Zethof, J., van der Weerden, G., Gerats, T., and Vandenbussche, M. (2006). Analysis of the Petunia TM6 MADS box gene reveals functional divergence within the DEF/AP3 lineage. Plant Cell **18,** 1819-1832.

Rosin, F.M., Hart, J.K., Van Onckelen, H., and Hannapel, D.J. (2003). Suppression of a Vegetative MADS Box Gene of Potato Activates Axillary Meristem Development. Plant Physiology **131,** 1613-1622.

Rounsley, S.D., Ditta, G.S., and Yanofsky, M.F. (1995). Diverse roles for MADS box genes in Arabidopsis development. Plant Cell **7,** 1259-1269.

Saedler, H., Becker, A., Winter, K.U., Kirchner, C., and Theissen, G. (2001). MADS-box genes are involved in floral development and evolution. Acta Biochim Pol **48,** 351-358.

Saito, T., Bai, S., Ito, A., Sakamoto, D., Ubi, B.E., Imai, T., and Moriguchi, T. (2013). Expression and genomic structure of the dormancy-associated MADS box genes MADS13 in Japanese pears (Pyrus pyrifolia Nakai) that differ in their chilling requirement for endodormancy release. Tree Physiol **33,** 654-667.

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

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

Schonrock, N., Bouveret, R., Leroy, O., Borghi, L., Kohler, C., Gruissem, W., and Hennig, L. (2006). Polycomb-group proteins repress the floral activator AGL19 in the FLC-independent vernalization pathway. Genes Dev **20,** 1667-1678.

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

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

Seoighe, C., and Gehring, C. (2004). Genome duplication led to highly selective expansion of the Arabidopsis thaliana proteome. Trends Genet **20,** 461-464.

Shore, P., and Sharrocks, A.D. (1995). The ETS-domain transcription factors Elk-1 and SAP-1 exhibit differential DNA binding specificities. Nucleic Acids Res **23,** 4698-4706.

Shulaev, V., Korban, S.S., Sosinski, B., Abbott, A.G., Aldwinckle, H.S., Folta, K.M., Iezzoni, A., Main, D., Arus, P., Dandekar, A.M., Lewers, K., Brown, S.K., Davis, T.M., Gardiner, S.E., Potter, D., and Veilleux, R.E. (2008). Multiple models for Rosaceae genomics. Plant Physiol **147,** 985-1003.

Shulaev, V., Sargent, D.J., Crowhurst, R.N., Mockler, T.C., Folkerts, O., Delcher, A.L., Jaiswal, P., Mockaitis, K., Liston, A., Mane, S.P., Burns, P., Davis, T.M., Slovin, J.P., Bassil, N., Hellens, R.P., Evans, C., Harkins, T., Kodira, C., Desany, B., Crasta, O.R., Jensen, R.V., Allan, A.C., Michael, T.P., Setubal, J.C., Celton, J.-M., Rees, D.J.G., Williams, K.P., Holt, S.H., Rojas, J.J.R., Chatterjee, M., Liu, B., Silva, H., Meisel, L., Adato, A., Filichkin, S.A., Troggio, M., Viola, R., Ashman, T.-L., Wang, H., Dharmawardhana, P., Elser, J., Raja, R., Priest, H.D., Bryant, D.W., Fox, S.E., Givan, S.A., Wilhelm, L.J., Naithani, S., Christoffels, A., Salama, D.Y., Carter, J., Girona, E.L., Zdepski, A., Wang, W., Kerstetter, R.A., Schwab, W., Korban, S.S., Davik, J., Monfort, A., Denoyes-Rothan, B., Arus, P., Mittler, R., Flinn, B., Aharoni, A., Bennetzen, J.L., Salzberg, S.L., Dickerman, A.W., Velasco, R., Borodovsky, M., Veilleux, R.E., and Folta, K.M. (2010). The genome of woodland strawberry (Fragaria vesca). Nature Genetics **43,** 109-116.

Smaczniak, C., Immink, R.G., Angenent, G.C., and Kaufmann, K. (2012). Developmental and evolutionary diversity of plant MADS-domain factors: insights from recent studies. Development **139,** 3081-3098.

Sommer, H., Beltran, J.P., Huijser, P., Pape, H., Lonnig, W.E., Saedler, H., and Schwarz-Sommer, Z. (1990). Deficiens, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. EMBO J **9,** 605-613.

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

Tapia-López, R., García-Ponce, B., Dubrovsky, J.G., Garay-Arroyo, A., Pérez-Ruíz, R.V., Kim, S.-H., Acevedo, F., Pelaz, S., and Alvarez-Buylla, E.R. (2008). An AGAMOUS-Related MADS-Box Gene, XAL1 (AGL12), Regulates Root Meristem Cell Proliferation and Flowering Transition in Arabidopsis. Plant Physiology **146,** 1182-1192.

Taylor, M., Treisman, R., Garrett, N., and Mohun, T. (1989). Muscle-specific (CArG) and serum-responsive (SRE) promoter elements are functionally interchangeable in Xenopus embryos and mouse fibroblasts. Development **106,** 67-78.

Theissen, G. (2001). Development of floral organ identity: stories from the MADS house. Curr Opin Plant Biol **4,** 75-85.

Theissen, G., and Saedler, H. (2001). Plant biology. Floral quartets. Nature **409,** 469-471.

Theissen, G., Kim, J.T., and Saedler, H. (1996). Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J Mol Evol **43,** 484-516.

Tiwari, S., Spielman, M., Schulz, R., Oakey, R.J., Kelsey, G., Salazar, A., Zhang, K., Pennell, R., and Scott, R.J. (2010). Transcriptional profiles underlying parent-of-origin effects in seeds of Arabidopsis thaliana. BMC Plant Biol **10,** 72.

Uimari, A., Kotilainen, M., Elomaa, P., Yu, D., Albert, V.A., and Teeri, T.H. (2004). Integration of reproductive meristem fates by a SEPALLATA-like MADS-box gene. Proceedings of the National Academy of Sciences of the United States of America **101,** 15817-15822.

Vandenbussche, M., Zethof, J., Royaert, S., Weterings, K., and Gerats, T. (2004). The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in Petunia hybrida flower development. Plant Cell **16,** 741-754.

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., Fontana, P., Bhatnagar, S.K., Troggio, M., Pruss, D., Salvi, S., Pindo, M., Baldi, P., Castelletti, S., Cavaiuolo, M., Coppola, G., Costa, F., Cova, V., Dal Ri, A., Goremykin, V., Komjanc, M., Longhi, S., Magnago, P., Malacarne, G., Malnoy, M., Micheletti, D., Moretto, M., Perazzolli, M., Si-Ammour, A., Vezzulli, S., Zini, E., Eldredge, G., Fitzgerald, L.M., Gutin,

N., Lanchbury, J., Macalma, T., Mitchell, J.T., Reid, J., Wardell, B., Kodira, C., Chen, Z., Desany, B., Niazi, F., Palmer, M., Koepke, T., Jiwan, D., Schaeffer, S., Krishnan, V., Wu, C., Chu, V.T., King, S.T., Vick, J., Tao, Q., Mraz, A., Stormo, A., Stormo, K., Bogden, R., Ederle, D., Stella, A., Vecchietti, A., Kater, M.M., Masiero, S., Lasserre, P., Lespinasse, Y., Allan, A.C., Bus, V., Chagné, D., Crowhurst, R.N., Gleave, A.P., Lavezzo, E., Fawcett, J.A., Proost, S., Rouzé, P., Sterck, L., Toppo, S., Lazzari, B., Hellens, R.P., Durel, C.-E., Gutin, A., Bumgarner, R.E., Gardiner, S.E., Skolnick, M., Egholm, M., Van de Peer, Y., Salamini, F., and Viola, R. (2010). The genome of the domesticated apple (Malus × domestica Borkh.). Nature Genetics **42,** 833-839.

Vernoux, T., Brunoud, G., Farcot, E., Morin, V., Van den Daele, H., Legrand, J., Oliva, M., Das, P., Larrieu, A., Wells, D., Guedon, Y., Armitage, L., Picard, F., Guyomarc'h, S., Cellier, C., Parry, G., Koumproglou, R., Doonan, J.H., Estelle, M., Godin, C., Kepinski, S., Bennett, M., De Veylder, L., and Traas, J. (2011). The auxin signalling network translates dynamic input into robust patterning at the shoot apex. Mol Syst Biol **7,** 508.

Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W., and Giovannoni, J. (2002). A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science **296,** 343-346.

Walia, H., Josefsson, C., Dilkes, B., Kirkbride, R., Harada, J., and Comai, L. (2009). Dosage-dependent deregulation of an AGAMOUS-LIKE gene cluster contributes to interspecific incompatibility. Curr Biol **19,** 1128-1132.

Wang, J.W., Czech, B., and Weigel, D. (2009a). miR156-regulated SPL transcription factors define an endogenous flowering pathway in Arabidopsis thaliana. Cell **138,** 738-749.

Wang, R., Farrona, S., Vincent, C., Joecker, A., Schoof, H., Turck, F., Alonso-Blanco, C., Coupland, G., and Albani, M.C. (2009b). PEP1 regulates perennial flowering in Arabis alpina. Nature **459,** 423-427.

Wang, R., Albani, M.C., Vincent, C., Bergonzi, S., Luan, M., Bai, Y., Kiefer, C., Castillo, R., and Coupland, G. (2011). Aa TFL1 confers an age-dependent response to vernalization in perennial Arabis alpina. Plant Cell **23,** 1307-1321.

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

Wellmer, F., and Riechmann, J.L. (2010). Gene networks controlling the initiation of flower development. Trends Genet **26,** 519-527.

White, L.M. (1979). Relationship between meteorological measurements and flowering of index species to flowering of 53 plant species. Agricultural Meteorology **20,** 189-204.

Winter, K.U., Saedler, H., and Theissen, G. (2002a). On the origin of class B floral homeotic genes: functional substitution and dominant inhibition in Arabidopsis by expression of an orthologue from the gymnosperm Gnetum. Plant J **31,** 457-475.

Winter, K.U., Becker, A., Munster, T., Kim, J.T., Saedler, H., and Theissen, G. (1999). MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. Proc Natl Acad Sci U S A **96,** 7342-7347.

Winter, K.U., Weiser, C., Kaufmann, K., Bohne, A., Kirchner, C., Kanno, A., Saedler, H., and Theissen, G. (2002b). Evolution of class B floral homeotic proteins: obligate heterodimerization originated from homodimerization. Mol Biol Evol **19,** 587-596.

Wuest, S.E., Vijverberg, K., Schmidt, A., Weiss, M., Gheyselinck, J., Lohr, M., Wellmer, F., Rahnenfuhrer, J., von Mering, C., and Grossniklaus, U. (2010). Arabidopsis female gametophyte gene expression map reveals similarities between plant and animal gametes. Curr Biol **20,** 506-512.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G., and Hirano, H.Y. (2006). Functional diversification of the two C-class MADS box genes OSMADS3 and OSMADS58 in Oryza sativa. Plant Cell **18,** 15-28.

Yan, L., Loukoianov, A., Blechl, A., Tranquilli, G., Ramakrishna, W., SanMiguel, P., Bennetzen, J.L., Echenique, V., and Dubcovsky, J. (2004). The wheat VRN2 gene is a flowering repressor down-regulated by vernalization. Science **303,** 1640-1644.

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

Yant, L. (2012). Genome-wide mapping of transcription factor binding reveals developmental process integration and a fresh look at evolutionary dynamics. Am J Bot **99,** 277-290.

Yant, L., Mathieu, J., and Schmid, M. (2009). Just say no: floral repressors help Arabidopsis bide the time. Curr Opin Plant Biol **12,** 580-586.

Yoo, S.K., Wu, X., Lee, J.S., and Ahn, J.H. (2011). AGAMOUS-LIKE 6 is a floral promoter that negatively regulates the FLC/MAF clade genes and positively regulates FT in Arabidopsis. The Plant Journal **65,** 62-76.

Yu, H., Xu, Y., Tan, E.L., and Kumar, P.P. (2002). AGAMOUS-LIKE 24, a dosage-dependent mediator of the flowering signals. Proc Natl Acad Sci U S A **99,** 16336-16341.

Zhang, H., and Forde, B.G. (1998). An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science **279,** 407-409.

Zhang, J.Z., Li, Z.M., Mei, L., Yao, J.L., and Hu, C.G. (2009). PtFLC homolog from trifoliate orange (Poncirus trifoliata) is regulated by alternative splicing and experiences seasonal fluctuation in expression level. Planta **229,** 847-859.

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

Zimmerman, R.H. (1972). Juvenility and flowering in woody plants: a review. HortScience **10,** 447-455.

Chapter II

Identification of two dormancy-associated MADS-box genes in apple

II.1 INTRODUCTION

Plants as sessile organisms evolved to sense and adapt to temperature changes to protect themselves from harsh winter and frost. In this way they ensure that flowering occurs only when optimal environmental conditions return (Rohde et al., 2000; 2007). Brassicaceae and cereals control flowering time by vernalization, which inhibits the transition from the vegetative to the reproductive phase until exposure to a prolonged cold period (Andres and Coupland, 2012). Similarly, during autumn and winter many perennial species keep the meristem tissue in a dormant stage (endodormancy, from now on referred to as dormancy) inside protective structures named buds, which are then activated after a prolonged period of chilling (Vegis, 1964; Anderson et al., 1986; Lang, 1987). Lateral buds are usually formed in early summer and enter a paradormant state that is mainly caused by apical dominance. During autumn, with timing that varies depending on species, bud internal signals inhibit its growth; these buds are referred to as endodormant (Lang, 1987). Certain plant hormones that mediate the induction of endodormancy, such as abscisic acid, gibberellic acid, and ethylene, have been identified and characterized (Rohde et al., 2002; Rohde and Bhalerao, 2007). Endodormant buds require a species-dependent amount of chilling to enter an ecodormant state, in which buds are capable of resuming growth in favourable environments. In contrast to endodormancy, ecodormancy is controlled by external environmental factors such as cold or drought stress, which prevent bud growth (Lang, 1987; Horvath et al., 2003). The lack of sufficient chilling hours to break bud dormancy in warm areas hinders the commercial production of temperate fruit trees and results in the use of chemicals, some of them toxic for human health, to induce flowering initiation.

Dormancy release in fruit trees is due to a common regulatory mechanisms controlled by a set of related MADS-box transcription factors (Hemming and Trevaskis, 2011). The Dormancy Associated MADS-box (DAM) group of transcription factors clustering with StMADS11 clade of *A. thaliana*, have been proposed to regulate bud dormancy processes in peach, leafy spurge, Japanese apricot, and Japanese pear (Bielenberg et al., 2008; Yamane et al., 2008; Horvath et al., 2010; Sasaki et al., 2011; Saito et al., 2013). Expression profiling during dormancy induction, maintenance and release were analyzed also in different *Populus* species, Norway spruce, oak, raspberry, and grapevine (Schrader et al., 2004; Derory et al., 2006; Yakovlev et al., 2006; Mazzitelli et al., 2007; Rohde et al., 2007; Ruttink et al., 2007; Park et al., 2008; Mathiason et al., 2009). Comparative studies of genes involved in dormancy indicated an expansion of the

StMADS11 clade of MIKC^c-type MADS-box genes in perennial tree (Leseberg et al., 2006). Only few copies are present in *A. thaliana*, tomato and rice, whereas duplication events led to eight genes in poplar, six in peach, five in *V. vinifera*, and three in pear (Alvarez-Buylla et al., 2000; Hileman et al., 2006; Leseberg et al., 2006; Arora et al., 2007; Bielenberg et al., 2008; Díaz-Riquelme et al., 2009; Saito et al., 2013). It is known that low temperature control dormancy release also in apple, but no DAM genes have been characterized in this species so far (Jonkers, 1979; Heide and Prestrud, 2005).

Vernalization in Brassicaceae is regulated by FLC (Michaels and Amasino, 1999). The *FLC* transcript decreases quantitatively during cold exposure by an epigenetic mechanism involving chromatin modifications at the histone level, including trimethylation of histone H3 at lysine 27 (H3K27me3) (Doyle and Amasino, 2009). Similar epigenetic modifications regulate DAM genes in peach during bud dormancy (Leida et al., 2012).

In this study we report the characterization of two StMADS11 *DAM* genes, *MdDAM1* and the novel *MdDAM2*, found in apple (*Malus x domestica* Borkh.) cv. 'Golden Delicious'. We performed a whole transcriptome screen in dormant buds followed by gene expression analysis and demonstrate that MdDAM1 and MdDAM2 transcripts highly correlate to exposure to cold. To investigate the epigenetic mechanism involved in *MdDAM* genes regulation, we localized and analyzed the loci associated with histone H3 modifications.

II.2 MATERIAL AND METHODS

II.2.1 Plant material and environmental parameters

Unless otherwise indicated, *Malus* × *domestica* Borkh cultivar 'Golden Delicious' was used in this study. Apple dormant buds were harvested from five year-old trees growing in the "Giaroni" orchard in San Michele all'Adige in Italy (latitude 46.181539°, longitude 11.119877°). Buds were collected during the period from the 2nd of November 2011 to the 2nd of March 2012. Setting up the 2nd of November as the starting point (t0), buds were collected after 16 (t1), 47 (t2), 78 (t3), 110 (t4), and 121 (t5) days corresponding to 300, 478, 777, 1020, 1255 CU, respectively. Chilling units were determined by the "progressive Utah" model for the period January 2011 to June 2012 using data collected from the IASMA meteorological station located in the orchard as described by (Rea and

Eccel, 2006). Temperatures and day lengths collected during this period were used to build the graph in *Figure 1* using Microsoft Office Excel.

II.2.2 RNA analysis and qRT-PCR

Only terminal flower buds were considered in this study. Immediately after harvesting, the wooden part of the buds was quickly removed and the green tissues were flash frozen in liquid nitrogen and homogenized. Total RNA from buds was extracted using the Plant spectrum kit followed by on-column DNase digestion with DNase I (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instruction. RNA integrity was verified by capillary electrophoresis (Agilent Technologies, Santa Clara, CA, USA) and only samples with RIN values higher than eight were analyzed. *Actin* (Li and Yuan, 2008) and *Md_4592:1:a* (Botton et al., 2011) were found to be the most stably expressed in the studied experimental conditions and therefore used as reference genes. The results were analyzed using the comparative Ct method (Pfaffl, 2001) on three biological and three technical replicates.

