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CLONING, EXPRESSION ANALYSIS, AND TRANSFORMATION VECTOR CONSTRUCTION OF DAM HOMOLOGS IN PEACH AND POPLAR

Yuhui Xie
Clemson University, yxie@clemson.edu

CLONING, EXPRESSION ANALYSIS, AND TRANSFORMATION VECTOR CONSTRUCTION OF *DAM* HOMOLOGS IN PEACH AND POPLAR

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Yuhui Xie
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Dr. Douglas G. Bielenberg, Committee Chair
Dr. Haiying Liang
Dr. Hong Luo

ABSTRACT

Genetic fine mapping and sequencing of the *EVG* locus in peach [*Prunus persica* (L.) Batsch] identified six tandem arrayed *Dormancy-Associated MADS-box* (*DAM*) genes as candidates for regulating growth cessation and terminal bud formation in the non-dormant *evergrowing* (*evg*) mutant. Since the mutant is lacking expression of six genes in the mapped locus, further functional analysis is needed to narrow the list of gene candidates for the non-dormant *evg* phenotype. Here I report three sets of experiments designed to functionally test *DAM* genes in peach and their homologs in a model tree, hybrid poplar.

First I constructed overexpression and knockdown vectors for the reverse genetic experiments needed to assign *DAM* gene function in peach via *Agrobacterium*-mediated transformation of plants. Peach *DAM* full-length open reading frame (ORF) cDNAs were cloned for construction of overexpression vectors. The 3′ UTR sequences of *DAM* genes, which are gene-specific, were cloned and used for the construction of hairpin-forming inverted repeat cassettes which are known to reduce endogenous gene expression via RNA interference (RNAi).

Second, I report results from experiments where I have constructed overexpression and knockdown vectors for putative *DAM* homologs from a hybrid poplar clone (*Populus tremula×alba*, INRA 717-1B4). The six peach *DAM*s are members of the *SVP/StMADS11* clade of type-II MADS-box genes and the sequenced

P. trichocarpa genome contains eight genes in this clade. However, only six *DAM* homologs were isolated from the INRA 717-1B4 clone. Full-length ORF cDNAs of these six hybrid poplar homologs of the peach *DAM* genes were cloned. Overexpression and knockdown vectors were constructed using full-length ORF cDNAs and 3′ UTR sequences, respectively, as described above for peach. These vectors were used for *Agrobacterium*-mediated transformation of the hybrid poplar clone INRA 717-1B4. Putative transgenic plants were obtained for three of the six poplar *DAM* homologs: *PtMADS7*, *PtMADS26* and *PtMADS48*.

Finally, I measured gene expression of the six *PtMADS* genes over a six month period from summer to winter solstice in order to find correspondences between the expression patterns of these genes in peach and hybrid poplar. The expression pattern was notably different from that observed in the peach *DAM*s. This suggests the function of *PtMADS* in poplar may be different from the *DAM*s in peach. Additionally, the expression patterns of all of the six *PtMADS*s genes were very similar, which opens the possibility that these highly similar genes may be functionally redundant.

This work is the first to specifically monitor hybrid poplar *DAM* homolog gene expression through a seasonal transition from active growth through growth cessation, bud set, and endodormancy entrance. The seasonal behavior of the hybrid poplar genes differs dramatically from that of the peach *DAM*s. The role of these genes in hybrid poplar is possibly divergent from that of peach. The function of the

hybrid poplar *DAM* homologs is currently not known; the overexpression and gene silencing vectors construction in this work is a necessary first step to understand the function of the *DAM* homologs in hybrid poplar.

DEDICATION

I dedicate this dissertation to my grandmother, parents, and wife, Ting Huang.

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CHAPTER ONE

LITERATURE REVIEW

As sessile organisms, plants have evolved elaborate mechanisms for surviving unfavorable growing conditions experienced in nature (Horvath et al., 2003). Dormancy, one of these mechanisms, assures a seasonally synchronized growth in trees, shrubs and perennial herbaceous species (Rohde et al., 2000). Physiological events that take place during dormancy have been extensively studied in agricultural and horticultural species because the dormancy has important implications for crop bud set and storage longevity. Disparate crops studied have included potato (*Solanum tuberosum* L.) tubers (Suttle, 2000) and the vegetative buds of perennial woody plants such as apple (*Pyrus malus* L.) and poplar (*Populus* sp.) (Crabbe and Barnola 1996; Petel and Gendraud 1996; Howe et al., 1998; Schrader et al., 2004; Ingvarsson et al., 2006; Bohlenius et al., 2006; Hoenicka et al., 2008; Ruonala et al., 2008; Mohamed et al., 2010; Resman et al., 2010; Rinne et al., 2011).

Dormancy duration is important to the health and productivity of forestry species. For example, Western Balsam Poplar (*P. trichocarpa*), a woody perennial, sets vegetative buds in the fall which acclimate and develop endodormancy, a mechanism enabling them to resist freezing and dehydration stress in the winter. The growing season, the time between spring bud break and fall bud set, largely determines tree productivity and wood quality. An adequate duration of bud dormancy, determined by the date of bud set and an appropriate bud flushing date, is of economic

significance (Rohde et al., 2007).

Definition and Classification of Dormancy

Despite much research on dormancy, a consistent definition of this phenomenon is still not agreed upon. The classic definition from Lang (1987) refers to dormancy as the "absence of visible growth in any plant structure containing a meristem". Focusing on the molecular components involved in dormancy at the cellular level, Rohde and Bhalerao (2007) referred to dormancy as "the inability to initiate growth from meristems (and other organs and cells with the capacity to resume growth) under favorable conditions".

Lang (1987) distinguished three types of dormancy: (i) paradormancy, in which growth suspension in a dormant structure is caused by another organ within the plant but outside the dormant tissue; (ii) ecodormancy, the inhibition of growth by temporary unfavorable environmental conditions, with growth being resumed when conditions become favorable; and (iii) endodormancy, in which growth suspension is caused by endogenous factors within the dormant tissue, no growth being possible even under environmentally favorable conditions. Although Lang's classification has become widely used because of its pragmatic nature, it underestimates the overlap between the different types of dormancy (Junttila, 1988). Apical and axillary buds differ at entry into endodormancy in autumn: the apical bud changes from an ecodormant to an endodormant state, whereas the axillary buds change from a paradormant to an endodormant state (Rohde et al., 2000).

Paradormancy, also referred to as apical dominance or correlative inhibition, is imposed by factors produced in the growing meristems and young expanding leaves (Horvath, 1999). These actively growing apical buds prevent the growth of axillary buds below the apical meristem, and the subtending leaves of the axillary buds have an effect on the growth of their axillary buds as well (Cline, 1991; Zieslin and Halevy, 1976). This process allows the plant to devote resources to reproduction and to control plant architecture, maximizing light harvesting while allowing for regeneration should the individual shoots become damaged (Horvath et al., 2003). Many studies have suggested basipetally transported auxin as the primary signal regulating paradormancy (Horvath et al., 2003). However, grafting studies have indicated signals other than auxin produced in the roots and stem have a profound effect on shoot outgrowth (Cline, 1999; Beveridge et al., 2000). In addition, light has been found to play a role in the regulation of growth in these buds as well (Zieslin and Halevy, 1976; Horvath, 1999). A compound produced by photosynthesizing leaves has been shown to play a significant role in inhibition of adventitious bud growth in leafy spurge (Horvath, 1998).

Ecodormancy is imposed by external environmental factors such as cold or drought stress, both of which induce critical signals that prevent bud growth. These extended periods of environmentally unfavorable growing conditions generally are required to signal the breaking of endodormancy, while at the same time imposing ecodormancy (Horvath et al., 2003). Environmental factors, including day length,

temperature, water, and nutrient availability, play major roles in the control of ecodormancy in apical buds (Nooden and Weber, 1978). The plant hormone abscisic acid (ABA) induced during both cold and drought stresses have long been recognized as a key signal of this type of dormancy (Gilmour et al., 1991).

Endodormancy is a non-growing (resting, quiescent or inactive) phase caused by conditions or factors within a plant itself. When environmental conditions fluctuate between permissive and inhibitory to growth during seasonal transitions, endodormancy is the result of physiological changes internal to the bud that prevent untimely growth (Horvath et al., 2003). This mechanism is important for protecting vegetative buds by ensuring that meristems will not resume growth until the return of stable permissive conditions.

Regulation of Endodormancy

In perennial plants, endodormancy development can be divided into four main steps: growth cessation, establishment of a quiescent state with an endogenous requirement for an environmental trigger for release, maintenance of quiescence and the resumption on growth (Rohde and Bhalerao, 2007). The first step in establishing endodormancy, growth cessation, is provoked by environmental cues, such as photoperiod, cold or drought (Rohde and Bhalerao, 2007). For more than 70 years, photoperiod has been known to govern growth cessation of many trees in temperate climates (Sylven, 1940; Nitsch, 1957). Leaves perceive the shortening of the day length, sending a signal to the apex to cease active growth (Hemberg, 1949; Wareing,

1956). However, some species, such as *Malus* and *Pyrus*, do not respond to short days but instead require low temperature (Heide et al., 2005) while some species such as *Populus* respond to both short days and low temperature (Karen et al., 2010).

Several factors have been identified as mediators of short-day signals for growth cessation. Phytochrome, *FLOWERING LOCUS T* (*FT*) and *CONSTANS* (*CO*) are important components of the signal transduction chain acting downstream of the critical day length (Rohde and Bhalerao, 2007). In *Populus*, phytochrome genes have been found to play a role in the regulation of short-day-induced bud set and growth cessation (Howe et al., 1998; Frewen et al., 2000; Ingvarsson et al., 2006). For example, transgenic *Populus* trees overexpressing the oat *PHYTOCHROME A* (*PHYA*) gene fail to initiate growth cessation and bud set as a response to the short-day signal (Olsen et al., 1997). *FT* and *CO* have also been identified as mediators of short-day-induced dormancy in poplar (Bohlenius et al., 2006). Research has shown that when *FT1* and *CO* homologues of poplar (*Populus trichocarpa*) were overexpressed in transgenic hybrid aspen (*Populus tremula*×*tremuloides*), growth did not cease upon exposure to short days (Bohlenius et al., 2006). On the other hand, the down-regulation of *FT1* by interference RNA (RNAi) led to growth cessation and bud set independent of day length, suggesting that *FT1* transcription is an important regulator of short-day-induced growth cessation and bud set (Bohlenius et al., 2006). In addition, a *PHYA* overexpressing poplar continued to grow under short days while the expression levels of *CO* and *FT* did not decrease, indicating that phytochrome

regulation of short-day-induced endodormancy, at least in part, is mediated through the regulation of *CO* and *FT* in this tree (Bohlenius et al., 2006).

Endodormancy establishment renders the meristem insensitive to growth-promoting signals. Once growth has ended and buds formed, the dormant state becomes progressively established, resulting in the inability of the meristem cells to respond to growth-promoting environmental signals (Rohde and Bhalerao, 2007). In poplar, a RNA-binding protein FCA may work with abscisic acid (ABA) to establish endodormancy (Rohde and Bhalerao, 2007). ABA may be involved in dormancy establishment because it peaks in poplar apical buds after growth cessation but before bud set (Rohde et al., 2002). The induction of *FCA-like* genes during apical dormancy is particularly significant in the context of the recent finding that FCA can bind ABA (Razem et al., 2006). Hybrid poplar (*Populus tremula×alba*) overexpressing *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) do not form buds and yet enter endodormancy (Rohde et al., 2002). A role for ethylene in endodormancy establishment has been indicated by the experiment that birch (*Betula pendula*) with a dominant-negative version of *ETHYLENE TRIPLE RESPONSE 1* (*ETR1*) does not form endodormancy (Ruonala et al., 2006).

Once endodormancy is established, it is maintained by mechanisms not yet fully understood. *FLOWERING LOCUS C* (*FLC*) may be involved in dormancy maintenance. In *Arabidopsis*, *FLC* is mitotically stable repressed after prolonged exposure to low temperatures (Sung and Amasino, 2005). This epigenetic repression

of *FLC* involves a series of histone modifications that implicate the gene products of *VERNALIZATION1*, *VERNALIZATION2*, *VERNALIZATION INSENSITIVE3*, and *LIKE HETEROCHROMATIN PROTEIN 1* (Bastow et al., 2004; Sung and Amasino, 2005; Mylne et al., 2006; Sung et al., 2006). Release from endodormancy requires exposure to chilling temperatures, which restores the ability to grow but does not promote growth (Rohde and Bhalerao, 2007). However, less is known about genes involved in the perennial endodormancy release. *FLC-like* genes have been shown to be differentially regulated before the completion of the chilling requirement in the vegetative buds of poplar (Chen and Coleman, 2006). Even vernalization is thought to occur effectively only in actively dividing cells but endodormancy is released by exposure to low temperatures after termination of cell division, the similarities between these two phenomena may provide a clue to illuminate the mechanism of perennial endodormancy release (Wellensiek, 1962).

Dormancy-Associated MADS-box (*DAM*) Genes

The MADS-box gene family encodes transcription factors which play fundamental roles in developmental control and signal transduction in eukaryotes (Leseberg et al., 2006). The MADS-box gene family is especially important in plant development (Becker and Theissen, 2003). Most MADS-box genes belong to the type II MADS-box category. In most plants, the MADS-box protein is composed of a MADS-box domain (M), a intervening (I) domain, a keratin (K) domain and a C-terminal (C) domain. Among these domains, the M is the signature domain that is

conserved even across the kingdoms (Leseberg et al., 2006). M domains are DNA-binding domains which involved in protein-protein interactions, a common trait of transcription factors (Riechmann et al., 1996). The K domain, similar to the coiled-coil domain of keratin, is the major region involved in protein-protein interactions (Ma et al., 1991; Pnueli et al., 1991; Fischer et al., 1995; Davies et al., 1996). The I domain that is located between the MADS-box and the K domain may affect the specificity of a DNA-binding dimer formation (Riechmann et al., 1996). The function of C domains is diverse, with some MADS-box genes having a C domain capable of activating transcription in yeast cells (Kramer et al., 1998). For more than 20 years, considerable research has been focused on the function of MADS-box genes. In addition to their role in floral organ development (Leseberg et al., 2006), MADS-box genes have also been found in roots (Zhang and Forde, 1998), in leaves (Samach et al., 2000), and in physiological processes such as vernalization and flowering time control (Ratcliffe et al., 2001, 2003; Hepworth et al., 2002).

The *evergrowing* (*evg*) peach [*Prunus persica* (L.) Batsch] mutant, a dormancy impaired genotype identified in Mexico (Rodriguez et al., 1994; Werner and Okie, 1998), fails to respond to winter dormancy cues, exhibiting persistent shoot growth, no bud formation and a lack of leaf abscission when experiencing short days and low temperatures in the fall (Rodriguez et al., 1994). Researchers found that the *evg* lacks a gene cluster of six MIKC-type MADS-box genes (Bielenberg et al., 2008; Li et al., 2009). These results suggest that these genes may play a critical role in the

dormancy process. Forward genetics methods have been conducted to locate these six MADS-box genes on *evg* locus. After a series of genetics and physical mapping studies, a bacterial artificial chromosome (BAC) contig was found to contain the EAT/MCAC marker (Wang et al., 2002a), which is located at a distance of 1 cM from the *EVG* gene (Wang et al., 2002b). Subsequently, two simple sequence repeat (SSR) markers were identified from this BAC that hybridized with the EAT/MCAC STS fragment. One of these SSR markers flanks the *evg* trait at a distance of 2.0 cM and the other marker co-segregates with this trait (Bielenberg et al., 2008). Bielenberg et al. (2008) then physically mapped, sequenced, and annotated 132-kb base pairs of genomic DNA encompassing the region controlling the *evg*. A 41,746-bp deletion in this region of the mutant genome results in the loss of all or part of four of the six MADS-box genes and the elimination of expression of all six genes (Bielenberg et al., 2008). The results from his study suggest that these deleted genes, which have been named *Dormancy-Associated MADS-box* (*DAM*) genes, are candidates for the regulation of growth cessation and terminal bud formation in peach in response to dormancy-inducing conditions (Bielenberg et al., 2008).

Further functional research has been conducted to determine the relationship between *DAM* genes and dormancy. Quantitative gene expression studies conducted by Li et al. (2009) revealed that three of the six *DAM* genes, *PpDAM1*, *PpDAM2* and *PpDAM4*, are the ones most likely responsible for the continuous growth phenotype of the *evg* mutant based on the coincidence of gene expression timing and cessation of

terminal growth in field condition. Suppression subtractive hybridization (SSH) PCR detected the differential expression of *PpDAM1* and *PpDAM6* in wild-type and *evg*; their expression was up-regulated after the change in photoperiod from long day to short day, and continually increasing during bud development under short-day condition (Li et al., 2009).

These six *DAM* genes belong to the general clade of MIKC-type MADS-box genes represented by the *StMADS11*/*AGL24*/*AtSVP* group of MADS-box transcription factors (Jimenez et al., 2009). Genes in this group, which are primarily expressed in vegetative tissues such as roots, stems, leaves and meristem, regulate vegetative growth or the transition from vegetative to reproductive meristems (Alvarez-Buylla et al., 2000; Garcia-Maroto et al., 2003). In *Arabidopsis*, the *AGL22/SVP* gene and its close homolog *AGL24* play an important role in flowering as flowering repressor and promoter (Hartmann et al., 2000; Yu et al., 2004). In rice, *OsMADS22*, *OsMADS47* and *OsMADS55* act as negative regulators of brassinosteroid responses and modulators of meristem identity (Duan et al., 2006; Lee et al., 2008). In tomato, in addition to the flowering time regulation, the *JOINTLESS* is involved in leaf and abscission zone development (Mao et al., 2000; Szymkowiak and Irish, 2006).

In perennial species, *SVP/StMADS11* genes are expressed in most vegetative tissues and often in bud tissues. *StMADS11* and *StMADS16* are preferentially expressed in vegetative tissues of potato (Carmona et al., 1998) while *IbMADS3* and *IbMADS4* genes of sweet potato are preferentially expressed in root tissue (Kim et al.,

2002). *IbMADS4* was demonstrated to increase leaf and axillary shoot number when ectopically overexpressed in chrysanthemum (Aswath et al., 2004). Additionally, when putative orthologues of *AtAGL24*/*AtSVP* genes *PkMADS* and *EgSVP* were ectopically overexpressed in *Paulownia kawakamii* and *Eucalyptus grandis*, precocious growth of lateral meristems was observed (Prakash and Kumar, 2002; Brill and Watson, 2004). Furthermore, an *SVP-like* MADS-box factor exhibited endodormancy-associated expression in the lateral buds of Japanese apricot (*Prunus mume*) (Yamane et al., 2008), while two putative *SVP-like* genes were down-regulated during dormancy release in *Rubus idaeus* L. buds (Mazzitelli et al., 2007). The review of the research on this group of genes suggests that the MADS-box genes in *StMADS11*/*AGL24*/*AtSVP* group, including *DAM* genes, seem to play an important role in the regulation of growth cessation and terminal bud formation during endodormancy.

Populus as a Model System for Trees

Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) are model plants for dicots and monocots, respectively, since both have the traits of small genomic DNA, sequenced genomic DNA and rapid generation time (Jansson et al., 2007). However, as annual herbaceous plant models, they are not good models for woody plants because they do not have such appropriate traits as wood development, seasonal growth and dormancy.

The genus *Populus*, which includes poplars, cottonwoods and aspens, has

many advantages as a model system for the molecular biology of forest trees, including a small genome, the completion of a draft sequence, facile transgenesis, and many of genomic and molecular biology tools and resources (Bradshaw et al., 2000). Table 1.1 below shows the contrasts of poplar as a model system from peach and *Arabidopsis*.

Table 1.1. Contrasts between *Arabidopsis*, poplar and peach.

	Arabidopsis (A. thaliana)	Poplar *(P. trichocarpa)*	Peach (*P. persica*)
Genome size	157 Mbp	550 Mbp	220 Mbp
Genome sequence	Available since 2000	Available since 2006	Available since 2010
Growth cycle	Annual	Perennial	Perennial
Time to get transgenic plant	2-3 months	6-12 months	none

The haploid genome size of *Populus* is only 550 Mbp, similar to rice, only four times larger than *Arabidopsis* and 40 times smaller than pine. In 2006, the draft genomic sequence of *P. trichocarpa* (Nisqually-1 genotype) was completed (Tuskan et al., 2006). This information will facilitate studies on the comparative biology of *Populus* and other species such as *Arabidopsis* and peach (Jansson et al., 2007). A good example is the *CO/FT* module. In *Arabidopsis*, this module regulates the photoperiod-dependent induction of flowering (Koornneef et al., 1998) while in *Populus*, however, it not only regulates flowering time but also controls bud set under dormancy conditions (Bohlenius et al., 2006). In addition, based on genome sequence information, bioinformatics can be a good tool for analyzing the evolution of genes.

