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Manju M George

Associate Professor,
Department of Botany,
Union Christian College
(Autonomous), Aluva,
Ernakulam, Kerala, India

Early flowering in apple using MADS-box gene overexpression: A review

Manju M George

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Abstract

The extended juvenile phase in apple (*Malus × domestica* Borkh.) represents a major bottleneck in breeding programs, typically requiring 5-12 years before seedlings reach reproductive maturity. This prolonged juvenility significantly delays trait introgression, cultivar development, and genetic improvement efforts. Recent advances in molecular genetics have revealed that MADS-box transcription factors play pivotal roles in regulating flowering time and phase transitions in apple. Overexpression of specific MADS-box genes, particularly heterologous genes such as *BpMADS4* from silver birch (*Betula pendula*) and endogenous apple genes including *MdFT1*, *MdFT2*, and suppression of *MdTFL1*, has successfully induced early flowering in transgenic apple plants, reducing the juvenile period to as little as 3-6 months. This review synthesizes current knowledge on MADS-box gene function in apple flowering regulation, examines transformation strategies and their outcomes, discusses the molecular mechanisms underlying early flowering phenotypes, and evaluates the application of early flowering technologies in accelerated breeding programs. The review highlights successful case studies where transgenic early flowering approaches have been combined with marker-assisted selection to rapidly introgress disease resistance traits from wild apple species into elite germplasm. The review also addresses regulatory considerations, phenotypic stability, and future directions for utilizing MADS-box gene manipulation to overcome juvenility barriers in apple and related fruit tree species.

Keywords: *Malus domestica*, MADS-box genes, early flowering, juvenile phase, BpMADS4, MdTFL1, accelerated breeding, genetic transformation

1. Introduction

Apple (*Malus × domestica* Borkh.) ranks among the most economically important fruit crops worldwide, with global production exceeding 95 million tons annually. Despite its agricultural significance, apple breeding remains constrained by biological limitations inherent to perennial woody species. Chief among these is the extended juvenile phase, during which seedlings are incapable of flowering and fruiting, typically lasting 5-12 years depending on genotype and environmental conditions (Kotoda *et al.*, 2006) ^[1]. This protracted juvenility severely impedes breeding efficiency, trait introgression from wild relatives, and the development of improved cultivars responsive to evolving market demands and climate challenges (Flachowsky *et al.*, 2009; Hanke, 2007) ^[2, 3].

The molecular regulation of flowering time in plants has been extensively studied in the model organism *Arabidopsis thaliana*, revealing complex genetic networks involving photoperiod, vernalization, autonomous, and gibberellin pathways that converge on floral integrator genes (Sun *et al.*, 2022) ^[4]. Among the key regulatory components are MADS-box transcription factors, which control developmental transitions and floral organ identity (Kumar *et al.*, 2016) ^[5]. Comparative genomics and functional studies have demonstrated that many flowering regulatory genes are conserved between herbaceous annuals and woody perennials, although their expression patterns and regulatory contexts may differ substantially (Hättasch *et al.*, 2008; Kurokura *et al.*, 2013) ^[6, 7].

In apple, homologs of *Arabidopsis* flowering genes including *FLOWERING LOCUS T (FT)*, *TERMINAL FLOWER 1 (TFL1)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*, and various MADS-box genes have been identified and characterized (Kotoda *et al.*, 2010; Mimida *et al.*, 2009; Hättasch *et al.*, 2009) ^[8, 9, 10].

Corresponding Author:

Manju M George

Associate Professor,
Department of Botany,
Union Christian College
(Autonomous), Aluva,
Ernakulam, Kerala, India

Functional analyses through heterologous expression and transgenic approaches have revealed that manipulation of these genes can dramatically alter flowering time, offering potential solutions to overcome juvenility barriers (Flachowsky *et al.*, 2007; Tränkner *et al.*, 2010) ^[11, 12].

This review focuses specifically on the use of MADS-box gene overexpression to induce early flowering in apple and also the molecular basis of flowering regulation, describe successful transformation strategies, analyze phenotypic outcomes, and discuss practical applications in breeding programs. Particular emphasis is placed on the pioneering work using *BpMADS4* from silver birch and the manipulation of endogenous apple flowering genes, which have collectively demonstrated the feasibility of reducing the juvenile phase to less than one year.