II.2.3 RNAseq analysis

Total RNA from dormant buds was extracted as described above and subjected to ribosomal RNA (rRNA) depletion using the Ribo-Zero rRNA magnetic kit for plant leaf (Epicentre Biotechnologies, Madison, WI, USA). Illumina libraries were prepared using the ScriptSeq-v2-RNA-Seq library preparation kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions. The libraries were sequenced in multiplexing (six libraries per lane) using a HiSeq2000 apparatus (Illumina, San Diego, CA, USA) at Fasteris (Geneva, Switzerland) using the single read 1x100 bp mode. After cleaning of reads from adapter sequences the residual rRNA sequences (4 % of total reads) were filtered. The total number of reads obtained for each library is indicated in *Table 1*. *De novo* assembly using reads of all libraries (137,705,353) was performed using Trinity pipeline with defaults parameters (Grabherr et al., 2011). Clustering of *de novo* transcripts was performed using CDHIT-EST with a homology parameter of 90% (Fu et al., 2012). The longest transcripts were used to perform a six-frame translation and to query the UNIPROT database (http://www.uniprot.org/) using the tblastx algorithm (Altschul et al., 1997). Only proteins belonging to the *Malus x domestica* subset (MALDO) and carrying a complete MADS-box domain were taken in consideration for further analysis. Reads from each library were mapped to transcripts corresponding to

the selected MADS-box proteins and fold changes in gene expression at each time point were compared to t0 using DEseq (Anders and Huber, 2010). The fold change values expressed in log2 were used to build a heatmap by hierarchical clustering of genes using MATLAB (Mathworks).

II.2.4 Chromatin immunoprecipitation and qPCR on DNA

Chromatin immunopreciptations were performed according to abcam protocol available at http://www.abcam.com/ps/pdf/protocols/ChIP_plant_arabidopsis.pdf (Cambridge, United Kingdom) with minor modifications. Twelve apple buds cleaned from wooden material were used for cross-linking using a 1% formaldehyde solution. After homogenization of frozen plant material chromatin was isolated according to the abcam protocol. Chromatin was sheared by sonication using a Bioruptor (Diagenode, Belgium).The chromatin was precipitated using a 7 µg antibody/30 µg chromatin ratio. The antibodies used were the anti-H3K4me3 (abcam, ab8580), anti-H3K27me3 (EMD-Millipore, 07-449), and Anti-histone H3 Antibody (EMD-Millipore, 06-755). As control mock immunoprecipitation were performed using a ratio 2 µg of normal mouse IgG (EMD-Millipore, 12-371) per 30 µg chromatin. Immunoprecipitated DNA was purified using the Ipure Kit according to manufacturer's inctructions (Diagenode, Belgium). One µl of diluted DNA (1:100, v/v) was used for real time PCR mix using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 300 nM of FH3 (5' -GAGATTCAGTCATCTGGGTCAA-3') and RH3 (5' -TTCCTTTTTCCTCTCCCTCTC-3') primers. The PCR reactions were run on an C1000 Touch Thermal Cycler with CFX96™-Real Time System (Bio-Rad) using the following cycling conditions: denaturation at 95°C for 3 min, 40 cycles of amplification and quantification program (95°C for 10 s, 61°C for 10 s, and 72°C for 30 s), followed by 95°C for 60 s, and melting curve program from 65°C to 95°C with a heating rate of 0.5°C/0.05 sec. Relative quantification of methylation state in chromatin immunoprecipitated with anti-H3K4me3 and anti-H3K27me3 were analyzed using immunoprecipitated DNA associated with histone H3 as reference with the Bio-Rad CFX Manager 3.0 software.

II.2.5 Phylogenetic analysis

Both sequence alignment using MUSCLE and phylogenetic analysis by the Maximum Likelihood method were conducted using MEGA5 (Tamura et al., 2011). The WAG model was used to estimate the tree. A discrete gamma distribution with invariant sites (G+I)

was used to model evolutionary rate differences among sites. All positions with less than 0% site coverage were eliminated. The bootstrap consensus tree was build using 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.

II.3 RESULTS

II.3.1 Identification of two *DAM* genes in apple

The apple 'Golden Delicious' trees cultivated in the geographic area where our study was performed requires about 1075 chilling units for acquiring competency to flower (Rea and Eccel, 2006). The accumulation of chilling units usually starts at the beginning of the autumn season around middle of October. To study transcriptional changes of MADS-box genes occurring during dormancy in apple we performed a differential gene expression by RNA-seq using dormant buds harvested monthly starting from middle of October (t0) until the bud break stage (t5) that occurs usually beginning of March (*Figure 1*). The number of reads obtained from the twelve Illumina libraries representing the six time points (t0 to t5) contained between 6,489,970 and 18,538,110 reads (*Table 1*).

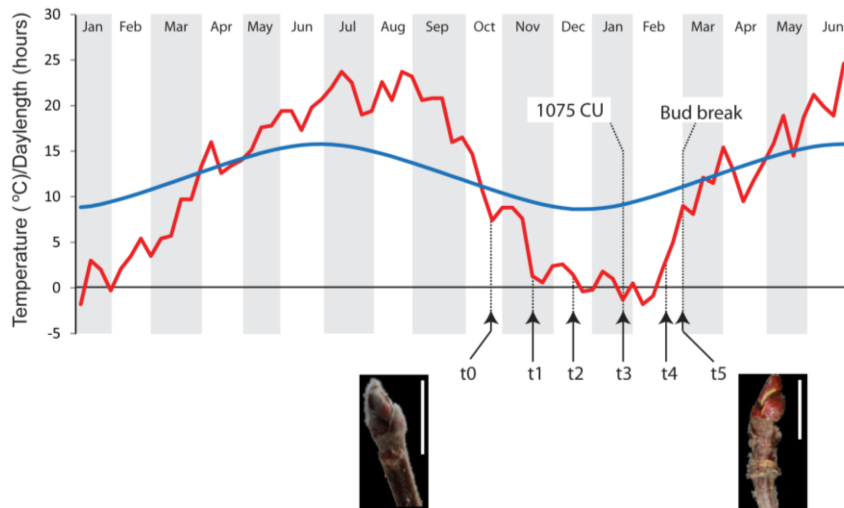


Figure 1 - Dormant bud time course.

Temperatures in °C and daylengths in hours, indicated by a red and a blue line respectively, were recorded each month from January 2011 to June 2012. Months are indicated by abbreviations. Dormant buds were harvested at six time points (t0 to t5) as indicated by vertical arrows. The 1075 chilling unit (CU) requirements corresponded during this season to the time point t3 and the bud break to time point t5. Representative picture of buds harvested at t0 and t5 are indicated (bar =1 cm).

Table 1 - Number of reads in Illumina libraries used in this study

Library name	Number of reads
t0_1	18,538,110
t0_2	6,489,970
t1_1	12,559,866
t1_2	7,001,296
t2_1	13,039,752
t2_2	8,697,373
t3_1	17,302,403
t3_2	8,733,527
t4_1	14,566,913
t4_2	8,907,308
t5_1	13,441,809
t5_2	8,427,026
t5_2	8,427,026
Total	**137,531,572**

A *de novo* transcript assembly of these reads using Trinity pipeline (Grabherr et al., 2011) retrieved 420,461 contigs and among them 609 aligned to a known subset of MADS-box coding genes predicted *in silico* using the 'Golden Delicious' reference genome (Velasco et al., 2010). 49 non-redundant contigs were coding for proteins containing at least a conserved MADS-domain with a hit value ≤ 0.00001. Transcriptional activities were found for fifteen of those MADS-box genes compare to the reference time point t0. However, only MDP0000322567 and a newly identified gene annotated MDP0000952188 showed a significant change ($p<0.001$) in expression throughout the chilling period to reach 0.03 and 0.004 fold change at bud break (*Figure 2A*). Since these genes are associated with dormancy we named them *MdDAM1* for MDP0000322567 and *MdDAM2* for MDP0000952188. The down regulation of *MdDAM1* and *MdDAM2* was verified by qRT-PCR analysis validating therefore the RNA-seq data (*Figure 2B*). The coding DNA sequences of *MdDAM1*, *MdDAM2*, and the MADS-box genes identified by RNA-seq were confirmed by RT-PCR analysis followed by sequencing. All proteins contain a MADS-domain adjacent to a K-box motif suggesting that they belong to the MIKC^{c}-type MADS domain protein family.

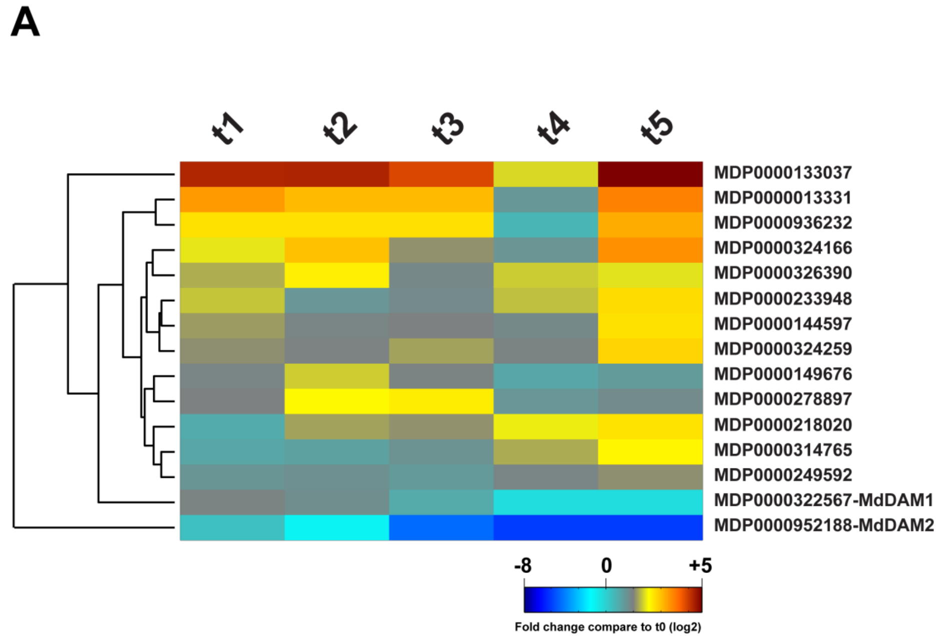


Figure 2 - MADS-box gene expression levels during dormancy

A heatmap indicating expression levels of genes transcriptionally active during bud dormancy at t1, t2, t3, t4 and t5 normalized to t0 (**A**). The fold induction expression of MDP0000322567_MdDAM1 (grey bars) and MDP0000952188_MdDAM2 (black bars) at the time points t1-t5 compared to t0 were determined by qRT-PCR.

II.3.2 Apple DAM genes are members of the StMADS11 lineage.

We determined the phylogenetic relationship between the MADS-box genes that we identified by RNA-seq, the *Pyrus* and *Prunus* DAM genes, and the well-studied Arabidopsis MIKC-type MADS domain proteins as a reference. This analysis revealed that *MdDAM1* and *MdDAM2* cluster together with peach and pear DAM genes to form a

well-supported clade (bootstrap value of 100) within the StMADS11 lineage that is also comprised of two Arabidopsis MIKC^c-type genes *AtAGL24* and *AtSVP.* MDP0000233948 shows high homology with AtSVP and therefore renamed MdSVP (*Figure 3*).

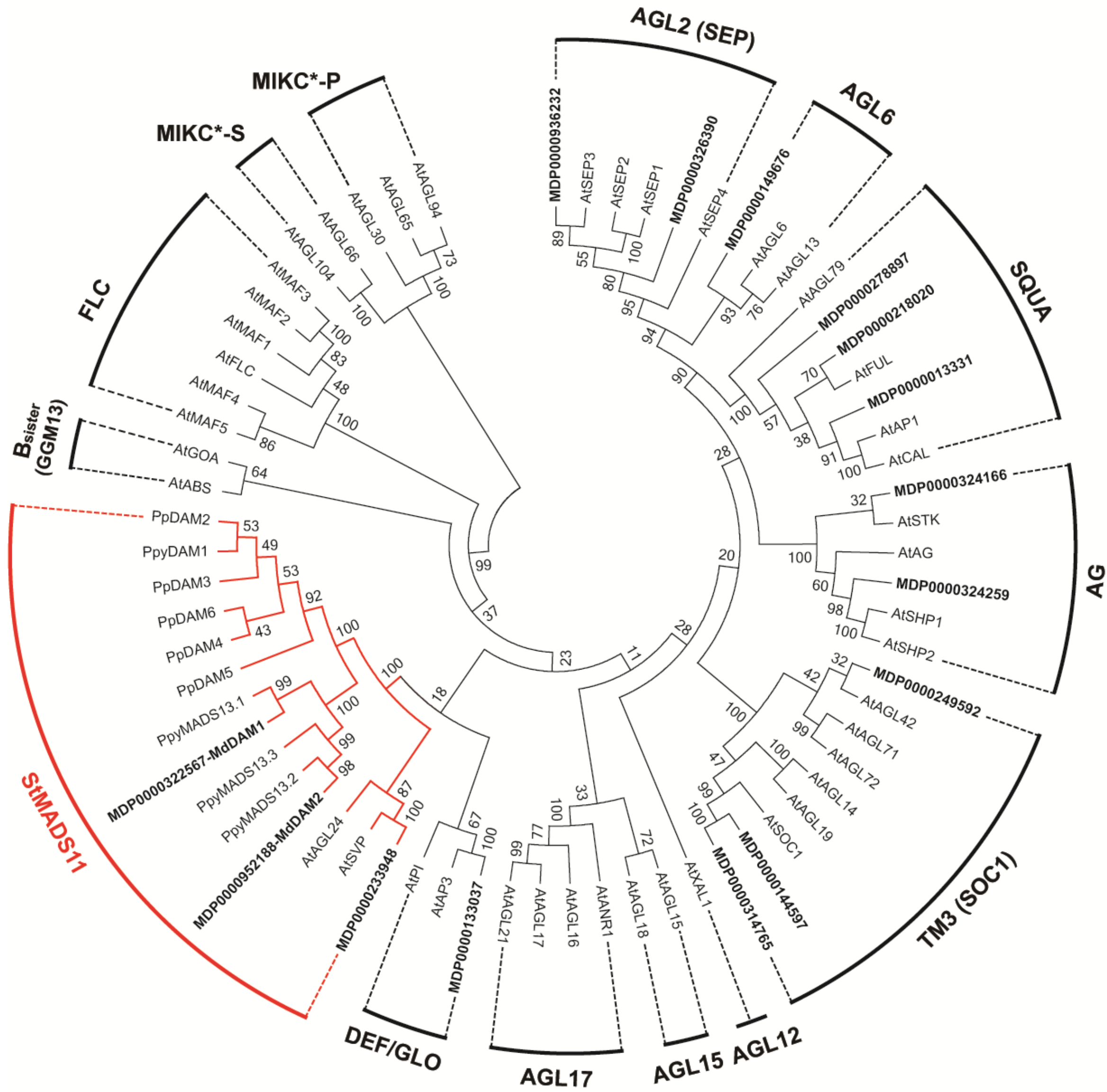


Figure 3 - Phylogenetic analysis of apple MADS-box genes expressed during bud dormancy

Maximum likelihood rooted tree of 39 *Arabidopsis thaliana* (At), 6 peach (*Prunus persica*, Pp), 2 pear (*Pyrus pyrifolia*, Ppy) and 15 apple (*Malus x domestica*, Md) MIKC^c-type MADS-box genes. Additionally, 5 Arabidopsis MIKC*-type MADS-box genes were included. The numbers at each interior branch indicate bootstrap support of 500 replicates. Branches with less than 50% bootstrap support are collapsed. Branches lengths are proportional to the number of nucleotide changes and are represented in red for the StMADS11 clade.

MdDAM1 and *MdDAM2* closest homologs are the *Pyrus pyrifolia* DAM proteins *PpyMADS13.1* and *PpyMADS13.2* with which they share 92% and 97% identity on the entire amino acid sequence, respectively. The remaining twelve MADS-box genes transcriptionally active in buds during dormancy cluster in all other Arabidopsis lineages except in AGL15, AGL17, and AGL12. They all show high homology to Arabidopsis proteins (60 to 77%) except for *MdDAM1* and *MdDAM2* (43 and 40%) that appear to have diverged to acquire functions specific to *Spiraeoideae* (*Table 2*).

Table 2 - Description and homologies of the genes identified in this study.

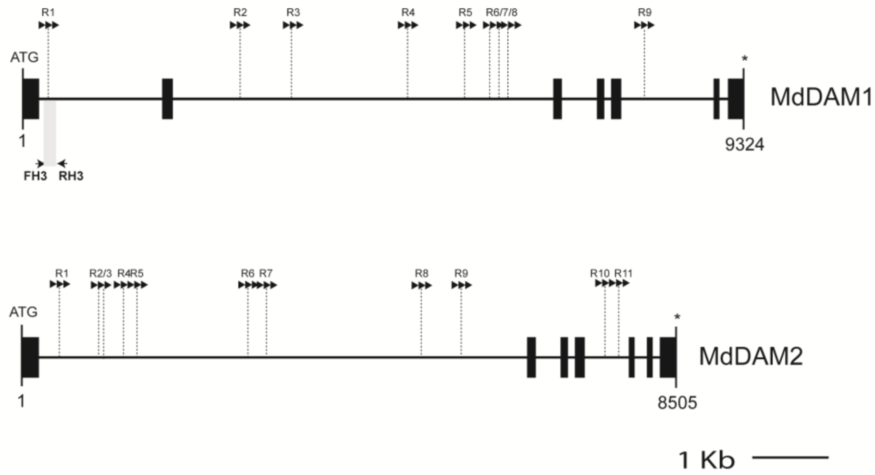
Gene ID[a]	Clade[b]	Homology[c]	Name and accession number[d]	Reference
MDP0000133037	DEF/GLO	AtAP3 (60%)	MdTM6 (AB081093.1)	(Kitahara et al., 2004)
MDP0000324166	AG	AtSTK (76%)	MdMADS221 (ADL36745.1)	-
MDP0000324259	AG	AtSHP1 (68%)	MdMADS14 (ADL36737.1)	-
MDP0000149676	AGL6	AtAGL6 (65%)	MdMADS11 (CAA04325.1)	(Yao et al., 1999)
MDP0000326390	AGL2 (SEP)	AtSEP1 (77%)	MdMADS3 (AAD51422.1)	(Sung et al., 2000)
MDP0000936232	AGL2 (SEP)	AtSEP3 (76%)	MdMADS18 (ADL36740.1)	-
MDP0000013331	SQUA	AtAP1 (62%)	MdMADS5 (CAA04321.1)	(Yao et al., 1999)
MDP0000218020	SQUA	AtFUL (53%)	MdFUL (ABB22022.1)	(Cevik et al., 2010)
MDP0000278897	SQUA	AtFUL (64%)	MdMADS12 (CAC86183.1)	(van der Linden et al., 2002)
MDP0000233948	StMADS11	AtSVP (61%)	MdSVP (ABD66219.2)	This work
MDP0000322567	StMADS11	AtAGL24 (43%)	MdDAM1 (NCBIXXXXX)	This work
		PpyMADS13.1 (92%)	PpyMADS13.1 (AB504716.1)	(Saito et al., 2013)
MDP0000952188	StMADS11	AtAGL24 (40%)	MdDAM2 (NCBIXXXXX)	This work
		PpyMADS13.2 (97%)	PpyMADS13.2 (AB504717.1)	(Saito et al., 2013)
MDP0000144597	TM3/SOC1	AtSOC1 (66%)	MdSOC1 (ABI20790.1)	(Mahna et al., 2006)
MDP0000249592	TM3/SOC1	AtAGL42 (40%)	MdMADS21 (ADL36744.1)	-
MDP0000314765	TM3/SOC1	AtSOC1 (66%)	MdSOC1a (AB501124.1)	(Kotoda et al., 2010)

[a] The gene ID number was retrieved from the apple genome website (http://genomics.research.iasma.it/)
[b] The Arabidopsis clade were determined by phylogenetic analyzed as indicated in *Figure 3*
[c] Percentage of sequence identity to Arabidopsis, *Pyrus* closest homologs are indicated between brackets.
[d] The name of genes and their accession number was taken from the GenBank sequence database NCBI.