Phylogenetic analyses show *Arabidopsis*, poplar and peach MIKC^{C} type MADS-box genes have different evolution processes, perhaps leading to a functional diversification in these lineages even though these MADS-box genes belong to the same group (Jimenez et al., 2009).

Another advantage of hybrid poplar is its relative ease of genetic transformation, which is important for the detection and characterization of gene function. Certain, but not all, *Populus* genotypes can be subject to *Agrobacterium*-mediated transformation to overexpress or down-regulate target genes (Busov et al., 2005). In general, *Populus* is easier to transform and regenerate *in vitro* than most other trees (Meilan and Ma, 2006). Reliable transformation systems have been developed for both pure species and hybrids in *Populus* (Ma et al., 2004; Meilan and Ma, 2006).

In addition to genome DNA sequence information, several other genomic and molecular biology tools and resources facilitating the study of tree biology are available for this genus. These include expressed sequence tags (ESTs), DNA microarrays and proteomics. Since 1998, numerous groups have contributed to EST sequencing efforts, using multiple species or hybrids, organs, tissues, and treatments, with 600,928 *Populus* sequences in the National Center for Biotechnology Information (NCBI) EST database as of 2011. Functional genomics tools such as DNA microarrays and proteomic data have been developed for *Populus* (Jansson et al., 2007).

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CHAPTER TWO

RNAi VECTOR CONSTRUCTION OF *DAM* GENES IN PEACH

Introduction

The *evergrowing* (*evg*) peach (*Prunus persica* L.) is a mutant that continues to grow and does not form terminal buds in response to dormancy-inducing conditions. These characteristics of the *evg* peach make it a useful tool for gaining a full understanding of the bud dormancy processes. Genetic and physical mapping have revealed that the wild-type *EVG* locus contains six MADS-box genes (Wang et al., 2002; Bielenberg et al., 2004). A 41,746-bp deletion in this region of the mutant genome results in the loss of all or part of four of these six MADS-box genes and elimination of expression of all six of them (Bielenberg et al., 2008). These six MADS-box genes, which belong to *SVP/StMADS11* clade of $MIKC^C$ MADS-boxes, were named as *Dormancy-Associated MADS-box* (*DAM*) genes (Bielenberg et al., 2008). These *DAM* genes are candidates for the cause of the *evg* phenotype.

To determine the function of these specific genes, overexpression and the silencing of *DAM* genes in wild-type peach are necessary (Figure 2.1). This chapter discusses the isolation of *DAM* genes from peach and the construction of the overexpression and the RNAi vectors which can be used for transient or heterologous transformation.

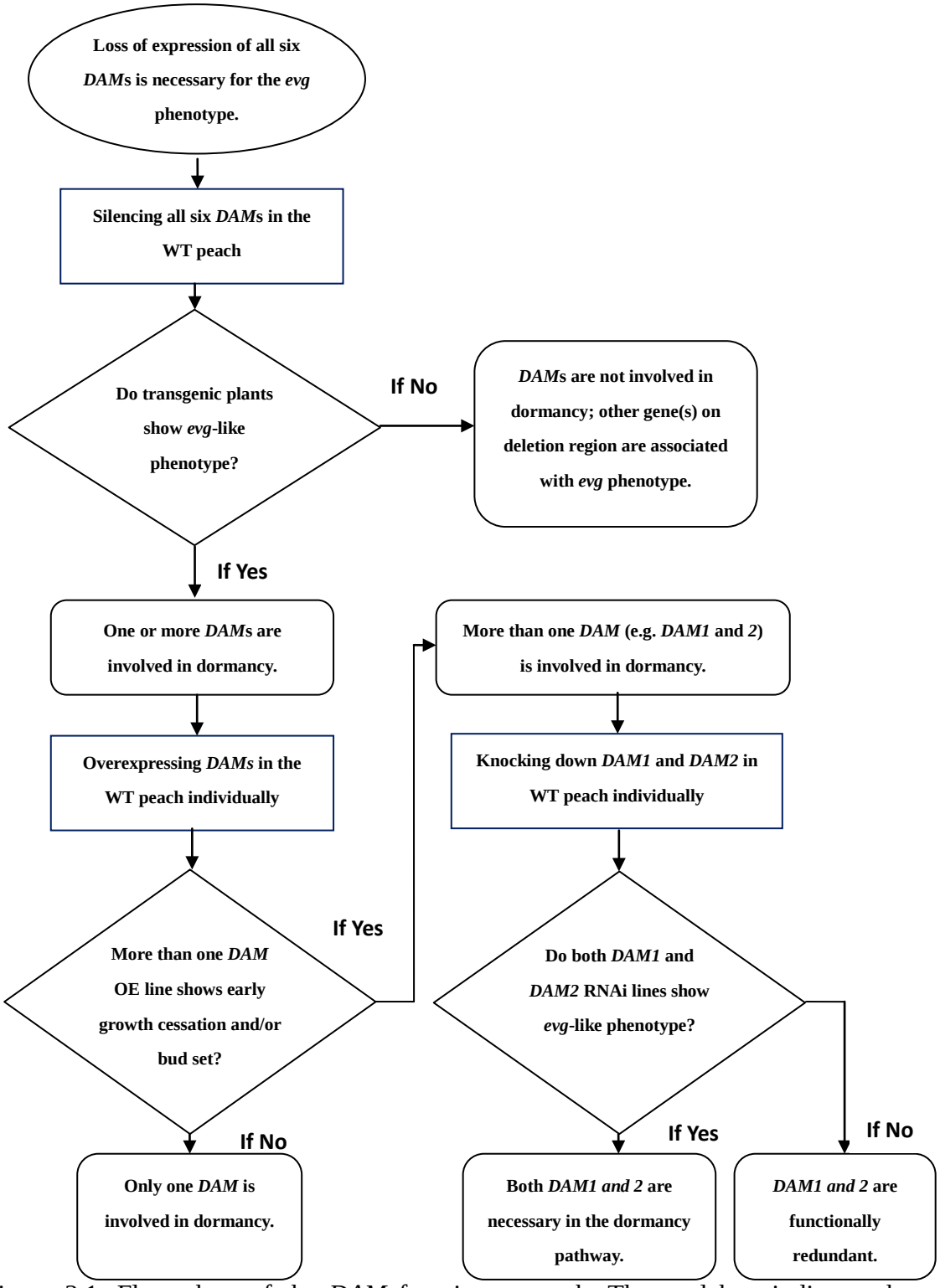

Figure 2.1. Flow chart of the *DAM* function research. The oval box indicates the initial hypothesis of the experiment; the rectangle boxes indicate experimental processes; the diamond boxes indicate the possible experiments results and rounded rectangle boxes indicate conclusions. *DAM1* and *DAM2* were used as examples to represent any two of the six *DAM*s.

At the start of this work, it was assumed that a viable peach transformation method would shortly be available. To date, this method has not been proven successful because of the difficulty in regenerating transformed plantlets (Padilla et al., 2006). Transformation events in peach have been reported using particle bombardment or *Agrobacterium*-mediated transformation of immature embryos (Scorza et al., 1990; Ye et al., 1994). However, the regeneration of plants from transgenic tissues remains difficult and the recovery of non-chimeric plants has not been reported to date (Padilla et al., 2006). The vectors designed in this work will, therefore, not be able to be directly used in peach transformation experiments to test *DAM* function. However, these constructs do still have utility for use in experiments with heterologous transformation of another *Prunus* species with a viable transformation system. Additionally, transient knockdown of endogenous peach genes has been successfully reported in peach and this method could also have potential for testing the loss of expression of the *DAM* genes (Testone et al., 2008).

Among *Prunus* species, regeneration and transformation protocols have been most successfully developed for plum (*Prunus domestica* L) (Bassi and Cossio, 1991; Escalettes and Dosba, 1993; Csanyi et al., 1999; Mikhailov and Dolgov, 2007; Nowak et al., 2004). Recently, an improved *Agrobacterium*-mediated transformation protocol for plum hypocotyls slices increased transformation efficiency up to 42% (Petri et al., 2008). As a demonstration of the use of plum for functional analysis of genes from other species, a *PHYTOENE DESATURASE* (*PDS*) gene from peach was

used to successfully silence the endogenous *PDS* gene from plum (Petri et al., 2008). This suggests that this system is amenable for the study of gene function in *Prunus* since a high level of similarity exists between members of the genus (Arus et al., 2006; Jung et al., 2006). In summary, the plum system could provide a rapid, high throughput system for analyzing the function of peach genes. Until there is a breakthrough in peach transformation technology, the plum system is a potential tool to illuminate peach *DAM* gene function.

Transient expression of transgene constructs can be triggered by infiltration of *Agrobacterium* into the leaf air spaces resulting in transient expression of the introduced constructs. This method is commonly used in model systems as a functionally assaying to test candidate genes of interest (Dinesh-Kumar et al., 2003; Lu et al., 2003). Unlike the stable transformation which integrates the T-DNA containing a transgene construct into host chromosomes, transient expression assays do not integrate into the chromosomes. Instead, *Agrobacterium* with a T-DNA vector is infiltrated into the leaf mesophyll air spaces by pressure. Although the exact mechanism is not understood, the T-DNA segments carried by the infiltrated *Agrobacterium* are transcriptionally competent (Voinnet et al., 2003). The transcriptionally active T-DNA is then able to produce dsRNA forming sequence which can locally or systemically silence endogenous gene targets. *Agrobacterium* infiltration has been successfully applied to several plant species, including *Nicotiana benthamiana* (Goodin et al., 2008), *Arabidopsis thaliana* (Wroblewski et al., 2005),

tobacco (Yang et al., 2000; Sparkes et al., 2006), tomato (Orzaez et al., 2006) lettuce (Wroblewski et al., 2005; Joh et al., 2005), and grapevine (Santos-Rosa et al., 2008; Zottini et al., 2008). Although there is no report of peach leaf infiltration, an *Agrobacterium*-mediated transient gene expression system was developed for ripe peach fruits (Spolaore et al., 2001). In order to establish and test the transient expression system in peach, we cloned peach *PDS* and constructed a *PpPDS* RNAi vector.

Materials and Methods

Plant materials and growth conditions

Clones of wild-type peach trees [*Prunus persica* (L.) Batsch] from an F_2 population used to map the *evg* mutation (Wang et al., 2002; Bielenberg et al., 2008) were propagated by bud grafting onto peach Guardian rootstock and planted in an irrigated nursery in 2003 at the Clemson University Musser Fruit Research Center located near Seneca, South Carolina, USA. A pooled sample of RNA from wild-type root, stem, leaf, branch tips, flowers, and fruit tissues grown in the greenhouse and from shoot tissues grown in the field were collected; the field tissues were collected at four times during the annual growth cycle: post-chilling, pre-budbreak (March); actively growing, long days (June); shortening days, budset (September); and dormant (December) (Bielenberg et al., 2008). cDNA reversely-transcribed from this pooled RNA was used as template for RNAi and overexpression targets application.

Primer Design and Polymerase Chain Reaction (PCR)

Gene-specific primers for *DAM* 3' untranslated regions (UTRs) and open reading frame (ORF) cDNA were designed based on the *DAM* cDNA sequences (GenBank accession numbers: *DAM1*, DQ863253; *DAM2*, DQ863255; *DAM3*, DQ863256; *DAM4*, DQ863250; *DAM5*, DQ863251; *DAM6*, DQ863252) using Primer Premier 5.00 (PREMIER Biosoft International, Palo Alto, CA). Primers were ordered from Integrated DNA Technologies (Coralville, IA, USA). Primers for peach *PDS* were designed based on the *phytoene desaturase* cDNA sequence from the closely-related species, *Prunus armeniaca* (GenBank accession numbers: AY822065). Since the unique sequences for *DAM1* and *DAM5* 3' UTR are only 80-90 bp and this length is thought to be too short for effective silencing of the endogenous targets, two pairs of primers with isocaudomer enzyme *Nhe*I and *Spe*I adapters were designed to ligate two sequences together as the target region. For constructing RNAi vectors, two pairs of primers named “sense” and “anti-sense”, respectively, were designed for each gene with restriction enzyme adapters. Sense primers contain *Xho*I and *Kpn*I while anti-sense primers contain *Bam*HI and *Cla*I. For cloning *DAM* ORF cDNA, one pair of primers was designed for each gene with *Kpn*I and *Cla*I adapters. Primer sequences are listed in the Table 2.1 and Table 2.2.

PCR was performed with 0.5 μl of first strand cDNA in a volume of 25 μl, containing 1 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 μM of each primer and 1×Taq polymerase

reaction buffer. An initial denaturation step of 120 s at 95 °C was followed by 39 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. The PCR products were fractioned on a 2% agarose gel and scored for the presence or absence of amplification products.

Table 2.1. Gene-specific primer pairs used for cloning *DAM* and *PpPDS* RNAi inverted repeat regions. The underlined sequences are the restriction enzyme sites.

Gene	Primer name	Primer sequence	Product length (bp)
PpDAM1 3' UTR	*PpDAM1* F(*Nhe*I)	5'-TGCTAGCTCCTATGTATTTTATGCAACCTGACA	85
	PpDAM1 R(*Spe*I)	5'-TACTAGTTTCTCCAAAGCAGGCTGCATTGAT	
PpDAM1 3' UTR×2	*PpDAM1* sense F(*Xho*I)	5'-TCTCGAGTGGAATTCGATTTGCTAGCTCCTA	178
	PpDAM1 sense R(*Kpn*I)	5'-TGGTACCTCGAATTCACTAGTGATTTACTAGTTTCTC	
	PpDAM1 anti-sense F(*Bam*HI)	5'-TGGATCCTGGAATTCGATTTGCTAGCTCCTA	178
	PpDAM1 anti-sense R(*Cla*I)	5'-TATCGATTCGAATTCACTAGTGATTTACTAGTTTCTC	
PpDAM2 3' UTR	*PpDAM2* sense F(*Xho*I)	5'-TCTCGAGTAGCTAATCTTAATTTCTATGGTTAA	193
	PpDAM2 sense R(*Kpn*I)	5'-TGGTACCTGTAATAGATAGGTTTAGATACAACA	
	PpDAM2 anti-sense F(*Bam*HI)	5'-TGGATCCTAGCTAATCTTAATTTCTATGGTTAA	193
	PpDAM2 anti-sense R(*Cla*I)	5'-TATCGATTGTAATAGATAGGTTTAGATACAACA	
PpDAM3 3' UTR	*PpDAM3* sense F(*Xho*I)	5'-TTCTCGAGGTGTGTGTTAACTCGACTTTGTTT	171
	PpDAM3 sense R(*Kpn*I)	5'-TGGTACCTACGTTATCCTGTGCTGTGACATTGA	
	PpDAM3 anti-sense F(*Bam*HI)	5'-TGGATCCTGTGTGTGTTAACTCGACTTTGTTT	171
	*PpDAM3*anti-sense R(*Cla*I)	5'-TATCGATTACGTTATCCTGTGCTGTGACATTGA	
PpDAM4 3' UTR	*PpDAM4* sense F(*Xho*I)	5'-TCTCGAGTCTCAAACTGGGGCTAACGGTA	282
	PpDAM4 sense R(*Kpn*I)	5'-TGGTACCTATGGCACTTTGACACAGGTCAT	
	PpDAM4 anti-sense F(*Bam*HI)	5'-TGGATCCTCTCAAACTGGGGCTAACGGTA	282
	PpDAM4 anti-sense R(*Cla*I)	5'-TATCGATTATGGCACTTTGACACAGGTCAT	
PpDAM5 3' UTR	*PpDAM5* isocaudomer F(*Nhe*I)	5'-TGCTAGCTCTAAACCTATGCGTTACATGCAA	82
	PpDAM5 isocaudomer R(*Spe*I)	5'-TACTAGTTGCAAGCTGCATTAATACAGAGTTT	
PpDAM5 3' UTR×2	*PpDAM5* sense F(*Xho*I)	5'-TCTCGAGTGGGAATTCGATTTGCTAGCTCTAAA	170
	PpDAM5 sense R(*Kpn*I)	5'-TGGTACCTGCGAATTCACTAGTGATTTACTAGTTGC	
	PpDAM5 anti-sense F(*Bam*HI)	5'-TGGATCCTGGGAATTCGATTTGCTAGCTCTAAA	170
	PpDAM5 anti-sense R(*Cla*I)	5'-TATCGATTGCGAATTCACTAGTGATTTACTAGTTGC	
PpDAM6 3' UTR	*PpDAM6* sense F(*Xho*I)	5'-TCTCGAGTGGGGCTTCCCTAGTTTCTTGGTT	261
	PpDAM6 sense R(*Kpn*I)	5'-TGGTACCTCAACATCTAGTAGTCTCCGTGCA	
	PpDAM6 anti-sense F(*Bam*HI)	5'-TGGATCCTGGGGCTTCCCTAGTTTCTTGGTT	261
	PpDAM6 anti-sense R(*Cla*I)	5'-TATCGATTCAACATCTAGTAGTCTCCGTGCA	
PpDAM4 Conserve Region	*PpDAM4* C sense F(*Xho*I)	5'-TCTCGAGTGGATGGTGAAAATGATGAGG	213
	PpDAM4 C sense R(*Kpn*I)	5'-TGGTACCTCGCACTTCGTACCTTTCAATAACAT	
	PpDAM4 C anti-sense F(*Bam*HI)	5'-TGGATCCTGGATGGTGAAAATGATGAGG	213
	PpDAM4 C anti- sense R(*Cla*I)	5'-TATCGATTCGCACTTCGTACCTTTCAATAACAT	
PpPDS	*PpPDS* RNAi sense(*Xho*I)F	5'-TCTCGAGTGCACAAAGCTTCCCTGATAGAACAG	1312
	PpPDS RNAi sense(*Kpn*I)R	5'-AGGTACCAAGGTTTATCGACTGCAAAATATTTGG	
	PpPDS RNAi anti-sense(*Bam*HI)F	5'-TGGATCCTGCACAAAGCTTCCCTGATAGAACAG	1312
	PpPDS RNAi anti-sense(*Cla*I)R	5'-AATCGATAGGTTTATCGACTGCAAAATATTTGG	

Table 2.2. Gene-specific primer pairs used for cloning *DAM* ORF cDNA. The underlined sequences are the restriction enzyme sites.

Gene	Primer name	Primer sequence	Product length (bp)
PpDAM1	*PpDAM1* F(*Kpn*I)	5'-TGGTACCTACCCTGAAACTCCCCGACAAAG	762
	PpDAM1 R(*Cla*I)	5'-TATCGATTCACAACCCTCCACTTTTTAACCAG	
PpDAM2	*PpDAM2* F(*Kpn*I)	5'-TGGTACCTAAAACTTCAGACCCTGAAACCC	766
	PpDAM2 R(*Cla*I)	5'-TATCGATTTTCATCTTTCACCATTTCTTAACCA	
PpDAM3	*PpDAM3* F(*Kpn*I)	5'-TGGTACCTAGACCTGAAACCTCCCAACGAAG	761
	PpDAM3 R(*Cla*I)	5'-TATCGATTATCGTTCTCCACTTCTTAACCAGAG	
PpDAM4	*PpDAM4* F(*Kpn*I)	5'-TGGTACCTGTACGCACAACTTCAGACCCTGA	937
	PpDAM4 R(*Cla*I)	5'-TATCGATTTTGATTTAATCCACGCAGACAACTA	
PpDAM5	*PpDAM5* F(*Kpn*I)	5'-TGGTACCTCCCCGAAACCCACCAACGAAGATG	739
	PpDAM5 R(*Cla*I)	5'-AATCGATATTCTTCACTTCTTAACGCCCCAGTT	
PpDAM6	*PpDAM6* F(*Kpn*I)	5'-TGGTACCTACCCCGACAAAGGGAAACGGTG	769
	PpDAM6 R(*Cla*I)	5'-AATCGATATTCCTTGTCCACTTCTTAACCAAGA	

Subcloning/Sequencing

PCR products of *DAM* fragments and *PpPDS* were ligated into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into competent *E.coli* DH5α cells using heat shock method following the pGEM-T Easy vector manual. Transformed cells were plated onto Luria-Bertani medium (LB, 16 g/L Bacto-tryptone, 8 g/L Bacto-yeast extract, 5 g/L NaCl, 15 g/L agar, pH=7.0) plates which had been treated with 100 mg/ml ampicillin; prior to plating cells, all plates were coated with 75-100 µl IPTG (isopropyl-beta-D-thiogalactopyranoside) and 75-100 µl X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) for blue-white screening. Plates were then incubated at 37°C overnight. White colonies were selected, streaked onto new ampicillin containing agar plates, and grown in LB media at 37°C overnight. Plasmids were isolated from cultures using alkaline lysis minipreps according to the

method described by Sambrook and Russell (2001). Isolated plasmids were used as template for PCR by using gene-specific primers to confirm insertion of the gene of interest, and sent to Clemson University Genomics Institute (CUGI, Clemson, SC) for sequencing. SP6 and T7 primers were used for forward and reverse reactions in sequencing.