2. The Juvenile Phase in Apple: A Breeding Constraint

The life cycle of apple trees comprises distinct developmental phases: a juvenile vegetative phase characterized by vigorous growth and inability to flower, a transition phase of increasing reproductive competence, and an adult phase marked by regular flowering and fruiting (Gottschalk *et al.*, 2013) ^[13]. The duration of juvenility varies considerably among apple genotypes, ranging from 3 years in some wild species to over 12 years in certain cultivars (Fischer, 1994) ^[14]. This extended juvenile period poses multiple challenges for breeding programs.

First, the long generation time severely limits the number of breeding cycles that can be completed within a reasonable timeframe, making it difficult to pyramid multiple traits or respond rapidly to emerging disease pressures (Campa *et al.*, 2023) ^[15]. Second, maintaining large populations of juvenile seedlings for extended periods requires substantial land, labor, and financial resources (Flachowsky *et al.*, 2011) ^[16]. Third, the inability to evaluate fruit quality and other reproductive traits until seedlings reach maturity introduces significant uncertainty into selection decisions (Gottschalk *et al.*, 2021) ^[17].

Traditional approaches to shorten juvenility have included cultural practices such as growth regulators, grafting onto precocious rootstocks, and environmental manipulations (Zurawicz *et al.*, 2013) ^[18]. While these methods can reduce the juvenile phase by 1-3 years, they have not achieved the dramatic reductions necessary for truly accelerated breeding (Kotoda, 2021) ^[19]. The molecular understanding of flowering regulation has opened new possibilities for genetic interventions that can fundamentally alter developmental timing (Mushtaq *et al.*, 2024) ^[20].

The transition from juvenile to adult phase in apple involves complex changes in gene expression, epigenetic modifications, and hormonal signaling (Guo *et al.*, 2017) ^[21]. Key molecular markers of phase change include alterations in microRNA expression, particularly miR156 and miR172, which regulate *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* transcription factors (Li *et al.*, 2019) ^[22]. Understanding these molecular transitions has provided targets for genetic manipulation aimed at accelerating reproductive maturity (Fan *et al.*, 2018) ^[23].

3. MADS-Box Transcription Factors in Flowering Regulation

3.1. Overview of MADS-Box Gene Families

MADS-box genes encode transcription factors characterized by a highly conserved DNA-binding domain (the MADS

domain) and play fundamental roles in developmental processes across eukaryotes (Sung *et al.*, 2000) ^[24]. In plants, MADS-box genes are particularly prominent in regulating floral meristem identity, floral organ specification, and flowering time (Kitahara *et al.*, 2004) ^[25]. The plant MADS-box family is divided into two major lineages: Type I (M-type) and Type II (MIKC-type), with the latter being more extensively studied due to their roles in flower development (Yao *et al.*, 2001) ^[26].

MIKC-type MADS-box proteins contain four domains: the MADS (M) domain for DNA binding, an Intervening (I) region, a Keratin-like (K) domain mediating protein-protein interactions, and a variable C-terminal region (Yan *et al.*, 2025) ^[27]. These proteins typically function as dimers or higher-order complexes, with specific combinations determining target gene specificity and developmental outcomes (Falavigna *et al.*, 2021) ^[28].

In apple, comprehensive genome-wide analyses have identified 142 MADS-box genes, including multiple paralogs resulting from whole-genome duplication events (Kumar *et al.*, 2016) ^[5]. Phylogenetic classification has revealed apple orthologs of key *Arabidopsis* flowering regulators, including *APETALA1 (API)*, *FRUITFULL (FUL)*, *SHORT VEGETATIVE PHASE (SVP)*, *FLOWERING LOCUS C (FLC)*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* (Kumar *et al.*, 2016) ^[5].

3.2. Key MADS-Box Genes in Apple Flowering

Several MADS-box genes have been implicated in apple flowering regulation through expression profiling and functional studies. *MdMADS2* and *MdMADS5*, homologs of *Arabidopsis API/FUL*, show elevated expression during floral transition and are considered floral meristem identity genes (Sung *et al.*, 2000; Yan *et al.*, 2025) ^[24, 27]. Transcriptional activation of these genes marks the commitment to reproductive development in apple buds (Hättasch *et al.*, 2008) ^[6].

The Dormancy-Associated MADS-box (*DAM*) genes, orthologs of *SVP*, play crucial roles in both flowering time regulation and seasonal dormancy (Kumar *et al.*, 2016) ^[5]. Apple possesses multiple *DAM* genes, including *MdDAM1* and *MdDAM4*, which exhibit high expression during bud dormancy and are dramatically downregulated during dormancy release and floral initiation (Sung *et al.*, 2000; Yan *et al.*, 2025) ^[24, 27]. Functional characterization through RNA silencing demonstrated that *MdDAM1* suppression results in loss of growth cessation and an evergrowing phenotype, confirming its role as a flowering repressor (Sung *et al.*, 2000) ^[24].