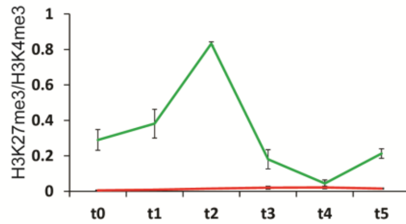
II.3.3 Genomic structure of *MdDAM1* and *MdDAM2* loci and associated chromatin changes

The *MdDAM1* and *MdDAM2* genomic loci have a size of 9324 and 8505 bp, respectively. *MdDAM1* locus contains seven exons and six introns with lengths ranging from 98 to 4994 bp whereas *MdADM2* largest intron reaches a size of 6424 bp. The only genomic features present in both loci are DNA repeats, remnants of retrotransposon and transposon sequences found in introns (*Figure 4A, Table 3 and Table 4*). We searched

for chromatin changes at these loci that could explain the drastic suppression of *MdDAM1* and *MdDAM2* gene activity at bud break (t5). Chromatin associated with histone H3 were immunoprecipitated using antibodies specific for K27me3 and K4me3 marks from the five time points. Several primer pairs were designed throughout *MdDAM1* and *MdDAM2* loci to allow the measurement of immunoprecipitated DNA by real time PCR in a quantitative manner. A signal above background associated with H3K4me3 was found in one region only located at the beginning of *MdDAM1* first intron. In contrast, no traces of histone methylation were found on *MdDAM2* locus. This analysis reveals that histone H3 is dynamically methylated at lysine 4 at the beginning of dormancy to reach a peak at t2 and gradually dropping down to reach background levels at t4 when chilling requirements were fulfilled. However, this suppression is not associated with an increase in H3K27 methylation in field conditions (*Figure 4B*). To verify if cold temperatures could trigger methylation of histone 3 at lysine 27 apple buds were constantly kept at 4°C for 42 days, or 1008 hours, and a chromatin immunoprecipitation was performed. Despite important fluctuations H3K27 methylation in apple buds showed high activity at 4°C correlating with a reduction in H3K4 methylation (*Figure 4C*). This result suggests that chilling temperatures are sufficient to repress *MdDAM1* gene expression by a decrease in H3K4 activity, however, not through an increase in H3K27me3 in field conditions.



B



C

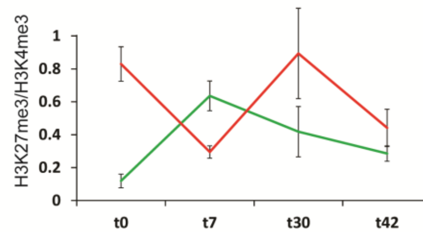


Figure 4 - Genomic structure of *MdDAM1* and *MdDAM2* loci and associated chromatin changes

Exon (black boxes) and intron structure of *MdDAM1* and *MdDAM2* loci. The start codon (ATG) at position 1 and stop codon (*) at positions 9324 for *MdDAM1* and 8505 for *MdDAM2* are indicated. Repeats are indicated by arrows and their respective positions on the locus by a dashed line. Primers (FH3-RH3) used for real time (PCR) measurement of immunoprecipitated DNA on *MdDAM1* are shown and the corresponding amplified amplicon indicated by a grey box (**A**). The abundance of DNA immunoprecipitated using H3K27me3 (red lines) and H3K4me3 (green lines) normalized to the histone H3 abundance from buds in (**B**) orchard conditions (t0 to t5) or exposed to constant temperature of 4°C during 42 days (t0 to t42) (C). The value 1 in y-axes in (**B**) and (**C**) represents the maximum abundance value.

Table 3 - Genomic structure of *MdDAM1* locus (MDP0000322567)

Genomic feature[a]	**Start**	**End**	**Length (bp)**
Exon 1	**1**	**173**	**172**
Intron 1	174	1801	**1627**
R1 (LTR/Gypsy)	*333*	*489*	***156***
Exon 2	**1802**	**1906**	**104**
Intron 2	1907	6901	**4994**
R2 (NonLTR/RTE)	*2851*	*3535*	***684***
R3 (NonLTR/RTE)	*3536*	*4129*	***593***
R4 (DNA/Harbinger)	*5052*	*5160*	***108***
R5 (DNA/Harbinger)	*5794*	*5994*	***200***
R6 (Sommer et al.)	*6119*	*6219*	***100***
R7 (Sommer et al.)	*6227*	*6332*	***105***
R8 (Sommer et al.)	*6339*	*6447*	***108***
Exon 3	**6902**	**6980**	**78**
Intron 3	6981	7473	**492**
Exon 4	**7474**	**7535**	**61**
Intron 4	7536	7634	**98**
Exon 5	**7635**	**7734**	**99**
Intron 5	7735	8989	**1254**
Exon 6	**8980**	**9021**	**41**
Intron 6	9020	9144	**124**
Exon 7	**9145**	**9324**	**179**
R9 (DNA/hAT)	*8146*	*8538*	***392***

[a] The repeats were found using CENSOR available at http://www.ebi.ac.uk/Tools/so/censor/

Table 4 - Genomic structure of *MdDAM2* locus (MDP0000952188)

Genomic feature	Start	End	Lentgh (bp)
Exon 1	**1**	**189**	**188**
Intron 1	**190**	**6614**	**6424**
R1 (DNA/hAT)	*474*	*531*	*57*
R2 (DNA/EnSpm)	*984*	*1062*	*78*
R3 (LTR/Copia)	*1071*	*1122*	*51*
R4 (DNA)	*1354*	*1382*	*28*
R5 (DNA/Helitron)	*1520*	*1615*	*95*
R6 (NonLTR/RTE)	*2962*	*3036*	*74*
R7 (NonLTR/RTE)	*3195*	*3325*	*130*
R8 (LTR/Gypsy)	*5236*	*5336*	*100*
R9 (DNA)	*5742*	*5922*	*180*
Exon 2	**6615**	**6695**	**81**
Intron 2	**6696**	**7085**	**390**
Exon 3	**7086**	**7148**	**63**
Intron 3	**7147**	**7148**	**100**
Exon 4	**7249**	**7345**	**97**
Intron 4	**7346**	**7952**	**607**
Exon 5	**7953**	**7995**	**43**
Intron 5	**7996**	**8158**	**163**
Exon 6	**8159**	**8202**	**44**
Intron 6	**8203**	**8322**	**120**
Exon 7	**8323**	**8505**	**183**
R10 (DNA)	*7622*	*7717*	*95*
R11 (LTR/Gypsy)	*7796*	*7865*	*69*

[a] The repeats were found using CENSOR available at http://www.ebi.ac.uk/Tools/so/censor/

II.4 DISCUSSION

The expression MADS-box genes transcript from 'Golden delicious' genome was screened in dormant buds at different stages using a genome-wide transcriptomic approach. Fifteen genes were found to be differentially expressed during dormancy. Two genes, *MdDAM1* (MDP0000322567) and *MdDAM2* (MDP0000952188) are down regulated in response to cold and therefore classified as apple DAM genes. *MdDAM1* was present in the predicted apple gene set, while *MdDAM2* was found *ex novo*. qRT-PCr expression profile demonstrated that repression of both genes starts immediately after the fulfillment of the chilling requirement for 'Golden Delicious' (1075 CU). The gradual decrease in their expression raises the possibility that these genes are suppressed by cold exposure or CU accumulation.

Our study demonstrate that the MADS-box genes MdSVP (MDP0000233948), MdDAM1 (MDP0000322567) and the newly identified MdDAM2 (MDP0000952188), all cluster with StMADS11 lineage together with *Arabidopsis AtSVP* and *AtAGL24*, and peach and pear DAM genes. Each of these three genes has ubiquitous expression in apple (data not shown), as it is commonly found in the StMADS11 clade in Arabidopsis and peach (Hileman et al., 2006; Díaz-Riquelme et al., 2009; Li et al., 2009). Moreover they are all differentially expressed in buds during dormancy, with *MdDAM1* and *MdDAM2* being down regulated.

Gene expression in response to cold accumulation is mediated by epigenetic gene silencing in Arabidopsis *FLC* and peach *DAM6*. Histone H3 methylation profile changes during vernalization in *FLC* as it does in *DAM6* during bud dormancy (Michaels and Amasino, 1999; Doyle and Amasino, 2009; Leida et al., 2012). Transcriptionally active peach *DAM6* contains H3K4me3 and H3ac; during dormancy a gradual H3K4 demethylation and H3 deacetylation and an increasing H3K27me3 mediate the stable repression of the gene. In this work we demonstrated that, in conditions of controlled temperature, *MdDAM1* locus is associated with chromatin modifications similar to peach *DAM6*. In particular, H3K4me3 and H3K27me3 are inversely correlated, with the latter increasing during CU accumulation. However, a different histone H3 modification profile is present in buds harvested from the field. In this case, only decrease of H3K24me3 is detectable. To clarify this ambiguous finding, the experiment should be implemented. The comparison with other cultivars with different chilling requirement would be useful to set a reference baseline for histone modification accumulation.

Moreover, since H3K27me3 is not the only mark for transcriptional repression, it would be beneficial to detect changes of H3K27me or H3K27me2 during dormancy.

To functionally validate the role of apple *MdDAM1* and *MdDAM2* in dormancy, the first step would be the complementation of *Arabidopsis svp* and *agl24* mutants to verify if their function is conserved in annual plants and the silencing of these genes in apple or alternatively in pear.

II.5 References Chapter II

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research **25,** 3389-3402.

Alvarez-Buylla, E.R., Liljegren, S.J., Pelaz, S., Gold, S.E., Burgeff, C., Ditta, G.S., Vergara-Silva, F., and Yanofsky, M.F. (2000). MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. Plant J **24,** 457-466.

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biology **11,** R106.

Anderson, J.L., Richardson, E.A., and Kesner, C.D. (1986). Validation of chill unit and flower bud phenology models for “Montmorency” sour cherry. . Acta Hortic **184,** 71-78.

Andres, F., and Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. Nat Rev Genet **13,** 627-639.

Arora, R., Agarwal, P., Ray, S., Singh, A.K., Singh, V.P., Tyagi, A.K., and Kapoor, S. (2007). MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics **8,** 242.

Bielenberg, D., Wang, Y., Li, Z., Zhebentyayeva, T., Fan, S., Reighard, G., Scorza, R., and Abbott, A. (2008). Sequencing and annotation of the evergrowing locus in peach [Prunus persica (L.) Batsch] reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. Tree Genetics & Genomes **4,** 495-507.

Botton, A., Eccher, G., Forcato, C., Ferrarini, A., Begheldo, M., Zermiani, M., Moscatello, S., Battistelli, A., Velasco, R., Ruperti, B., and Ramina, A. (2011). Signaling Pathways Mediating the Induction of Apple Fruitlet Abscission. Plant Physiology **155,** 185-208.

Cevik, V., Ryder, C., Popovich, A., Manning, K., King, G., and Seymour, G. (2010). A FRUITFULL-like gene is associated with genetic variation for fruit flesh firmness in apple (Malus domestica Borkh.). Tree Genetics & Genomes **6,** 271-279.

Derory, J., Leger, P., Garcia, V., Schaeffer, J., Hauser, M.T., Salin, F., Luschnig, C., Plomion, C., Glossl, J., and Kremer, A. (2006). Transcriptome analysis of bud burst in sessile oak (Quercus petraea). New Phytol **170,** 723-738.

Díaz-Riquelme, J., Lijavetzky, D., Martínez-Zapater, J.M., and Carmona, M.J. (2009). Genome-Wide Analysis of MIKCC-Type MADS Box Genes in Grapevine. Plant Physiology **149,** 354-369.

Doyle, M.R., and Amasino, R.M. (2009). A single amino acid change in the enhancer of zeste ortholog CURLY LEAF results in vernalization-independent, rapid flowering in Arabidopsis. Plant Physiol **151,** 1688-1697.

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics **28,** 3150-3152.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotech **29,** 644-652.

Heide, O.M., and Prestrud, A.K. (2005). Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. Tree Physiol **25,** 109-114.

Hemming, M.N., and Trevaskis, B. (2011). Make hay when the sun shines: the role of MADS-box genes in temperature-dependant seasonal flowering responses. Plant Sci **180,** 447-453.

Hileman, L.C., Sundstrom, J.F., Litt, A., Chen, M., Shumba, T., and Irish, V.F. (2006). Molecular and Phylogenetic Analyses of the MADS-Box Gene Family in Tomato. Molecular Biology and Evolution **23,** 2245-2258.

Horvath, D.P., Anderson, J.V., Chao, W.S., and Foley, M.E. (2003). Knowing when to grow: signals regulating bud dormancy. Trends Plant Sci **8,** 534-540.

Horvath, D.P., Sung, S., Kim, D., Chao, W., and Anderson, J. (2010). Characterization, expression and function of DORMANCY ASSOCIATED MADS-BOX genes from leafy spurge. Plant Mol Biol **73,** 169-179.

Jonkers, H. (1979). Bud dormancy of apple and pear in relation to the temperature during the growth period. Scientia Horticulturae **10,** 149-154.

Kitahara, K., Ohtsubo, T., Soejima, J., and Matsumoto, S. (2004). Cloning and Characterization of Apple Class B MADS-box Genes Including a Novel AP3 Homologue MdTM6. J. Japan. Soc. Hort Sci. **73,** 208-215.

Kotoda, N., Hayashi, H., Suzuki, M., Igarashi, M., Hatsuyama, Y., Kidou, S.-i., Igasaki, T., Nishiguchi, M., Yano, K., Shimizu, T., Takahashi, S., Iwanami, H., Moriya, S., and Abe, K. (2010). Molecular Characterization of FLOWERING LOCUS T-Like Genes of Apple (Malus × domestica Borkh.). Plant and Cell Physiology **51,** 561-575.

Lang, G.A. (1987). Dormancy: a new universal terminology. HortScience **22,** 817–820.

Leida, C., Conesa, A., Llacer, G., Badenes, M.L., and Rios, G. (2012). Histone modifications and expression of DAM6 gene in peach are modulated during bud dormancy release in a cultivar-dependent manner. New Phytol **193,** 67-80.

Leseberg, C.H., Li, A., Kang, H., Duvall, M., and Mao, L. (2006). Genome-wide analysis of the MADS-box gene family in Populus trichocarpa. Gene **378,** 84-94.

Li, J., and Yuan, R. (2008). NAA and Ethylene Regulate Expression of Genes Related to Ethylene Biosynthesis, Perception, and Cell Wall Degradation During Fruit Abscission and Ripening in 'Delicious' Apples. Journal of Plant Growth Regulation **27,** 283-295.

Li, Z., Reighard, G.L., Abbott, A.G., and Bielenberg, D.G. (2009). Dormancy-associated MADS genes from the EVG locus of peach [Prunus persica (L.) Batsch] have distinct seasonal and photoperiodic expression patterns. J Exp Bot **60,** 3521-3530.

Mahna, N., Dreesen, R., Baghban Kohneh Rouz, B., Ghareyazie, B., Valizadeh, M., Grigorian, V., and Keulemans, J. (2006). Isolation and characterization of a MADS-box type gene from apple (*Malus x domestica*). Commun. Agric. Appl. Biol. Sci. **71,** 187-191.

Mathiason, K., He, D., Grimplet, J., Venkateswari, J., Galbraith, D.W., Or, E., and Fennell, A. (2009). Transcript profiling in Vitis riparia during chilling requirement fulfillment reveals coordination of gene expression patterns with optimized bud break. Funct Integr Genomics **9,** 81-96.

Mazzitelli, L., Hancock, R.D., Haupt, S., Walker, P.G., Pont, S.D., McNicol, J., Cardle, L., Morris, J., Viola, R., Brennan, R., Hedley, P.E., and Taylor, M.A. (2007). Co-ordinated gene expression during phases of dormancy release in raspberry (Rubus idaeus L.) buds. J Exp Bot **58,** 1035-1045.