RNAi Target Region Design

Because the mechanism of silencing depends on sequence homology, there is potential for cross-silencing of related mRNA sequences, especially if the target is a member of a gene family. According to the gene local alignment search (BLASTn) results, the coding regions of *DAM* genes are highly conserved; nucleotide sequence homology among them ranged from 85% to 89%, with contiguous stretches of nucleotides in which the 25 to 101 bases being identical between *DAM*s (Table 2.3).

Table 2.3. Nucleotide homology between the highly conserved coding sequences of *DAM1* to *DAM6*.

	DAM1		*DAM2*		*DAM3*		*DAM4*		*DAM5*		*DAM6*	
	Homology	Longest perfect match length	Homology	Longest perfect match length	Homology	Longest perfect match length	Homology	Longest perfect match length	Homology	Longest perfect match length	Homology	Longest perfect match length
DAM1	100%	723										
DAM2	88%	55	100%	711								
DAM3	88%	56	89%	83	100%	720						
DAM4	89%	61	88%	48	87%	52	100%	714				
DAM5	87%	60	87%	55	89%	30	89%	101	100%	708		
DAM6	85%	34	88%	34	87%	25	89%	77	89%	83	100%	717

However, the 3' ends of the coding region and the 3' UTRs are divergent among these *DAM*s. To reduce the cross-silencing, blocks of sequence with identity over 20 bases between the construct and non-target gene sequences were avoided. To

silence specific *DAM* genes, fragments of DNA from 3' UTRs were chosen as the inverted repeat (IR) forming sequences (Figure 2.2).

A 213bp DNA fragment from *DAM4* MADS-box domain was used to silence all *DAM* genes. This region is highly conserved in all six *DAM*s, and has contiguous stretches of nucleotides in which 49 to 102 bases are identical between *DAM4* and the other members of the gene family. To ensure no other gene was silenced, a BLASTn search was conducted in the peach genome database (http://www.rosaceae.org/peach/genome) and no other hit except these six *DAM* genes was found.

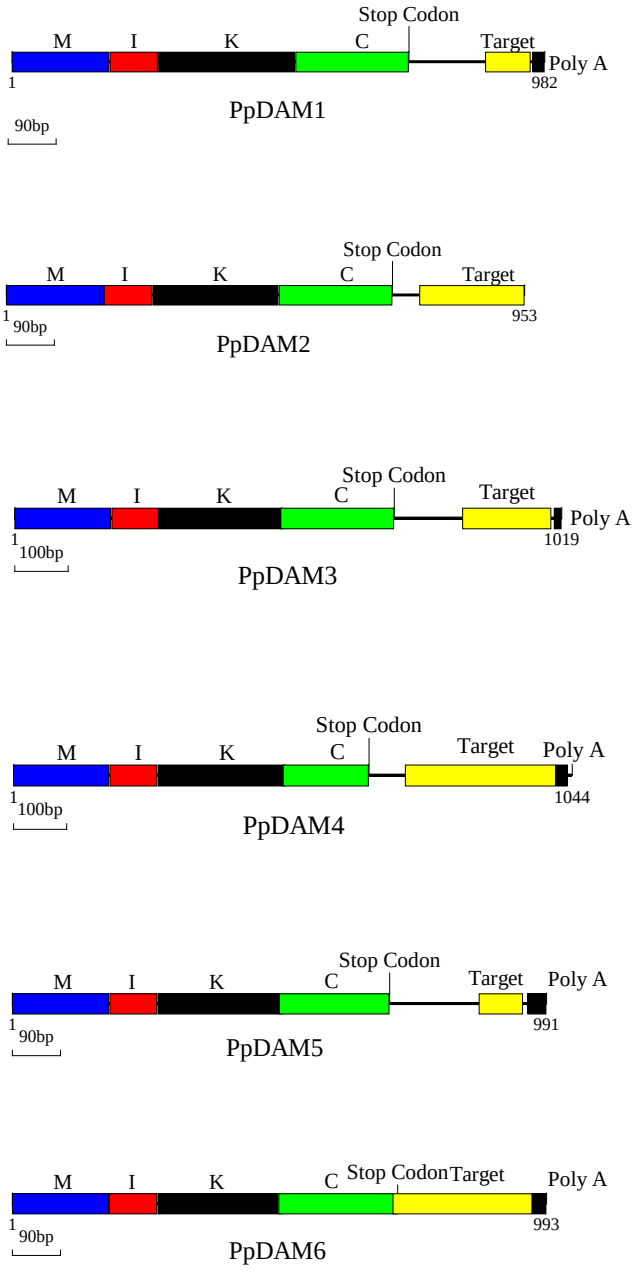

Figure 2.2. Structures of *PpDAM* genes. *PpDAM* gene M, I, K and C domains are represented by blue, red, black and green boxes separately. The RNAi IR regions are represented by yellow boxes. The poly A tails are represented by black boxes. The numbers under the genes represent the length of the genes (bp). All domains and genes are drawn in scale.

For *DAM1* and *DAM5*, only 80-90 bp of unique sequence are available for the RNAi target region. In order to ensure silencing efficiency, a target of over 100 bp

in length may be necessary (Wesley et al., 2001). Therefore, 3' UTRs of *DAM1* and *DAM5* were first amplified with gene-specific primers contained isocaudomer enzymes, *Nhe*I and *Spe*I, and then ligated together (2×*DAM1* and 2×*DAM5* 3' UTR). This method, connecting two targets together, has been used before and obtained a better silencing efficiency than using single target (Miki et al., 2005).

RNAi Vector Construction

Vector pBSKS^{+}-KANNIBAL, kindly provided by Dr. Julia Frugoli at Clemson University, was chosen to provide the RNAi cassette, which contains a CaMV35S promoter, an octopine synthase (OCS) terminator, an 800 bp pyruvate dehydrogenase kinase (pdk) intron and restriction enzyme sites between the intron and the promoter/terminator as seen in Figure 2.3.

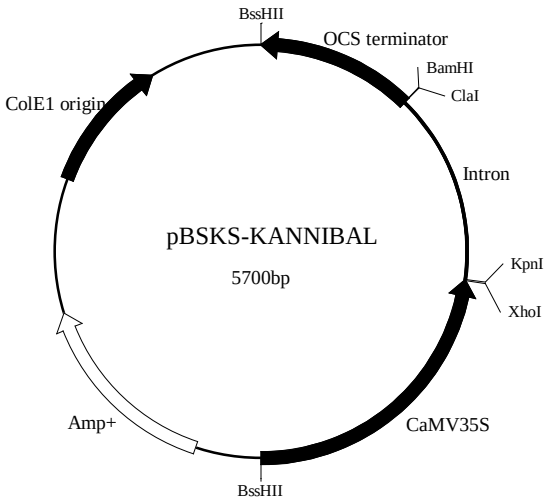

Figure 2.3. pBSKS^{+}-KANNIBAL vector map.

In the subcloning steps, RNAi target sequences were cloned to pGEM-T Easy vector and verified by sequencing. The first sequence of RNAi target was excised from pGEM-T Easy vector by *Xho*I and *Kpn*I and ligated to vector pBSKS^{+}-KANNIBAL between the 35S promoter and the intron. Then the second sequence of RNAi target region was excised from pGEM-T Easy vector by *Bam*HI and *Cla*I and ligated to the pBSKS^{+}-KANNIBAL between the intron and the OCS terminator; these two target sequences with opposite direction formed the IR region for the RNAi vector (Figure 2.4).

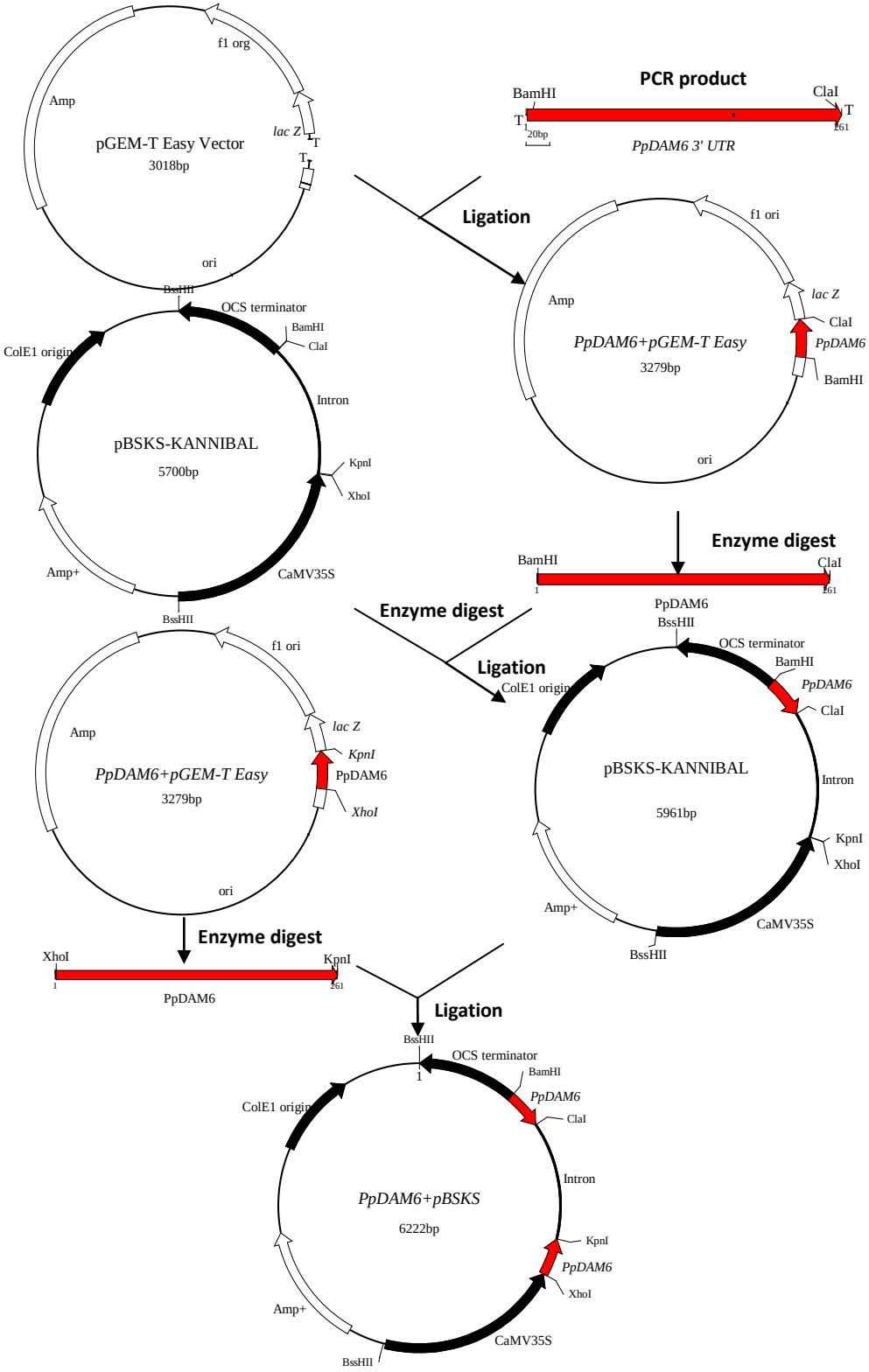

Figure 2.4. Flow chart the peach RNAi IR construction. The *PpDAM6* gene was excised from the *PpMADSX*+pGEM-T-easy vector by a double digest of *Bam*HI and *Cla*I and ligated with the pBSKS vector. This process was repeated with *Xho*I and *Kpn*I enzymes.

Results

A series of vectors were constructed to provide a resource for functional testing of the *DAM* genes from peach. To verify that these six peach *DAM* genes are candidates for the *evg* mutant phenotype, RNA interference (RNAi) vectors were constructed to silence all six *DAM* genes in the wild-type peach. If knocking out *DAM* gene(s) by RNAi results in a phenotype similar to the *evg* mutant, i.e. no terminal bud formation and continued growth under short-day conditions, it would suggest that these genes are responsible for the *evg* phenotypes (Figure 2.1).

If knocking out all six *DAM* genes successfully recreates the *evg* phenotype, then we need to determine if all or only a subset of these genes are involved in growth cessation and bud dormancy. RNAi vectors were constructed for each *DAM* gene and will be transferred to the wild-type peach. If silencing a single *DAM* results in a similar phenotype to the *evg* mutant, it would suggest that this *DAM* alone may be responsible for the *evg* phenotype.

Gene overexpression is another method to verify the function of a particular gene. Since loss of *DAM*s results in no growth cessation and bud set in *evg* mutant under dormancy-inducing condition, these genes may be involved in essential steps of growth inhibition or bud set. Overexpression of these genes should therefore accelerate the growth cessation and bud formation seen in response to dormancy-inducing conditions. For example, if overexpressing a single *DAM* results in a phenotype opposite to the *evg* mutant, i.e. earlier growth cessation and bud set under short days or independent of day length, it would also suggest that this *DAM*

has a role as a promoter in the peach bud dormancy process (Figure 2.1).

Third, if more than one *DAM* is found to be involved in the dormancy process, it is important to discover whether they are functionally redundant. For example, if overexpressing lines of *DAM1* or *DAM2* both show dormancy-related phenotypes, both genes are involved in the dormancy process. As a result, these two *DAM*s must be silenced separately to determine if they are functionally redundant. If the RNAi lines of *DAM1* or *DAM2* do not show dormancy-related phenotypes, it can be concluded that although both *DAM1* or *DAM2* are involved in dormancy process, either can fulfill the requirement of dormancy, meaning they are functionally redundant. However, if both RNAi lines of *DAM1* and *DAM2* show dormancy-related phenotypes, both *DAM1* and *DAM2* are concluded to be necessary for dormancy (Figure 2.1).

For each knockdown target sequence (six *DAM*s, one *DAM* universal knockdown, and the *PpPDS*) two sequences were amplified differing only in the restriction enzyme sites engineered into the amplification products. These eight pairs of nucleotide sequences obtained were 178 bp to 1312 bp in length (Table 2.4-2.11). For each sequence a BLASTn search showed no hit in the peach genome database (http://www.rosaceae.org/peach/genome) or NCBI except the target itself. The nucleotide sequences obtained for *DAM1, 2, 3, 4, 5* and *6* ORF cDNA are 762, 766, 761, 937, 739 and 769 bp in length, respectively.

Table 2.4. Nucleotide sequence of the *DAM1* 3' UTR used for RNAi target.

```
1     CCTATGTATT TTATGCAACC TGACATATTG CTCTTTGACT TGTATTGTCT TTAGAACTCT
61    GTATCAATGC AGCCTGCTTT GGAGAAACTA GCTCCTATGT ATTTTATGCA ACCTGACATA
121   TTGCTCTTTG ACTTGTATTG TCTTTAGAAC TCTGTATCAA TGCAGCCTGC TTTGGAGA
```

Table 2.5. Nucleotide sequence of the *DAM2* 3' UTR used for RNAi target.

```
1     CTAATCTTAA TTTCTATGGT TAAGAAGTGA AGAAAGATGA AGATTTACTC TCCTTATAGC
61    TAAATAAAAT TAATGTATTG TAAGATATTA GTGATTATAT GTCTTAACTC GATACAAAAT
121   TAAAAAATAT TGAAGGGAGA GTAACTTTGT GTTGTATCTA AACCTATCTA TTACATGCCA
181   GCCCTAACCA TTT
```

Table 2.6. Nucleotide sequence of the *DAM3* 3' UTR used for RNAi target.

```
1     TTGTGTGTGT TAACTCGACT TTGTTTTGTA TCTAAACCTA TGTATTACGT CCAGCCTGAC
61    AAATTGCGCT TTGACTTGTA TTGTATTTAG AACTTTGTAT CAAAATAGTC TGCATGGAGA
121   CTCTTGTGGA TTTTGTATTG CTTCAATGTC ACAGCACAGG ATAACGTTAC G
```

Table 2.7. Nucleotide sequence of the *DAM4* 3' UTR used for RNAi target.

```
1     TCTCAAACTG GGGCTAACGG TACGCGCGGG TCGTAGACCG ATGTGCTTAA AGACTTAGAT
61    GTCTTTCTTC ATTGTTTTCA CGTTACAAAG CTCATTTCAA GATATCGTGA AGTTTAGTCC
121   CACGAATGAC ATTATAGGAA CCAGAATGTT TAAGAAGAAA CTCATATAAT TGTCTGCGTG
181   GATTAAATCA ACACAAGTGG CCTGGGGAAA GTAACCTGAG CAAGATAAAG ACGAAGCCGT
241   GTGTCATGGT GGGCTCAATC AAGTGGCTTG ACAGCGCATG ACCTGTGTCA AAGTGCCAT
```

Table 2.8. Nucleotide sequence of the *DAM5* 3' UTR used for RNAi target.

```
1     TCTAAACCTA TGCGTTACAT GCAAGCTTGA AATTGCTCTG ACTTTGTATT GTATTTAAAA
61    CTCTGTATTA ATGCAGCTTG CAACTAGTAC TAAACCTATG CGTTACATGC AAGCTTGAAA
121   TTGCTCTGAC TTTGTATTGT ATTTAAAACT CTGTATTAAT GCAGCTTGCA
```

Table 2.9. Nucleotide sequence of the *DAM6* 3' UTR used for RNAi target.

```
1     CTGGGGCTTC CCTAGTTTCT TGGTTAAGAA GTGGACAAGG AAGAAAGTTT ACTCACTTTA
61    TAGCTAAATA AACAAAATCG AGATAATGTG AGACGCATCA GTAGTGATCG TGTGCCTTAA
121   CTCGATATGA CATTGAAGGG AGAGTAACTT TCTGTGTATC TAAACCTATG GTATTACATG
181   CAGCCTGACA TACTGCTCTT CGACTTTGTA TTGTATGTAT TAATGCAGCC TGCACGGAGA
241   CTACTAGATG TTG
```

Table 2.10. Nucleotide sequence of the *DAM4* conserve region used for RNAi target.

```
1     CGCACTTCGT ACCTTTCAAT AACATCCTCG ATACTTGAGC TTGAATAATC AAAAAGCTTG
```

```
61    CCAGTAGCAG AAAAGATGAC AACTGCCACC TCAGATTCAC ACAGAACAGA TAGCTCTGCA
121   GCTTTCTTGA AGATCCCTCT TCTCCTCTTT GAGAAGGTCA CCTGCCTTGC TGGCAGGTAG
181   TCAATCTTCT TGATCTTGAT CTTCTCCCTC ATCATTTTCA CCATCC
```

Table 2.11. Nucleotide sequence of the *PpPDS* used for RNAi target.

```
1     GTGCACAAAG CTTCCCTGAT AGAACAGCAC CTTCCATTGG GGCTAAATAC TTTTGTTTTG
61    TGTAATCACC AGCTAAATAG AAACCCTCTA GGGGAGATCT TTGCAAGGGA CGGCAAGGTT
121   CACAACCTGG TACAGTTTTG TAAACCGACC TTGGTGTTTT CACCACATGG TACTTCAAAA
181   TCTTTGCTTT GCTTTGATCT ACAGCTATCT CATCTGGAAA GAGTTTTGCA AGTTCTTTGA
241   GTGTAGCATC AATAATTTCT GAATCACTGC ATGATATCCA TTCTTCTGCT GGTGCAAAAA
301   CCAACTCCAG CATTGACTGG TTTGGATTGT AATATTCCTT ACATGTTACG GACATGTCGG
361   CATAGACACT TAAAAGAGGA CTTCTGCTAA AAAGTAGATG ATCATATGTG TTCTTCAGCT
421   TTCTGTCAAA CCATATGTGA ACATTGATAA CTGGAACGCC AACCAGTTTC TCCAATTTCT
481   TGAAATATGG GATCTCTTTC CAGTTATCAG GCAATAGAAG CTTTAGGATA TCAACTGGAG
541   TGGCGAATAC ATAGGCATCT GCTTCAATCA TGCTCCCATT ATTTAGTACA AAACTCTTCA
601   CGGTCCCATC TTTATTTAGC TCAATTCTCT GTATTCGGGA ATTAATTCGG ACTTCACCGC
661   CTAATGACTG GATATGATCA ACAATTGGTG CACAGAGTCT CTCAGGGGGA CTACCATCCA
721   AAAAAGCCAT CTTGGAACCG TGTTTCTCCT GAAGGAATCG GTTCAAAGCA ATCAATATGC
781   ATTGCATTGA AAGTTCATCA GGGTTAATAA AGTTCAGGGC CTTTGACATG GCAATAAACA
841   CCTCAGTAGT CACTCGATCC GGTATGCCCT GTTTCCTCAT CCAATCTTTT ACACTCAAGC
901   CATCTTGGGC TTCAACATAA GCCTGCCCAC CAAGAATTGC TGGCAGTAGT CCAATTGCAA
961   ACTTGATTTT CTCTGGCCAA GTCAGCATCT CATTGTTCTT CAATATGGCC CATATTCCAT
1021  TTAAGGGTGC TGGTAAAACT TCAGGGAAAT CAAACCGGCT GAACTCTCCT GGTTTGCTTG
1081  GCATTGCAAA TATCATAGAA TGCTCCTTCC ACTGCAATCG ATCATCAATA CCAAGCTCAC
1141  CAAACAGGTT CTGAATATTC GGATAAGCCC CAAAGAAGAT ATGTAGGCCT GTTTCGTACC
1201  AGTCTCCATC CTTATCTTTC CACGCTGCCA CCTTTCCGCC CAGAACATCT CTTGCTTCCA
1261  GTAAGATAGG TTTATGACCT GCATCAGCCA AATATTTTGC AGT
```

Targets of RNAi were successfully cloned into pBSKS vector and verified by sequencing. In total seven RNAi vectors were constructed for *DAM* genes (Table 2.12).