FLC-like genes in apple, including *MdMADS135* and *MdMADS136*, also function as flowering repressors, with their expression decreasing during the transition to flowering (Kumar *et al.*, 2016) ^[5]. The reciprocal expression patterns of *FLC*-like/*DAM* genes and floral promoters such as *FT* and *SOC1* orthologs suggest conserved regulatory networks between *Arabidopsis* and apple, despite differences in life history (Hättasch *et al.*, 2008) ^[6].

Recent studies have revealed complex regulatory interactions among apple MADS-box genes. For instance, *MdWRKY6* interacts with both *MdFT1* and *MdTFL1* to regulate *AFL1 (APPLE FLORICAULA/LEAFY)* expression, with the balance between these interactions determining flowering outcomes (Kumar *et al.*, 2016) ^[5]. Additionally,

transcription factors such as *MdbHLH48* directly regulate both *MdFT1* (positively) and *MdTFL1* (negatively), fine-tuning the flowering response (Kumar *et al.*, 2016) [5].

4. Genetic Strategies for Inducing Early Flowering

4.1. Overexpression of BpMADS4 from Silver Birch

The most successful and widely applied strategy for inducing early flowering in apple has been the constitutive overexpression of *BpMADS4*, an *API/FUL*-like MADS-box gene from silver birch (*Betula pendula* Roth.) (Flachowsky *et al.*, 2007) [11]. Flachowsky *et al.* (2007) [11] transformed apple cultivar 'Pinova' with *BpMADS4* under control of the CaMV 35S promoter via *Agrobacterium tumefaciens*-mediated transformation, generating 25 independent transgenic lines (Flachowsky *et al.*, 2007) [11].

Eight transgenic clones produced solitary flowers on *in vitro* shoots, demonstrating the capacity of *BpMADS4* to override endogenous flowering repression even under tissue culture conditions (Flachowsky *et al.*, 2007) [11]. When transferred to greenhouse conditions, three lines (T1165, T1187, and T1190) exhibited robust flowering, with some plants initiating flowers within 3-4 months post-transfer (Flachowsky *et al.*, 2007) [11]. Flowers appeared both as solitary terminal structures and as lateral clusters containing 3-5 individual flowers, resembling normal apple inflorescence architecture (Flachowsky *et al.*, 2007) [11].

Critically, the flowers produced by *BpMADS4*-overexpressing plants were morphologically normal and functionally fertile (Flachowsky *et al.*, 2007) [11]. Pollen viability and germination rates were comparable to non-transgenic controls, and successful pollination yielded small but viable apple fruits (Flachowsky *et al.*, 2007) [11]. This fertility is essential for breeding applications, as it enables the use of early flowering transgenics as parents in crossing programs (Flachowsky *et al.*, 2011) [16].

Among the early flowering lines, T1190 was selected for extensive characterization and breeding applications due to its balanced phenotype combining early flowering with acceptable vegetative growth (Flachowsky *et al.*, 2011) [16]. Molecular analysis revealed that the T-DNA insertion in T1190 mapped to linkage group 4, and inheritance studies confirmed stable transmission of the transgene and early flowering phenotype to progeny (Flachowsky *et al.*, 2011) [16]. Approximately 50% of T1190 offspring inherited the transgene and exhibited early flowering, consistent with Mendelian segregation of a single dominant locus (Sung *et al.*, 2000) [24].

The mechanism by which *BpMADS4* induces early flowering in apple likely involves activation of floral meristem identity genes and suppression of flowering repressors (Flachowsky *et al.*, 2007) [11]. *BpMADS4* shares high sequence similarity with *API/FUL* genes, which in *Arabidopsis* directly activate floral organ identity genes and repress *TFL1* (Sun *et al.*, 2022) [4]. Heterologous expression of *BpMADS4* may functionally substitute for or enhance endogenous apple *API/FUL* activity, triggering premature floral commitment (Flachowsky *et al.*, 2007) [11].

