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

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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 agronomically 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 *AGAMOUS* 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 do not show altered flower phenotype, suggesting a different mechanism of flower development in strawberry, probably due to the peculiar flower structure.
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) 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’esposizione 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 PISTILLATA 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- 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--type, the former usually span from four to five exons, while the latter are rather encoded by one exon (Henschel et al.,
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 \(\text{M}_{\alpha}, \text{M}_{\beta}, \text{M}_{y}\) and \(\text{M}_{\delta}\), based on the phylogenetic relationships between MADS-box regions (Parenicova, 2003). The \(\text{M}_{\delta}\) group, can also be referred as the MIKC\(^*\) class.

![Figure 1 - Type I and type II MADS-box proteins](image)

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,
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 paleoAP3/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<sup>a</sup>) and ancestral PLENA/SHATTERPROOF (PLE/SHP<sup>a</sup>) 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).
1.2 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*-type and MIKC*-type on the basis of structural features (Henschel et al., 2002). The MIKC* 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 SEP3 (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). ANRI 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>
<thead>
<tr>
<th>Clade</th>
<th>Gene name</th>
<th>Arabidopsis Gene ID</th>
<th>Functions in Arabidopsis (References)</th>
<th>Functions in other plant species (References)</th>
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<tbody>
<tr>
<td><strong>AGAMOUS (AG)</strong></td>
<td><strong>AGAMOUS (AG)</strong></td>
<td>At4g18960</td>
<td>Homeotic C-class gene, carpel and stamen specification (Yanofsky et al., 1990); Carpel, ovule and fruit development; dehiscence; periodic lateral root formation (Lijegren et al., 2000; Moreno-Risueno et al., 2010).</td>
<td>Subfunctionalization of homeotic C-function; fruit development (Causier et al., 2005; Arold et al., 2010; 2012)</td>
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<td></td>
<td><strong>SHATTERPROOF1,2 (SP1, 2)</strong></td>
<td>At3g58780</td>
<td>Carpel, ovule and fruit development; periodic lateral root formation (Pinyopich et al., et al., 2003; Moreno-Risueno et al., 2010).</td>
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<td><strong>SEEDSTICK (STK)</strong></td>
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<td>At4g09960</td>
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<td><strong>SEPALLATA1-4 (SEP1-4)</strong></td>
<td>At5g15800</td>
<td>Homeotic E-class gene; sepal, petal, stamen and carpel specification (Mandel and Yanofsky, 1998; Pelaz et al., 2000; Ditta, et al., 2004).</td>
<td>Inflorescence meristem identity in Gerbera; tomato fruit ripening (Vrebavalo et al., 2002; Uimari et al., 2004)</td>
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<td>Root development cell cycle regulation; transition to flowering (activator) (Tapia-López et al., 2008).</td>
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<td><strong>AGAMOUS-LIKE 15 (AGL15)</strong></td>
<td>At5g13790</td>
<td>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).</td>
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<td><strong>AGL17</strong></td>
<td><strong>AGAMOUS-LIKE 18 (AGL18)</strong></td>
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<td>Transition to flowering (repressor) with AGL15 (Adamczyk et al., 2007).</td>
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<td><strong>AGAMOUS-LIKE 16 (AGL16)</strong></td>
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<td>Seed pigmentation and endothelium development (Nesi et al., 2002; Kaufmann et al., 2005; de Fotel et al., 2006).</td>