Table 2.12. RNAi vectors constructed for *DAM*s.

Source of Target	Vector	Function
DAM1	RNAi	Silencing *DAM1*
DAM2	RNAi	Silencing *DAM2*
DAM3	RNAi	Silencing *DAM3*
DAM4	RNAi	Silencing *DAM4*
DAM5	RNAi	Silencing *DAM5*
DAM6	RNAi	Silencing *DAM6*
DAM4	RNAi	Silencing six *DAM*s

Discussion

The six *DAM* genes are candidates for the *evg* non-dormant phenotype in peach (Bielenberg et al., 2008). Since the molecular and genetic regulation of perennial dormancy is not well-understood, analysis of *DAM* gene function is critical for a clearer understanding of this important life history trait of perennial species. In this study, the gene 3' UTRs and full-length ORF cDNA sequences were cloned for each of the six *DAM* genes in peach.

RNAi Strategy for *DAM*s

RNA silencing is a useful tool for the functional analysis of highly conserved multi-gene families in plants. Intron-containing 'hairpin' RNA (ihpRNA) constructs, which contain sense and anti-sense arms separated by an intron, have been reported to provide 90-100% of transgenic lines silencing (Wesley et al., 2001). The degree of silencing with these constructs was much greater than that obtained using either co-suppression or anti-sense constructs (Wesley et al., 2001). In this study, pBSKS, which contains an expression cassette with multiple cloning sites on either side of an 800 bp *pdk* intron for the formation of identical IR sequences, was used as

RNAi vector to construct the ihpRNA. RNAi target regions, which constitute the sense and anti-sense arms of the ihpRNA construct, are important for achieving specific gene silencing. Miki et al. (2005) found that RNA silencing can be used to suppress the expression of individual members of a gene family in rice that have a high sequence similarity with one another using diverged 3' UTR as a trigger for the formation of double-stranded RNA (dsRNA). Because *DAM* genes are highly conserved in coding region but divergent in the 3' UTR, 3' UTR sequences between 178 bp to 282 bp in length were selected as the RNAi target regions from *DAM1* to *DAM6*.

A universal RNAi construct was constructed to knockdown all six *DAM* genes as a group. A single IR construct of rice *OsRac1* gene with high sequence similarity in the conserved region with 72% to 100% homology with other members can effectively suppress the expression of other *OsRac* genes (Miki et al., 2005). In our study, the conserved region from *DAM4*, which has a higher degree of homology (87%-100%) with other *DAM*s than the *OsRac1* example above, should successfully silence the *DAM* family. Most importantly, Miki et al. (2005) also found that the IR constructs transcribing dsRNA containing two or more chimeric gene-specific regions of *OsRac* genes can be used for silencing the corresponding *OsRac* genes. The chimeric RNAi construct will be useful for our further *DAM* functional examination, such as silencing of two or more *DAM*s by one RNAi vector.

Obstacles during Vector Construction

DAM RNAi IR constructs needed to be transferred to the peach transformation vector pSBbarB #2 for peach transformation (Figure 2.5). The *DAM* RNAi IR constructs, which contain sense and anti-sense arms (178 bp to 282 bp), an *Arabidopsis* gene intron (800 bp), a 35S promoter (700 bp) and a terminator (1.3 kb), are about 3.2 kb in length. Here, the RNAi constructs were excised from pBSKS by *Pst*I and *Not*I. The pSBbarB #2 vector was digested by *Hind*III to provide the insertion site for the IR construct. Both insert and vector were digested by T_4 DNA polymerase to blunt the ends and subsequently ligated by T_4 DNA ligase. Although the length of insert and vector was verified to be correct on the agarose gel, ligation of the RNAi constructs into the pSBbarB #2 vector was not successful. Different molar ratios of vector and insert were tried but no positive clones were obtained.

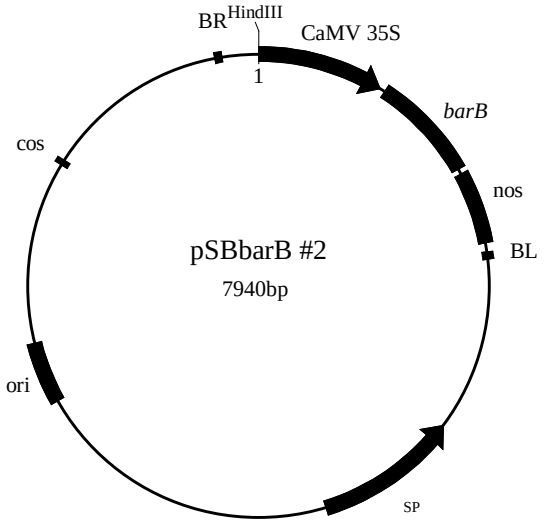

Figure 2.5. pSBbarB #2 vector map.

It is possible that most of inserts and vectors ligated themselves and inhibit the insert/vector ligation. To overcome this obstacle, we designed a linker contained an *Asc*I site in the middle and two *Hind*III sites on each ends. This linker was used to introduce *Asc*I to the pSBbarB #2. The boundaries of RNAi constructs contain *Bss*HII, which is compatible with *Asc*I. By introducing the *Asc*I to the pSBbarB #2, we were able to use the sticky ends instead of blunt ends for the DNA ligation. Using this strategy, the *PpPDS* RNAi construct was successfully ligated to the pSBbarB #2 vector, although the ligation efficiency was low. Due to the inefficiency at which the IR containing construct was able to be transferred to the transformation vector, future work should focus on developing a transformation vector which already contains the MCS sites flanking an intron for the rapid insertion of new sequences.

Future Perspectives

Although the *Agrobacterium*-mediated transformation in peach is not available now, other methods such as heterologous plant transformation and transient transformation can be used for *DAM* functional research. The transient transformation such as the leaf infiltration has been used as a quick assay in other species to find out whether the transgenes show phenotype(s) in a short period. The recently developed plum system can possibly be used for *DAM* functional research because of the close relationship between peach and plum (Petri et al., 2008). Unlike the leaf infiltration method where the level of transgene expression usually peaks at 60-72 h post-infiltration and declines rapidly thereafter (Voinnet et al., 2003), the

Agrobacterium-mediated transformation in plum stably integrates the transgene into the host genome. This trait of the plum system is important for a long-term phenotype observation. Because of this relative easy transformation method, overexpressing *DAM* in plum could be a quick way to understand the *DAM* function.

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CHAPTER THREE

CLONING, VECTOR CONSTRUCTION AND TRANSFORMATION OF POPLAR *DAM* HOMOLOGS

Introduction

DAM gene in other species

The peach *DAM* genes belong to the *SVP/StMADS11* clade of MIKC^{C} MADS-box genes (Bielenberg et al., 2008). Perennials like the peach have more genes in this group than annuals; for example, peach (six), poplar (eight) and grapevine (five) have more *SVP/StMADS11* genes than *Arabidopsis* (two) and other annual models such as tomato (two) and rice (three) (Mao et al., 2000; Leseberg et al., 2006; Arora et al., 2007; Diaz-Riquelme et al., 2009). This increased representation in three phylogeneticaly distinct species may indicate that perennials use the *SVP/StMADS11* genes for their plant perennial life history traits such as the regulation of endodormancy cycling, and/or regulation of the juvenile to mature transition (Jimenez et al., 2009a).

Most functional research of *SVP/StMADS11* genes has been conducted on annual species. Several genes of this group have been found to be involved in the transition from vegetative to reproductive meristem identity. In *Arabidopsis*, the *AGL22/SVP* gene acts as flowering negative regulator (Hartmann et al., 2000), whereas it's close homolog *AGL24* has an opposite function in flower development (Yu et al., 2004). In rice, *OsMADS22*, *OsMADS47* and *OsMADS55* act as negative regulators of brassinosteroid responses and modulators of meristem identity (Duan et al., 2006, Lee et al., 2008), and in tomato, the MADS-box gene *JOINTLESS* is

involved in leaf and abscission zone development in addition to regulation of the flowering time (Mao et al., 2000; Szymkowiak et al., 2006). In *Paulownia kawakamii* and *Eucalyptus grandis*, overexpression of putative orthologues of *AtAGL24/AtSVP* genes *PkMADS* and *EgSVP* resulted in precocious growth of lateral meristems (Prakash and Kumar, 2002; Brill and Watson, 2004). In *Ipomoea batatas*, *IbMADS3* and *IbMADS4* genes are preferentially expressed in root tissue and *IbMADS1* is involved in tuberous root initiation (Kim et al., 2002; Ku et al., 2008).

The function of *SVP/StMADS11* genes is more fully understood in annual than in perennial species. In perennial species, *SVP/StMADS11* genes are expressed in most vegetative tissues and often in bud tissues. For example, in poplar, at least one of the *SVP/StMADS11-like* genes is expressed in cambium tissue (Leseberg et al., 2006) and qRT-PCR has shown that the five grapevine *SVP/StMADS11-like* genes are preferentially expressed in bud tissue, although two of them are also detected in vegetative organs (Diaz-Riquelme et al., 2009). The peach *DAM* genes (Bielenberg et al., 2008), a raspberry gene putatively encoding an *SVP/StMADS11-like* transcription factor (Mazzitelli et al., 2007) and Japanese apricot *SVP/StMADS11-like* genes (Yamane et al., 2008) are all expressed in bud tissues. Although the roles of *SVP/StMADS11-like* genes are not clear, their vegetative and/or bud localization of expression perhaps supports the hypothesis that a perennial habit of growth requires additional regulatory pathways to control seasonal life history traits.

In addition to the functional research in peach discussed in Chapter Two, a parallel approach is to use a model species to study the function of *DAM*s since no reliable peach transformation method is currently available. Poplar species are good model for gene functional research in part because a transformation system is well established for several species. The key is to determine if the model has *DAM* homologs closely related to the peach genes. Phylogenetic analysis of peach and poplar (*Populus trichocarpa*) MIKC^{C}-type MADS-box genes has confirmed that eight *P. trichocarpa* (*PtMADS7*, *PtMADS21*, *PtMADS26*, *PtMADS27*, *PtMADS28*, *PtMADS29*, *PtMADS47* and *PtMADS48*) MADS-box genes belong to the *SVP/StMADS11* group (Jimenez et al., 2009a). The peach *DAM* genes diverged sequentially through tandem duplications from a common ancestor most closely related to two of the *P. trichocarpa* genes, *PtMADS7* and *PtMADS21* (Jimenez et al., 2009a). Synteny conservation analysis of the *SVP-like* gene loci among peach, apricot, *Arabidopsis* and poplar has confirmed that *PtMADS7* and *PtMADS21* are more related to the peach *DAM* genes than the others (Jimenez et al., 2009b).

The research reported here silenced or overexpressed hybrid poplar (*P. tremula*×*alba*, clone INRA 717-1B4) *DAM* homologs to determine if they have a similar function to the *DAM* in peach. *P. tremula*×*alba* (clone INRA 717-1B4) is the most widely used poplar clone for transformation due to its relatively high transformation efficiency (Leple et al., 1992). The transformation method is mature in clone INRA 717-1B4 and the transformation efficiency in this hybrid is higher than in

P. trichocarpa (Ma et al., 2004; Meilan and Ma, 2006). Sequences of six *PtMADS* genes in poplar clone INRA 717-1B4 were cloned based on the sequences of *P. trichocarpa PtMADS* homologs in DOE JOINT GENOME INSTITUTE (JGI) poplar genome database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) and sequence tags (EST) database (NCBI).

The method reported here for investigating the function of *DAM* gene homologs in the poplar hybrid *P. tremula*×*alba* has an important advantage over the leaf infiltration and heterologous sequence silencing procedures discussed in Chapter Two. *Agrobacterium*-mediated transformation can be applied to obtain stable transgenic plants, which is necessary for long-term phenotype observation. In the experiments reported here, the poplar hybrid *P. tremula*×*alba* (clone INRA 717-1B4) was selected as the model plant because of its mature transformation and regeneration method.

The strategy for *DAM* gene function research in *Populus*

Our goal is to determine if the eight *SVP/StMADS11* group *PtMADS* genes are the *DAM* genes orthologs involved in the bud dormancy signaling pathway. If knocking down all eight *PtMADS* genes results in an *evg*-like phenotype, i.e., no bud formation and continued growth under short-day conditions, it would suggest that these genes are responsible for this *evg*-like phenotype (Figure 3.1).

If knocking out all eight *PtMADS* genes successfully generates the *evg*-like phenotype, then we need to determine which are involved in the bud dormancy

process. RNAi vectors were constructed for each *PtMADS* gene and will be transferred to the wild-type poplar. If silencing a single *PtMADS* results in a similar phenotype to the peach *evg* mutant, it would suggest that this *PtMADS* alone may be responsible for the *evg*-like phenotype.

Since it is possible that more than one *PtMADS*s are involved in the bud dormancy pathway, overexpression genes individually can find out the target gene from eight candidates. For example, if one *PtMADS* OE line shows dormancy-related phenotypes, such as earlier bud set or dormancy entrance, this gene is the only one of these eight *PtMADS* genes involved in dormancy pathway(s). If more than one *PtMADS* OE lines show dormancy-related phenotype, these genes could be functionally redundant or complementary. This determination can be verified by silencing the candidate genes separately. For example, if both OE lines of *PtMADS7* and *PtMADS26* show dormancy-related phenotypes, while neither RNAi lines of *PtMADS7* nor *PtMADS26* show *evg*-similar phenotype, these two genes are functionally redundant (Figure 3.1).

The six *PtMADS* RNAi and overexpression vectors constructed here are the first step in this ongoing transgenic work. In the *PtMADS* knockdown lines, it is expected to see phenotypes similar to the peach *evg* mutant, including failure to cease growth and enter dormancy under dormancy-inducing conditions. We expect the degree of these phenotypes to correlate with the gene silencing level which can be detected through Northern blot or quantitative RT-PCR. For example, transgenic lines

with 50% target gene knocking down should show late dormancy entrance while those lines with 90% target gene knocking down should show no dormancy entrance. On the other hand, we expect to see early growth cessation, bud formation or entrance of dormancy when overexpressing *PtMADS* genes.

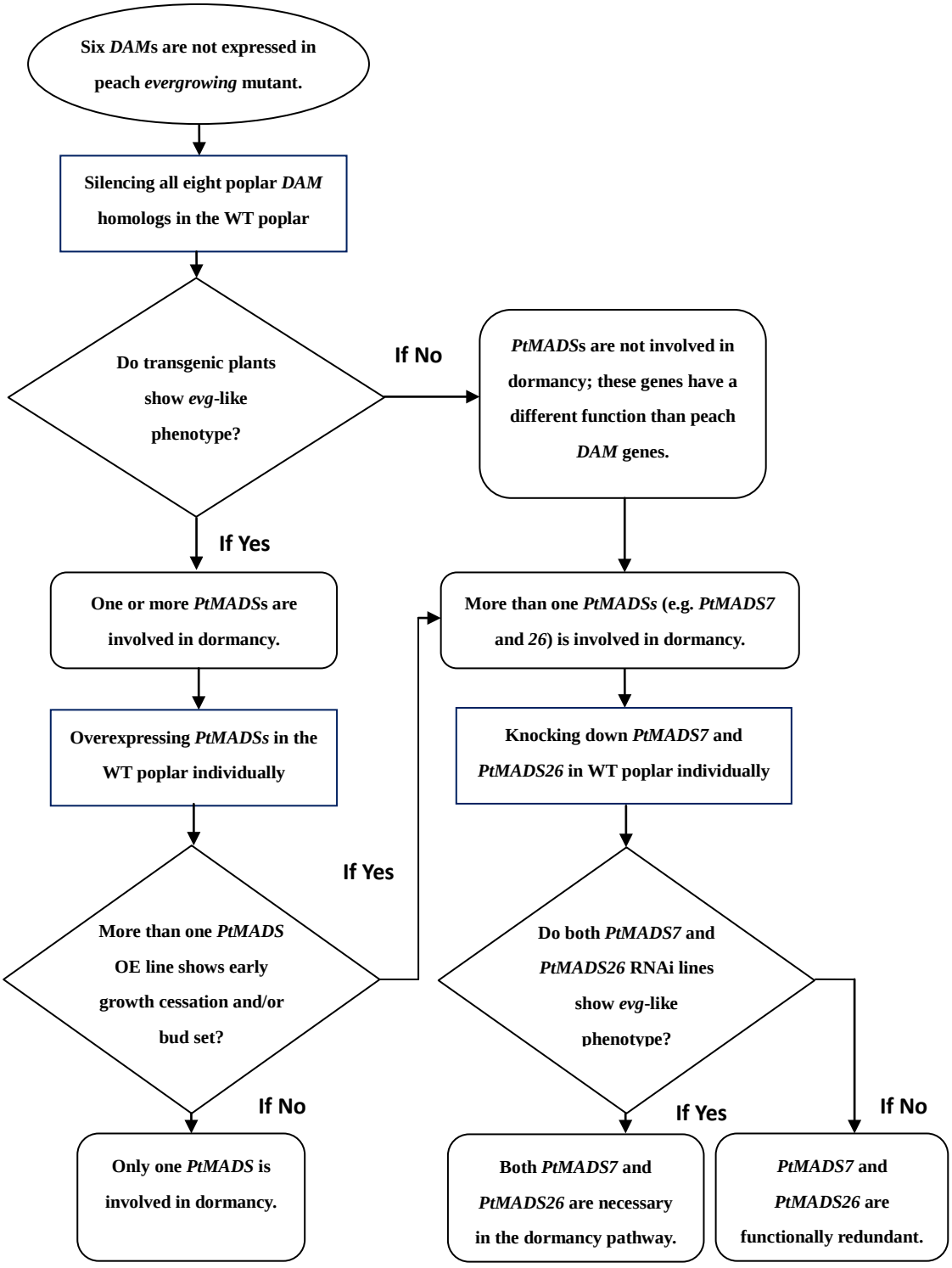

Figure 3.1. Flow chart of the *PtMADS* function research. The rectangle boxes show experimental processes; the diamond boxes show the potential experimental results and the round edge rectangle boxes show the conclusions. *PtMADS7* and *PtMADS26* were used as examples to represent any two of the eight *PtMADS*s.

Materials and Methods

Plant Material and Growth Conditions

P. tremula×alba (clone INRA 717-1B4) was obtained from Dr. Steven H. Strauss, Oregon State University. Forty- to fifty-day-old, *in vitro* grown plantlets served as explant sources. Micro-cuttings of 717-1B4 were initially cultured on hormone-free, half-strength Murashige and Skoog medium (MS) (Murashige and Skoog, 1962). Shoot cultures were maintained on the same medium and grown at 25°C under a 16-h photoperiod. *P. trichocarpa* (Nisqually-1 genotype) leaf tissue was obtained from Dr. Haiying Liang, Clemson University.