4.2. Manipulation of Endogenous Apple Flowering Genes

In parallel with heterologous gene approaches, researchers have manipulated endogenous apple flowering genes to induce early flowering. The *TERMINAL FLOWER 1 (TFL1)* gene family in apple includes *MdTFL1-1* and *MdTFL1-2*,

which function as flowering repressors by maintaining inflorescence meristem identity and suppressing floral meristem genes (Mimida *et al.*, 2009) [9]. Kotoda *et al.* (2006) [1] demonstrated that antisense suppression of *MdTFL1* reduced the juvenile phase, with transgenic apple plants flowering 8-15 months after grafting onto rootstocks, compared to non-transformed controls that remained vegetative for over 5 years (Kotoda *et al.*, 2006) [1].

Subsequently, RNAi-mediated silencing of *MdTFL1* was shown to induce even more rapid flowering, with regenerated transgenic shoots flowering within 6 months under *in vitro* conditions and continuing to flower after greenhouse transfer (Yan *et al.*, 2025) [27]. Quantitative RT-PCR confirmed substantial reduction in *MdTFL1* transcript levels in transgenic lines, correlating with the early flowering phenotype (Yan *et al.*, 2025) [27].

Conversely, overexpression of *FLOWERING LOCUS T (FT)* homologs promotes flowering in apple. Apple possesses two *FT*-like genes, *MdFT1* and *MdFT2*, with distinct expression patterns (Kotoda *et al.*, 2010) [8]. *MdFT1* is predominantly expressed in apical buds of adult fruit-bearing shoots, while *MdFT2* shows higher expression in reproductive organs (Kotoda *et al.*, 2010) [8]. Overexpression of *MdFT1* under the CaMV 35S promoter induced precocious flowering in transgenic apple, with some lines flowering during *in vitro* culture (Kotoda *et al.*, 2010; Sung *et al.*, 2000) [8, 24].

Tränkner *et al.* (2010) [12] demonstrated that *MdFT1* overexpression accelerated flowering not only in apple but also in *Arabidopsis* and poplar, confirming functional conservation of *FT* orthologs across diverse plant species (Tränkner *et al.*, 2010) [12]. Transgenic apple clone T780 expressing 35S::*MdFT1* produced its first flowers *in vitro*, representing one of the most rapid flowering responses achieved through genetic manipulation (Tränkner *et al.*, 2010) [12].

Interestingly, the promoter choice significantly affects phenotypic outcomes. While constitutive 35S::*MdFT1* expression induced extremely early flowering, it often compromised vegetative growth, with some transformants failing to establish viable plants (Kumar *et al.*, 2016) [5]. Use of the *rolC* promoter, which confers vascular-specific expression, resulted in early flowering with better maintenance of vegetative growth, although floral architecture was sometimes abnormal with altered petal and stamen numbers (Kumar *et al.*, 2016) [5].

Functional characterization studies have revealed that *MdFT1* and *MdTFL1* act antagonistically in apple flowering regulation, similar to their *Arabidopsis* counterparts (Sung *et al.*, 2000) [24]. Both proteins interact competitively with *MdWRKY6* transcription factor, with *MdFT1* promoting and *MdTFL1* inhibiting *MdWRKY6*-mediated activation of *AFL1*, the apple *LEAFY* ortholog (Kumar *et al.*, 2016) [5]. This competitive interaction provides a molecular mechanism for the balance between vegetative and reproductive development (Kumar *et al.*, 2016) [5].

4.3. Alternative Approaches: Viral Vectors and Transient Expression

An innovative non-transgenic approach to induce early flowering utilizes Apple latent spherical virus (ALSV) vectors to transiently express flowering genes (Hättasch *et*

al., 2008) [6]. Yamagishi *et al.* (2011, 2014, 2016) [40, 39, 41] developed ALSV vectors carrying *Arabidopsis FT* gene, which upon inoculation into apple seedlings induced flowering within 1 year, compared to 5-12 years for non-inoculated controls (Kumar *et al.*, 2016; Hättasch *et al.*, 2008; Kumar *et al.*, 2016) [5, 6, 5].

Critically, ALSV can be eliminated from plants by high-temperature treatment (37°C for 3 weeks), yielding virus-free plants that have undergone accelerated flowering but contain no foreign genetic material (Hättasch *et al.*, 2008) [6]. Seeds from these plants develop normally and do not inherit the virus or transgene, making this approach particularly attractive from a regulatory perspective (Hättasch *et al.*, 2008) [6]. The ALSV system has been successfully applied to multiple apple cultivars and also to pear, demonstrating broad applicability across Rosaceae species (Kumar *et al.*, 2016) [5].