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<td><strong>GORDITA (GOA)</strong></td>
<td>At1g31140</td>
<td>Fruit development (Prasad et al., 2010).</td>
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<td><strong>APETALA 3 (AP3)</strong></td>
<td>At3g54340</td>
<td>Homeotic B-class gene; petal and stamen specification (Jack et al., 1992).</td>
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<td><strong>PISTILLATA (PI)</strong></td>
<td>At5g20240</td>
<td>Homeotic B-class gene; petal and stamen specification (Goto and Meyerowitz, 1994).</td>
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<td><strong>FLOWERING LOCUS C (FLC)</strong></td>
<td>At5g10140</td>
<td>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).</td>
<td>Role in floral bud dormancy; perennial life cycle of A. alpina (Du et al., 2008; Wang et al., 2009b; Zhang et al., 2009)</td>
</tr>
<tr>
<td><strong>MADS AFFECTING FLOWERING 1-4 (MAF1-4)</strong></td>
<td>At1g77080, At5g65050, At5g65060, At5g65070</td>
<td>Transition to flowering (repressors) (Ratcliffe et al., 2001; 2003).</td>
<td></td>
<td></td>
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<tr>
<td><strong>SQUA</strong></td>
<td><strong>MADS AFFECTING FLOWERING 5 (MAFS) APETALA1 (AP1)</strong></td>
<td>At5g65080</td>
<td>Transition to flowering (activator) (Ratcliffe et al., 2003).</td>
<td>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; Elio et al., 2017; Nakano et al., 2012).</td>
</tr>
<tr>
<td></td>
<td><strong>CAULIFLOWER (CAL)</strong></td>
<td>At1g69120</td>
<td>Meristem identity specification; homeotic A-class gene (Mandel et al., 1992; Weigel et al., 1992; Ferrandiz et al., 2000).</td>
<td></td>
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<td></td>
<td><strong>FRUITFULL (FUL)</strong></td>
<td>At1g26310</td>
<td>Meristem identity specification; annual life cycle regulator with SOC1, fruit development; caluine leaf growth (Gu et al., 1998; Ferrandiz et al., 2000; Ferrández et al., 2000; Melzer et al., 2008).</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>At5g60910</td>
<td></td>
<td></td>
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<tr>
<td><strong>STMAD511</strong></td>
<td><strong>AGAMOUS-LIKE 24 (AGL24)</strong></td>
<td>At4g24540</td>
<td>Transition to flowering (activator) (Michaels et al., 2003).</td>
<td>Floral bud dormancy in <em>Prunus</em> and <em>Pyrus</em>; flower abscission in tomato; role in prophyll development in <em>Antirrhinum</em>; inflamed calyx syndrome in <em>Physalis</em> (Mao et al., 2000; Masiero et al., 2004; He and Saedler, 2005; Li et al., 2009; Saito et al., 2013).</td>
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<tr>
<td></td>
<td><strong>SHORT VEGETATIVE PHASE (SVP)</strong></td>
<td>At2g22540</td>
<td>Transition to flowering (repressor) (Hartmann et al., 2000).</td>
<td></td>
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<tr>
<td><strong>TM3/SOC1</strong></td>
<td><strong>AGAMOUS-LIKE 19 (AGL19)</strong></td>
<td>At4g22950</td>
<td>Transition to flowering (activator) (Schonrock et al., 2006).</td>
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<td></td>
<td><strong>AGAMOUS-LIKE 42 (AGL42) (FOREVER YOUNG FLOWER, FYF)</strong></td>
<td>At5g62165</td>
<td>Transition to flowering (activator); flower organ senescence and abscission; root development (Nawy et al., 2005; Chen et al., 2011; Dorca-Fornell et al., 2011).</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>AGAMOUS-LIKE 71, 72 (AGL71, 72)</strong></td>
<td>At5g51870, At5g51860, At2g45660</td>
<td>Transition to flowering (activators) with AGL42 (Dorca-Fornell et al., 2011).</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)</strong></td>
<td>At5g51870, At5g51860, At2g45660</td>
<td>Transition to flowering (activator); periodic lateral root formation (Lee et al., 2000; Moreno-Risueno et al., 2010).</td>
<td></td>
</tr>
</tbody>
</table>
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](image)

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., 2013).
(1997), all the other genes encode MIKCc-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* (*SHP1* 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](image)