Michaels, S.D., and Amasino, R.M. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949-956.
Park, S., Keathley, D.E., and Han, K.H. (2008). Transcriptional profiles of the annual growth cycle in Populus deltoides. Tree Physiol **28,** 321-329.
Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research **29,** e45.
Rea, R., and Eccel, E. (2006). Phenological models for blooming of apple in a mountainous region. Int J Biometeorol **51,** 1-16.
Rohde, A., and Bhalerao, R.P. (2007). Plant dormancy in the perennial context. Trends Plant Sci **12,** 217-223.
Rohde, A., Prinsen, E., De Rycke, R., Engler, G., Van Montagu, M., and Boerjan, W. (2002). PtABI3 Impinges on the Growth and Differentiation of Embryonic Leaves during Bud Set in Poplar. The Plant Cell Online **14,** 1885-1901.
Rohde, A., Ruttink, T., Hostyn, V., Sterck, L., Van Driessche, K., and Boerjan, W. (2007). Gene expression during the induction, maintenance, and release of dormancy in apical buds of poplar. J Exp Bot **58,** 4047-4060.
Rohde, A., Howe, G.T., Olsen, J.E., Moritz, T., Montagu, M., Junttila, O., and Boerjan, W. (2000). Molecular Aspects of Bud Dormancy in Trees. In Molecular Biology of Woody Plants, S.M. Jain and S. Minocha, eds (Springer Netherlands), pp. 89-134.
Ruttink, T., Arend, M., Morreel, K., Storme, V., Rombauts, S., Fromm, J., Bhalerao, R.P., Boerjan, W., and Rohde, A. (2007). A molecular timetable for apical bud formation and dormancy induction in poplar. Plant Cell **19,** 2370-2390.
Saito, T., Bai, S., Ito, A., Sakamoto, D., Saito, T., Ubi, B.E., Imai, T., and Moriguchi, T. (2013). Expression and genomic structure of the dormancy-associated MADS box genes MADS13 in Japanese pears (Pyrus pyrifolia Nakai) that differ in their chilling requirement for endodormancy release. Tree Physiology.
Sasaki, R., Yamane, H., Ooka, T., Jotatsu, H., Kitamura, Y., Akagi, T., and Tao, R. (2011). Functional and expressional analyses of PmDAM genes associated with endodormancy in Japanese apricot. Plant Physiol **157,** 485-497.
Schrader, J., Moyle, R., Bhalerao, R., Hertzberg, M., Lundeberg, J., Nilsson, P., and Bhalerao, R.P. (2004). Cambial meristem dormancy in trees involves extensive remodelling of the transcriptome. Plant J **40,** 173-187.
Sommer, H., Beltran, J.P., Huijser, P., Pape, H., Lonnig, W.E., Saedler, H., and Schwarz-Sommer, Z. (1990). Deficiens, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. EMBO J **9,** 605-613.
Sung, S.-K., Yu, G.-H., Nam, J., Jeong, D.-H., and An, G. (2000). Developmentally regulated expression of two MADS-box genes, MdMADS3 and MdMADS4, in the morphogenesis of flower buds and fruits in apple. Planta **210,** 519-528.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution **28,** 2731-2739.
van der Linden, C.G., Vosman, B., and Smulders, M.J.M. (2002). Cloning and characterization of four apple MADS box genes isolated from vegetative tissue. Journal of Experimental Botany **53,** 1025-1036.
Vegis, A. (1964). Dormancy in Higher Plants. Annual Review of Plant Physiology **15,** 185-224.
Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., Fontana, P., Bhatnagar, S.K., Troggio, M., Pruss, D., Salvi, S., Pindo, M., Baldi, P., Castelletti, S., Cavaiuolo, M., Coppola, G., Costa, F., Cova, V., Dal Ri, A., Goremykin, V., Komjanc, M., Longhi, S.,

Magnago, P., Malacarne, G., Malnoy, M., Micheletti, D., Moretto, M., Perazzolli, M., Si-Ammour, A., Vezzulli, S., Zini, E., Eldredge, G., Fitzgerald, L.M., Gutin, N., Lanchbury, J., Macalma, T., Mitchell, J.T., Reid, J., Wardell, B., Kodira, C., Chen, Z., Desany, B., Niazi, F., Palmer, M., Koepke, T., Jiwan, D., Schaeffer, S., Krishnan, V., Wu, C., Chu, V.T., King, S.T., Vick, J., Tao, Q., Mraz, A., Stormo, A., Stormo, K., Bogden, R., Ederle, D., Stella, A., Vecchietti, A., Kater, M.M., Masiero, S., Lasserre, P., Lespinasse, Y., Allan, A.C., Bus, V., Chagné, D., Crowhurst, R.N., Gleave, A.P., Lavezzo, E., Fawcett, J.A., Proost, S., Rouzé, P., Sterck, L., Toppo, S., Lazzari, B., Hellens, R.P., Durel, C.-E., Gutin, A., Bumgarner, R.E., Gardiner, S.E., Skolnick, M., Egholm, M., Van de Peer, Y., Salamini, F., and Viola, R. (2010). The genome of the domesticated apple (Malus × domestica Borkh.). Nature Genetics **42,** 833-839.

Yakovlev, I., Fossdal, C.-G., Johnsen, Ø., Junttila, O., and Skrøppa, T. (2006). Analysis of gene expression during bud burst initiation in Norway spruce via ESTs from subtracted cDNA libraries. Tree Genetics & Genomes **2,** 39-52.

Yamane, H., Kashiwa, Y., Ooka, T., Tao, R., and Yonemori, K. (2008). Suppression subtractive hybridization and differential screening reveals endodormancy-associated expression of an SVP/AGL24-type MADS-box gene in lateral vegetative buds of japanese apricot. Journal of the American Society for Horticultural Science **133,** 708-716.

Yao, J.-L., Dong, Y.-H., Kvarnheden, A., and Morris, B. (1999). Seven MADS-box Genes in Apple are Expressed in Different Parts of the Fruit. Journal of the American Society for Horticultural Science **124,** 8-13.

Chapter III

Functional characterization of *PISTILLATA*- and *AGAMOUS*-like genes in strawberry (*Fragaria vesca* L.)

III.1 INTRODUCTION

The diversity of flower morphology within angiosperms is due to the evolutionary plasticity of four organs that are sepals, petals, stamens and carpels (Theissen and Melzer, 2007). The formation and architecture of these organs is genetically controlled following the ABC(D)E model (Coen and Meyerowitz, 1991; Theissen and Saedler, 2001).

Although the genetic mechanisms governing flower formation are widely conserved, differences are still observed in many plant species (reviewed by Krizek and Fletcher, 2005). For instance, Liliaceae flowers contain stamen and carpel whereas the sepals and petals are replaced by two outer whorls of identical organs called tepals. Using tulip (*Tulipa gesneriana*) as exemplification model a modified ABC model was proposed to elucidate Liliaceae flower morphology (van Tunen et al., 1993). According to this model A- and B-function overlaps in whorls 1 and 2, which develop into tepals (Kanno et al., 2003). Another example is found in grasses flowers where sepals and petals are substituted by leaf-like organs called paleas and lemmas as well as by lodicules. Lodicules are determined by B-function conserved genes in rice (*SUPERWOMAN1*) and in maize (*SILKY 1*). In addition, rice contains *DROOPING LEAF* (*DL*) which controls carpel identity and that acts antagonistically to the class B gene whereas maize contains two putative class C genes named ZAG1 and ZMM2 (Kang et al., 1998; Nagasawa et al., 2003; Whipple et al., 2004; Yamaguchi et al., 2004; 2006; Li et al., 2011).

In perennial plants only few mutants of *PI* homeotic genes have been described in grapevine and apple and few flowering homologous genes have been identified in various species. Recently Fernandez et al. (2013) showed that the insertion of a transposable element in the promoter of the grapevine *PISTILLATA* gene *VvPI* is responsible of its ectopic expression and cause the fleshless berry phenotype in *Vitis*. On the other hand, the work of Yao et al. (2001) demonstrated that the expression of *MdPI* is required for the development of petals and stamens in apple. The naturally occurring apple mutants 'Rae Ime' develop an apetalous flower without stamens which can produce parthenocarpic fruits. This phenotype is conferred by the insertion of a LTR-type retrotransposon into intron 4 of *MdPI* gene of 'Rae Ime'. The floral organ transformation occurring in apple mirrors what happens in *Arabidopsis w*ith the exception that *MdPI* mutant produces parthenocarpic fruit in apple but *pi* mutant in *Arabidopsis* does not (Yao et al., 2001; Krizek and Fletcher, 2005). Ten genes differentially expressed during flower and fruit development in Japanese pear (*Pyrus*

pyrifolia) were characterized by Ubi et al. (2013). Among them, four A-class genes, five members of the E-class and one C-class gene were identified. Surprisingly, no B-class genes such as *AP3*/*PI*-like were identified (Ubi et al., 2013).

The identification of floral homeotic genes in tree species has been achieved by analyzing natural occurring mutants or by expressing candidate genes in heterologous species. This points out the need to put in place a model species for studying flower development in plant bearing fruits. Strawberry (*Fragaria spp.*) is the ideal plant to achieve this goal since it is a small plant in size, has a short life cycle of 3.5 months, and is a perennial plant similar to the agronomically important family of Rosaceae comprising apple and other important fruits trees. Furthermore, the genome of woodland strawberry has a small size (~240 Mb) and is diploid (Shulaev et al., 2010). Most genotypes of woodland strawberry (*F. vesca* L.) and garden strawberry (*Fragaria* x *ananassa* Duch.) are junebearing short day plants, whose seasonal flowering is induced by decreasing photoperiod in autumn (Heide and Sønsteby, 2007). In addition, everbearing genotypes are present both in garden and wild strawberry such as 'Baron Solemacher', 'Hawaii-4', and 'Rügen'. 'Baron Solemacher' cultivar carries recessive alleles of *SEASONAL FLOWERING LOCUS* (*SFL*) gene that has been shown to cause everbearing flowering habit (Albani et al., 2004). The gene seems to encode a key repressor of flowering in wild strawberry.

From the experimental point of view, the biology of sexual and asexual reproduction of strawberry is important and practical. *F. vesca* is self-pollinating and can produce many seeds per fruits. Moreover, it is also capable of asexual clonal propagation through the production of runners and by the production of branch crowns. Flower architecture is similar in garden and wild strawberry and is conserved in many Rosaceae crops (Hollender et al., 2011). In the last years inbred lines of *F. vesca* cultivars, such as 'Rügen', 'Yellow wonder' and 'Hawaii-4' have been developed specifically for genetic and genomic studies (Oosumi et al., 2006; Shulaev et al., 2008; Mouhu et al., 2009; Slovin et al., 2009; Slovin and Michael, 2011). Some varieties, such as 'Rügen' are runnerless and permit growing large number of plants in a small space.

These varieties were used to investigate flowering genes through gene expression studies. *AP1* (A-class) is expressed in everbearing apices at early stages of leaf development and accumulates through time in later developmental stages (Mouhu et al., 2009). The expression profile of *STAG1*, the class c gene *AG* homolog found in *Fragaria* x *ananassa*, has been detected by *in situ* hybridization and histochemical assay (Rosin et al., 2003). At early stages of flower development *STAG1* is expressed

throughout stamen and carpel primordia, while in late stages is detectable in specific differentiated cells such as achene endothelium and vascular connection between achenes and the receptacle. *In situ* hybridization studies in *F. vesca* revealed that also *FvAG* homolog is uniquely expressed in stamen and carpel primordia, while it is absent in sepals and petals, mimicking the expression of its homolog in *Arabidopsis* (Hollender et al., 2011). *SEPALLATA* E-class homeotic genes *FaMADS9* and *FaMADS4* were investigated regarding their role in flower and fruit development in *Fragaria* x *ananassa* (Seymour et al., 2011). Severe repression of *FaMADS9* gene results in complete inhibition of receptacle development suggesting the maintenance of a vegetative state and the arrest of ripening in this organ. To our knowledge, no B-class genes were isolated in *Fragaria* until now.

Our study aims to study and to provide functional characterization of B- and C-class homeotic genes in strawberry by using a reverse genetic approach. We produced silencing mutant lines for *PI* and *AG* strawberry homologs and demonstrated that silencing of single gene is not sufficient to impair flower development in the woodland strawberry ‘Rügen’. This suggests that B- and C-class strawberry MADS-box gene functions is likely not conforming to the ABC model used for *Arabidopsis* and differs from other Rosaceae species such as apple.

III.2 MATERIALS AND METHODS

III.2.1 Phylogenetic analysis

MADS-box protein sequences containing an N-terminal MADS domain followed by a K-box domain were aligned using MUSCLE alignment software with default parameters (Edgar, 2004). The alignment was manually edited to remove badly aligned and gap-rich positions resulting in an alignment of 73 sequences for a total length of 177 residues. The maximum likelihood tree shown in *Figure 2* was obtained by running PhyML 200 times on bootstraps of this alignment (Guindon and Gascuel, 2003; 2010). We used the JTT model Γ+F+I (Jones et al., 1992). The parameter alpha for gamma distribution was estimated from the data and the distribution was discretized using six rate categories.

III.2.2 Cloning of floral homeotic genes

Specific primers (*Annex 1*) were used to amplify the full-length coding regions from cDNAs prepared from leaves, sepals, petals, stamens, and carpels of *F. vesca* cultivars 'Hawaii-4' and 'Rügen'. The PCR products were cloned into pCR®II-TOPO® (*Annex 3*) using TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) and verified by sequencing.

III.2.3 Transformation vectors

Genomic DNA extracted from *F. vesca* cv. 'Hawaii-4' was used as template to amplify the ~250bp DNA fragments specific for *FvPISTILLATAlike-1* (*FvPIlike-1*), *FvPISTILLATAlike-2* (*FvPIlike-2*), and *FvAGAMOUSlike* (*FvAGlike*) genes using the primers listed in *Annex 1*. The resulting fragments were named *hpFvPIlike-1*, *hpFvPIlike-2,* and *hpFvAGlike* as shown in *Annex 2B*. The fragments were cloned into the pENTR™/D-TOPO® vector (*Annex 4*) using the pENTR™ directional TOPO® Cloning Kit (Invitrogen) according to the manufacturer's instruction. The hairpins were then inserted by LR recombination reaction into the expression vector pK7GWIWG2(II),0 (*Annex 5*) using the Gateway® LR Clonase II enzyme mix (Invitrogen). The resulting plasmids were called pK7GWIWG2(II),0::*hpFvPIlike-1,* pK7GWIWG2(II),0::*hpFvPIlike-2,* and pK7GWIWG2(II),0::*hpFvAGlike*. The maps of the three T-DNAs are shown in *Figure 1*.

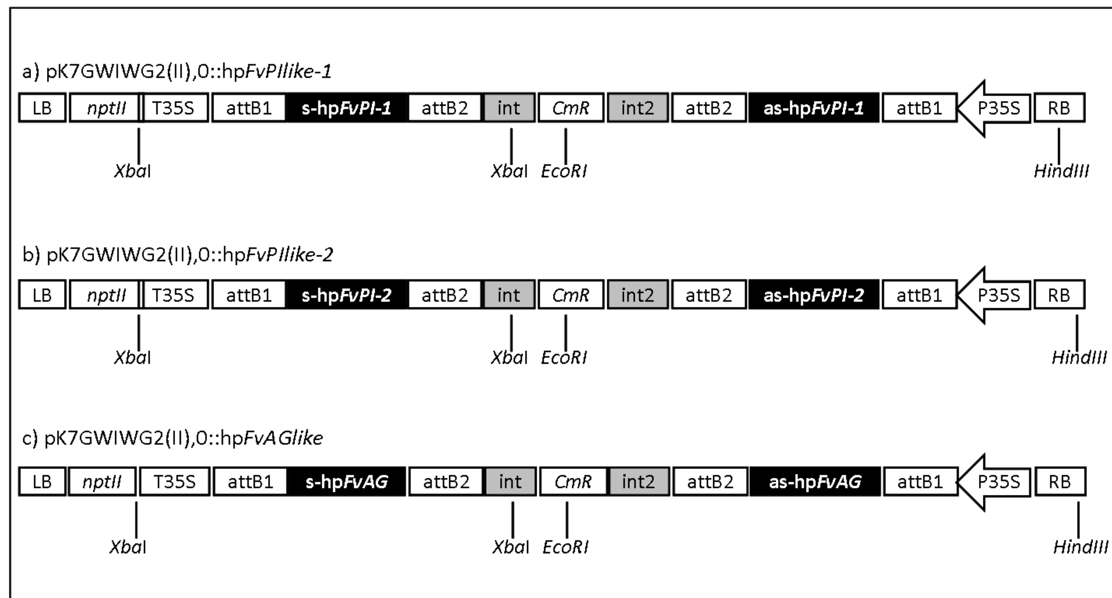


Figure 1 – Binary vectors constructed in this study.

Sense (s) and antisense (as) hairpins specific for *FvPI-1* (a), *FvPI-2* (b), and *FvAG* (c) are inserted into the binary vector pK7GWIWG2(II),0 using gateway technology.

III.2.4 Plant material and transformation

The cultivar used in this study is strawberry *Fragaria vesca* L. cv. 'Rügen'. Plants were propagated *in vitro* from leaf explants in proliferation medium containing MS medium with a pH of 5.7) supplemented with 0.1 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L indole-3-butryc acid (IBA), 3% sucrose, and 0.88% agar. Plants were grown in a culture chamber (16 h light at 21°c and 8 h dark at 16°c) and sub-cultured every 4 weeks. Young leaves from wild type strawberry were transformed using *A. tumefaciens* strain AGL0 carrying the binary plasmid vectors pK7GWIWII,(0)::*hpFvPIlike-1*, pK7GWIWII,(0)::*hpFvPIlike-2*, and pK7GWIWII,(0)::*hpFvAGlike.* A novel transformation protocol was developed in this study. First, a colony containing the vector was inoculated overnight in 2 mL liquid LB medium supplemented with 100µg/L rifampicin and 50 µg/L spectinomycin. 30 µl of the liquid culture were spread on five LB plates and incubated for two days at 28 °C. After centrifugation the bacterial pellet was resuspended in 25 ml simplified induction medium (SIM) containing 2% sucrose, 20 mM sodium citrate, 0.1 mM acetosyringone, and 1 mM betaine hydrochloride at pH 5.2. The bacterial culture was incubated at 28°C with shaking at 220 rpm on a horizontal shaker to a final OD_{600} of 0.8 to 1. Co-cultivation was performed in the dark at 25°C on regeneration medium containing 4.4 g/L MS salts and vitamins (M0222, Duchefa), 2% sucrose, 2 mg/L thidiazuron (TDZ), 1.8 mg/L indole-3-acetic acid (IAA) and 0.3% gelrite at a pH of 5.7 for three days. Leaf explants were washed in MSO medium containing 2.15 g/L MS salts (M0221, Duchefa) and 500 mg l^{-1} timentin for 10 min, in ddH_2O for 5 min, and finally in MSO for 10 min again. Shoot induction was performed on selective regeneration medium supplemented with 500 µg/L timentin and 300 µg/L kanamycin. The plates were cultured for three weeks in the dark at 25 °C, then under 16/8 h photoperiod at the same temperature. Regenerated shoots were transferred to proliferation medium with 500 µg/L timentin and 300 µg/L kanamycin at a light/dark photoperiod of 16 h at 21°C/ 8 h at 16 °C and sub-cultured every 4 weeks.

III.2.5 Rooting and acclimatization

Rooted transgenic plantlets grown in proliferation medium without antibiotics were transferred into soil after 3-4 weeks. The acclimatization of rooted plantlets in the glasshouse was done as described by Bolar et al. (1998) under long day (LD) conditions at a 16 h photoperiod and a day/night temperature of 20°C/18°C.