RNA Extraction and cDNA synthesis

Wild-type hybrid poplar 717-1B4 mixed tissue (leaves and tips) samples were frozen in liquid N_2 immediately following harvest and stored at -80 °C until processed. Total RNA was isolated according to the method described by Meisel et al. (2005) and modified by Dr. Zhigang Li (Li et al., 2009) for use in our laboratory. Tissues were finely ground in liquid N_2 and 100 mg of powdered tissue was transferred to a 2.0 ml centrifuge tube. Then 13 µl of β-mercaptoethanol and 600 µl of 85 °C extraction buffer (2% SDS, 1.4 M NaCl, 50 mM EDTA pH 8.0, 0.5% PVP, 0.1 M TRIS-HCl, pH 8.0) were added sequentially. The mixture was homogenized by vortexing, followed by incubation at 65 °C for 15-20 min. After the tubes cooled to room temperature, 60 µl of 5 M potassium acetate, 180 µl of cold (-20 °C) 100% ethanol, 600 µl of equilibrated phenol, and 120 µl of chloroform were added sequentially, mixing well after each addition. Tubes were vortexed and incubated for

30 min on ice. Tubes were then centrifuged at 12,000 g at 4 °C for 30 min. The supernatant was transferred to a clean tube and an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added. Tubes were mixed by vortexing and centrifuged at 12,000 g at 4 °C for 20 min. The supernatant was transferred to a new tube and precipitated with LiCl at a final concentration of 3 M at -20 °C for 3 h. The RNA was pelleted by centrifugation at 12,000 g at 4 °C for 20 min. The pellet was dissolved completely into 400 µl of DEPC-treated SS buffer (1 M NaCl, 0.5% SDS, 10 mM TRIS-HCl, pH 8.0). The sample solution was extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) by vortexing. Phases were separated by centrifugation at 14,000 g for 10 min at 4 °C. The aqueous phase was transferred to a clean tube and the RNA was ethanol precipitated at -80 °C for 30 min. RNA was pelleted by centrifugation at 12,000 g for 20 min at 4 °C and the pellet was washed with 75% ethanol twice and resuspended in 20 µl of RNase free water. Total RNA was quantified using spectrophotometry, and the quality was assessed by the ratio of A260 nm: A280 nm (>1.8) and A260 nm: A230 nm (>2.1) followed by electrophoresis in a denaturing 1.3% agarose gel. Two µg of DNase I-treated total RNA were reverse transcribed with oligomer (dT30) as a primer using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) by following the manufacturer's instructions. Aliquots of first strand cDNA were stored at -20 °C.

Primer Design and Polymerase Chain Reaction

Gene-specific primers for *PtMADS* 3' UTR and the full-length open reading frame (ORF) cDNA were designed based on the genomic sequences of poplar genome database (JGI) using Primer Premier 5.00 (PREMIER Biosoft International, Palo Alto, CA). Primers were ordered from Integrated DNA Technologies (Coralville, IA, USA). For *PtMADS* 3' UTR, two pairs of primers named “sense” and “anti-sense”, were designed for each gene with restriction enzyme adapters. Sense primers contain *Asc*I and *Kpn*I while anti-sense primers contain *Spe*I and *Bam*HI. For *PtMADS* ORF cDNA, primers with *Xho*I and *Bam*HI adapters were designed for each gene. Primer sequences are listed in the Table 3.1 and Table 3.2.

Table 3.1. Gene-specific primer pairs used for cloning the *PtMADS* RNAi region. The underlined sequences are the restriction enzyme sites.

Gene	Primer name	Primer sequence	Product length (bp)
PtMADS7	*PtSVP1* sense primer F(*Asc*I)	5'-TGGCGCGCCTGATTTAGACACAGCAGTTCAGGAA	386
	PtSVP1 sense primer R(*Kpn*I)	5'-AGGTACCAGATCAATTCACAGTTGATTTATTTAGGA	
	PtSVP1 Anti-sense F(*Spe*I)	5'-TACTAGTTGATTTAGACACAGCAGTTCAGGAA	386
	PtSVP1 Anti-sense R(*Bam*HI)	5'-AGGATCCAGATCAATTCACAGTTGATTTATTTAGGA	
PtMADS26	*PtSVP3* sense primer F(*Asc*I)	5'-TGGCGCGCCTGTTACGAGGAAGGGCAGTCATCA	366
	PtSVP3 sense primer R(*Kpn*I)	5'-AGGTACCAGCAAAAACAATAAGCGCATTTGGT	
	PtSVP3 Anti-sense F(*Spe*I)	5'-TACTAGTTGTTACGAGGAAGGGCAGTCATCA	366
	PtSVP3 Anti-sense R(*Bam*HI)	5'-AGGATCCAGCAAAAACAATAAGCGCATTTGGT	
PtMADS27	*PtSVP4* sense primer F(*Asc*I)	5'-TGGCGCGCCTTTGATCCTCGCCAGGACTGTGA	368
	PtSVP4 sense primer R(*Kpn*I)	5'-AGGTACCAGACGTCCAAATCTGCTACAATACCA	
	PtSVP4 Anti-sense F(*Spe*I)	5'-TACTAGTTTTGATCCTCGCCAGGACTGTGA	368
	PtSVP4 Anti-sense R(*Bam*HI)	5'-AGGATCCACGTCCAAATCTGCTACAATACCA	
PtMADS29	*PtSVP5* sense primer F(*Asc*I)	5'-TGGCGCGCCTAGGATTGAACTTGGAAGAACTACAT	506
	PtSVP5 sense primer R(*Kpn*I)	5'-AGGTACCAAACAATATTAACGTGTAAATTAATTCAA	
	PtSVP5 Anti-sense F(*Spe*I)	5'-TACTAGTTAGGATTGAACTTGGAAGAACTACAT	506
	PtSVP5 Anti-sense R(*Bam*HI)	5'-AGGATCCAAACAATATTAACGTGTAAATTAATTCAA	
PtMADS28	*PtSVPX* sense primer F(*Asc*I)	5'-TGGCGCGCCTCCCCAGATTCTTTAGTGACCAATAT	294
	PtSVPX sense primer R(*Kpn*I)	5'-AGGTACCAGAATCAACAAGCAGACATGAATATTAT	
	PtSVPX Anti-sense F(*Spe*I)	5'-TACTAGTTCCCCAGATTCTTTAGTGACCAATAT	294
	PtSVPX Anti-sense R(*Bam*HI)	5'-AGGATCCAGAATCAACAAGCAGACATGAATATTAT	
PtMADS48	*PtSVP7* sense primer F(*Asc*I)	5'-TGGCGCGCCTTGCAGGGACTGAACATGGAAGA	684
	PtSVP7 sense primer R(*Kpn*I)	5'-AGGTACCAAGCAAAGAATCTCAAACTGATAATTAAA	
	PtSVP7 Anti-sense F(*Spe*I)	5'-TACTAGTTTGCAGGGACTGAACATGGAAGA	684
	PtSVP7 Anti-sense R(*Bam*HI)	5'-AGGATCCAAGCAAAGAATCTCAAACTGATAATTAAA	

Table 3.2. Gene-specific primer pairs used for cloning *PtMADS* full-length ORF cDNA. The underlined sequences are the restriction enzyme sites.

Gene	Primer name	Primer sequence	Product length (bp)
PtMADS7	*PtSVP1* full cDNA F(*Xho*I)	5'-TCTCGAGTTAACTCAAGCCAGCTCCCA	752
	PtSVP1 full cDNA R(*Bam*HI)	5'-AGGATCCATCGCAAGATTCTACTGGATCA	
PtMADS21	*PtSVP2* full cDNA F(*Xho*I)	5'-TCTCGAGTGGTGTTTGTTTTACTTTCTGAAATT	763
	PtSVP2 full cDNA R(*Bam*HI)	5'-AGGATCCATGCCTCCAGCTTTCAGCTTA	
PtMADS26	*PtSVP3* full cDNA F(*Xho*I)	5'-TCTCGAGTATGGCAAGAGAGAGGATTCAGATAA	738
	PtSVP3 full cDNA R(*Bam*HI)	5'-AGGATCCAGCAATTATTTGTGTCCGCATTT	
PtMADS29	*PtSVP5* full cDNA F(*Xho*I)	5'-TCTCGAGTATGACTCGAAGGAAAATCCAGATCA	671
	PtSVP5 full cDNA R(*Bam*HI)	5'-AGGATCCATCCATCTTTCCCATTCAATCA	
PtMADS28	*PtSVPX* full cDNA F(*Xho*I)	5'-TCTCGAGTATGACTCGAAAGAAAATCCAGAT	798
	PtSVPX full cDNA R(*Bam*HI)	5'-AGGATCCAACAAGCAGACATGAAAAGTGCTCAT	
PtMADS48	*PtSVP7* full cDNA F(*Xho*I)	5'-TCTCGAGTAGAAGAAAGACAACATAGAAATAGG	762
	PtSVP7 full cDNA R(*Bam*HI)	5'-AGGATCCATCCATCTTTCGGATTCAAT	

PCR was performed with 0.5 μl of first strand cDNA in a volume of 25 μl, containing 1 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 μM of each primer and 1×Taq polymerase reaction buffer. An initial denaturation step of 120 s at 95 °C was followed by 39 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. The PCR products were fractioned on a 2% agarose gel and scored for the presence or absence of amplification products.

Subcloning/Sequencing

PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into competent *E.coli* DH5α cells with heat shock method by following pGEM-T Easy vector manual. Transformed cells were plated onto agar plates which had been treated with 100 mg/ml ampicillin; prior to plating cells, all

plates were coated with 75-100 µl IPTG (isopropyl-beta-D-thiogalactopyranoside) and 75-100 µl X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) for blue-white screening. Plates were incubated at 37°C overnight. White colonies were selected, streaked onto new ampicillin containing agar plates, and grown in LB media at 37°C overnight. Plasmids were isolated from cultures using alkaline lysis minipreps as mentioned in Chapter Two. Isolated plasmids were used as template for PCR using gene-specific primers to confirm insertion of the gene of interest, and sent to Clemson University Genomics Institute (CUGI, Clemson, SC) for sequencing. SP6 and T7 primers were used in the forward and reverse reactions of sequencing.

RNAi Target Region Design

Similar to peach *DAM*s, the 5' end regions of the six *PtMADS*s used here are highly conserved while the 3' ends of the coding region and the 3' UTRs are divergent enough to construct RNAi vectors. To reduce cross-silencing and silence specific *PtMADS*, fragments of DNA from the end of the coding region and the 3' UTRs of each gene (from 294-684bp) were selected as the inverted repeat (IR) forming sequence (Figure 3.2).

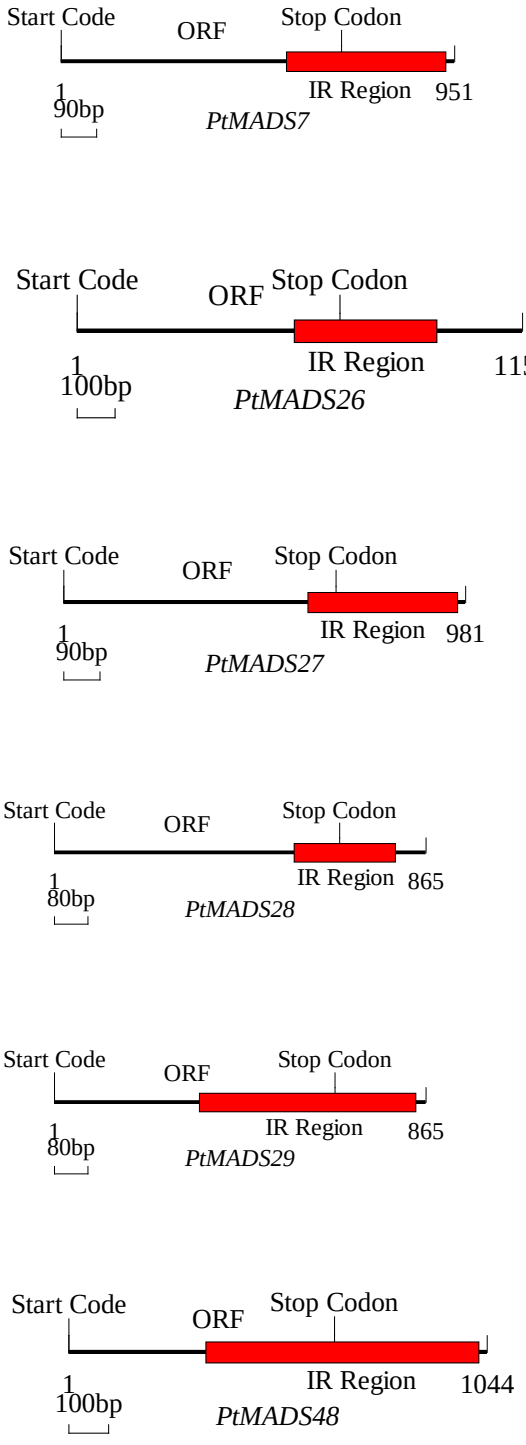

Figure 3.2. Sequences used for *Populus* RNAi IR construction. *PpMADS* genes IR regions for RNAi are represented by the red bar area. The predicted open reading frames are located between the start and stop codons.

RNAi Vector Construction

Vector pRR2222, which was derived from pFGC1008 and modified by Dr. Ramesh Raina (Syracuse University, Syracuse), was chosen as RNAi vector. This vector contains a CaMV35S promoter, an OCS terminator, a 400 bp *Arabidopsis GPA1* gene intron, restriction enzyme sites between the intron and the promoter/terminator, and hygromycin phosphotransferase II (HPTII) as the selection marker for plant transformation (Figure 3.3).

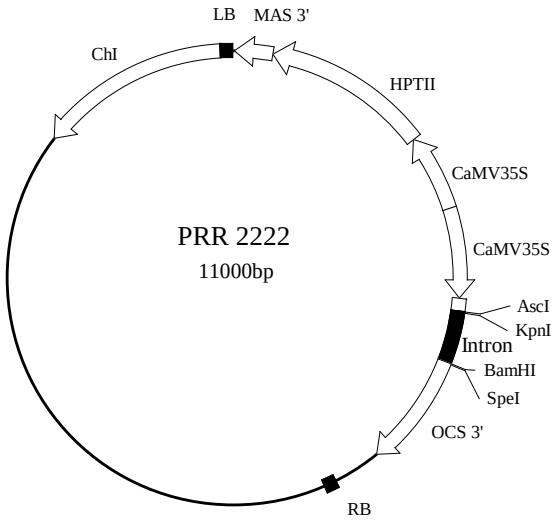

Figure 3.3. pRR2222 vector map.

RNAi target sequences were cloned to pGEM-T Easy vector and verified by sequencing. The sense strand of RNAi target was excised from pGEM-T Easy vector by *Asc*I and *Kpn*I and ligated to previously digested vector pRR2222 between the 35S promoter and the intron. The anti-sense strand of the RNAi target region was excised from pGEM-T Easy vector by *Bam*HI and *Spe*I and ligated with previously digested

vector pRR2222 between the intron and the OCS terminator. These two target sequences with opposite orientation formed the IR region for the RNAi vector (Figure 3.4).

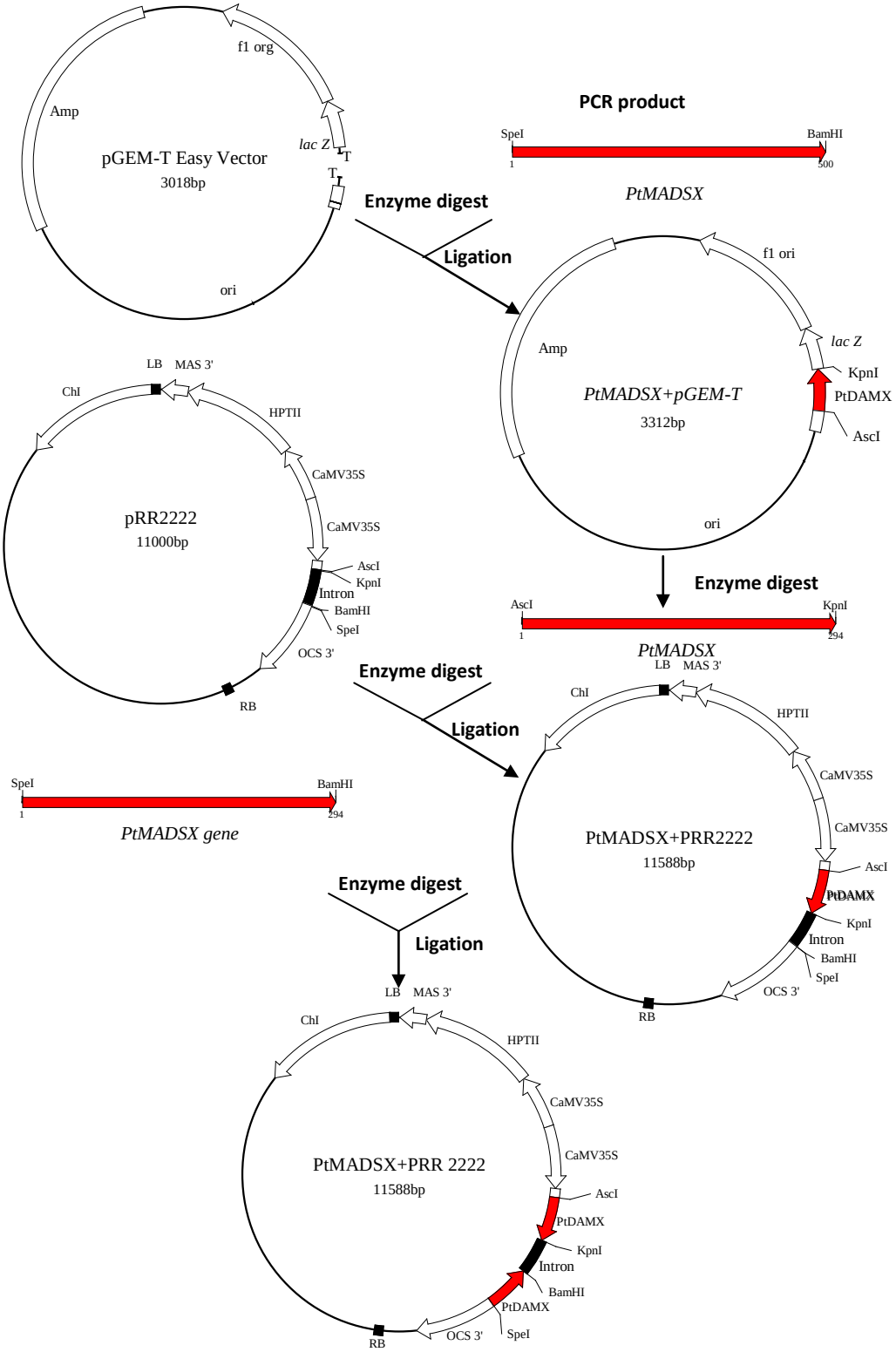

Figure 3.4. Flow chart of the *Populus* RNAi vector construction. The *PtMADSX* gene was excised from the *PpMADSX*+pGEM-T-easy vector by a double digest of *Asc*I and *Kpn*I and ligated into the pRR2222 vector. This process was repeated with *Spe*I and *Bam*HI enzymes. *PtMADSX* represents any one of the six *PtMADS* genes and 500bp was selected as the length of IR region for this figure.

Overexpression Vector Construction

The pGEM-T Easy vector containing *PtMADS* gene full-length ORF cDNA and pRR2222 were digested by *Xho*I and *Bam*HI. *PtMADS* ORF cDNA was then ligated into the pRR2222 between CaMV35S promoter and OCS terminator (Figure 3.5).

