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Analysis of ADC2 and SAMDC1 expression and the effects of transgenic manipulation of polyamine biosynthesis on flower development in Arabidopsis

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ANALYSIS OF *ADC2* AND *SAMDC1* EXPRESSION AND THE EFFECTS OF TRANSGENIC MANIPULATION OF POLYAMINE BIOSYNTHESIS ON FLOWER DEVELOPMENT IN ARABIDOPSIS

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THESIS

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Date

DEDICATION

I would like to dedicate this thesis to my parents, Krishna M. Challa and Lakshmi S. Challa, whose love and encouragement have always been a great boost to my career and my husband Rajashekar R. Kasireddy for his tremendous support and extreme patience throughout my academics in New Hampshire.

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ABBREVIATIONS

ADC = Arginine decarboxylase, AG = AGAMOUS, AP1 = APETALA1, AP3 = APETALA3, dcSAM = decarboxylated S-adenosylmethionine, DFMO = difluoromethylornithine, EDTA = Ethylene diamine tetra acetic acid, GUS = β-glucuronidase, LB = Luria bertani, LFY = LEAFY, LiCl = Lithium chloride, MGBG = Methylglyoxal-bis guanyl hyrazone, ODC= Ornithine decarboxylase, ORF = Open Reading Frame, RT-PCR = Reverse Transcription Polymerase Chain Rreaction, SAMDC= S-adenosyl-L-Methionine decarboxylase, SPDS = Spermidine synthase, UTR = Untranslated region

ABSTRACT

ANALYSIS OF *ADC2* AND *SAMDC1* EXPRESSION AND THE EFFECTS OF TRANSGENIC MANIPULATION OF POLYAMINE BIOSYNTHESIS ON FLOWER DEVELOPMENT IN ARABIDOPSIS

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Polyamines are low molecular weight, aliphatic cationic molecules playing a vital role in all cellular and physiological processes such as cell growth, differentiation, abiotic stress, and somatic embryogenesis. The present study is aimed at unraveling their role during floral development in *Arabidopsis thaliana* by studying the temporal and spatial expression of *ADC2* and *SAMDC1* genes by promoter-reporter fusion analysis, and by the transgenic expression of mouse *ODC* and carrot *SAMDC* under tissue specific promoters AP1 and AP3, respectively. The expression of *SAMDC1* was found to be prominent during different stages of development and in different whorls of flowers whereas *ADC2* expression was almost undetectable in all stages and in various whorls. The heterologous expression of mouse *ODC* and carrot *SAMDC* under the control of tissue specific promoters AP1 and AP3 resulted in altered polyamine levels leading to abnormalities like delayed flowering, bolting, abnormal sepal formation and a reduction in stamen number.

CHAPTER I

INTRODUCTION

I.1.Polyamines and their functions

Polyamines are low molecular weight, aliphatic cationic molecules. They are ubiquitous cellular constituents, playing a vital role in all physiological processes such as cell growth and differentiation (Cohen, 1998; Igarashi, 2000). They contain two terminal amino groups and one or more imino groups (Cohen, 1998). The most common polyamines are: putrescine (diamine), spermidine (triamine) and spermine (tetraamine). They occur both as free cations as well as conjugates bound with phenolic acids, alkaloids and macromolecules (Galston & Sawnhey, 1990). Free polyamines and soluble conjugates are extractable in perchloric acid (PCA) whereas insoluble conjugates are neither disassociated with salt nor can be exchanged with free polyamines.

At physiological pH, polyamines are positively charged, and some of their functions in the cell are direct result of charge-to-charge interactions. For example, they interact with the anionic molecules such as DNA, RNA, proteins, cell walls and membranous structures and thus are involved in normal physiology and growth of all cells. They bind to the phosphate groups strongly in each strand of the DNA duplex and reduce the repulsion between phosphate groups of the two DNA strands. Polyamine binding sites in DNA are found in both major and minor grooves (Jain et al., 1989; Tari et al., 1995; Egli et al., 1998; Shui et al.,