III.2.6 PCR analysis

Genomic DNA was extract from *in vitro* leaves using a modified protocol of the Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich). Leaf discs of 0.5 cm were put into 50 µL extraction solution. After incubation at 95 °C for 10 min 50 µl of Extract-N-Amp Plant Dilution Solution were added. PCR screening of the transgenic plants was performed in a 25 µL volume reaction containing 2 µL of the extracted DNA solution, 1xDreamTaq™ buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1xQ-Solution (QIAGEN), and 2U DreamTaq™ DNA polymerase (MBI Fermentas). All primers used to detect the presence of the sense and anti-sense fragment contained in the binary vector the *hairpins*, the *nptII* marker gene, and the housekeeping gene elongation factor 1-alpha (*EF1α*) are listed in *Annex 1*. The PCR conditions are: an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final elongation at 72 °c for 7 min. Reactions with primer for *nptII* and *EF* differ for the annealing step at 56°C for 1 min and the elongation step at 72 °c for 1 min. All PCR reactions were performed in a MyCycler™ thermocycler (Bio-Rad, Hercules, CA, USA). PCR products were detected by electrophoresis on 1% agarose gel.

III.2.7 Southern blot analysis

Genomic DNA was extracted from *in vitro* leaves using the DNeasy® Plant Mini Kit (QIAGEN, Venlo, Netherlands). Southern hybridization experiments were performed using 5-10 µg of DNA digested with 70 U of *Xba*I (MBI Fermentas, Waltham, Massachusetts, USA) at 37 °C overnight. The restricted DNA was separated on a 1% agarose gel and transferred onto a nylon membrane (Roche Diagnostics, Basel, Switzerland). PCR amplified, digoxigenin-labeled probes from the coding region of the *nptII* marker gene were generated using the primers nptII F/R and the PCR DIG Probe Synthesis Kit (Roche Diagnostics). Hybridization and detection were performed using the ECF Random Prime Labeling and Detection Kit (Amersham Biosciences, Amersham, UK) according to the manufacturer's manual.

III.2.8 Phenotypic evaluation

Twenty flowers per genotype of eight transgenic lines and one control wild type line were collected. The number of anthers, petals, and sepals per flower was counted to

test statistically using ANOVA test the eventual difference into the different groups. The pollen was collected and stained with carmine acetic acid to perform a vitality test. Two droplets per genotype containing the stained pollen were evaluated. For each droplets ten areas, each containing ~100 pollen grains, were counted under the microscope. The average of the two counts was used to calculate the vitality percentage.

III.3 RESULTS

III.3.1 Phylogenetic identification of *Fragaria* flowering genes

A phylogenetic analysis enabled the identification of strawberry homologs of Arabidopsis AP1, AP3, PI, and AG and named *FvAPETALA1*like (*FvAP1*), *FvAPETALA3*like (*FvAP3*), *FvPISTILLATA*like-1 (*FvPI-1*), *FvPISTILLATA*like-2 (*FvPI-2*), and *FvAGAMOUS*like (*FvAG*), respectively (*Figure 2*). *FvAP1* was identified in the PLAZA database (Proost et al., 2009) as FV0G03660, *FvAP3* as *FV1G14200*, *FvPI-1* as FV2G30320, *FvPI-2* as FV2G30330, and *FvAG* as FV3G08310 (previously identified as Hybrid Gene Model #24852 by Hollender et al., 2011). The amino acid sequences obtained with ExPASy Translate Tool (http://www.expasy.org/) confirmed that *FvPI-1* and *FvAG* are proteins containing a MADS domain as well as I and K-box and therefore classified as MIKC-type. The sequences of the genes (*Annex 2A*) identified with phylogenetic approach were confirmed by EST sequences retrieved from the NCBI EST database (http://www.ncbi.nlm.nih.gov/dbEST/) and by the annotations found in the strawberry genome v1.0 (http://www.strawberrygenome.org). They display some differences in comparison to the prediction, *FvPI-1* sequence is 621 bp instead of 606 bp due to a 15 bp longer forth exon and has seven exons as predicted, *FvPI-2* coding sequence is 616 bp and has seven exons while the predicted size was 424 bp and four exons, at last *FvAG* sequence lenght and exons number were confirmed with minor mismatches in the nucleic acid sequence (*Table 1*).

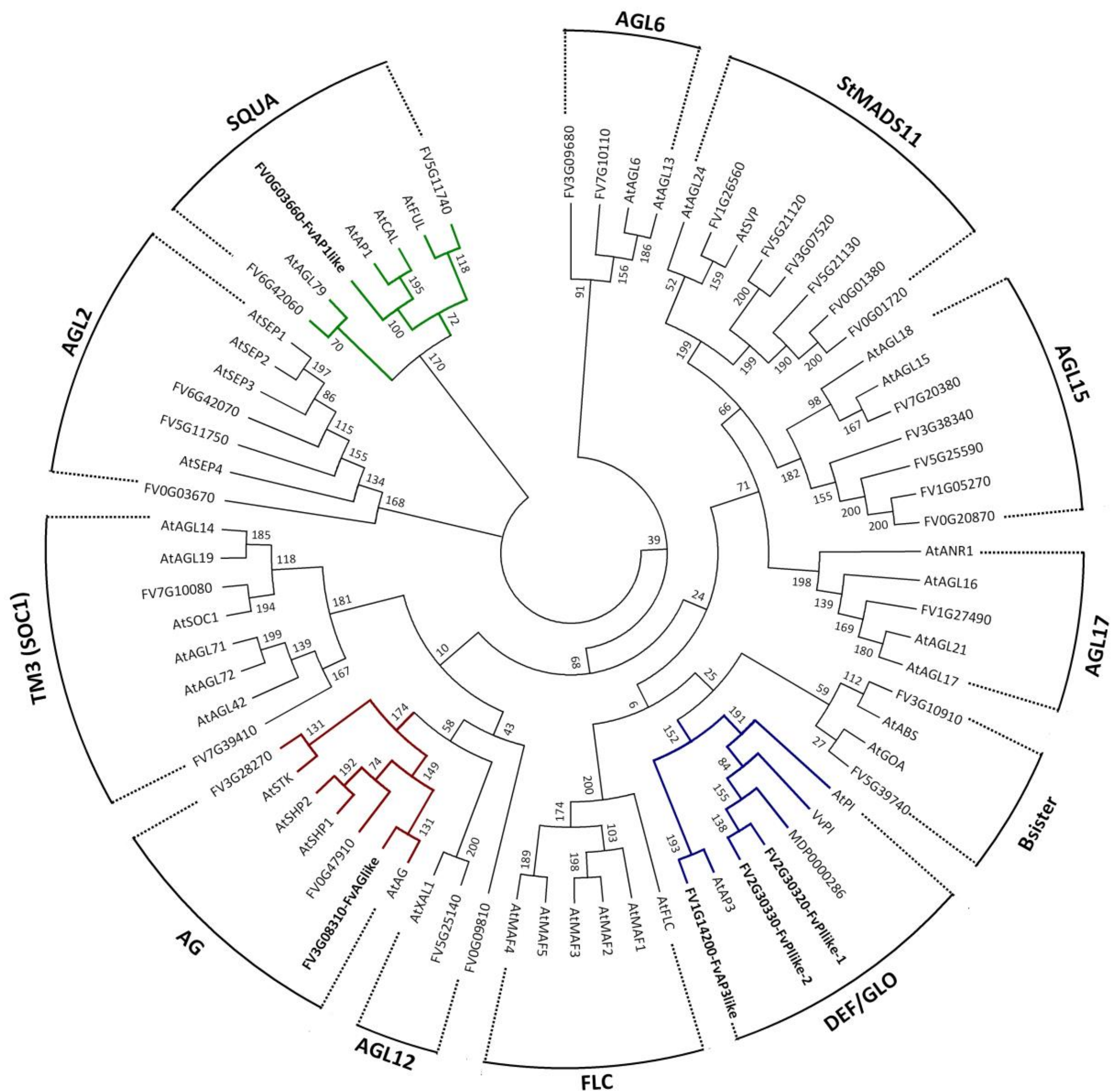


Figure 2 - Phylogenetic tree of MIKC-type MADS domain proteins[a].

The phylogenetic tree was built using the MIKC^c-type MADS-box amino acid sequences, containing MADS domain and K-box. Maximum likelihood tree of 30 proteins from *Arabidopsis thaliana* (bold, *At* prefix), 31 from *Fragaria vesca (FV* prefix), one from *Malus* x *domestica* (MD prefix) and one from *Vitis* (VvPI). The branches of the SQUA, DEF/GLO (B-function), and AG (C-function) clades are represented in green, blue, and brown respectively. The *F. vesca* putative flowering genes are highlighted in bold.

[a] Gene names retrieved by PLAZA database (available at http://bioinformatics.psb.ugent.be/plaza/) for strawberry and by TAIR for Arabidopsis.

Table 1 – Exons structure of the selected MADS-box genes

Exon Nr.	Exon size[a]					
	PI-1 pred	*PI-1* seq	*PI-2* pred	*PI-2* seq	*AG* pred	*AG* seq
Exon 1	196 bp	196 bp	192 bp	192 bp	236 bp	236 bp
Exon 2	65 bp	65 bp	67 bp	67 bp	82 bb	82 bb
Exon 3	63 bp	63 bp	-	63 bp	63 bp	63 bp
Exon 4	84 bp	99 bp	-	99 bp	99 bp	99 bp
Exon 5	30 bp	30 bp	-	30 bp	43 bp	43 bp
Exon 6	45 bp	45 bp	44 bp	40 bp	47 bp	47 bp
Exon 7	123 bp	123 bp	126 bp	126 bp	183 bp	183 bp

[a]The differences in the nucleotide number are marked in grey. Pred.: predicted; seq: sequenced.

In order to clone the coding sequence of the MADS-box genes we performed a RT-PCR on RNA extracted from different floral organs. A strong PCR band was obtained for the class-A gene *FvAP1* only in sepals whereas the class C gene *FvAG* is expressed exclusively in carpels. The class B genes *FvPI-1* and *FvPI-2* are mostly expressed in petals and stamens (*Figure 3*). As a control, the class B gene *FvAP3* is expressed in all four floral organs, with a stronger band obtained in petals and carpels materials. None of the genes is expressed in leaves confirming the flower-specificity of their expression.

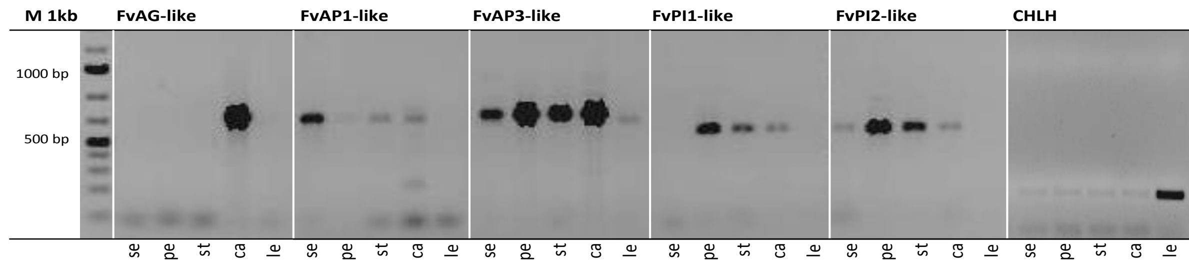


Figure 3 - Strawberry MADS-box genes detected by RT-PCR in floral organs.

The expression of the strawberry putative homeotic gene is tested in different organs of strawberry *F. vesca* cv 'Hawaii-4'. *Magnesium chelatase H subunit* (*CHLH*) gene is used as control. [sepal *se*, petal *pe*, stamen *st*, carpel *ca*, and leaf *le*].

To investigate the B and C function genes in strawberry and the impact of their respective mutants on flower and fruit development, *FvPI-1/2* and *FvAG* were selected as candidates for RNA interference.

III.3.2 Molecular characterization of the transgenic lines

Three independent transformations were performed using the silencing vectors described previously. The leaf explants produced plantlets in almost 4 weeks after transformation. Unlike in apple, tobacco and other plant species, in wild strawberry

multiple plantlets or subclones originates from a single callus. That probably reflects the bushy architecture of the wild strawberry but makes it difficult to understand whether all the subclones share the same genotype. Therefore we decided to test up to 8 subclones for each callus regenerant in order to establish whether they all contain the T-DNA. For each transformation we tested at least 20 regenerants, and for each of them from 4 up to 8 subclones. The genomic DNA of the subclones was screened with PCR. We considered positive the regenerants where the presence of the marker *nptII* and of the *sense* and *antisense* strand of the hairpin was verified (*Figure 4A*). Among the regenerants obtained, 96% were positive for T-DNA insertion, even if different PCR profiles were detected among the subclones indicating variation in the genotype. We obtained 25 pK7GWIWG2(II),0::*hpFvPIlike-1*, 35 pK7GWIWG2(II),0::*hpFvPIlike-2*, and 40 pK7GWIWG2(II),0::*hpFvAGlike* positive clones (*Table 2*). The presence of the T-DNA and the number of insertion were tested by Southern Blot on at least 18 clones per transformation (*Figure 4B*).

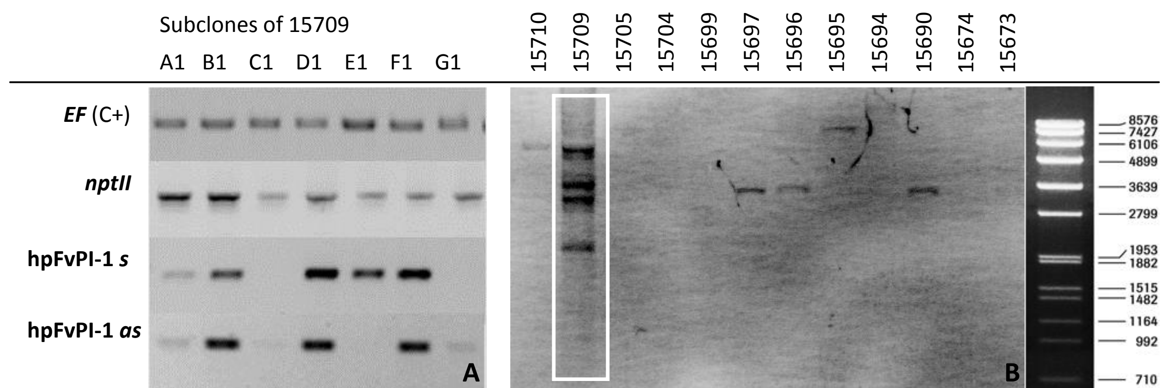


Figure 4 - Molecular characterization of the obtained transgenic lines.

PCR screening on different subclones of regenerant 15709 transformed with *hpFvPI-1*. The presence of the selection marker (*nptII*), and the sense (*s*) and antisense (*as*) hairpin RNA sequences was tested (A). After Southern Blot hybridization with *nptII* probe, positive clones present one or more dark bands according to the number of copies of T-DNA inserted and are then referred as transgenic lines. The results for regenerant 15709, line F81, are indicated in the white square (B).

Different methods of genomic DNA extraction were tested in order to obtain higher purity in the material extracted. CTAB method results in high yield but low quality genomic DNA rich in phenols and sugars unsuitable for Southern Blot Hybridization, while the DNeasy® Plant Mini Kit (Invitrogen) guaranteed a high quality DNA at a lower yield. Therefore we decided to use the DNA obtained from cumulative extraction using the Invitrogen kit. Once the quality and amount of the starting material were optimized,

we tested the lines with Southern Blot. Among the positive strawberry lines, we selected 10 transgenic lines for each transformation and proceed with the acclimatization in the greenhouse (*Table 3*).

Table 2 - Molecular screening of strawberry regenerants

Construct	Positive /Tested regenerants	Positive / Tested subclones	SB tested	SB positive
hpFvPI-1	25/26	66/156	30	10
hpFvPI-2	35/36	147/216	18	10
hpAG	40/44	152/352	29	10

Table 3 - Positive transgenic lines in strawberry

hpFvPI-1	Clone	T-line	hpFvPI-2	Clone	T-line	hpFvAG	Clone	T-line
1	15690	F71	1	16002	F96	1	15817	F93
2	15695	F73	2	15996	F114	2	15904	F95
3	15697	F75	3	15998	F115	3	15809	F98
4	15709	F81	4	15896	F125	4	15833	F101
5	15671	F83	5	15898	F126	5	15861	F108
6	15685	F85	6	15902	F127	6	15812	F120
7	15738	F116	7	16004	F128	7	15824	F121
8	15879	F132	8	16045	F129	8	15864	F122
9	16028	F118	9	16048	F130	9	15866	F123
10	16029	F119	10	16058	F131	10	16121	F124

III.3.3 Phenotypic evaluation of transgenic lines

The positive transgenic lines were preliminary tested in order to evaluate eventual major phenotypical differences in comparison to the wild-type plants. Not all the plants were transferred at the same time, therefore the phenotypic evaluation is limited to the *wt* plants, six lines of *hpFvPI-1*, F71, F73, F75, F81, F83, F85, and two lines of *hpFvAG*, F93 and F98. The phenotype of the wild type and the *hpFvPI* flower were compared and no striking alterations of flower development are detectable at the macroscopic level

(*Figure 5*). Furthermore, microscopic observation of twenty flowers for each transgenic line did not show any anomaly compared to the *wt*. The number of anthers, petals, and sepals per flower were counted and the data statistically analyzed showing no significant difference between the transgenic lines and the wild-type (*Table 4*). We next verified if these lines show differences in pollen structure and viability. Two counts per genotype were performed and the average from two values was used to calculate the vitality (*Table 5*). This data indicates reduced pollen vitality in seven out of eight transgenic lines in comparison to the wild-type. However, this result should be repeated next spring which in the season were plants growing under natural conditions in woods produce pollen.

Table 4 - Statistical analysis of flower organs from transgenic plants.

T-Line	Average nr. of anthers	Average nr. of petals	Average nr. of sepals	Std.dev. anthers	Std.dev. petals	Std.dev. sepals
F71	20.00	5.30	10.25	0.92	0.66	0.64
F73	19.70	5.00	10.05	0.47	0.32	0.22
F75	19.80	5.00	10.25	0.41	0.00	0.44
F81	19.80	5.00	10.10	0.52	0.00	0.45
F83	20.25	5.15	10.20	0.64	0.37	0.41
F85	19.75	5.10	10.10	0.79	0.31	0.31
F93	19.80	5.05	10.15	0.95	0.22	0.37
F98	19.95	5.10	10.00	0.51	0.31	0.00
Rügen	19.85	5.30	10.15	0.88	0.66	0.37

Table 4. Pollen Vitality evaluation

T-Line	Mean of Vitality %	Std. Dev of Vitality %
F71	55.27	1.54
F73	57.35	2.93
F75	53.71	1.24
F81	61.23	0.19
F83	63.49	2.42
F85	49.24	8.15
F93	39.92	0.90
F98	79.50	3.84
Rügen	85.87	1.50

Figure 5 - Transformation and regeneration steps and phenotypic evaluation of transgenic lines.