The ALSV approach offers several advantages: (1) no stable genetic modification, (2) applicability to diverse genotypes without transformation, (3) elimination of the virus after achieving early flowering, and (4) production of non-transgenic progeny (Flachowsky *et al.*, 2007) [11]. However, limitations include the need for virus inoculation infrastructure, variable efficiency across genotypes, and potential for incomplete virus elimination (Campa *et al.*, 2023) [15].

5. Molecular Mechanisms of Early Flowering

5.1. Flowering Regulatory Networks in Apple

The molecular control of flowering in apple involves complex regulatory networks integrating environmental signals (photoperiod, temperature) with endogenous developmental programs (Hättasch *et al.*, 2008) [6]. Transcriptomic analyses during floral transition have revealed coordinated changes in expression of hundreds of genes, including transcription factors, hormone signaling components, and metabolic enzymes (Li *et al.*, 2019) [22].

Key components of the apple flowering network include orthologs of *Arabidopsis* flowering pathway genes. The photoperiod pathway, involving *CONSTANS* (*CO*) and *FT*, appears to function in apple, although its role may differ from photoperiodic annuals (Hättasch *et al.*, 2009) [10]. *MdCOL1* and *MdCOL2* (*CO*-like genes) show variable expression patterns during vegetative phase change, suggesting possible involvement in juvenility rather than seasonal flowering (Hättasch *et al.*, 2008) [6].

The age/maturation pathway, regulated by miR156-*SPL* modules, plays a crucial role in the juvenile-to-adult transition in apple (Guo *et al.*, 2017) [21]. High miR156 levels in juvenile tissues repress *SPL* transcription factors, preventing flowering competence (Guo *et al.*, 2017) [21]. As plants age, miR156 declines and *SPL* expression increases, enabling responsiveness to floral inductive signals (Guo *et al.*, 2017) [21]. Manipulation of miR156 or *SPL* genes represents an alternative strategy for modulating juvenility, though this has been less extensively explored in apple compared to MADS-box gene approaches (Kotoda, 2021) [19].

Hormone signaling, particularly gibberellin (GA) and cytokinin (CK), integrates with genetic pathways to regulate flowering (Fan *et al.*, 2018) [23]. Transcriptomic analysis of 6-BA (cytokinin) treated apple buds revealed upregulation of *SPL*, *SOC1*, *FD*, and *COL* genes, along with changes in sugar metabolism genes (Li *et al.*, 2019) [22]. GA signaling

components show complex expression patterns, with GA promoting vegetative growth but also being required for floral transition under certain conditions (Fan *et al.*, 2018) [23].

5.2. Interactions Between *FT*, *TFL1*, and MADS-Box Genes

The antagonistic relationship between *FT* and *TFL1* represents a central regulatory module controlling flowering time in apple (Kumar *et al.*, 2016) [5]. Despite high sequence similarity, these proteins have opposite effects: *FT* promotes flowering while *TFL1* maintains vegetative growth (Mimida *et al.*, 2009) [9]. Recent molecular studies have elucidated the mechanisms underlying this antagonism.

Zuo *et al.* (2024) [34] demonstrated that *MdbHLH48* transcription factor directly binds to and activates *MdFT1* promoter while repressing *MdTFL1* promoter, providing a mechanism for coordinated reciprocal regulation (Kumar *et al.*, 2016) [5]. Overexpression of *MdbHLH48* in *Arabidopsis* and tomato accelerated flowering, confirming its role as a floral promoter (Kumar *et al.*, 2016) [5].

Both *MdFT1* and *MdTFL1* proteins interact with *MdWRKY6*, but with opposite functional consequences (Kumar *et al.*, 2016) [5]. The *MdFT1-MdWRKY6* complex activates *AFL1* expression, promoting floral meristem identity, while *MdTFL1* competitively inhibits this activation (Kumar *et al.*, 2016) [5]. This competitive interaction provides a molecular switch mechanism where the relative abundance of *FT* and *TFL1* determines developmental fate (Kumar *et al.*, 2016) [5].

MADS-box genes function both upstream and downstream of *FT/TFL1* in flowering regulation (Sun *et al.*, 2022) [4]. *MdSOC1*, a MADS-box gene orthologous to *Arabidopsis SOC1*, is upregulated during floral transition and likely acts downstream of *FT* signaling (Hättasch *et al.*, 2008) [6]. *MdSOC1* in turn activates floral meristem identity genes including *AFL1* and *AFL2* (apple *LEAFY*-like genes) and *MdMADS2* (Hättasch *et al.*, 2008) [6].