1998). Theoretical modeling results suggest that polyamine-binding sites are AT-rich duplexes in minor grooves (Feuerstein et al., 1986; Zakrzewska & Pullman, 1986) and GC-rich duplexes in major grooves (Feuerstein et al., 1990). At molecular level, polyamines are involved in DNA replication (Pohjanpelto et al., 1986), transcription, translation, DNA condensation (Pelta et al., 1996; Deng et al., 2000), chromatin stabilization (Smirnov IV et al., 1987), DNA-RNA hybrid formation (Antony et al., 1999), triple-helical DNA formation (Musso & Van Dyke, 1995; Musso et al., 1997; Saminathan et al., 1999) and other double-helical structure formations like stems and loops in rRNA, mRNA, and tRNA (Kusama-Eguchi et al., 1991). They also play a role in changing the DNA conformations within the cell. Feuerstein et al. (1990) reported the role of spermidine and spermine in DNA bending. Under specific conditions, it was found that they induce the transition of B-DNA to A-DNA and to Z-DNA (Ohishi et al., 1996; Gao et al., 1999).

At cellular level, polyamines are essential for cell proliferation, differentiation, growth transformation, stabilization of cellular membranes and cytoskeletal structures. Polyamines have been reported to have an effect on mitotic cell cyle (Rupnaik et al., 1978) and cytokinesis (Pohjanpelto et al., 1985). It was reported that their exogenous addition allows the progression of cells to the S phase and inhibition of spermidine and spermine biosynthesis blocks the cells in G1 stage of the cell cycle (Rupnaik et al., 1978).

In higher plants, polyamines are involved in many processes such as response to stress (Minocha et al., 1992, 2000; Zhou et al., 1995; Watson & Malmberg, 1996; Bouchereau et al., 1999; Liu et al., 2000), somatic embryogenesis (Robie & Minocha, 1989; Minocha & Minocha, 1995; Singh &

Rajam, 1998; Shoeb et al., 2001), pollen formation, fruit development (Slocum & Flores, 1991; Rodriguez et al., 1999) and senescence (Srivastava, 1987; Pandey et al., 2000). In plants, in response to environmental stress, many nitrogenous compounds like amino acids, amides and polyamines, especially putrescine accumulate and this increase is considered to be an indicator of the stress level (Bohnert & Jensen, 1996; Holmberg & Bülow, 1998; Minocha et al., 2000, 2002, 2004). Although the cause for their increase in response to stress is unclear, it is thought to be either the means of defensive mechanism against stress or due to stress-induced injury.

An important role of polyamines in somatic embryogenesis and organogenesis in carrot (Montague et al., 1978; Feinberg et al., 1984; Robie & Minocha, 1989; Minocha & Minocha, 1995), *Hevea brasiliensis* (El Hadrami et al., 1989), egg plant (Yadav & Rajam, 1998), *Pinus radiata* (Minocha et al., 1999b) has been reported. These studies have suggested that inhibition of polyamine biosynthesis prior to or during somatic embryogenesis causes an inhibition of this process whereas enhancement of polyamine biosynthesis via the transgenic manipulation of polyamine biosynthetic genes results in increased somatic embryogenesis (Bastola & Minocha, 1995).

Polyamines are also found to play a major role in several human disorders like cystic fibrosis (Rennet et al., 1978) and psoriasis (Russell et al., 1978). Intracellular concentrations of polyamines range from few micromolar to high millimolar (Tabor & Tabor, 1984) and their elevated levels in the body fluids are often an indication of malignant tissue. Depletion of polyamines appears to reduce cell growth, and their increased expression is associated with the cancer

development and progression, making polyamine biosynthesis a target for the control of cancer (Pegg, 1988; Gupta et al., 2000).

I.2. Polyamine biosynthesis

The diamine putrescine in almost all organisms is synthesized by the decarboxylation of ornithine (Orn) in a single step reaction catalyzed by ornithine decarboxylase (ODC, EC 4.1.1.17). In plants, bacteria and some fungi, putrescine is also synthesized via arginine decarboxylase (ADC, EC 4.1.1.19) from arginine through the intermediates agmatine and N-carbamoylputrescine (Figure 1). Agmatine is converted into N-carbamoylputrescine by agmatine iminohydrolase. Putrescine is formed by the conversion of N-carbamoyl putrescine by the action of N-carbamoylputrescine amidohydrolase. All plants except *Arabidopsis* utilize both pathways for the putrescine production. Arabidopsis was demonstrated to lack ODC activity and totally relies on the ADC pathway for putrescine production (Hanfrey et al., 2001).