In vitro culture of *wild-type* 'Rügen' ready for transformation (A) are inoculated with *Agrobacterium*, after the transformation, the regenerants develop from callus in selective medium (B). The clones are then transferred into bigger glass until the plantlets are grown enough to extract DNA (C) for screening. Flower development from buds, to blossom, to open flower in wild-type 'Rügen' (D, E, and F, respectively) compared to transgenic line F81 transformed with *hpFvPI-1* (H, I, and J, respectively). The typical short internodes between flower and leaves are shown in both wild-type (G) and F81 line (K). White fruit, wild-type (L) compared to F81 line (N), and wild-type pink fruit (M) compared to F81 line (O) developed 5 weeks after the transfer in the greenhouse.

III.4 DISCUSSION

In this study we successfully transformed *F. vesca* 'Rügen' using RNAi. *Fragaria x ananassa* was shown to be transformable however, our method showed higher efficiencies that can be explained by the intrinsic differences between the two varieties or by the difference in ploidy (Fischer et al., 2014). Three independent transformations were performed to obtain *pistillata-1*, *pistillata-2* and *agamous* silencing mutants resulting in twenty silenced lines each. To our knowledge, this is the first example of reverse genetic approach used in strawberry to study the function of floral homeotic genes.

Few apple mutants ('Rae Ime', 'Spencer Seedless' and 'Wellington Bloomless') containing a disrupted *MdPI* gene produce only apetalous flowers that resemble the *pi* mutant phenotype observed in *Arabidopsis* (Coen and Meyerowitz, 1991; Yao et al., 2001; Krizek and Fletcher, 2005). Normal apple flowers contain all the four whorls of floral organs. In contrast, the flowers of mutant 'Rae Ime' have no petals or stamens but a doubled whorl of five sepals and an increased number of styles and carpels (Yao et al., 2001). Without either pollination or fertilization these apple flowers can develop into seedless fruits of almost normal size. In addition, seeds are produced on hand pollination in the apetalous flowers, indicating that the carpels are still functional (Pratt, 1988; Tobutt, 1994). In contrast, none of the two independent silenced *F. vesca* lines *pi-1* and *pi-2*, showed macroscopic alteration of the flower architecture. In fact, no homeotic transition of one organ into another was observed, and the flowers produced are not distinguishable from the *wt* ones. Thus, indicating that repressing the activity of one out of the two copies of *FvPI* gene is not sufficient to complete B-function in wild strawberry. Possibly, both *FvPI* copies retained the same original function or other flowering genes such as FvAP3 take over their function. In a similar way, *FvAG* silenced lines showed absence of abnormal flower phenotype suggesting the presence of a redundant pathway also for C-function genes. The preliminary statistical data on flower architecture indicated no significant difference between transgenic and *wt* plants. To investigate the effects of RNAi at the molecular level, real time PCR experiments are necessary. The relative abundance of the flowering genes (silenced and not) should be measured in selected flower organs in order to compare gene expression between transgenic and wild-type plants and evaluate the effect of RNAi. Furthermore, to overcome the redundancy effect, we crossed the transgenic *pi* and *ag* lines to obtain a *pistillata x agamous* double mutant. The evaluation of the double mutant flower and

fruit phenotype in comparison to wild-type and single mutant will provide new information about the role played by B- and C-function genes in strawberry.

III.5 References Chapter III

Albani, M.C., Battey, N.H., and Wilkinson, M.J. (2004). The development of ISSR-derived SCAR markers around the SEASONAL FLOWERING LOCUS (SFL) in Fragaria vesca. Theoretical and Applied Genetics **109,** 571-579.

Angenent, G.C., Franken, J., Busscher, M., van Dijken, A., van Went, J.L., Dons, H.J., and van Tunen, A.J. (1995). A novel class of MADS box genes is involved in ovule development in petunia. Plant Cell **7,** 1569-1582.

Bolar, J.P., Norelli, J.L., Aldwinckle, H.S., and Hanke, V. (1998). An Efficient Method for Rooting and Acclimation of Micropropagated Apple Cultivars. HortScience **33,** 1251-1252.

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

Colombo, L., Van Tunen, A.J., Dons, H.J.M., and Angenent, G.C. (1997). Molecular control of flower development in Petunia hybrida. Adv. Bot. Res. **26,** 229-250.

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C., and van Tunen, A.J. (1995). The petunia MADS box gene FBP11 determines ovule identity. Plant Cell **7,** 1859-1868.

de Folter, S., Shchennikova, A.V., Franken, J., Busscher, M., Baskar, R., Grossniklaus, U., Angenent, G.C., and Immink, R.G. (2006). A Bsister MADS-box gene involved in ovule and seed development in petunia and Arabidopsis. Plant J **47,** 934-946.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S., and Yanofsky, M.F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Curr Biol **14,** 1935-1940.

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32,** 1792-1797.

Fernandez, L., Chaib, J., Martinez-Zapater, J.M., Thomas, M.R., and Torregrosa, L. (2013). Mis-expression of a PISTILLATA-like MADS box gene prevents fruit development in grapevine. Plant J **73,** 918-928.

Fischer, T.C., Mirbeth, B., Rentsch, J., Sutter, C., Ring, L., Flachowsky, H., Habegger, R., Hoffmann, T., Hanke, M.-V., and Schwab, W. (2014). Premature and ectopic anthocyanin formation by silencing of anthocyanidin reductase in strawberry (Fragaria × ananassa). New Phytologist **201,** 440-451.

Guindon, S., and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol **52,** 696-704.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol **59,** 307-321.

Heide, O.M., and Sønsteby, A. (2007). Interactions of temperature and photoperiod in the control of flowering of latitudinal and altitudinal populations of wild strawberry (Fragaria vesca). Physiologia Plantarum **130,** 280-289.

Hollender, C.A., Geretz, A.C., Slovin, J.P., and Liu, Z. (2011). Flower and early fruit development in a diploid strawberry, Fragaria vesca. Planta **235,** 1123-1139.

Jimenez, S., Lawton-Rauh, A.L., Reighard, G.L., Abbott, A.G., and Bielenberg, D.G. (2009). Phylogenetic analysis and molecular evolution of the dormancy associated MADS-box genes from peach. BMC Plant Biol **9,** 81.

Jones, D.T., Taylor, W.R., and Thornton, J.M. (1992). The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci **8,** 275-282.

Kang, H.G., Jeon, J.S., Lee, S., and An, G. (1998). Identification of class B and class C floral organ identity genes from rice plants. Plant Mol Biol **38,** 1021-1029.

Kanno, A., Saeki, H., Kameya, T., Saedler, H., and Theissen, G. (2003). Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (Tulipa gesneriana). Plant Mol Biol **52,** 831-841.

Krizek, B.A., and Fletcher, J.C. (2005). Molecular mechanisms of flower development: an armchair guide. Nat Rev Genet **6,** 688-698.

Li, H., Liang, W., Hu, Y., Zhu, L., Yin, C., Xu, J., Dreni, L., Kater, M.M., and Zhang, D. (2011). Rice MADS6 interacts with the floral homeotic genes SUPERWOMAN1, MADS3, MADS58, MADS13, and DROOPING LEAF in specifying floral organ identities and meristem fate. Plant Cell **23,** 2536-2552.

Mouhu, K., Hytönen, T., Folta, K., Rantanen, M., Paulin, L., Auvinen, P., and Elomaa, P. (2009). Identification of flowering genes in strawberry, a perennial SD plant. BMC Plant Biology **9,** 122.

Murashige, T., and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum **15,** 473-497.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H., and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. Development **130,** 705-718.

Nesi, N., Debeaujon, I., Jond, C., Stewart, A.J., Jenkins, G.I., Caboche, M., and Lepiniec, L. (2002). The TRANSPARENT TESTA16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. Plant Cell **14,** 2463-2479.

Oosumi, T., Gruszewski, H.A., Blischak, L.A., Baxter, A.J., Wadl, P.A., Shuman, J.L., Veilleux, R.E., and Shulaev, V. (2006). High-efficiency transformation of the diploid strawberry (Fragaria vesca) for functional genomics. Planta **223,** 1219-1230.

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

Pratt. (1988). Apple Flower and Fruit: Morphology and anatomy. Hort Rev **10**.

Proost, S., Van Bel, M., Sterck, L., Billiau, K., Van Parys, T., Van de Peer, Y., and Vandepoele, K. (2009). PLAZA: a comparative genomics resource to study gene and genome evolution in plants. Plant Cell **21,** 3718-3731.

Rosin, F.M., Aharoni, A., Salentijn, E.M.J., Schaart, J.G., Boone, M.J., and Hannapel, D.J. (2003). Expression patterns of a putative homolog of AGAMOUS, STAG1, from strawberry. Plant Science **165,** 959-968.

Saedler, H., Becker, A., Winter, K.U., Kirchner, C., and Theissen, G. (2001). MADS-box genes are involved in floral development and evolution. Acta Biochim Pol **48,** 351-358.

Seymour, G.B., Ryder, C.D., Cevik, V., Hammond, J.P., Popovich, A., King, G.J., Vrebalov, J., Giovannoni, J.J., and Manning, K. (2011). A SEPALLATA gene is involved in the development and ripening of strawberry (Fragaria×ananassa Duch.) fruit, a non-climacteric tissue*. Journal of Experimental Botany **62,** 1179-1188.

Shulaev, V., Korban, S.S., Sosinski, B., Abbott, A.G., Aldwinckle, H.S., Folta, K.M., Iezzoni, A., Main, D., Arus, P., Dandekar, A.M., Lewers, K., Brown, S.K., Davis, T.M., Gardiner, S.E.,

Potter, D., and Veilleux, R.E. (2008). Multiple models for Rosaceae genomics. Plant Physiol **147,** 985-1003.

Shulaev, V., Sargent, D.J., Crowhurst, R.N., Mockler, T.C., Folkerts, O., Delcher, A.L., Jaiswal, P., Mockaitis, K., Liston, A., Mane, S.P., Burns, P., Davis, T.M., Slovin, J.P., Bassil, N., Hellens, R.P., Evans, C., Harkins, T., Kodira, C., Desany, B., Crasta, O.R., Jensen, R.V., Allan, A.C., Michael, T.P., Setubal, J.C., Celton, J.-M., Rees, D.J.G., Williams, K.P., Holt, S.H., Rojas, J.J.R., Chatterjee, M., Liu, B., Silva, H., Meisel, L., Adato, A., Filichkin, S.A., Troggio, M., Viola, R., Ashman, T.-L., Wang, H., Dharmawardhana, P., Elser, J., Raja, R., Priest, H.D., Bryant, D.W., Fox, S.E., Givan, S.A., Wilhelm, L.J., Naithani, S., Christoffels, A., Salama, D.Y., Carter, J., Girona, E.L., Zdepski, A., Wang, W., Kerstetter, R.A., Schwab, W., Korban, S.S., Davik, J., Monfort, A., Denoyes-Rothan, B., Arus, P., Mittler, R., Flinn, B., Aharoni, A., Bennetzen, J.L., Salzberg, S.L., Dickerman, A.W., Velasco, R., Borodovsky, M., Veilleux, R.E., and Folta, K.M. (2010). The genome of woodland strawberry (Fragaria vesca). Nature Genetics **43,** 109-116.

Slovin, J.P., and Michael, T.P. (2011). Strawberry. Part 3 structural and functional genomics. In Genetics, Genomics and Breeding of Berries (Science Publishers), pp. 162-193.

Slovin, J.P., Schmitt, K., and Folta, K.M. (2009). An inbred line of the diploid strawberry Fragaria vesca f. semperflorens for genomic and molecular genetic studies in the Rosaceae. Plant Methods **5,** 15.

Theissen, G., and Saedler, H. (2001). Plant biology. Floral quartets. Nature **409,** 469-471.

Theissen, G., and Melzer, R. (2007). Molecular mechanisms underlying origin and diversification of the angiosperm flower. Ann Bot **100,** 603-619.

Tobutt, K.R. (1994). Combining apetalous parthenocarpy with columnar growth habit in apple. Euphytica **77,** 51-54.

Ubi, B.E., Saito, T., Bai, S., Nishitani, C., Ban, Y., Ikeda, K., Ito, A., and Moriguchi, T. (2013). Characterization of 10 MADS-box genes from Pyrus pyrifolia and their differential expression during fruit development and ripening. Gene **528,** 183-194.

van Tunen, A.J., Eikelboom, W., and Angenent, G. (1993). Floral organogenesis in Tulipa. Flow. News Lett. **16,** 33-37.

Whipple, C.J., Ciceri, P., Padilla, C.M., Ambrose, B.A., Bandong, S.L., and Schmidt, R.J. (2004). Conservation of B-class floral homeotic gene function between maize and Arabidopsis. Development **131,** 6083-6091.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y., and Hirano, H.Y. (2004). The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. Plant Cell **16,** 500-509.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G., and Hirano, H.Y. (2006). Functional diversification of the two C-class MADS box genes OSMADS3 and OSMADS58 in Oryza sativa. Plant Cell **18,** 15-28.

Yao, J., Dong, Y., and Morris, B.A. (2001). Parthenocarpic apple fruit production conferred by transposon insertion mutations in a MADS-box transcription factor. Proc Natl Acad Sci U S A **98,** 1306-1311.

Chapter IV

General Discussion

IV.1 Discussion

Since the isolation of the first members more than 20 years ago, MADS-box gene family has been one of the most investigated transcription factors family in plants. Particularly relevant is their role in flower initiation and flower organ development that has been deeply studied in model plant Arabidopsis as well as in cereals and some perennials. Nevertheless, their function in agronomically relevant Rosaceae family has been poorly investigated so far. In this study we started to fill this lack of information describing two apple genes involved in bud dormancy and presenting preliminary data on functional characterization of strawberry floral homeotic genes.

MADS-box genes are involved in cold response during dormancy

As previously described, seasonal temperature changes are sensed by plants and allow correct timing of flowering (King and Heide, 2009). Many annual plants, both dicots and monocots, originating from temperate climate require a prolonged exposure to cold during winter to be able to flower in spring, a phenomenon called ‘vernalization’. Perennial fruit trees instead inhibit growth seasonally during winter, a process known as ‘bud dormancy’ that is divided into three phases: paradormancy, endodormancy and ecodormancy (Lang, 1987). To release bud dormancy and initiate flowering, plants require a period of exposition to chilling temperatures. Genes controlling flowering in response to cold have been identified in Arabidopsis, cereals and perennial fruit trees; in all the three cases, MADS-box genes play a central role. *FLC* is key regulator of vernalization in annual *A. thaliana*; in perennials species related to Arabidopsis, FLC-like genes, for instance *PEP1* from *A. alpina*, control vernalization (Michaels and Amasino, 1999; Wang et al., 2009). The clade of SVP-like genes seems to play also a conserved role in seasonal regulation of flowering (Hemming and Trevaskis, 2011). In both eudicots and monocots, they repress floral meristem identity genes and delay flowering, also, their activity seem to be temperature dependent (Hartmann et al., 2000; Masiero et al., 2004; Trevaskis et al., 2007). In deciduous trees, they more likely regulate bud dormancy. An expansion of StMADS11-like genes, mainly due to gene duplication, has been observed in peach (six genes), poplar (eight), grapevine (five), and pear (three). We can now include apple among them. Here we reported three apple SVP/StMADS11-like genes, one showing a high similarity to AtSVP, and the other two closely related to pear *DAM* genes. This suggests the evolution of different strategies to adapt and respond to seasonal changes between annual and perennial, mostly due to the different plant life cycle and structure, resulting in different gene clades carrying over the same function.

All the genes involved in cold response seem to share a similar regulative fate: epigenetic control at the histone H3 level. *FLC* undergo a stable repression through PRC-2 complex that accumulates H3K27 tri-methylation during vernalization. In *A. alpina*, *PEP1* shows the same mechanism, with the level of H3K27me3 gradually increasing during vernalization, however, it does not persist after. On the contrary, the epigenetic modification is unstable and the gene is reactivated by warm temperatures (Hemming and Trevaskis, 2011). Temporal changes in *PEP1* expression regulates the perennial life cycle of *A. alpine*, as *DAM6* gene does in peach. According to these findings, it is evident that in perennials and deciduous tree, the bud dormancy pattern must adapt to the cyclic succession of vegetative and reproductive phases. The *DAM* genes have to be repressed in winter after cold exposure to initiate flowering in spring, but they must return active during periods of warm temperatures to permit the production of new buds and continue plant vegetative growth. This fine modulation seem to be only due to epigenetic changes at the histone level, analysis on pear *DAM* genes promoter revealed that changes in DNA methylation status were not correlated to changes in *DAM* gene expression (Saito et al., 2013).

Nevertheless, the mechanism by which plants sense temperature changes and cold remains to understand. Few mechanisms for temperature sensing have been proposed, including calcium influx into the cytoplasm or transcription factors involved in rapid response to cold temperature, such as C-repeat Binding Factors (Viswanathan and Zhu, 2002; Penfield, 2008; Thomashow, 2010). However, is not clear how these mechanisms contribute to the gradual changes in transcript level of MADS-box transcription factors. Future research in this key field could help to elucidate how seasonal changes are sensed and how these signals trigger quantitative changes in MADS-box genes expression.

An emerging problem for fruit trees cultivation comes from the global climate change. Apple cultivars chilling requirements (CR) are classified into low, intermediate and high and can span from less than 200 hours (‘Anna’) to almost 1500 hrs (‘Wright’) (Hauagge and Cummins, 1991). However, the majority of cultivated apples have from intermediate to high CR and poorly adapted to warm climate. Changes in apple tree blooming date in response to temperature changes have been already observed (Legave et al., 2013). For example, ‘Golden Delicious’ mean flowering dates have increased by seven to nine days in the last years. If this phenomenon persist in the future phenological alteration could be observed, including irregular bud break and poor fruit production as it has already been observed in warm countries. The current

countermeasures involve the employment of dormancy breaking chemicals, which have negative environmental effects and can be toxic for human health. Our study can be the starting point to produce apple of superior market quality even where the necessary chilling hours are not reached, exploiting natural plant adaptation. The comparative analysis of MdDAM1 and MdDAM2 loci in apple varieties with different CR will lead to the identification of a molecular marker for chilling requirement and dormancy control. The increasing availability of new generation sequencing tools would allow a faster and extensive comparative screening of transcriptome and ChIP chromatin and implement the research in this field.