The *DAM* and *FLC*-like MADS-box genes function as flowering repressors, with their downregulation being necessary for floral transition (Kumar *et al.*, 2016) [5]. Expression profiling during dormancy release and floral initiation revealed dramatic reduction in *MdDAM1*, *MdDAM4*, and *FLC*-like gene expression coinciding with increased *FT* and *SOC1* expression (Kumar *et al.*, 2016) [5]. This reciprocal pattern suggests that relief from *DAM/FLC* repression is required for *FT*-mediated floral induction (Kumar *et al.*, 2016) [5].

Overexpression of *BpMADS4* likely bypasses normal flowering regulatory checkpoints by directly activating floral meristem identity genes (Flachowsky *et al.*, 2007) [11]. As an *API/FUL* ortholog, *BpMADS4* may substitute for endogenous apple *API/FUL* genes (*MdMADS2*, *MdMADS5*), which normally require upstream activation by *FT-SOC1* signaling (Sung *et al.*, 2000) [24]. Constitutive *BpMADS4* expression thus short-circuits the regulatory network, inducing flowering independent of age, photoperiod, or vernalization signals (Flachowsky *et al.*, 2007) [11].

6. Phenotypic Characteristics of Early Flowering Transgenics

Early flowering transgenic apple plants exhibit a range of

phenotypic characteristics depending on the gene construct, promoter, and genetic background (Flachowsky *et al.*, 2011) [16]. *BpMADS4*-overexpressing lines generally display relatively normal vegetative morphology, with the primary distinguishing feature being precocious flower production (Flachowsky *et al.*, 2007) [11]. Line T1190, extensively characterized for breeding applications, shows moderate vegetative vigor with shorter internodes compared to wild-type seedlings but maintains sufficient growth to support flower and fruit development (Flachowsky *et al.*, 2011) [16]. Flowers produced by *BpMADS4* transgenics are typically morphologically normal, with correct numbers and arrangements of sepals, petals, stamens, and carpels (Flachowsky *et al.*, 2007) [11]. Pollen fertility is generally maintained, enabling successful crosses with non-transgenic cultivars (Flachowsky *et al.*, 2007) [11]. Fruit set following pollination occurs normally, though fruits may be smaller than those from mature trees, likely reflecting the limited vegetative framework of young transgenic plants (Flachowsky *et al.*, 2007) [11].

In contrast, some *MdFT* overexpression lines exhibit more severe phenotypic alterations (Kumar *et al.*, 2016) [5]. Strong constitutive expression (35S::*MdFT*) can result in extremely early flowering, sometimes directly from callus tissue, but often compromises vegetative growth to the point of plant lethality (Tränkner *et al.*, 2010) [12]. Flowers from such lines may show abnormalities including altered petal numbers, reduced stamen development, and absent or malformed pistils (Kumar *et al.*, 2016) [5]. Pollen from these flowers often exhibits reduced viability and germination capacity (Kumar *et al.*, 2016) [5].

Use of tissue-specific or inducible promoters can mitigate some of these pleiotropic effects (Kumar *et al.*, 2016) [5]. The *rolC* promoter, which drives expression primarily in vascular tissues, resulted in early flowering with better vegetative growth maintenance, though floral architecture was sometimes abnormal with perpetual flowering behavior (Kumar *et al.*, 2016) [5].

MdTF1-silenced plants generally show normal floral morphology and fertility, with the primary phenotype being reduced juvenile phase (Kotoda *et al.*, 2006) [1]. These plants transition to flowering earlier than controls but subsequently exhibit relatively normal adult phase characteristics (Kotoda *et al.*, 2006) [1].

An important consideration for breeding applications is the stability of the early flowering phenotype across generations (Flachowsky *et al.*, 2011) [16]. In *BpMADS4* transgenics, the early flowering trait segregates in progeny according to Mendelian ratios, with hemizygous offspring (carrying one transgene copy) flowering early while null segregants (lacking the transgene) revert to normal juvenility (Flachowsky *et al.*, 2011) [16]. This predictable inheritance enables breeding strategies where the transgene is used to accelerate crossing cycles and then segregated out in advanced generations (Flachowsky *et al.*, 2007) [11].