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.
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 an 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$_2$N$_2$) 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$_2$N$_2$ 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:

$$GDH(k) = \sum_{i=r}^{k} f_f (T_n(i)) f_o (GDH(i-1)) f_p (i);$$
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:

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

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

<table>
<thead>
<tr>
<th>Chilling requirement</th>
<th>Apple variety</th>
<th>Chilling Hours</th>
</tr>
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<tbody>
<tr>
<td>High and intermediate chilling</td>
<td>'Golden Delicious'</td>
<td>1000</td>
</tr>
<tr>
<td>requirement</td>
<td>'Red Delicious'</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>'Jonagold'</td>
<td>700-800</td>
</tr>
<tr>
<td>Low chilling requirement</td>
<td>'Gala'</td>
<td>&lt; 600</td>
</tr>
<tr>
<td></td>
<td>'Pink Lady'</td>
<td>200-400</td>
</tr>
<tr>
<td></td>
<td>'Fuji'</td>
<td>200-400</td>
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<td></td>
<td>'Ein Shemer'</td>
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<td></td>
<td>'Anna'</td>
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</tr>
<tr>
<td></td>
<td>'Dorsett Golden'</td>
<td>&lt; 100</td>
</tr>
</tbody>
</table>
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,
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.
References Chapter I


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; Mathiasson et al., 2009). Comparative studies of genes involved in dormancy indicated an expansion of the
StMADS11 clade of MIKC-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 x 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 immunoprecipitations were performed according to abcam protocol available at [http://www.abcam.com/ps/pdf/protocols/ChIP_plant_arabidopsis.pdf](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 instructions (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’ - GAGATTCCAGTCATCTGGGTCAA-3’) and RH3 (5’ -TTCTTTTTTCCTCTCCCTCTC-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

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<th>Library name</th>
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</tr>
<tr>
<td>t0_2</td>
<td>6,489,970</td>
</tr>
<tr>
<td>t1_1</td>
<td>12,559,866</td>
</tr>
<tr>
<td>t1_2</td>
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<td>t2_1</td>
<td>13,039,752</td>
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<tr>
<td>t2_2</td>
<td>8,697,373</td>
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<tr>
<td>t3_1</td>
<td>17,302,403</td>
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<tr>
<td>t3_2</td>
<td>8,733,527</td>
</tr>
<tr>
<td>t4_1</td>
<td>14,566,913</td>
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<tr>
<td>t4_2</td>
<td>8,907,308</td>
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<tr>
<td>t5_1</td>
<td>13,441,809</td>
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<td>t5_2</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>137,531,572</strong></td>
</tr>
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</table>