Strawberry flower development differs from classical ABC model

Comparative analysis of the genetic regulation of flower development between Arabidopsis and Antirrhinum showed a conserved genetic pattern despite their distance within the eudicots. This led to the postulation of a universally applicable ABC-model of flower development (Coen and Meyerowitz, 1991; Schwarz-Sommer et al., 1992). The E-function was not included in the original ABC-model, but added later as it became clear that the A-, B-, and C-function genes need other co-factors to produce floral organs (Angenent et al., 1994; Pelaz et al., 2000). The ABC model was a major breakthrough in the understanding of floral development and represented a trailhead for comparative floral architecture studies (Rijpkema et al., 2010; Heijmans et al., 2012). These have resulted in a progressive better understanding of the variation in the molecular control of the development of different types of flowers. Diversification can be found between species with similar flower architecture which can be explained by lineage-specific differences in gene duplications and the subsequent functional diversification, such as functional redundancy, gene loss, and subfunctionalization (Airoldi and Davies, 2012). Moreover, there are indications that some aspects of the regulatory network are not conserved between certain species, although the final result is the same sepals-petals-stamens-carpels flower set-up (Garay-Arroyo et al., 2012). A second aspect is the variation between species with flowers with different architectures. Considering the tremendous variation in floral forms among angiosperms, it is evident that important changes to the general ABC model can be expected in flower types that deviate from the classical flower architecture. Typical examples can be found in the monocots, such as the tulip flower, and the flowers of grasses which develop palea/lemma and lodicules rather than sepals and petals (van Tunen et al., 1993; Kang et al., 1998; Ambrose et al., 2000). These data contributed to a comparative perspective of the ABC model. The A-function, with *AP1* and *AP2* strictly acting as perianth organ identity genes has been

questioned by Litt (2007). According to this author these genes more likely specify organ identity indirectly by establishing the floral meristem and by antagonizing C-function gene expression (Drews et al., 1991; Schultz and Haughn, 1993; Gregis et al., 2009). Functional studies on *AP1* lineage genes from other species indicate that the role of *AP1* in floral meristem specification is most likely conserved in other eudicot species, while the contribution of the gene to perianth formation is not confirmed except for Arabidopsis (Litt, 2007). Functional analyses of B- and C-lineage MADS-box protein genes in a diverse range of dicot and monocot species show that in general the B- and C-functions are very well conserved at the molecular level. Differences are usually found at the level of subfunctionalization between paralogs in the same gene lineage (Vandenbussche et al., 2004; Geuten and Irish, 2010). The variability observed within the classical A-function and, in contrast, the well-conserved B- and C-function genes, led to the proposition of a revised (A)BC model in which the (A)-function comprises a plethora of genes that ultimately allow the B- and C-genes to determine floral organ identity (Causier et al., 2010). B- and C-function seem to be conserved also in perennials such as apple, garden strawberry (*Fragaria* x *ananassa*) and grapevine (Yao et al., 2001; Mouhu et al., 2009; Fernandez et al., 2013), but there is still a lack of experimental data.

New data are provided for *F. vesca* in this work. Even if our results must be considered as preliminary, since they are based on RT-PCR data and silenced mutant phenotype analysis, we shown that single silenced *pi* and *ag* mutants does not conform to the ABC model from Arabidopsis, and also differ from other Rosaceae such as apple. In fact, the spatial boundaries due to antagonistic action between the different classes of homeotic genes seem not to be valid for *F. vesca*. The duplication of *FvPI* in the strawberry genome, an event already observed in tomato and petunia, results in mutants with not affected petals or stamen development. Another possible explanation to why *PI* B-function genes alone are not sufficient to specify petal and stamen development, is that they act in complex with *AP3* which is able to complete this function even when one of the *PI* alleles is not active. As *AP3* seemed to be expressed in all floral organs, it can also rescue stamen and carpel development in the *ag* silenced mutant. Thus, implying an extension of B-function gene *AP3* to the first and fourth whorls in *F. vesca*. According to these data, we propose a model for flower development in wild strawberry (*Figure 1*) but comparative expression data of flowering genes between wild type and mutants, in addition to the screening of the double silenced *pi x ag* mutants, will provide a complete view.

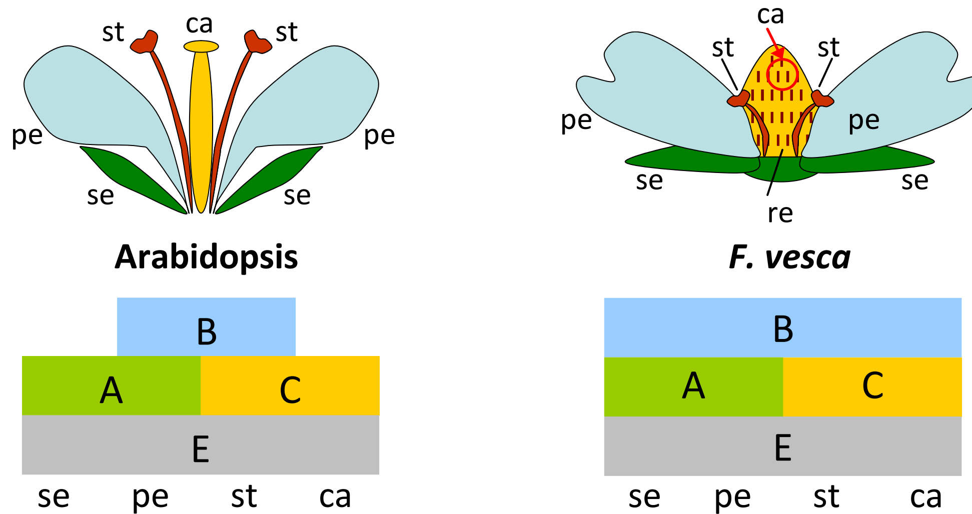


Figure 1 - Comparative representation of ABC model in different plants

In Arabidopsis was postulated the ABCE model for flower development. In *F.vesca*, B-function (completed by AP3/PI) seems to extend to first and fourth whorls. The following abbreviations for floral organs were used: se, sepals; pe, petals; st, stamens; ca, carpels; re, receptacle

Given that the ABC-genes control the identity of the floral organs, their spatial and temporal expression patterns are of main importance, since they are subject to tight regulation. Most research in this regard has focused on transcriptional regulation. In Arabidopsis, the expression of the floral organ identity genes is directly promoted by LEAFY (LFY), which is expressed throughout the emerging floral meristem (Parcy et al., 1998; Siriwardana and Lamb, 2012). To regulate these genes, LFY appears to require co-factors. Among them, SEP3 has been shown to interact *in vitro* with LFY and it appears that a SEP3/LFY complex activates B- and C-class gene expression (Liu et al., 2009). AP1 expression is directly promoted by LFY at the floral meristem (Wagner et al., 1999), while the restriction of AP1 expression in the outer two whorls is mediated by the class C gene AGAMOUS (Gustafson-Brown et al., 1994).

Regulation of B function, AP3 and PI heterodimers in Arabidopsis, is more complex. Although AP3/PI expression is reduced in *lfy* mutants, it is not abolished, indicating that other factors are needed to regulate their expression (Weigel and Meyerowitz, 1993). The F-box protein UNUSUAL FLORAL ORGANS (UFO) can be considered as co-regulator of B class gene expression (Wilkinson and Haughn, 1995; Lee et al., 1997; Chae et al., 2008). The expression of AP3/PI is also promoted by AP1, as indicated by the absence of their transcripts in *lfy ap1* double-mutant flowers (Weigel and Meyerowitz, 1993). Several regulators of the C class gene *AG* have been identified.

These include repressors, such as the polycomb group protein CURLY LEAF, that prevent AG expression outside of the flower through epigenetic mechanisms (Goodrich et al., 1997; Calonje et al., 2008), as well as activators, such as the homeodomain transcription factor WUSCHEL (WUS), that promote the transcription of *AG* in the center of the flower (Liu and Mara, 2010).

As the original genetic ABC model predicted, A- and C-function genes act in a mutually antagonistic manner (Bowman et al., 1991). In fact, AP2 suppresses AG in the outer two floral whorls; however, AG does not appear to control AP2 expression levels (Wollmann et al., 2010). Rather, it has been shown that this function is mediated by the microRNA *miR*172, which prevents the accumulation of AP2 mRNA and protein in the third and fourth whorls (Chen, 2004). In contrast, in Petunia and Antirrhinum, homologous microRNA from the *miR169*-family are required to indirectly restrict C-class gene expression (Cartolano et al., 2007).

The gene network controlling flowering is yet to be elucidated in Rosaceae. So far, mainly for technical and experimental reasons (for instance the difficulty to perform functional analysis), it has often been challenging to work with non model plants, especially perennials. We propose to use *F. vesca* as plant model for reverse genetic studies in perennial plants and that can be representative for other Rosaceae species. Additionally, thanks to affordable next generation sequencing technologies, sequencing the entire genome of large series of individual plants will enable broader molecular screenings for genes involved in the network regulating flower organ formation and global transcriptome profiling.

IV.2 References Chapter IV

Airoldi, C.A., and Davies, B. (2012). Gene duplication and the evolution of plant MADS-box transcription factors. J Genet Genomics **39,** 157-165.

Ambrose, B.A., Lerner, D.R., Ciceri, P., Padilla, C.M., Yanofsky, M.F., and Schmidt, R.J. (2000). Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. Mol Cell **5,** 569-579.

Angenent, G.C., Franken, J., Busscher, M., Weiss, D., and van Tunen, A.J. (1994). Co-suppression of the petunia homeotic gene fbp2 affects the identity of the generative meristem. Plant J **5,** 33-44.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1991). Genetic interactions among floral homeotic genes of Arabidopsis. Development **112,** 1-20.

Calonje, M., Sanchez, R., Chen, L., and Sung, Z.R. (2008). EMBRYONIC FLOWER1 participates in polycomb group-mediated AG gene silencing in Arabidopsis. Plant Cell **20,** 277-291.

Cartolano, M., Castillo, R., Efremova, N., Kuckenberg, M., Zethof, J., Gerats, T., Schwarz-Sommer, Z., and Vandenbussche, M. (2007). A conserved microRNA module exerts

homeotic control over Petunia hybrida and Antirrhinum majus floral organ identity. Nature Genetics **39,** 901-905.

Causier, B., Schwarz-Sommer, Z., and Davies, B. (2010). Floral organ identity: 20 years of ABCs. Seminars in Cell & Developmental Biology **21,** 73-79.

Chae, E., Tan, Q.K., Hill, T.A., and Irish, V.F. (2008). An Arabidopsis F-box protein acts as a transcriptional co-factor to regulate floral development. Development **135,** 1235-1245.

Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science **303,** 2022-2025.

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

Drews, G.N., Bowman, J.L., and Meyerowitz, E.M. (1991). Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product. Cell **65,** 991-1002.

Fernandez, L., Chaib, J., Martinez-Zapater, J.M., Thomas, M.R., and Torregrosa, L. (2013). Mis-expression of a PISTILLATA-like MADS box gene prevents fruit development in grapevine. Plant J **73,** 918-928.

Garay-Arroyo, A., Pineyro-Nelson, A., Garcia-Ponce, B., Sanchez Mde, L., and Alvarez-Buylla, E.R. (2012). When ABC becomes ACB. J Exp Bot **63,** 2377-2395.

Geuten, K., and Irish, V. (2010). Hidden variability of floral homeotic B genes in Solanaceae provides a molecular basis for the evolution of novel functions. Plant Cell **22,** 2562-2578.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997). A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. Nature **386,** 44-51.

Gregis, V., Sessa, A., Dorca-Fornell, C., and Kater, M.M. (2009). The Arabidopsis floral meristem identity genes AP1, AGL24 and SVP directly repress class B and C floral homeotic genes. Plant J **60,** 626-637.

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

Hartmann, U., Hohmann, S., Nettesheim, K., Wisman, E., Saedler, H., and Huijser, P. (2000). Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. Plant J **21,** 351-360.

Hauagge, R., and Cummins, J.N. (1991). Phenotypic Variation of Length of Bud Dormancy in Apple Cultivars and Related Malus Species. Journal of the American Society for Horticultural Science **116,** 100-106.

Heijmans, K., Morel, P., and Vandenbussche, M. (2012). MADS-box genes and floral development: the dark side. J Exp Bot **63,** 5397-5404.

Hemming, M.N., and Trevaskis, B. (2011). Make hay when the sun shines: the role of MADS-box genes in temperature-dependant seasonal flowering responses. Plant Sci **180,** 447-453.

Kang, H.G., Jeon, J.S., Lee, S., and An, G. (1998). Identification of class B and class C floral organ identity genes from rice plants. Plant Mol Biol **38,** 1021-1029.

King, R.W., and Heide, O.M. (2009). Seasonal flowering and evolution: the heritage from Charles Darwin. Functional Plant Biology **36,** 1027-1036.

Lang, G.A. (1987). Dormancy: a new universal terminology. . HortScience **22,** 817–820.

Lee, I., Wolfe, D.S., Nilsson, O., and Weigel, D. (1997). A LEAFY co-regulator encoded by UNUSUAL FLORAL ORGANS. Curr Biol **7,** 95-104.

Legave, J.M., Blanke, M., Christen, D., Giovannini, D., Mathieu, V., and Oger, R. (2013). A comprehensive overview of the spatial and temporal variability of apple bud dormancy release and blooming phenology in Western Europe. Int J Biometeorol **57,** 317-331.

Litt, A. (2007). An Evaluation of A-Function: Evidence from the APETALA1 and APETALA2 Gene Lineages. International Journal of Plant Sciences **168,** 73-91.
Liu, C., Xi, W., Shen, L., Tan, C., and Yu, H. (2009). Regulation of Floral Patterning by Flowering Time Genes. Developmental Cell **16,** 711-722.
Liu, Z., and Mara, C. (2010). Regulatory mechanisms for floral homeotic gene expression. Seminars in Cell & Developmental Biology **21,** 80-86.
Masiero, S., Li, M.-A., Will, I., Hartmann, U., Saedler, H., Huijser, P., Schwarz-Sommer, Z., and Sommer, H. (2004). INCOMPOSITA: a MADS-box gene controlling prophyll development and floral meristem identity in Antirrhinum. Development **131,** 5981-5990.
Michaels, S.D., and Amasino, R.M. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949-956.
Mouhu, K., Hytönen, T., Folta, K., Rantanen, M., Paulin, L., Auvinen, P., and Elomaa, P. (2009). Identification of flowering genes in strawberry, a perennial SD plant. BMC Plant Biology **9,** 122.
Parcy, F., Nilsson, O., Busch, M.A., Lee, I., and Weigel, D. (1998). A genetic framework for floral patterning. Nature **395,** 561-566.
Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E., and Yanofsky, M.F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. Nature **405,** 200-203.
Penfield, S. (2008). Temperature perception and signal transduction in plants. New Phytologist **179,** 615-628.
Rijpkema, A.S., Vandenbussche, M., Koes, R., Heijmans, K., and Gerats, T. (2010). Variations on a theme: changes in the floral ABCs in angiosperms. Semin Cell Dev Biol **21,** 100-107.
Saito, T., Bai, S., Ito, A., Sakamoto, D., Ubi, B.E., Imai, T., and Moriguchi, T. (2013). Expression and genomic structure of the dormancy-associated MADS box genes MADS13 in Japanese pears (Pyrus pyrifolia Nakai) that differ in their chilling requirement for endodormancy release. Tree Physiol **33,** 654-667.
Schultz, E.A., and Haughn, G.W. (1993). Genetic analysis of the floral initiation process (FLIP) in Arabidopsis. Development **119,** 745-765.
Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.E., Saedler, H., and Sommer, H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J **11,** 251-263.
Siriwardana, N.S., and Lamb, R.S. (2012). The poetry of reproduction: the role of LEAFY in Arabidopsis thaliana flower formation. International Journal of Developmental Biology International Journal of Developmental Biology **56,** 207–221.
Thomashow, M.F. (2010). Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. Plant Physiol **154,** 571-577.
Trevaskis, B., Tadege, M., Hemming, M.N., Peacock, W.J., Dennis, E.S., and Sheldon, C. (2007). Short Vegetative Phase-like MADS-BOX genes inhibit floral meristem identity in barley. Plant Physiology **143,** 225-235.
van Tunen, A.J., Eikelboom, W., and Angenent, G. (1993). Floral organogenesis in Tulipa. Flow. News Lett. **16,** 33-37.
Vandenbussche, M., Zethof, J., Royaert, S., Weterings, K., and Gerats, T. (2004). The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in Petunia hybrida flower development. Plant Cell **16,** 741-754.
Viswanathan, C., and Zhu, J.-K. (2002). Molecular genetic analysis of cold–regulated gene transcription. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences **357,** 877-886.

Wagner, D., Sablowski, R.W., and Meyerowitz, E.M. (1999). Transcriptional activation of APETALA1 by LEAFY. Science **285,** 582-584.
Wang, R., Farrona, S., Vincent, C., Joecker, A., Schoof, H., Turck, F., Alonso-Blanco, C., Coupland, G., and Albani, M.C. (2009). PEP1 regulates perennial flowering in Arabis alpina. Nature **459,** 423-427.
Weigel, D., and Meyerowitz, E.M. (1993). Activation of Floral Homeotic Genes in Arabidopsis. Science **261,** 1723-1726.
Wilkinson, M.D., and Haughn, G.W. (1995). UNUSUAL FLORAL ORGANS Controls Meristem Identity and Organ Primordia Fate in Arabidopsis. The Plant Cell Online **7,** 1485-1499.
Wollmann, H., Mica, E., Todesco, M., Long, J.A., and Weigel, D. (2010). On reconciling the interactions between APETALA2, miR172 and AGAMOUS with the ABC model of flower development. Development **137,** 3633-3642.
Yao, J., Dong, Y., and Morris, B.A. (2001). Parthenocarpic apple fruit production conferred by transposon insertion mutations in a MADS-box transcription factor. Proc Natl Acad Sci U S A **98,** 1306-1311.