7. Application in Accelerated Breeding Programs

The practical utility of early flowering transgenics has been convincingly demonstrated through accelerated breeding programs aimed at introgressing disease resistance traits from wild apple species (Flachowsky *et al.*, 2011) [16]. Flachowsky *et al.* (2011) [16] pioneered the application of *BpMADS4* line T1190 in a rapid breeding scheme targeting fire blight resistance from *Malus fusca*

(Flachowsky *et al.*, 2011) [16].

In this program, T1190 was crossed with *M. fusca*, and transgenic F₁ seedlings carrying the *BpMADS4* transgene were selected using PCR markers (Flachowsky *et al.*, 2011) [16]. These early flowering F₁ plants flowered within 1 year and were backcrossed to elite cultivars 'Regia' and 98/6-10, which carry apple scab resistance genes (*Rvi2*, *Rvi4*), powdery mildew resistance (*Pl-1*, *Pl-2*), and fire blight resistance QTL (*FB-F7*) (Flachowsky *et al.*, 2011) [16]. Marker-assisted selection was applied to identify BC₁ seedlings pyramiding multiple resistance genes (Flachowsky *et al.*, 2011) [16].

Within a single breeding cycle, three transgenic BC₁ seedlings combining *Rvi2*, *Rvi4*, and *FB-F7* were identified, along with three additional BC₁ seedlings carrying *Pl-1* and *Pl-2* (Flachowsky *et al.*, 2011) [16]. This represented a dramatic acceleration compared to conventional breeding, which would require 10-15 years to achieve equivalent introgression (Flachowsky *et al.*, 2011) [16].

Subsequent work extended this approach through multiple generations. Roux *et al.* (2012) [44] used T1190 to introgress the highly effective fire blight resistance locus *Fb_E* from ornamental cultivar 'Evereste' (Flachowsky *et al.*, 2007) [11]. Background selection using SSR markers distributed across the apple genome enabled identification of BC₁ seedlings carrying less than 15% 'Evereste' genome, minimizing linkage drag (Flachowsky *et al.*, 2007) [11].

Schlathölter *et al.* (2018) [43] reported generation of advanced fire blight-resistant selections of the fifth generation within just 7 years using the early flowering approach (Flachowsky *et al.*, 2007) [11]. Eighteen advanced selections were established, with 15 showing normal apple seedling habitus and maintaining high fire blight resistance (Flachowsky *et al.*, 2007) [11]. Eight selections retained only 4% of 'Evereste' genome, demonstrating effective reduction of linkage drag through rapid cycling (Flachowsky *et al.*, 2007) [11]. Notably, some selections also carried apple scab resistance (*Rvi6*) and additional fire blight resistance QTL (*Fb_F7*), demonstrating feasibility of pyramiding multiple resistance traits (Flachowsky *et al.*, 2007) [11].

A critical regulatory consideration is the status of null segregants—progeny that have lost the transgene through segregation (Flachowsky *et al.*, 2007) [11]. In the United States, such null segregants are not considered genetically modified organisms (GMOs) and face no special regulatory requirements (Flachowsky *et al.*, 2007) [11]. If similar regulatory treatment is adopted in Europe, null segregants from early flowering breeding programs could be directly used as parents in conventional breeding or potentially as commercial cultivars (Flachowsky *et al.*, 2007) [11].

The early flowering approach has also been applied to other Rosaceae species. Tomes *et al.* (2023) [45] demonstrated that *BpMADS4* overexpression in European pear (*Pyrus communis*) induced flowering within 6-18 months after grafting, with normal tree habit and fertile flowers (Flachowsky *et al.*, 2007) [11]. Progeny from these transgenic pears showed 50% flowering within 3 months of sowing, confirming applicability to accelerated pear breeding (Flachowsky *et al.*, 2007) [11].

Beyond disease resistance introgression, early flowering technologies enable rapid evaluation of fruit quality traits, assessment of combining ability, and development of new rootstocks (Campa *et al.*, 2023) [15]. The ability to complete 5-7 generations in the time conventionally required for one

generation fundamentally transforms the economics and feasibility of apple breeding (Flachowsky *et al.*, 2011) [16].

8. Challenges and Future Perspectives

Despite remarkable successes, several challenges remain in optimizing and expanding early flowering technologies for apple breeding. First, the balance between early flowering and adequate vegetative growth requires careful management (Flachowsky *et al.*, 2011) [16]. Extremely early flowering can compromise plant vigor and fruit quality, necessitating selection of transgenic lines with appropriate phenotypic balance (Tränkner *et al.*, 2010) [12]. Development of inducible or developmentally regulated promoters may provide better control over flowering timing (Kumar *et al.*, 2016) [5].