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+ type MADS domain protein family.
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*-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*-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.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Clade</th>
<th>Homology</th>
<th>Name and accession number</th>
<th>Reference</th>
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<tr>
<td>MDP0000133037</td>
<td>DEF/GLO</td>
<td>AtAP3 (60%)</td>
<td>MdTM6 (AB081093.1)</td>
<td>(Kitahara et al., 2004)</td>
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<tr>
<td>MDP0000324166</td>
<td>AG</td>
<td>AtSTK (76%)</td>
<td>MdMADS221 (ADL36745.1)</td>
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<tr>
<td>MDP0000324259</td>
<td>AG</td>
<td>AtSHP1 (68%)</td>
<td>MdMADS14 (ADL36737.1)</td>
<td>-</td>
</tr>
<tr>
<td>MDP0000149676</td>
<td>AGL6</td>
<td>AtAGL6 (65%)</td>
<td>MdMADS11 (CAAO4325.1)</td>
<td>(Yao et al., 1999)</td>
</tr>
<tr>
<td>MDP0000326390</td>
<td>AGL2 (SEP)</td>
<td>AtSEP1 (77%)</td>
<td>MdMADS3 (AAD51422.1)</td>
<td>(Sung et al., 2000)</td>
</tr>
<tr>
<td>MDP0000936232</td>
<td>AGL2 (SEP)</td>
<td>AtSEP3 (76%)</td>
<td>MdMADS18 (ADL36740.1)</td>
<td>-</td>
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<tr>
<td>MDP0000133331</td>
<td>SQUA</td>
<td>AtAPI1 (62%)</td>
<td>MdMADS5 (CAAO4321.1)</td>
<td>(Yao et al., 1999)</td>
</tr>
<tr>
<td>MDP0000218020</td>
<td>SQUA</td>
<td>AtFUL (53%)</td>
<td>MdFUL (ABB22022.1)</td>
<td>(Cevik et al., 2010)</td>
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<tr>
<td>MDP0000278897</td>
<td>SQUA</td>
<td>AtFUL (64%)</td>
<td>MdMADS12 (CAAC86183.1)</td>
<td>(van der Linden et al., 2002)</td>
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<tr>
<td>MDP0000233948</td>
<td>StMADS11</td>
<td>AtSVP (61%)</td>
<td>MdSVP (ABD66219.2)</td>
<td>This work</td>
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<tr>
<td>MDP0000322567</td>
<td>StMADS11</td>
<td>AtAGL24 (43%)</td>
<td>MdDAM1 (NCBIXXXX)</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PpyMADS13.1 (92%)</td>
<td>PpyMADS13.1 (AB504716.1)</td>
<td>(Saito et al., 2013)</td>
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<td>MDP0000952188</td>
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<td>TM3/SOC1</td>
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<td>MdSOC1 (AB120790.1)</td>
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<td>TM3/SOC1</td>
<td>AtAGL42 (40%)</td>
<td>MdMADS21 (ADL36744.1)</td>
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<td>TM3/SOC1</td>
<td>AtSOC1 (66%)</td>
<td>MdSOC1a (AB501124.1)</td>
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</table>

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 analysis 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 MdDAM2 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.
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)

<table>
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<th>Length (bp)</th>
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<tr>
<td>Intron 1</td>
<td>174</td>
<td>1801</td>
<td>1627</td>
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<td>R1 (LTR/Gypsy)</td>
<td>333</td>
<td>489</td>
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<tr>
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<tr>
<td>R2 (NonLTR/RTE)</td>
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<td>684</td>
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<td>R3 (NonLTR/RTE)</td>
<td>3536</td>
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<td>593</td>
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<td>R4 (DNA/Harbinger)</td>
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<td>R9 (DNA/hAT)</td>
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* The repeats were found using CENSOR available at http://www.ebi.ac.uk/Tools/so/censor/
Table 4 - Genomic structure of *MdDAM2* locus (MDP0000952188)

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<tr>
<td>Intron 1</td>
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<td>R2 (DNA/EnSpm)</td>
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<tr>
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<td>R6 (NonLTR/RTE)</td>
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<td>R11 (LTR/Gypsy)</td>
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</table>

* 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


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 with 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
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 pK7GWIG2(II),0 (Annex 5) using the Gateway® LR Clonase II enzyme mix (Invitrogen). The resulting plasmids were called pK7GWIG2(II),0::hpFvPIlike-1, pK7GWIG2(II),0::hpFvPIlike-2, and pK7GWIG2(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 pK7GWIG2(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)::hpFvPllike-1, pK7GWIWII,(0)::hpFvPllike-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$_2$O 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 *XbaI* (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 FvAPETALA1like (FvAP1), FvAPETALA3like (FvAP3), FvPISTILLATAlike-1 (FvPI-1), FvPISTILLATAlike-2 (FvPI-2), and FvAGAMOUSlike (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.

The phylogenetic tree was built using the MIKC-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.

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

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<th>Exon Nr.</th>
<th>Exon size&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>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.](image)

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 pK7GW1WG2(II),0::hpFvPPlike-1, 35 pK7GW1WG2(II),0::hpFvPPlike-2, and 40 pK7GW1WG2(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).

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

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Table 3 - Positive transgenic lines in strawberry

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<td>F131</td>
<td>10</td>
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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.