Annexes

Annex 1 - Oligonucleotides used for PCR and RT-PCR in strawberry

Oligo name	**Sequence 5‘-3‘**	**Experiment**
EF for	ATTGTGGTCATTGGYCAYGT	
EF rev	CCAATCTTGTAVACATCCTG	
nptII for	ACAAGATGGATTGCACGCAGG	PCR, SB probe
nptII rev	AACTCGTCAAGAAGGCGATAG	PCR, SBprobe
F_FvPI1-fl	ATGGGAAGGGGTAAGATTGAGA	PCR
R_FvPI1-fl	TTAGCAGTCGTGGTGGAGATT	PCR
F_FvPI2-fl	ATGGGGAGGGGTAAGATTGA	PCR
R_FvPI2-fl	TTACATTATGTCGTGGAGATTGGG	PCR
F_FvAG-fl	ATGGCCTATGAAAACAAACCA	PCR
R_FvAG-fl	TTACACTAACTGAAGGGAAACTTG	PCR
FvhpPI1 for	*CACC*CTTCAGGCTCCAGCCTATTC	Vector
FvhpPI1 rev	AGTTCAGACACACCTCGATCA	Vector, PCR
FvhpPI2 for	*CACC*CATGGCCAGCAGATACCTTT	Vector
FvhpPI2 rev	ACAAGTCCCACAAACATTATTACTT	Vector, PCR
FvhpAG for	CACCGACTCAGCCCTTTCATGAGG	Vector, PCR
FvhpAG rev	AGAAACCAGGCCAACATACG	Vector, PCR
Fseq35S	ATGACGCACAATCCCACTATC	PCR
Rseq35Ster	TGATTTTTGCGGACTCTAGC	PCR
ChlH for	ACGAGGGTGTTCGGG	PCR
ChlH rev	CGTTCCTCGAGGCCAA	PCR

Annex 2 - Sequences of the MADS-box genes and of the hairpin construct for *FvAGlike*, *FvPIlike-1*, and *FvPIlike-2*. Sequences of three selected genes, *FvPISTILLATA-1* and *-2*, and *FvAGAMOUS*, sequenced in *F.vesca* cv 'Hawaii-4'. The regions not present in the prediction are underlined. Putative exon/exon junctions are in bold (A). Sequences of hpRNA obtained from *F.vesca* cv 'Hawaii-4'. The right and left primers used are underlined (B).

A)

>*FvPISTILLATA*like-1

ATGGGAAGGGGTAAGATTGAGATCAAGAGGATTGAGAACTCAAGCAACAGGCAGGTGACCTATTCTAAGAGAAGG
AATGGGATCATCAAGAAAGCCAAGGAAATCACTGTTCTTTGTGATGCCAAGGTTTCTCTTATTATCATTAATCCT
AGCTCTGGGAAAATGTCTGAGTACTGCAGCGGCTCTCAAGAAACG**TT**GCTGGAAATCTTAAGCAGATACCATTCA
CAAACTGGGTTGAGGTTGTGGGATACCAAGCATGA**GA**ACGTTTCCAATGAATTGGATAGAATCAAGAAGGAAAAT
GACAACATGCAAGTCCATCTCAG**GC**ATCTTAACGGGGAGGACATAACATCCACGAACCACATTGAGCTGGGGGAC
TTGGAGAAATCACTCGAGAACGGCCTTACTGCTGTCAGAGACAAGAA**GA**CAGAGGTCGCCCAGAGGCATAGAGAC
AG**TT**ACAAAGCTGTGGAGGCCGAGCATGATCGCCTCAATTATGAGCT**GC**ACAAACAGACGATAAAAACTGAAGAC
AATAACTTGAGGGACATAGAGTATCAACAGAGGATGTCCCATATGTCATTCTTCAGGCTCCAGCCTATTCAGCCT
AATCTCCACCACGACTGCTAA

>*FvPISTILLATA*like-2

ATGGGGAGGGGTAAGATTGAGATTAAGAGGATTGAAAACTCAAGCAACAGGCAGGTGACCTATTCTAAGAGAAGG
AATGGGATCATCAAGAAGGCTAAGGAAATCACTGTTCTCTGTGATGCTAAGGTTTCTCTTATTATCATTGCTAGC
TCTGGAAAAATGGTTGATTACTGCAGTGGCCCTCCGGAAAC**GC**CGAAGAAAATCTTGGACAAATACCACTCACAG
TCTGGAAAGAGGTTATGGGATGCAAAGCATGAGA**AC**CTCTCCAATGAAGTGGATAGAGTCAAGAAAGACAATGAT
AGCATGCAAATCGAGCTTCGGC**AT**TTGAAAGGGGTAGACATAACATCTTTGAACCATGTAGACCTGATGACCTTA
GAGGAAGCACTTGAAATTGGCCTTGCAAGTATCAGAGACAGAAAGT**CA**AAGTACGTAGAGGCGGTTATAGAAAAT
A**GC**TCTGGAGGAAGAGCGTAAGCGCCTCACATACCAGCTGT**AC**AAAGTGATGAAAATTGAAGAGAATTTGAGGGA
CATGAACTACAACACCACCACCCATGGCCAGCAGATACCTTTTGCCCTCCGTGTCCAGCCTAATCGGCCCAATCT
CCACGACATAATGTAA

>***FvAGAMOUS*like**

ATGGCCTATGAAAACAAACCAAACACTGACCTGGACGCTGATGCCCAAAGAAGATTGGGAAGGGGAAAGATAGAG
ATCAAGCGGATCGAAAACACCACCAATCGCCAAGTTACCTTCTGCAAAAGGCGCAATGGTTTGCTCAAGAAGGCC
TATGAGCTCTCTGTGCTCCGTGATGCTGAGGTTGCTCTCATAGTCTTCTCTAACCGTGGCCGCCTCTATGAGTAT
TCCAACAACA**GT**GTTAGAGAAACGATTGAACGATACAAGAAGGCATGTGCAGATACTTCAACTAATGGATCTGCC
TCAGAAGCTACTGCTCA**GT**ACTGTCAGCAAGAAGCTGCCAAGCTGCGCAACCAGATAAATGCTTTGCAGAACAGT
AACAG**GG**GTTATATGGCTGAGGGTTTAAGCAATATGAATATCAAGGAGCTCAAGGGCATGGAGAGCAAACTTGGG
AAAGCAATTACCAGAATTAGATCCAAGAA**GA**ATGAACTCTTGTTTGCCGAAATTGAGTACATGCAGAAAAGG**GA**A
CTTGACTTGCATAACAATAACCAGCTCCTCCGAGCAAAGGGGCA**GA**TAGCTGAGAATGAGAGGCAACAGCAGAGC
ATAAATGCAATTGCAGGAGGGCATGGAAGCCATGAGATCGTGCAGCCGACTCAGCCCTTTCATGAGGCTCGCAAC
TATTTTCAAGTGAATGCTTTGCAACCCAATATTCATCAGTACTCGCGCCATGACCAAGTTTCCCTTCAGTTAGTG
TAA

B)

>hpFvAGlike

CACCGACTCAGCCCTTTCATGAGGCTCGCAACTATTTTCAAGTGAATGCTTTGCAACCCAATATTCATCAGTACT
CGCGCCATGACCAAGTTTCCCTTCAGTTAGTGTAAGTACAGTAAAAACAGCTATAGCAATTGCCTGTTTGTACTA
TGTGTCACAGTTTAACTGTACTTTTTTTTTCTCTTTACAGTTGATATATACGTATGTTGGCCTGGTTTCT

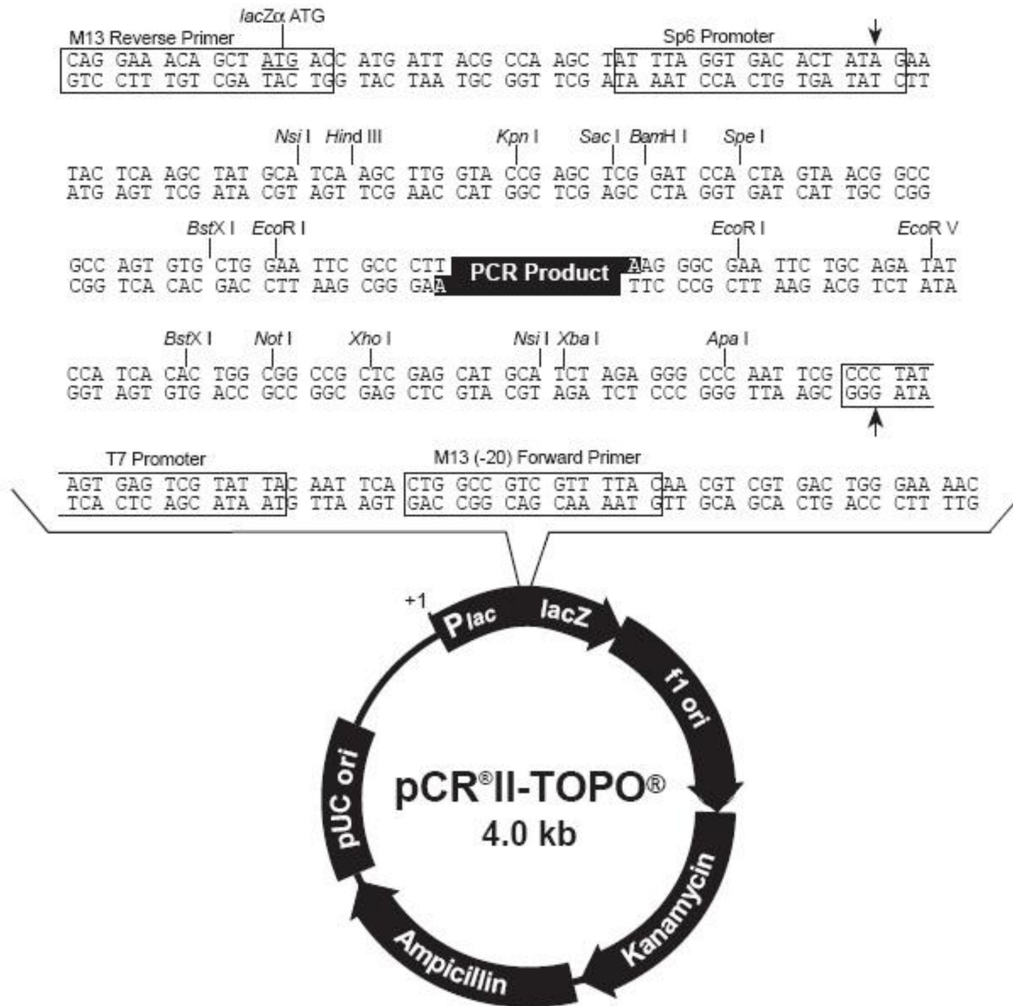
>hpFvPIlike-1

CACCCTTCAGGCTCCAGCCTATTCAGCCTAATCTCCACCACGACTGCTAAAAAGAGTGGAAGTTATTTCGATCAT
CAAGAACTAATATGCTTTATATCGATCAGTTCAGTTAAATTTCATGGTTTGTAACATTATCCAGTGCACTTAGTG
GTTAACTACTTAATCTTCTTAATTATGACTAATTAGTTATTAGTGATGATCGAAGAACTTGTAATAATATTGATC
GAGGTGTGTCTGAACT

>hpFvPIlike-2

CACCCATGGCCAGCAGATACCTTTTGCCCTCCGTGTCCAGCCTAATCAGCCCAATCTCCACGACATAATGTAATA
TTCATGCCGATATACATCCCTGTATATTTGCTATGTTTGAGCTCCCTATCGTCCTTCCCTCCAAGAACTGATTAA
TGCTTTTATGGTTTGTAATATTAAAGCACTTAAGAAGCTAGTCTGCTTATGACTAAGTAATAATGTTTGTGGGAC
TTGT

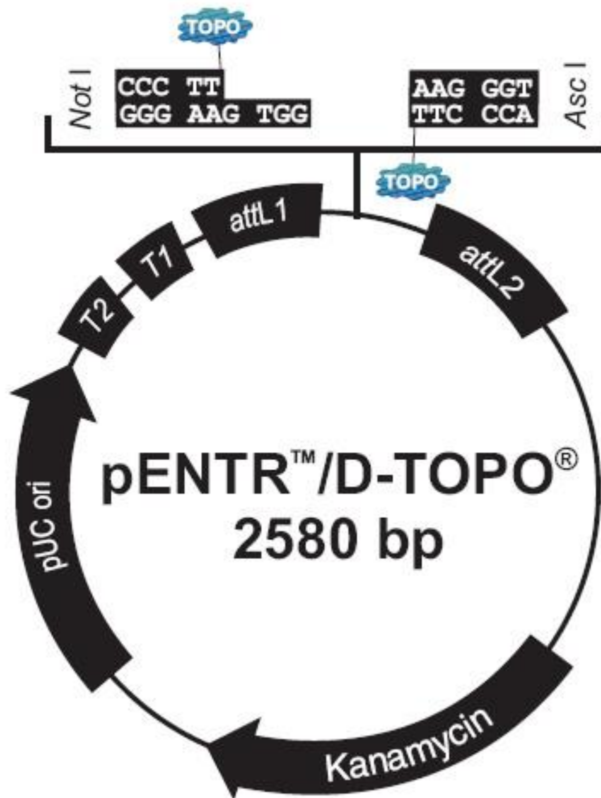
Annex 3 - Map and features of pCR®II-TOPO® vector



Comments for pCR®II-TOPO®: 3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

Annex 4 - Map and features of pENTR™/D-TOPO vector

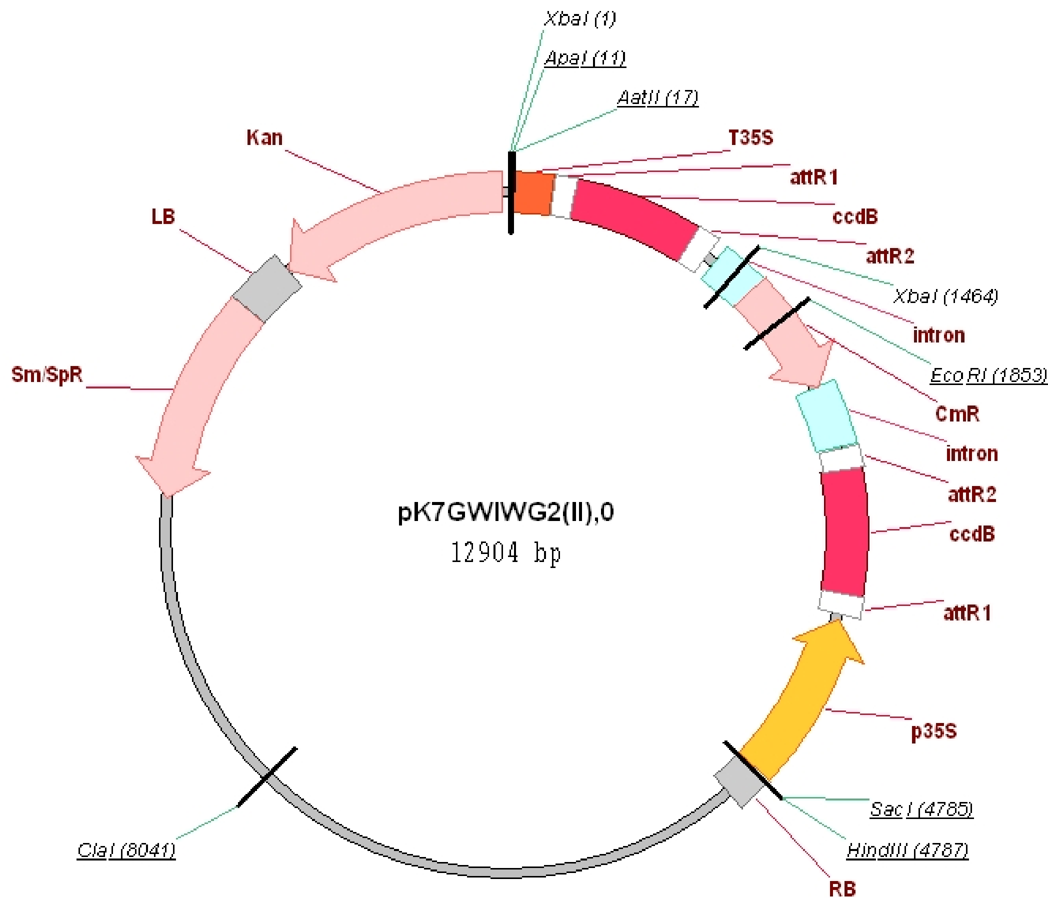


Comments for pENTR™/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
a*tt*L1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang : bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence

Annex 5 - Map and features of pK7GWIWG2(II),0 for RNAi



Comments for pK7GWIWG2(II): 12904 nucleotides

T35S transcription terminatror sequence: bases 28-253
*att*R1: bases 263-387
ccdB gene: bases 388-1140
*att*R2: bases 1140-1264
intron1: bases 1331-1606
Chloramphenicol resistance gene: bases 1607-2309
intron2: bases 2309-1685
*att*R2: bases 2701-2825
ccdB gene: bases 2825-3579
*att*R1: bases 3578-3702
p35S promoter: bases 3730-4765
Right Border: bases 4787-4986
Streptomycin and Spectinomycin resistance gene: bases 9890-11139
Left Border: bases 11145-11477
Kanamycin resistance gene: bases 11481-12877

Acknowledgements

I would like to thank the PhD School of Bioscience and biotechnologies of the University of Padua for accepting me in their PhD Program, in particular my supervisor Prof. Fiorella Lo Schiavo, and Edmund Mach Foundation (FEM) for granting the fellowship and supporting my research.
Thank to my supervisor at FEM, Dr. Azeddine Si Ammour, for his illuminating scientific advices and his help and support throughout these years.
Thank to my supervisors at Julius Kühn-Institute (JKI) for Breeding Research on Fruit Crops, Prof. Dr. Magda-Viola Hanke and Dr. Henryk Flachowsky for the kind welcome in their Institute and the stimulating scientific support.
I also want to thank all the reviewers of my thesis for their corrections and suggestions.
A special thank goes to Elisa Asquini (FEM), Ines Hiller, and Uta Hille (JKI) for the invaluable technical help and support and to the workers who take care of the plants in the greenhouse. Thank also to Mirko Moser for the help with the bioinformatic analyses, Matteo Brilli and Jörg Vogt for the help with phylogeny, and Alexandre Galvao for the phenotypic analysis.
A thank to my present and former colleagues of the Functional Genomic group, especially Marina Cavaiuolo and Jaap Wolters for their friendship “beyond the lab”.
Thank to all my work colleagues at FEM and at JKI, making an international working environment a continue stimulus for confrontation and dialogue.
At last but not at least, a huge thank to my family for their lifelong love and support.