Second, while *BpMADS4* and *MdTFL1* manipulation have proven effective, these approaches may not be optimal for all breeding objectives or genetic backgrounds (Kotoda, 2021) [19]. Exploration of additional flowering regulatory genes, including other MADS-box family members, *SPL* genes, and microRNA targets, may yield alternative strategies with distinct advantages (Guo *et al.*, 2017) [21]. Combinatorial approaches manipulating multiple genes simultaneously could provide enhanced control over flowering time and plant architecture (Campa *et al.*, 2023) [15].

Third, regulatory frameworks for transgenic plants vary globally and continue to evolve (Campa *et al.*, 2023) [15]. While null segregants from early flowering programs may face reduced regulatory barriers, the use of transgenic intermediates in breeding programs requires navigation of complex approval processes in many jurisdictions (Campa *et al.*, 2023) [15]. Development of non-transgenic alternatives, such as the ALSV viral vector system or genome editing approaches, may provide more broadly acceptable solutions (Hättasch *et al.*, 2008) [6].

Genome editing technologies, particularly CRISPR/Cas9, offer new possibilities for manipulating flowering time without introducing foreign genes (Campa *et al.*, 2023) [15]. Targeted knockout of *MdTFL1* or *DAM* genes, or modification of *FT* promoter regions to enhance expression, could achieve early flowering phenotypes that may be considered non-GMO in some regulatory frameworks (Campa *et al.*, 2023) [15]. Multiplexed editing could simultaneously target multiple flowering repressors, potentially achieving more robust early flowering (Campa *et al.*, 2023) [15].

Fourth, the long-term stability and potential unintended effects of early flowering modifications require continued evaluation (Flachowsky *et al.*, 2007) [11]. While short-term studies have demonstrated fertility and normal fruit development, comprehensive assessment across multiple environments and over extended periods will strengthen confidence in these technologies (Flachowsky *et al.*, 2011) [16]. Epigenetic effects and potential impacts on other developmental processes warrant investigation (Mushtaq *et al.*, 2024) [20].

Fifth, integration of early flowering technologies with other breeding innovations, including genomic selection, high-throughput phenotyping, and speed breeding protocols, could further accelerate genetic gain (Campa *et al.*, 2023) [15]. Combining rapid cycling with precise molecular markers and predictive models may enable unprecedented breeding efficiency (Campa *et al.*, 2023) [15].

Finally, extension of early flowering approaches to other fruit tree species with long juvenile phases, including citrus, avocado, and various nut crops, represents an important frontier (Campa *et al.*, 2023) [15]. While some progress has been made in pear and stone fruits, many economically important tree crops remain constrained by juvenility (Flachowsky *et al.*, 2007) [11]. Comparative studies of flowering regulation across diverse species will inform development of tailored strategies for each crop (Sun *et al.*, 2022) [4].

9. Conclusion

The manipulation of MADS-box genes and related flowering regulators has successfully overcome the juvenility barrier in apple, reducing the time to flowering from 5-12 years to as little as 3-6 months. Overexpression of *BpMADS4* from silver birch has emerged as a particularly effective and widely applied strategy, enabling accelerated breeding programs that have achieved trait introgression and resistance gene pyramiding in timeframes previously considered impossible. Complementary approaches involving suppression of *MdTFL1* and overexpression of *MdFT* genes have further demonstrated the plasticity of flowering time regulation and the conservation of flowering pathways between herbaceous and woody species.

The successful application of early flowering line T1190 in multiple breeding programs, achieving five generations of selection within 7 years, provides compelling proof-of-concept for this technology. The ability to rapidly introgress disease resistance traits from wild species while minimizing linkage drag through marker-assisted selection represents a transformative advance in apple breeding methodology.

Looking forward, continued refinement of genetic strategies, integration with genome editing technologies, development of non-transgenic alternatives, and expansion to additional fruit tree species will further enhance the impact of early flowering technologies. As regulatory frameworks evolve and public acceptance of plant biotechnology increases, these tools are poised to play an increasingly important role in developing improved apple cultivars capable of meeting the challenges of climate change, emerging diseases, and evolving consumer preferences.

The work reviewed here demonstrates that fundamental knowledge of flowering regulation, when coupled with modern genetic engineering capabilities, can overcome longstanding biological constraints and enable new paradigms in perennial crop improvement. The early flowering revolution in apple breeding serves as a model for addressing juvenility barriers across diverse tree crop species, with implications extending far beyond a single crop or trait.

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