<table>
<thead>
<tr>
<th>T-Line</th>
<th>Average nr. of anthers</th>
<th>Average nr. of petals</th>
<th>Average nr. of sepals</th>
<th>Std.dev. anthers</th>
<th>Std.dev. petals</th>
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<td>0.41</td>
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<td>0.44</td>
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<td>5.00</td>
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<td>F83</td>
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<td>0.88</td>
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Table 4. Pollen Vitality evaluation

<table>
<thead>
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<th>T-Line</th>
<th>Mean of Vitality %</th>
<th>Std. Dev of Vitality %</th>
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<td>F85</td>
<td>49.24</td>
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<tr>
<td>Rügen</td>
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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


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. alpina*, 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 cofactors. 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 Ify 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 Ify 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 miR172, 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


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


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

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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)

>FvPISTILLATAlike-1
ATGGGAAGGGGTAAAGTTGAGTCAACAGGATTGAGAATCTCAAGCAACAGGCAGGTGACCTATTCTAAGAGAAGG
AATGGGATCATCAAGAAAGCCAAGGAAATACCTGTCTCTTCTGATGACCGAGTTTCTCTTATTATCTTAATCTTCT
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>FvPISTILLATAlike-2
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>FvAGAMOUSlike
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AATCGGGAATCATCACAAGAAGCTAAGGAATAACACGTCTCTCCTGATGACCGAGTTTCTCTTATTATCTTAATCTTCT
AGCTCGGGAAAAATGTTGATGACTGCAGGGCTCTCTCAAAAGAACGTGCTGGGAAAATGTCTGAGTACTGCAGCGGCTC
TCAAGAAACGTTGGAAATCTTAAGCAGATACCATTCAAAACACTGGGTTGAGGTTGTGGGATACCAAGCATGAAC
ACGTTTCCAATGAAGTGGATAGAGTCAAGAAAGACAATGATAGCATGCAAATCGAGCTTCGGCAT
ATTTGCCTGTTTGAAGGGAAGGCATGGAAGCCATGAGATCGTGCAGCGACTCGTGTTTCATGAGGC
TGTTACTTGGACTTTGGCTTTACCAATATTCATCAGTACTCGCGCCATGACCAAGTTTCCCTTCAGTTAGTG

B)

>hpFvAGlike
CACCGAATCCGCTTCCTTCTCTAGGAGCTGCTGACACTATTTTTTCATAGTGAATGCTTTGCTGACAAAATATTTTCTACT
CTGGGGACAAGATTCTCCTTGAGTTTAGAAGTTAGGAAGGTTCTTTTCTTCTCTACTACGTGAACGTTAGGCGCTGTGTTTCT

>hpFvPIlike-1
CACCCCTCTCAGCCTCCAGCTATTCTACGCTTACCTACCTACCCAGACTTCTATAAAAGAGTTGAATGTTTCTTTTTTTTT
CATAGCAGCTCGTCTGCTGTTTCTTCTCTACTACGTGAACGTTAGGCGCTGTGTTTCT

>hpFvPIlike-2
CACCCATGCGCAGCAGATACCTTTTGCCTCCTGGTCGTCAGCCTTAAATCGCAGACTAACAAATTTACTATCTTATTGTTGT
AACGTTAGGCGCTGTGTTTCT

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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

*rmB* T2 transcription termination sequence: bases 268-295
*rmB* T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*attL1*: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*attL2*: 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 pK7GW1WG2(II),0 for RNAi

Comments for pK7GW1WG2(II): 12904 nucleotides

T35S transcription terminator sequence: bases 28-253
attrR1: bases 263-387
ccdB gene: bases 388-1140
attrR2: bases 1140-1264
intron1: bases 1331-1606
Chloramphenicol resistance gene: bases 1607-2309
intron2: bases 2309-1685
attrR2: bases 2701-2825
ccdB gene: bases 2825-3579
attrR1: 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
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