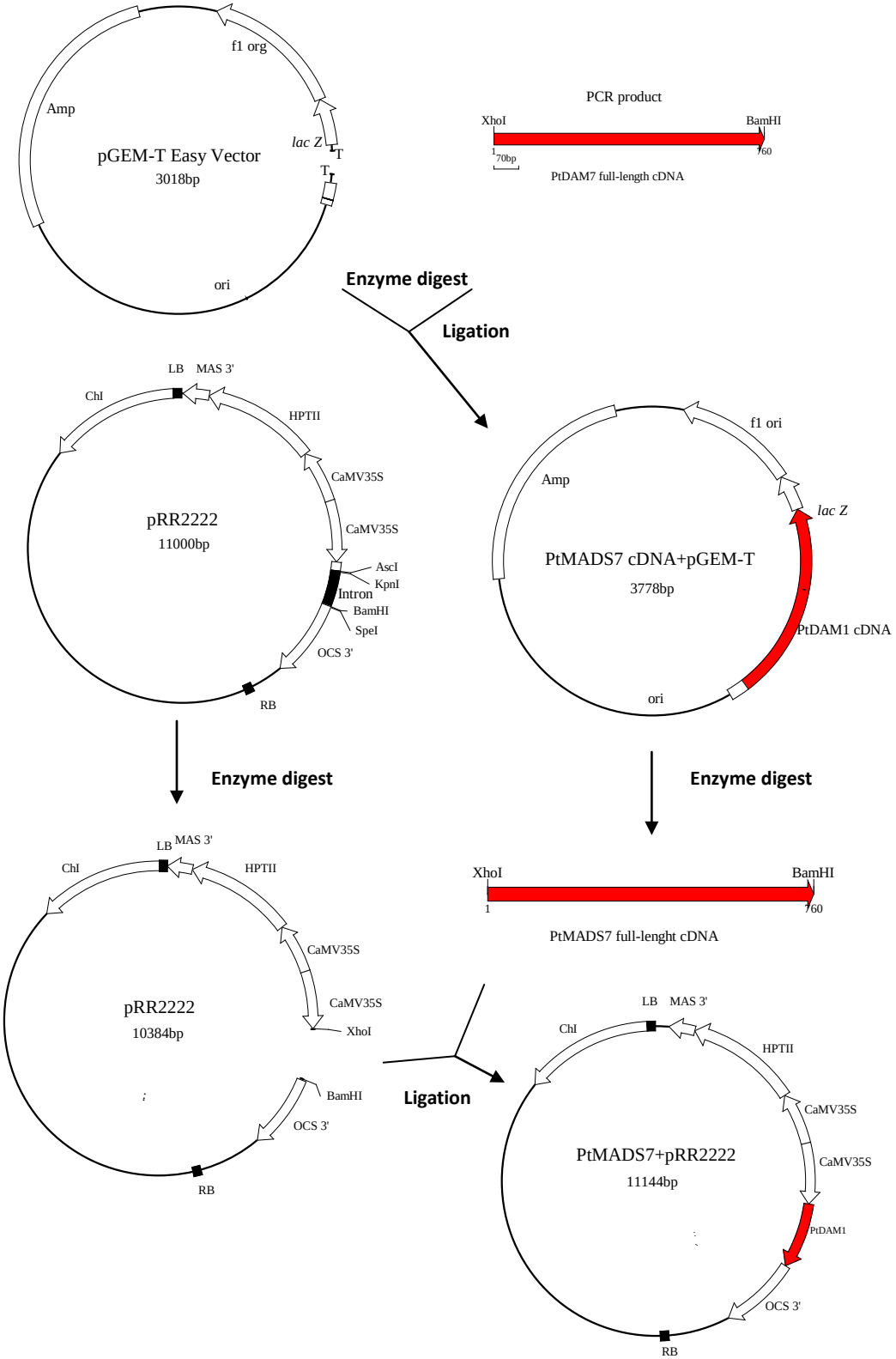

Figure 3.5. Flow chart of the *PtMADS7* overexpression vector construction. The *PtMADS7* gene excised from the PtMADS7+pGEM-T-easy vector by a double digest of *Xho*I and *Bam*HI. The intron sequence was excised from the pRR2222 vector by a double digest of *Xho*I and *Bam*HI. *PtMADS7* was ligated with pRR2222.

Agrobacterium Transformation

Agrobacterium tumefaciens strain C58/pMP90 (GV3101) was kindly provided by Dr. William Marcotte, Jr., Clemson University. DNA was transferred into *Agrobacterium* by electroporation method. Competent cells were taken out from -80 °C freezer and put on ice to thaw slowly. Then purified plasmid DNA (10-50 ng) was added directly to 50 µl competent cells and mixed by tapping the tubes. 50 µl competent cells with DNA were transferred to a 0.2-mm-gap chilled electroporation cuvette (Eppendorf, Westbury, NY). The cuvette with cells was put into chilled holder and then quickly inserted into chamber of the Eppendorf 2510 electroporator (Eppendorf, Westbury, NY). The voltage was set at 2.2 kV and then the PULSE button was pushed twice successively. One milliliter of LB medium was added to the cuvette immediately after the pulse and the cuvette was placed on ice. Contents of the cuvette were transferred to a 15 ml Falcon tube (VWR Scientific, Chicago, IL) and incubated on a roller drum at 30 °C for 2 hours. 100-200 µl of incubated cells were placed on media containing 10 µg/mL rifampicin and 30 µg/mL chloramphenicol. Plates were incubated for two days at 28-30 °C.

Transformation of Poplar

The 717-1B4 transformation protocol was based on the method described by Ma et al. (2004) and modified for use in our laboratory. Internodal stem segments (5-10 mm in length) and leaf discs (1 cm in diameter) were wounded with multiple fine cuts and then cultured at 25 °C for two days in the dark on callus-induction

medium 1 (CIM1, 4.91 g/L MS, 200 mg/L L-glutamine, 30 g/L sucrose, 5 g/L agar, 5 μM 2-(*N*-morpholino)-ethanesulfonic acid (2iP), 10 μM NAA, pH 5.7) plates (30-40 plantlets per plate). *Agrobacterium* cells carrying the binary vector were streaked on solid LB media (16 g/L Bacto-tryptone, 8 g/L Bacto-yeast extract, 5 g/L NaCl, 15 g/L agar, pH=7.0) containing chloramphenicol (30 μg/ml) and rifampicin (10 μg/ml) from frozen glycerol stock, and grown for 1 to 2 days at 28 °C. An individual colony was selected and cultured in 50 ml LB media (16 g/L Bacto-tryptone, 8 g/L Bacto-yeast extract, 5 g/L NaCl, pH=7.0) containing chloramphenicol (30 μg/ml) and rifampicin (10 μg/ml). *Agrobacterium* cells were grown for 24-36 h in LB medium (16 g/L Bacto-tryptone, 8 g/L Bacto-yeast extract, 5 g/L NaCl, pH=7.0) supplemented with the 25 mg/l chloramphenicol and 10 mg/l rifampicin on an orbital shaker at 28 °C and 250 rpm. The cells were pelleted by centrifugation at 4 °C and 3,500 rpm for 30-40 min and resuspended in *Agrobacterium* induction medium (4.91 g/L MS, 50 μM acetosyringone, 10 mM galactose, 1.28 mM 2iP, pH 5.0) at an A_{600} of 0.4-0.6. Explants (internodal stem segments and leaf discs) were transferred from CIM1 plate (30-40 plantlets per plate) to a tube containing 10-30 ml *Agrobacterium* suspension. Explants were inoculated by swirling in *Agrobacterium* suspension at 50 rpm for 1 h in the dark. The inoculated explants were dried on sterile paper for 5 min and co-cultivated on CIM1 containing 50 μM acetosyringone (30-40 explants per plate) at 25 °C in darkness for two days. Explants were washed four times in sterile deionized water and one time with washing solution (2.46 g/L MS, 1.0 μM α-naphthaleneacetic

acid (NAA), 1.0 μM 6-benzylaminopurine (BAP), 1.0 μM 2iP, 250 mg/L Ascorbic acid, 200 mg/L timentin, pH 5.7). Explants were dried on sterile paper for 5-10 min and cultured for 21 days in the dark on callus-induction medium 2 (CIM2, 4.91 g/L MS, 200 mg/L L-glutamine, 30 g/L sucrose, 5 g/L agar, 5μM 2iP, 10μM NAA, 15 mg/L hygromycin, 200 mg/L timentin, pH 5.7). Explants were transferred to shoot-induction medium 2 (SIM2, 4.91 g/L MS, 200 mg/L L-glutamine, 30 g/L sucrose, 5 g/L agar, 0.2 μM thidiazuron, 15 mg/L hygromycin, 200 mg/L timentin, pH 5.7) and cultured in sealed plates in growth room under lights. Explants were checked during this period for regenerated shoots and sub-cultured every 2-3 weeks. Explants with multiple small shoots were transferred to shoot-elongation medium (SEM, 4.91 g/L MS, 200 mg/L L-glutamine, 30 g/L sucrose, 5 g/L agar, 0.2 mg/L BAP, 15 mg/L hygromycin, 200 mg/L timentin, pH 5.7). Regenerated shoots were further screened for hygromycin resistance by rooting on half-strength MS medium supplemented with 0.5 uM IBA and 25 mg/l hygromycin.

Results

Cloning and Sequencing of the *PtMADS*s cDNA

The nucleotide sequences obtained for *PtMADS*s are 752, 738, 671, 798 and 762 bp in length, respectively, and were cloned from 717-1B4 (Table 3.4-3.8). The coding sequences begin at bp 29 for *PtMADS7* and at the first nucleotide for other *PtMADS*s. There is no other start codon before gene start codon to ensure the correct reading frame.

PtMADS21 and *PtMADS47* were not cloned from 717-1B4, but *PtMADS21* was cloned from *P. trichocarpa* Nisqually 1 clone by using primers designed for 717-1B4. *PtMADS21* are 763 bp in length and 100% match to the *P. trichocarpa* Nisqually 1 sequence (NCBI GI#: 224081932).

Table 3.4. Nucleotide sequence of the *PtMADS7* with amino acid translation.

```
1       TAACTCAAGCCAGCTCCCAATAAGTATAATGGCTAGAGAGAAGATCAAGATCAAGAAGAT
1                                    M  A  R  E  K  I  K  I  K  K  I

61      TGACAACGTGACTGCAAGACAAGTGACCTTCTCCAAGAGGAGGCGAGGACTTTTCAAGAA
12        D  N  V  T  A  R  Q  V  T  F  S  K  R  R  R  G  L  F  K  K

121     AGCTGAAGAGCTTTCTGTTCTTTGTGATGCTGAGGTTGCTGTCATCATCTTCTCTGCTAC
32        A  E  E  L  S  V  L  C  D  A  E  V  A  V  I  I  F  S  A  T

181     CGGCAAGCTCTTTGAGTATTCCAGCTCCAGCATGAAGGATGTGCTTGCAAGGTATAATCT
52        G  K  L  F  E  Y  S  S  S  S  M  K  D  V  L  A  R  Y  N  L

241     GCACTCCAATAACCTCGACAAAATAAATCAGCCGTCTCTTGAGTTGCAGCTAGAAAACAG
72        H  S  N  N  L  D  K  I  N  Q  P  S  L  E  L  Q  L  E  N  S

301     CAATCACATGCGATTGAGCAAGGAAGTTTCCGAGAAGAGTCATCAACTAAGGCGGATGAG
92        N  H  M  R  L  S  K  E  V  S  E  K  S  H  Q  L  R  R  M  R

361     AGGTGAAGATCTTCAAGGACTAAATATAGAGGAACTGCAGCAATTGGAAAAGGCGCTTGA
112       G  E  D  L  Q  G  L  N  I  E  E  L  Q  Q  L  E  K  A  L  E

421     AGTAGGACTTAGCCGTGTGCTTGAATCCAAGGGAGAACGAATTATGAATGAGATATCCAC
132       V  G  L  S  R  V  L  E  S  K  G  E  R  I  M  N  E  I  S  T

481     CCTTGAAAGGAAGGGAGTACAGCTTTTGGAAGAGAATAAGCAACTAAAACAGAAGATCGC
152       L  E  R  K  G  V  Q  L  L  E  E  N  K  Q  L  K  Q  K  I  A

541     AACCATTTGCAAGAGAAAAAGACCCGCCCTTGTTGATTTAGACACAGCAGTTCAGGAAGA
172       T  I  C  K  R  K  R  P  A  L  V  D  L  D  T  A  V  Q  E  E

601     AGGGATGTCATCGGAGTCTACAACCAATGTTTGCAGCTGCAGCAGTGGCCCTCCTGTGGA
192       G  M  S  S  E  S  T  T  N  V  C  S  C  S  S  G  P  P  V  E
```

```
661      GGACGATAGCTCCGATACCTCTCTCAAATTAGGGTTGGCCATCTGATGCTGAAAGCTGGA
212        D  D  S  S  D  T  S  L  K  L  G  L  A  I  *
```

Table 3.5. Nucleotide sequence of the *PtMADS26* with amino acid translation.

```
1        ATGGCAAGAGAGAGGATTCAGATAAAAAAGATCGATAACGCCACCGCTAGGCAAGTCACG
1          M  A  R  E  R  I  Q  I  K  K  I  D  N  A  T  A  R  Q  V  T

61       TTTTCAAAACGAAGAAGAGGGCTTTTCAAGAAAGCTGAGGAGCTTTCAGTTCTCTGTGAT
21         F  S  K  R  R  R  G  L  F  K  K  A  E  E  L  S  V  L  C  D

121      GCTGATGTTGCTGTCATCATCTTCTCCTCCACTGGCAAGCTCTTTGAGTTCTCCAGCTCA
41         A  D  V  A  V  I  I  F  S  S  T  G  K  L  F  E  F  S  S  S

181      AGCATGAAGAAAATACTGGAAAGGCATAATTTGCACTCGAAGAATCTTGAGAAGCTGGAG
61         S  M  K  K  I  L  E  R  H  N  L  H  S  K  N  L  E  K  L  E

241      CAACCATCTCTTGAGTTGCAGCTGGTAGAGGACAGCACCTGCTCCAGGTTGAGTAAGGAA
81         Q  P  S  L  E  L  Q  L  V  E  D  S  T  C  S  R  L  S  K  E

301      GTTGCGGAGAAAAGCCATCAGCTGAGGCAAATGAGAGGGGAAGATCTGCGAGGATTAAAT
101        V  A  E  K  S  H  Q  L  R  Q  M  R  G  E  D  L  R  G  L  N

361      ATAGATGAATTGCTGCAGCTAGAGAAGTCTCTTGAGGCTGGATTGAGCTGTGTGATAGAG
121        I  D  E  L  L  Q  L  E  K  S  L  E  A  G  L  S  C  V  I  E

421      AAGAAGGGTGAGAAGATTATGAACGAGATCACTGATCTTCAAAGAAAGGGAATGCAATTG
141        K  K  G  E  K  I  M  N  E  I  T  D  L  Q  R  K  G  M  Q  L

481      ATGGAAGAGAATGAGAGACTCAAACAGCAAGTGGTTGAGATAACTAATGGCCGAAAGCAA
161        M  E  E  N  E  R  L  K  Q  Q  V  V  E  I  T  N  G  R  K  Q

541      GTTACAGCTGATTCAGAGAATGTTGGTTACGAGGAAGGGCAGTCATCAGAGTCTGTAACC
181        V  T  A  D  S  E  N  V  G  Y  E  E  G  Q  S  S  E  S  V  T

601      AATGTCTGCAACTCAAATGGCCCCCTACATGATTATGAAAGCTCTGATACATCCCTCAAG
201        N  V  C  N  S  N  G  P  L  H  D  Y  E  S  S  D  T  S  L  K
```

```
661     TTGGGGTTGCCATTTTCAAACTGAATGGGAGATCGCAGGGAGATTTGTTCACCAGCAAAT
221       L  G  L  P  F  S  N  *
```

Table 3.6. Nucleotide sequence of the *PtMADS29* with amino acid translation.

```
1       ATGACTCGAAGGAAAATCCAGATCAAGAAGATAGACGACACAATCGCAAGACAAGTTACT
1         M  T  R  R  K  I  Q  I  K  K  I  D  D  T  I  A  R  Q  V  T

61      TTCTCAAAGAGGAGAAGAGGGCTTTTCAAGAAAGCTTATGAGCTCTCAACTCTATGTGAT
21        F  S  K  R  R  R  G  L  F  K  K  A  Y  E  L  S  T  L  C  D

121     GCTGAGATTGCTCTCATGGTGTTTTCTGCATCNGGCAAGCTTTTTGAGTACTCAAACTCA
41        A  E  I  A  L  M  V  F  S  A  S  G  K  L  F  E  Y  S  N  S

181     AGCATGGAGCAAGTGATTGAAAGGCGCAATCTACATCAAAAGAACATCGGTCAACCATCT
61        S  M  E  Q  V  I  E  R  R  N  L  H  Q  K  N  I  G  Q  P  S

241     CTTGAGCTGCAGCCTGATGATGATGTGCATGCCACGCTGAACAAAGAAATAGCCGAGAAA
81        L  E  L  Q  P  D  D  D  V  H  A  T  L  N  K  E  I  A  E  K

301     ACCCGTGAACTGAGCCAGTTGAGGGGAGAAGACCTACAAGGATTGAACTTGGAAGAGCTA
101       T  R  E  L  S  Q  L  R  G  E  D  L  Q  G  L  N  L  E  E  L

361     CATAAATTAGAAAAATTAATCAAAACAAGCTTGCGTCGTGTCGTGGAAGAAAAGGGGGGT
121       H  K  L  E  K  L  I  K  T  S  L  R  R  V  V  E  E  K  G  G

421     AAAATTATAAACGAGATCAATACTCTCAAGAACGAGGGGGAGCAATTAGTAGAAGAGAAC
141       K  I  I  N  E  I  N  T  L  K  N  E  G  E  Q  L  V  E  E  N

481     TGGCGATTGAAGCAGCAAGTGATGAATCTATCAGCAGGCCGAAGGCATTTGCTCCAACCA
161       W  R  L  K  Q  Q  V  M  N  L  S  A  G  R  R  H  L  L  Q  P

541     GACAAGTCATCAGATTCTCTGGTGACCACTGCCAGGAGCATGAGCTCAGCTGATCCTTGT
181       D  K  S  S  D  S  L  V  T  T  A  R  S  M  S  S  A  D  P  C

601     CAGGACTGTGACAGCCCCTGCGCTTTTCTTACACTAGGGTTACCTTTTCGTGATTGAATG
201       Q  D  C  D  S  P  C  A  F  L  T  L  G  L  P  F  R  D  *
```

Table 3.7. Nucleotide sequence of the *PtMADS28* with amino acid translation.

```
1        ATGACTCGAAAGAAAATCCAGATCAAGAAGATCGACAACATCGCTGCAAGGCAAGTTACT
1          M  T  R  K  K  I  Q  I  K  K  I  D  N  I  A  A  R  Q  V  T

61       TTCTCAAAGAGGAGAAGAGGGCTTTTCAAGAAAGCTTACGAGCTCTCAACTCTATGTGAT
21         F  S  K  R  R  R  G  L  F  K  K  A  Y  E  L  S  T  L  C  D

121      GCTGAGATTGCTCTCATTGTGTTTTCTGCAACTGGCAAGCTTTTTGAGTACTCAAACACA
41         A  E  I  A  L  I  V  F  S  A  T  G  K  L  F  E  Y  S  N  T

181      AGCATGGGGGAAGTGATTGAAAGGCGCAGTCTGCATCCAAAGAACATCAACACCCTTCAT
61         S  M  G  E  V  I  E  R  R  S  L  H  P  K  N  I  N  T  L  H

241      CAACTATCTCTTGGGCAGCAGCTTGATGGTGGTGTGCATGCCATGCTGATCAAAGAAATA
81         Q  L  S  L  G  Q  Q  L  D  G  G  V  H  A  M  L  I  K  E  I

301      GCAGAGAAAAACCGTGAACTGAGGCACATGAGGGGAGAAGACCTACAAGTATTGAGCTCG
101        A  E  K  N  R  E  L  R  H  M  R  G  E  D  L  Q  V  L  S  S

361      GAAGAACTGAAAAAATTAGAAAAATTAATTGAAGGAAGCTTGCGTCGAGTGGTCGAGGAA
121        E  E  L  K  K  L  E  K  L  I  E  G  S  L  R  R  V  V  E  E

421      AAGGAGGAAAAAAGCATGAAGGAGATCGATGCTCTCAAGGCCAAGGGGGAGCAATTAGCA
141        K  E  E  K  S  M  K  E  I  D  A  L  K  A  K  G  E  Q  L  A

481      GAAGAGAACCAGCGATTGAAGCAACAAGTGATGAATTTATCAGCAGCACAAGGGCATTTG
161        E  E  N  Q  R  L  K  Q  Q  V  M  N  L  S  A  A  Q  G  H  L

541      CTCGAACCAGGCCAGTCCTCAGATTCTTTAGTGACCAATATCAGCAGTATGAGCTCAGCT
181        L  E  P  G  Q  S  S  D  S  L  V  T  N  I  S  S  M  S  S  A

601      GATCCTCGCCAGGACAACGATAGCTGTTTTGCTTTTCTTACACTAGGGTTACCTTTTCCT
201        D  P  R  Q  D  N  D  S  C  F  A  F  L  T  L  G  L  P  F  P

661      GATTGAATCCGAAAGATGGAGGGTTATCCAGCAATATGTAATGGCTTAGAAGTCATCAGT
221        D  *
```

Table 3.8. Nucleotide sequence of the *PtMADS48* with amino acid translation.

```
1       ATGACTCGCAAGAAAATCCCGATCAAGAAGATAGACAACACAACTGCAAGGCAAGTAAGT
1         M  T  R  K  K  I  P  I  K  K  I  D  N  T  T  A  R  Q  V  S

61      TTCTCAAAGAGGAGAAGAGGGCTCTTCAAGAAAGCTTGCGAGCTCTCAATTCTATGTGAT
21        F  S  K  R  R  R  G  L  F  K  K  A  C  E  L  S  I  L  C  D

121     GCTGAGATTGCTCTCATGGTGTTTTCTGCAACTGGAAAGTTTTTTGAGTACTCAAACTCA
41        A  E  I  A  L  M  V  F  S  A  T  G  K  F  F  E  Y  S  N  S

181     AGCATTGGGCAAGTGATAGAAAGGCGTAATCTGCATCCCAAAAACCTTGACACATTTAGT
61        S  I  G  Q  V  I  E  R  R  N  L  H  P  K  N  L  D  T  F  S

241     CAACCATCGGTTGAGCTTCAGCTTGATAGTGCTGTGCATGCCATGTTGAACAAGGAAATA
81        Q  P  S  V  E  L  Q  L  D  S  A  V  H  A  M  L  N  K  E  I

301     GCAGAGAAAACCCGTGAACTGAGGCGAACAAGGGGAGAAGACCTGCAGGGATTGAACATG
101       A  E  K  T  R  E  L  R  R  T  R  G  E  D  L  Q  G  L  N  M

361     GAAGAATTAGAAAAATTAGAAAAATTAATTGAAGGAAGCTTGTGTCGTGTCATGGAAACA
121       E  E  L  E  K  L  E  K  L  I  E  G  S  L  C  R  V  M  E  T

421     AAGGGAGAGAAAATTCTAAAGGAGGTCGATGCTCTCAAGTCCAAGGAGCAACAACTAATG
141       K  G  E  K  I  L  K  E  V  D  A  L  K  S  K  E  Q  Q  L  M

481     GAAGAGAACCAGCGATTGAAGCAGAGATTAATGAATTTATCGAAGGGCCAAGGGCATTTG
161       E  E  N  Q  R  L  K  Q  R  L  M  N  L  S  K  G  Q  G  H  L

541     CTCGAACAAGGCCAGTCGTCAGATTCTATGGTGACCAATATCAGCAGCACCTCAGCAAAT
181       L  E  Q  G  Q  S  S  D  S  M  V  T  N  I  S  S  T  S  A  N

601     CCTCGTCAGGACCACGACAACGTGTGTTCTTTTCTTAGACTTGGGTTACCTTTTCCTGAT
201       P  R  Q  D  H  D  N  V  C  S  F  L  R  L  G  L  P  F  P  D

661     TGAATCCGAAAGATGGAGGATTTCCAACAAAATTTTTCAAATAAAACCTTCATCCTGGCT
221       *

721     TGTATTTTTTTAATATTTGCTTGTTGATTCAAGAAATTC
241
```

Cloning and Sequencing of the *PtMADS*s RNAi region

The six pairs of nucleotide sequences obtained for RNAi target by PCR ranged from 178 to 1312 bp in length (Table 3.9-3.14). BLASTn search showed no hit in the *Populus trichocarpa* genome sequence database (JGI) and ESTs database (NCBI) except target itself. Sequencing results showed RNAi target regions were inserted into pRR2222 between the 35S promoter/intron and the OCS terminator/intron with an opposite direction.

Table 3.9. Nucleotide sequence of the *PtMADS7* 3' UTR used for RNAi target.

```
1     GATTTAGACA CAGCAGTTCA GGAAGAAGGG ATGTCATCGG AGTCTACAAC CAATGTTTGC
61    AGCTGCAGCA GTGGCCCTCC TGTGGAGGAC GATAGCTCCG ATACCTCTCT CAAATTAGGG
121   TTGGCCATCT GATGCTGAAA GCTGGAGGAA AATGAAGTGA TCCAGTAGAA TCTTGCGAGG
181   AACAGATCTG CTATCGCTGG AAGATCGACT TATCCCTTGA GATACTGGTA TTTATATATG
241   TTTGAGTTGA TGTGACAATG AAGCTGGATT CGCTTTCTTC ACCTGAAGAA CTACTGTATC
301   TATTTGTGAT GTGTGTGAAA TCTAAAACTG AAAATATGTT CCCGGGACTT CAACACGGTC
361   CTAAATAAAT CAACTGTGAA TTGATC
```

Table 3.10. Nucleotide sequence of the *PtMADS26* 3' UTR used for RNAi target.

```
1     CTCTGATACA TCCCTCAAGT TGGGGTTGCC ATTTTCAAAC TGAATGGGAG ATCGCAGGGA
61    GATTTGTTCA CCAGCAAATG CGGACACAAA TAATTGCTTG TATATATAAA TAGAACAACT
121   CTGGCACATA GTACTTATGT ACCTTGGACA AGTGCAGGGA AACATTTGTG TTTCCTTCAC
181   CGGATGCTAC TCATGGAGTT TCTCTTATTG TGTGCACGAG GGTTTGCAGC CCGTCTTTGT
241   GTTACGAGGA AGGGCAGTCA TCAGAGTCTG TAACCAATGT CTCCAACTCA AATGGCCCCC
301   TGCATGATTA TGAAAGTGCA TAAGGAAGAA GAACAGAAAA AAACCAAATG CGCTTATTGT
361   TTTTGC
```

Table 3.11. Nucleotide sequence of the *PtMADS27* 3' UTR used for RNAi target.

```
1     TTGATCCTCG CCAGGACTGT GACAGCTCTT GTGCCTTTCT CAAACTAGGG CTACCATTTC
61    CTGATTGAAT CTGGGAGATG GAGGGTTCTA GCAAGTGTTT AATGCCTTAG CAGTCATCCA
121   TTTAACTTAA TTAACGGTGT GTCGCGATCG CCTTCAAATA CAATTTCATC TTTGCATGTA
181   GTTAATAAAA TTCCTGTGAG CCCTTTTCAT GTCTGCTTGT TGATTAGAGA AATTTGTAGT
241   GTTGTGTGTG TGTGTGTGTA GATGAACCAA GGCTTCTCTT GTTTCCTTAA ATTAGGAGGA
301   AATGAGTTGT AGCTAGTGGA AGGAGGAGTT GTATCTACCT TTTTATGGTA TTGTAGCAGA
```

```
361    TTTGGACG
```

Table 3.12. Nucleotide sequence of the *PtMADS29* 3' UTR used for RNAi target.

```
1      AGGATTGAAC TTGGAAGAAC TACATAAATT AGAAAAATTA ATCAAAACAA GCTTGCATCG
61     TGTCGTGGAA GAAAAGGGGG GTAAAATTAT AAACGAGATC AATACTCTCA AGAACGAGGG
121    GGAGCAATTA GTAGAAGAGA ACTGGCGATT GAAGCAGCAA GTGATGATAT CAGCAGGCCA
181    AAGGCATTTG CTCGAACCAG ACAAGTCATC AGATTCTCCG GTGACCAATA CCAGGAGCAT
241    GAGCTCAGCT GATCCTTGTC AGGACTGTGA CAGCCCTTGC GCTTTTCTTA CACTAGGGTT
301    ACCTTTTCGT GATTGAATGG GAAAGATGGA GGCTCATCCA GCAAAAGTGT TTTTAGCTTG
361    ATCTAAATAA ATAATAATAA AAAAGAACAT TTAATTTTAA CACATGTACC AATACTGAAC
421    AGAGAATATC CCCTGCATTA ATTGTGGTCT TCTTTTCTAA ATAGACCTTA AAGAATCCTT
481    GAATTAATTT ACACGTTAAT ATTGTT
```

Table 3.13. Nucleotide sequence of the *PtMADS28* 3' UTR used for RNAi target.

```
1      TTCTTACACT AGGGTTACCT TTTCCTGATT GAATCCGAAA GATGGAGGGT TATCCAGCAA
61     TATGTAATGG CTTAGAAGTC ATCAGTTTAA TTAATAGTGT ATTAACACTC CTTGCAAATA
121    AAATATATTA CATCTTGGCT TGTAGTTAAT AAAATTCATA CCCCAGATTC TTTAGTGACC
181    AATATCAGCA GTATGAGCTC AGCTGATCCT CGCCAGGACA ACGATAGCTG TTTTGCTTTG
241    AGCACTTTTC ATGTCTGCTT GTAGTTAATA ATATTCATGT CTGCTTGTTG ATTC
```

Table 3.14. Nucleotide sequence of the *PtMADS48* 3' UTR used for RNAi target.

```
1      AGGAAGCNTT GTGTCGTGTC ATAGAAACAA AGGGAGAGAA AATTCTCAAG GAGGTNGACG
61     CTCTCAAGTC CAAGGAGCAA CAACTAATAG AAGAGANCCA GCGATTGAAG CAGAGATTAA
121    TGAATTTATC AAAGGGCCAA GGGCATTTGC TCGAACAAGG CCAGTCATCA GATTCTATGG
181    TGACCAATAT CAGCAGCAAC TCAGCAAATC CTCGTCAGGA CTGCGACAAC GTGTGTTCTT
241    TTCTTACACT TGGGTTACCT TTTCCTGATT GAATCCGAAA GATGGAGGAT TTCCAACAAA
301    ATTTTTCAAA TAAAACCTTC ATCCTGGCTT GTACGTGGTT AATATTTGCT TGTTGATTCA
361    AGAAATTCAC AGGGTTTAAG ATACAGATCA TGTATGTGTG GTTGTGTATA CATGTATATG
421    TGGATCAAGG GTTCCCATAT TTCCTTAAGG TAAAAGGAAA TGAGGTGAAG CTGTTGAAAC
481    ATGTAGTAAA ATATATATTA ATATAGCTTG AGTTGAAGCT AAATTAGTAT ATATGTATGC
541    TATGTCTTGC TTAATTTTAT TGCAGGGACT GAACATGGAA GAANTAGAAA AATTAGAAAA
601    ATTAATNGAT TCAATCTCAG TGGGGGAAAG GATAGGAATA TGCATCCACA AGACTCTTTA
661    ATTATCAGTT TGAGATTCTT TGCT
```

Poplar Transformation

The resulting *PtMADS7* and *PtMADS26* RNAi vectors, *PtMADS7*, *PtMADS26* and *PtMADS48* overexpression vectors and the empty vector pRR2222 were

introduced into wild-type hybrid poplar clone 717-1B4 through *Agrobacterium*-mediated transformation. In total, 128 putative transgenic lines (141 plants in shoot form) survived on the selection media and most of them are *PtMADS7* OE lines (Table 3.15, Table 3.16 and Figure 3.6).

Table 3.15. Effect of different *PtMADS* gene vectors on the transformation efficiency.

Vectors	No. of Explants Inoculated	No. of Green Calli Formed (%)	No. of Putative Transgenic Shoots (%)
PtMADS7 OE	2460	227 (9.2)	130 (5.2)
PtMADS7 RNAi	2405	0 (0)	0 (0)
PtMADS26 OE	180	13 (7.2)	0 (0)
PtMADS26 RNAi	200	17 (8.5)	6 (3)
PtMADS48 OE	150	16 (10.7)	5 (3.3)
PtMADS48 RNAi	165	0 (0)	0 (0)

Table 3.16. Putative poplar transgenic plants on different media. SIM, shoot-induction medium; SEM, stem-elongation medium; OE, overexpression.

Genotype	Medium	Number of lines per genotype
PtMADS7 OE	SIM, SEM	117
PtMADS26 RNAi	SIM	6
PtMADS48 OE	SIM	5

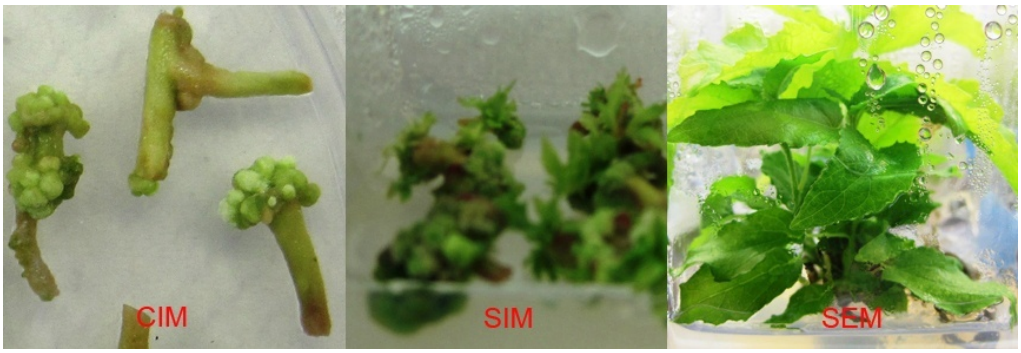

Figure 3.6. *PtMADS7* OE lines on CIM, SIM and SEM.

Discussion

Cloning of *PtMADS21* and *PtMADS47*

Eight *PtMADS* genes were identified in the sequenced *P. trichocarpa* genome. However, only six *PtMADS* genes were cloned from hybrid poplar clone 717-1B4. Cloning of *PtMADS21* and *PtMADS47* were unsuccessful, despite of the fact that several different tissues (root, leaf, and stem) were included for amplification. However, *PtMADS21* was successfully cloned from *P. trichocarpa* Nisqually 1 clone using same primers designed for 717-1B4 without any difficulty. Two hypotheses are proposed here based on this result.

First, *PtMADS21* and *PtMADS47* are expressed in 717-1B4 poplar but cDNA was not amplified by PCR in our experiment. This may caused by low expression level of these two genes in the tissues and sample conditions. In tomato most of the $MIKC^{C}$-type MADS-box genes were expressed only in reproductive tissues, while fewer genes showed a broad pattern of expression, with mRNA detected in all, or nearly all, tissues including both vegetative and floral tissues (Hileman et al., 2006). For the five grapevine *SVP/StMADS11* group MADS-box genes, *VvSVP2* and *VvSVP5* were expressed in shoots and leaves while the other three *SVP* genes (*VvSVP1*, *VvSVP3*, and *VvSVP4*) were preferentially expressed in buds Diaz-Riquelme et al., 2009). Expression of the *DAM* genes in peach is not limited to the terminal tissues or tissues preparing to undergo developmental changes associated with the formation of dormant structures such as vegetative and floral buds (Li et al., 2009). Each of the genes has ubiquitous expression in peach with the exception of *DAM1* and *DAM6*,

which were not expressed in flower structure and developing seed respectively (Li et al., 2009). Since the MADS-box genes were detected in various tissues, more tissues, such as tip, flower and buds should be used to isolate the *PtMADS* genes. In our experiment, because reproductively competent trees are not available for us, we only attempted to isolate these genes from the vegetative tissues of the plant. Therefore it is conceivable that the expression of these genes were not detected in our experiment if they are only expressed in reproductive tissues. This limitation of sampling tissue would preclude these genes involvement in seasonal dormancy of vegetative tissues and would reduce the number of genes of interest.

It is possible that the expression level of *PtMADS21* and *PtMADS47* is low when the tissues were sampled. In our experiment, poplar trees grown in greenhouse under 16 hours lights were sampled in May 2007. Semi-quantitative PCR showed the expression of peach *DAM1*, *DAM4*, *DAM5* and *DAM6* reached the lowest point in May under field condition (Li et al., 2009). The expression level of *PtMADS21* and *PtMADS47* may be affected by day length or other factors which are unavailable under our greenhouse condition with a constant 16 hours light. To solve this issue, poplar should be grown under natural lights and sampled in different times during a growth cycle.

Second, it is possible that *PtMADS21* and *PtMADS47* are not expressed in the 717-1B4 clone. This hypothesis can be divided into two sub-hypotheses: *PtMADS21* and *PtMADS47* do not exist in 717-1B4 genomic DNA or these two genes are present

but have lost their expression in the 717-1B4 clone. Genomic DNA PCR or Southern analysis could be conducted to check whether these two genes are present in 717-1B4. It is interesting to note that both *PtMADS21* and *PtMADS47* are members of a highly similar gene pairs, possibly arising from duplication events. *PtMADS21* is very similar to *PtMADS7* and *PtMADS47* appears to be a tandem duplication of *PtMADS46* (Jiminez et al., 2009). Poplar genome is highly duplicated and loss of gene function is one of the potential fates of duplicated genes (Tuskan et al., 2006). It is therefore possible that these similar, possibly duplicated genes have lost functionality in the 717-1B4 hybrid or were not functional in the parental germplasm.

Poplar Transformation

This study was not able to obtain the *PtMADS7* and *PtMADS48* RNAi vector transgenic plants. Although about the same amount of explants were used for transformation for RNAi and OE vectors, no putative transgenic lines were obtained for RNAi vector while 135 putative OE transgenic shoots survived on the selection media. It is possible that silencing of *PtMADS7* and *PtMADS48* affects the regeneration of the callus.

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CHAPTER FOUR

EXPRESSION PROFILE OF *PtMADS* GENES DURING DORMANCY ESTABLISHMENT IN POPLAR

Introduction

In peach, a deletion at the *EVG* locus leads to a loss of expression of six tandem-duplicated *DAM* genes in the mutant *evg* genotype (Bielenberg et al., 2008). Targeted gene expression profiling is an important method to illuminate the association of a specific gene or group of genes with a mutant phenotype. Bielenberg et al. (2008) evaluated expression of the six *DAM* genes in wild-type peach at March, June, September, and December during the annual growth cycle. *DAM1*, *2*, and *4* were expressed in June and September; *DAM3* was expressed in June, September and December, and *DAM5* and *6* were expressed only in September and December (Bielenberg et al., 2008). Although these six *DAM* genes have high sequence similarity and were tandem duplicated, the differing expression patterns suggest that they may not be functionally redundant (Jimenez et al., 2009).

Li et al. (2009) evaluated expression of the six *DAM* genes in wild-type peach every two weeks for an entire year in field-grown conditions and in a nine week controlled environment experiment. In field-grown conditions, *DAM2* expression peaks at the summer solstice while *DAM1* and *DAM4* peak approximately at the date of terminal bud set. *DAM5* and *DAM6* peak in expression at the winter solstice (Li et al., 2009). The coincidence of the expression timing of *DAM1*, *DAM2*, and *DAM4*

with the cessation of terminal growth and bud set suggests that these genes are stronger candidates for control of the *evg* non-dormant phenotype.

As outlined in the previous chapter, peach currently lacks a stable transformation system for functional testing of candidate genes. We are using the hybrid poplar (INRA 717-1B4) as a model system for testing the function of *DAM* gene homologs in this clone. In parallel to the creation of overexpression and knockdown vectors for the hybrid poplar *DAM* homologs, we investigated the seasonal expression profile of the hybrid poplar putative homologs. Phylogenetic analysis of the sequenced *P. trichocarpa* genome discovered eight genes of the *SVP/StMADS11* clade of MIKC^{C} MADS-box genes (Jimenez et al., 2009). Although *PtMADS7* and *PtMADS21* are more closely related to the peach *DAM* genes than other *SVP/StMADS11*family genes in *Arabidopsis* and *P. trichocarpa*, phylogenetic analysis has not identified clear *P. trichocarpa* homologs for specific peach genes, primarily because the peach genes form a monophyletic group (Jimenez et al., 2009). In this condition, corresponding expression patterns between peach and *P. trichocarpa* genes might provide a clue for identifying clear *P. trichocapa DAM* homologs and further connecting their function. However, there is no existing specific expression data for these genes in *P. trichocarpa* or any other *Populus* species. Similar patterns in expression profiling of specific peach and poplar genes may provide evidence that the genes share a regulatory role in these two species and allow for prioritizing of reverse genetics experiments to determine gene function.

As detailed in the previous chapter, only six of the putative eight *DAM* homologs were isolated from the hybrid poplar clone INRA 717-1B4. Here I report the results of an experiment profiling the expression of these six genes during the seasonal transition from summer to winter under natural photoperiod and temperature conditions.

Materials and Methods

Plant materials and growth conditions

Rooted cuttings of hybrid poplar clone INRA 717-1B4 were grown for three months in pots under natural light in the BRC facility greenhouse, located in Clemson University, Clemson, South Carolina (Figure 4.1). Trees were then transferred to a covered growth cage under ambient temperature and light conditions (Figure 4.2). Trees were watered as needed. Beginning in June 2009, three trees were randomly selected for sampling of the most recently mature leaf and the apical tip/bud every two weeks. Sampling continued through December 2009. At each sampling date, the elongation growth of the tree was measured by recording the length from the tip to the first internode marked at the beginning of the experiment. Elongation growth is shown as change in growth from the beginning of the experiment.

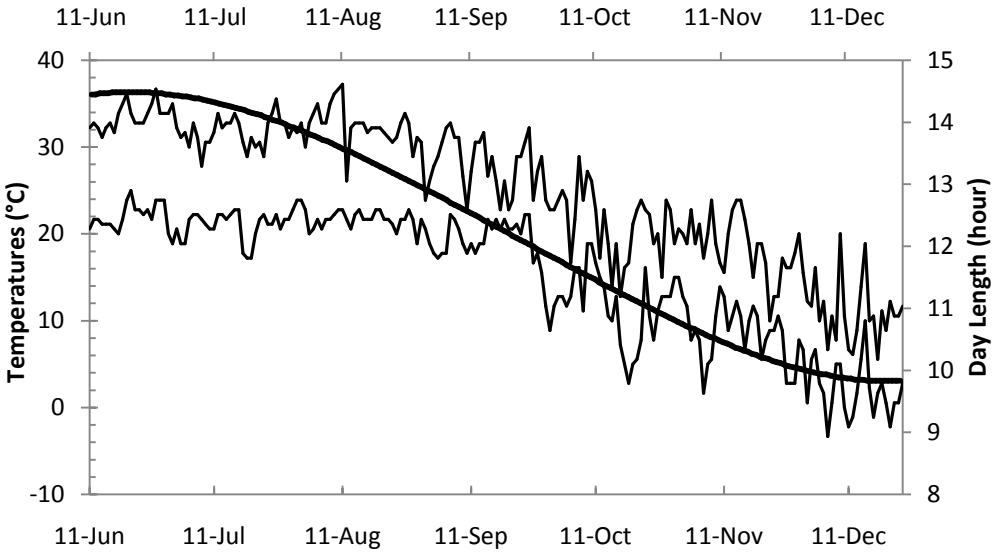

Figure 4.1. Temperatures and day length during experiment. Daily maximum (top line) and minimum (bottom line) temperatures and day length (smooth line) from June 11th to December 24th 2009, at the Clemson University, SC, USA. Temperature data were from www.wunderground.com (Clemson, US.) and day length data were from the U.S. Naval Observatory (Clemson, US.) (http://www.usno.navy.mil/).

RNA Extraction and cDNA Synthesis

RNA extraction and cDNA synthesis were conducted as in Chapter Three.

Designing primers

Quantitative Real Time PCR (RT-PCR) primers for the six *PtMADS* genes were designed from the cDNA sequences isolated from the INRA 717-1B4 clone. In order to avoid the cross amplification between the gene family members, gene-specific regions were chosen from 3' UTR or 3' end of coding region for each gene as amplification targets (Table 4.1). Since only six *PtMADS* genes were cloned from 717-1B4 clone (Chapter Three), I only designed primers for these genes. Primers of candidate reference genes were designed based on the *Populus* EST sequences from the NCBI database. The amplification product size for the RT-PCR was designed to between 70-200 bp with a target size of 100-150 bp. Larger or smaller

amplification products were required for some primer pairs to ensure gene specificity (Marino et al., 2003).

Table 4.1. Gene-specific primer pairs used for quantitative RT-PCR.

Gene	Primer pair	Sequence (5'->3')	RT-PCR product size (bp)
PtMADS7	*PtSVP1* RT F	CCAGTAGAATCTTGCGAGGAACAG	125
	PtSVP1 RT R	TCAGGTGAAGAAAGCGAATCCAG	
PtMADS26	*PtSVP3* RT F	AATGGGAGATCGCAGGGAGATT	162
	PtSVP3 RT R	GAGAAACTCCATGAGTAGCATCCG	
PtMADS27	*PtSVP4* RT F	CAGGACTGTGACAGCTCTTGT	73
	PtSVP4 RT R	CCTCCATCTCCCAGATTCAATCAG	
PtMADS29	*PtSVP5* RT F	CAGATTCTCTGGTGACCACTGC	124
	PtSVP5 RT R	GCCGCCATCTTTCCCATTCAA	
PtMADS48	*PtSVP7* RT F	TCTCAAGTCCAAGGAGCAACAACT	193
	PtSVP7 RT R	ACCCAAGTCTAAGAAAAGAACACACG	
PtMADS28	*PtSVPX* RT F	GATCCTCGCCAGGACAACGATA	82
	PtSVPX RT R	CCTCCATCTTTCGGATTCAATCAGG	
PtACT2	*PtACT2* RT F	CTGAAGAGCACCCAGTCCTC	176
	PtACT2 RT R	AGAATCCCACCCCGATACCAG	
CF228206	*CF228206* RT F	GCTAAAGTTGGCAGTTGAGAAGAAG	115
	CF228206 RTR	GGCAGCATCCAAACTCAGTTC	
PtEF1	*PtEF1* RT F	AAGAGGACAAGAAGGCAGCAG	145
	PtEF1 RT R	CTGACTGCCTTCTCCAACTCC	
PtTUA	*PtTUA* RT F	TCTGGTTTGGGGTCTTTGTTGTTAG	112
	PtTUA RT R	GCTCCACAACTGCTGTTGAGA	

Validation of RT-PCR primers

RT-PCR primers were first verified by normal PCR. The PCR products were cloned into pGEM-T Easy vector according to the manufacturer's protocol (Promega, Madison, WI). Cloned fragments were sequenced with SP6 and T7 primers using the ABI Dye Terminator kit.

RT-PCR

RT-PCR was performed as described in Jimenez et al. (2009). cDNA samples were diluted with water to obtain a concentration of 2.3 ng/µl of the original total RNA sample. RT-PCR reactions were performed on an iCycler iQ^{TM} system (Bio-Rad, Hercules, CA, USA) using the iQ SYBR-Green Supermix (Bio-Rad, Hercules, CA, USA). Gene-specific primers for each of the selected genes were used to amplify products from cDNA samples, which were synthesized with the SuperScript III first strand synthesis system (Invitrogen, Carlsbad, CA, USA) for reverse transcription PCR (Table 4.1). Three technical replications for each of the three biological replicates were performed. PCR reactions were set up in 25 µl volumes. PCR was conducted with the following program: an initial DNA denaturation step at 95°C for 180 s, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Finally, a melting curve was performed on each reaction to check for multiple PCR products. PCR products were checked with 2% agarose gel in 1× TAE with ethidium bromide.

Fluorescence values were baseline-corrected and averaged efficiencies for each gene and Ct values were calculated using LinRegPCR program (Ruijter et al., 2009). Relative gene expression levels were determined with the Gene Expression C_T Difference (GED) formula which was derived from the GED formula in Schefe et al. (2006).

Selecting a Reference Gene

The expression of *Elongation factor 1* (*PtEF1*) and *α-tubulin* (*PtTUA*) from *Populus*, *actin2* (*PaAct2*) from *Picea abies* and *at5g12240* from *Arabidopsis* are highly stable and their C_T values are between 20-30, which are close to the C_T value of the six *PtMADS* genes in our study (Brunner et al., 2004; Yakovlev et al., 2006; Czechowski et al., 2005). *PtEF1, PtTUA, PtACT2* (*PaAct2 Populus* homolog) and *CF228206* (*Populus* homolog of *at5g12240*) were chosen as candidate reference genes (Table 4.2). geNorm software identified *PtEF1* as the most stable reference gene among these four candidates (Vandesompele et al., 2002). *PtEF1* was selected as the reference gene for this experiment against which to normalize target gene expression levels.

Table 4.2. Quantitative RT-PCR reference genes.

Reference gene	**Genebank accession #**	**PCR product size (bp)**	**Tested homolog genes accession #**	**Species in which homolog genes were tested**
PtACT2	CF233920.1	176	AY961918	*Picea abies*
CF228206	CF228206.1	115	at5g12240	*Arabidopsis*
PtEF1	CX659475.1	145	BI125345	*Populus*
PtTUA	CX660288.1	112	CA822230	*Populus*

Statistical Analysis

Statistical testing of quantitative expression level between 0 week (June 11th) and the following sampling dates was performed with the Mann-Whitney-Wilcoxon test ($P < 0.05$). Analyses were performed using the statistical software version package of SAS v.9.1.3 (SAS Institute Inc., Cary, NC, USA).

Results

Elongation growth of 717-1B4 during the transition from summer to winter

Hybrid poplar clone 717-1B4 was actively growing at the beginning of the experiment, prior to summer solstice. Elongation growth of 717-1B4 ceased between July 9th and July 23rd, approximately three weeks following summer solstice (June 21st). Terminal buds became visible immediately following growth cessation (Figure 4.2 and 4.3).

Figure 4.2 Hybrid poplar apical tip growth under natural photoperiods from June to December, 2009.

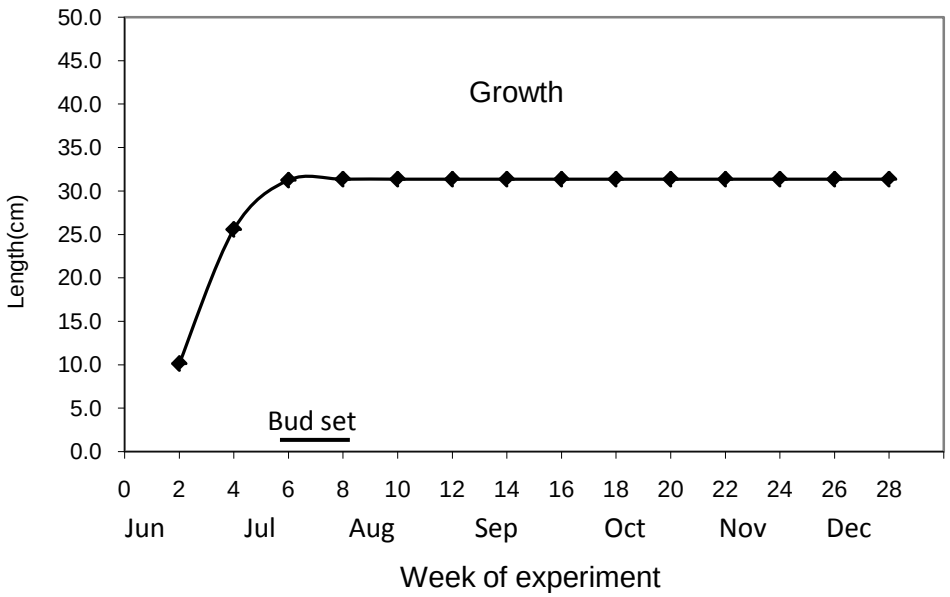

Figure 4.3. The growth of poplar during seasonal transition. The lengths from first internodes to the tip were matured from 0 week (06/11/09) to 28 week (12/24/09).

*PtMADS*s Expression in 717-1B4

Expression of the six *PtMADS* genes was measured in leaves and apical tips/buds. In leaf tissue, the six *PtMADS* genes all showed a similar pattern of slowly increasing gene expression with time (Figure 4.4). In apical tissues, five of the genes showed a very low and steady level of expression throughout the sampling dates (Figure 4.5). *PtMADS28* showed a higher relative expression than the other genes in apical tips/buds and the expression level fluctuated with time, but none of these changes was statistically significant analyzed by the Mann-Whitney test.

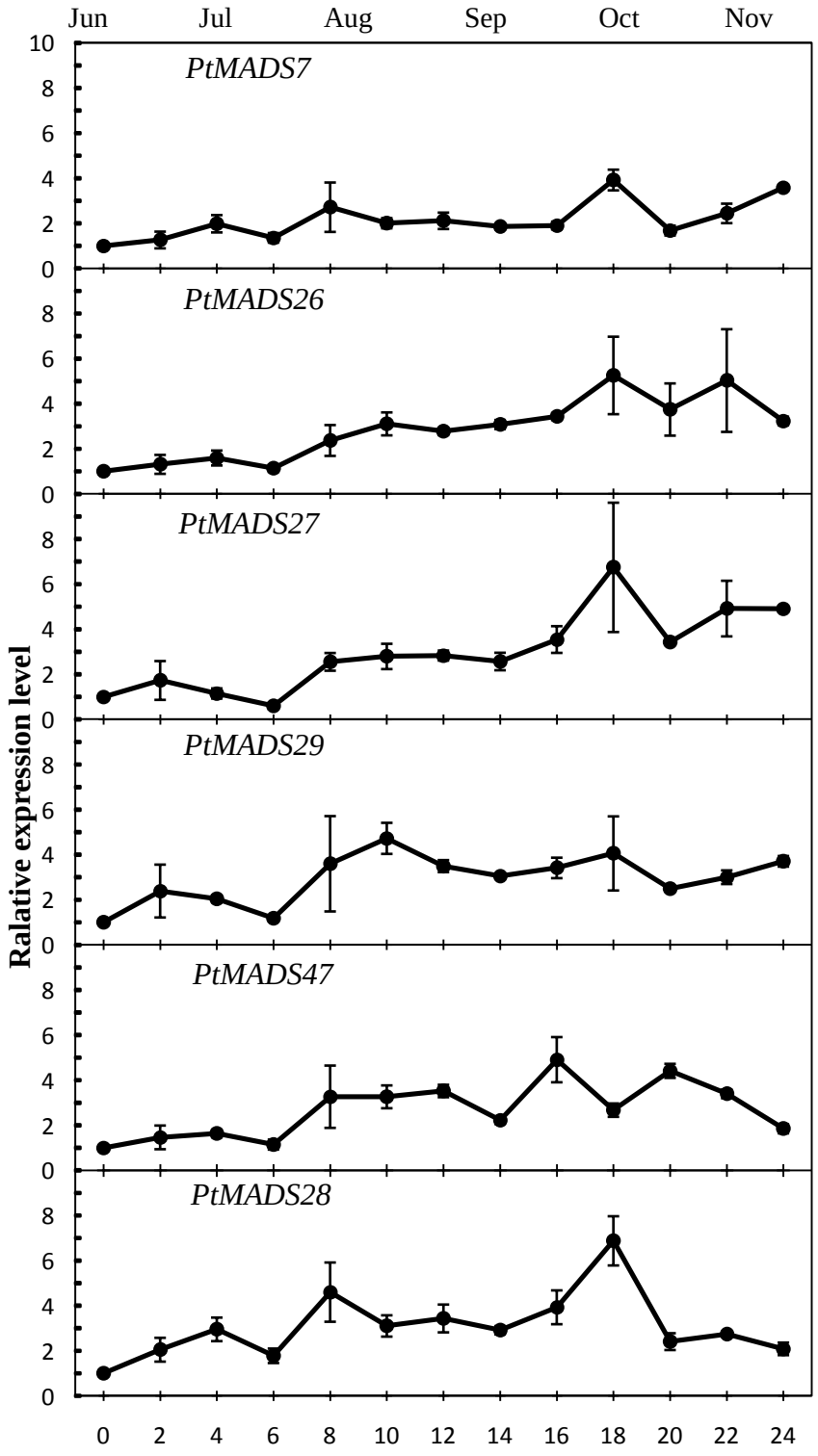

Figure 4.4. RT-PCR expression profile of *PtMADS* genes in poplar leaf tissues under natural light conditions. Expression levels were calculated relative to the poplar *EF1* gene. Each column represents the relative fold change to the first sampling date (June 11th, 2009).

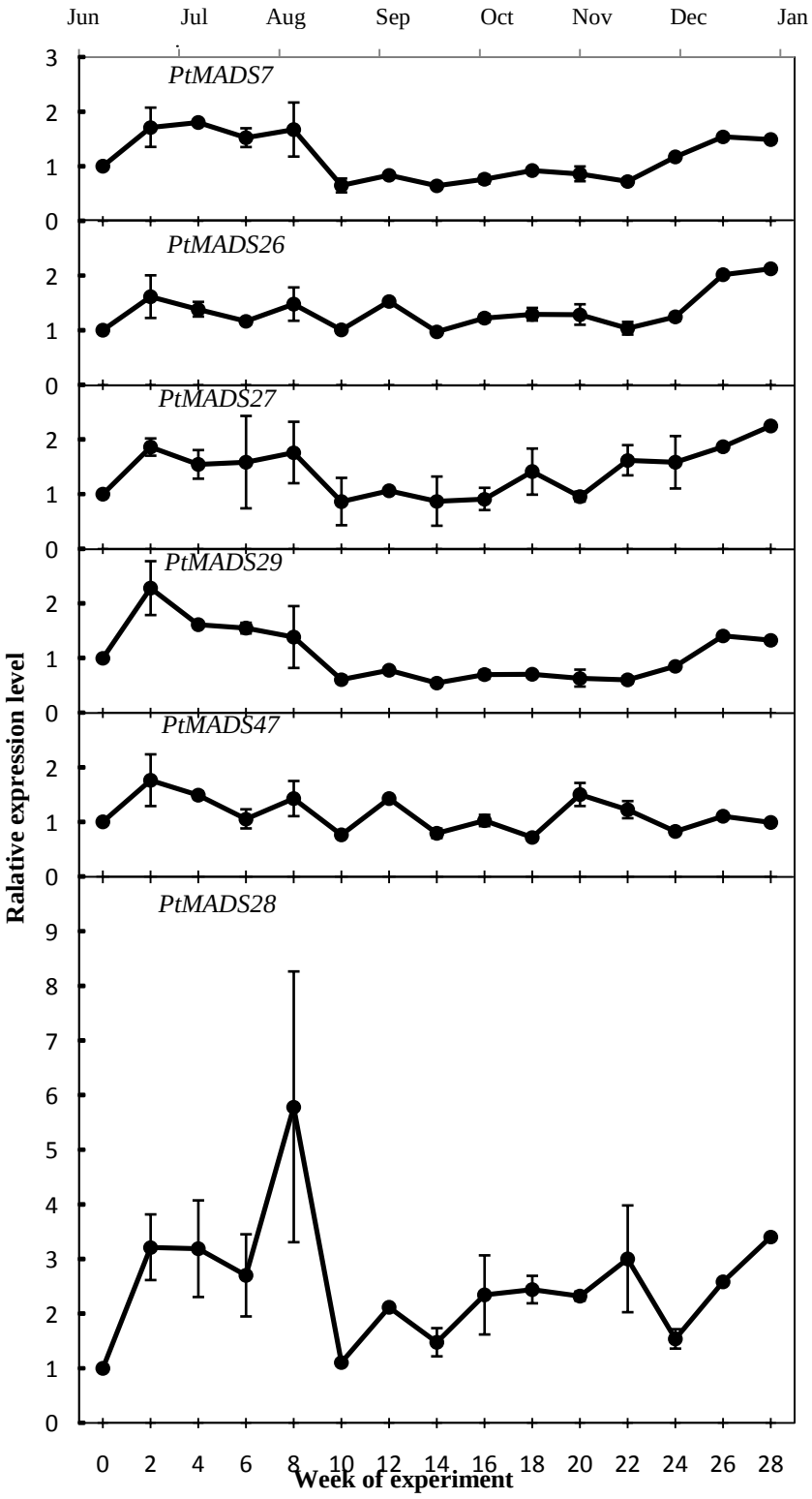

Figure 4.5. RT-PCR expression profile of *PtMADS* genes in poplar tip/bud tissues under natural light conditions. Expression levels were calculated relative to the poplar *EF1* gene. Each column represents the relative fold change to the first sampling date (June 11, 2009).

Discussion

In peach, six MIKC^C MADS-box genes were identified as candidates for *evg* phenotype (Bielenberg et al., 2008). By doing gene local alignment search (BLASTn) and phylogenetic analysis, eight *PtMADS* genes were identified as putative homologs of peach *DAM*s in the *Populus trichocarpa* genome (Jimenez et al., 2009). Only six of these *PtMADS* genes were found in hybrid poplar clone (INRA 717-1B4) in our experiments (Chapter Three).

PtMADS expression in response to the natural seasonal transition from summer to winter

Several recent studies have identified *DAM-like* genes from the *SVP/StMADS11* clade of MIKC^C MADS-box genes as responding to dormancy-inducing conditions in bud tissues of perennial species by differential display or global transcriptome analysis methods (Ruttink et al., 2007; Campbell et al., 2008; Horvath et al., 2008; Diaz-Riquelme et al., 2009; Li et al., 2009). These genes have been shown to respond to either photoperiod or temperature (Ruttink et al., 2007; Horvath et al., 2008; Li et al., 2009). *DAM*s are thought to be involved in the transcriptional regulation of developmental programs that need to occur to cease growth and develop new structures like the bud, which will protect the meristem through the winter (Horvath et al., 2008). Regulation of these genes by changes in photoperiod and temperature provides the beginnings of a pathway of response from environmental perception to growth arrest and bud formation.

In contrast to the strong regulation of *DAM*s by temperature or photoperiod seen in other systems, the expression of six *PtMADS* genes does not appear to have a significant response to the change of day length or temperature. In particular, the expression patterns of *PtMADS* genes do not appear to match the patterns of *DAM* genes in peach. The very different environmental responsiveness of the *PtMADS* genes relative to peach, therefore, argues that the *PtMADS* genes may be poor models for understanding the role of the *DAM*s in peach. Although a similar role of the *PtMADS* genes in the hybrid poplar 717-1B4 cannot be ruled out, it is apparent that the induction or repression of these genes is not a regulatory step in the process of growth arrest and bud set in this genotype. Functional testing of the peach *DAM*s may have to be performed in a more closely related species. Transformation protocols have been developed for several species within the Rosaceae, generally with low efficiency, and these should be explored as alternatives to the hybrid poplar system (Shulaev et al., 2008).

This work is the first specific expression study of the *SVP/StMADS11* clade of MADS-box genes in hybrid poplar. These genes do not currently have a known function in hybrid poplar. Transgenic plants produced from the functional experiments detailed in Chapter Three will provide the first evidence of the role of this group of genes in *Populus* and may uncover new roles for this unique group of MADS-box genes.

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