The presence of two pathways for the synthesis of putrescine in plants complicates their metabolic regulation since the substrates of the two pathways are interconvertible. However, it was reported that the two mechanisms are not mutually exclusive and can operate simultaneously (Knight et al., 1996). The utilization of one substrate over the other for the synthesis of putrescine depends on the enzymes and other catabolic machinery. ODC is the first and the rate-limiting enzyme in polyamine biosynthesis and is regulated by feed back control of the enzyme in animals. Although the activities of ADC and ODC are regulated developmentally in a tissue-specific manner in plants also (Walden et al., 1997), the biochemical mechanism of their turnover is unknown.

The triamine spermidine is synthesized by the enzyme spermidine synthase (EC 2.5.1.16) from putrescine by the transfer of aminopropyl moiety from decarboxylated S-adenosyl- methionine (*dcSAM*). Spermine is synthesized by the enzyme spermine synthase where, an additional aminopropyl moiety is transferred from dcSAM to spermidine. S-adenosyl-methionine is converted into dcSAM by SAM decarboxylase (SAMDC, EC 4.1.1.21). S-adenosyl-methionine is formed from L-methionine and ATP by SAM synthase. Polyamine biosynthetic pathway also interacts with other pathways, such as those of alkaloids, proline, and ethylene (Bhatnagar et al., 2001; Figure 1).

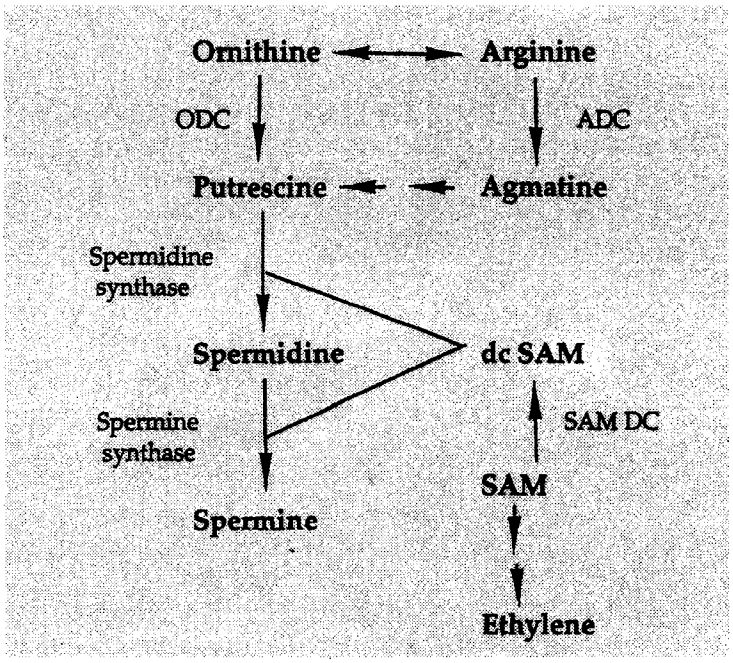


Figure 1: Polyamine biosynthesis pathway (Slocum, 1991). ADC – Arginine decarboxylase; ODC- Ornithine decarboxylase; SAMDC- S-adenosylmethionine decarboxylase; SAM- S-adenosylmethionine

I.3. Polyamines and flowering

Since polyamines are present in all living cells with a wide range of functions, it can be argued that they also play a crucial role in flower development, either in determining floral timing or organ identity and/or organ development. Although studies involving analysis of polyamines in relation to floral induction and development are limited, a few studies that have been carried out indeed show correlations between changing cellular polyamine levels during floral initiation and also in different organs of the flower. However, none of the studies has reported their absolute requirement during flower development. It was not possible to do classical genetic analysis or mutational analysis in these lines because many of the mutants were unable to produce flowers. Their role could involve interactions with RNA or facilitating the interactions of transcription regulators with DNA. Non-flowering tobacco mutants RMB7 did not produce amides under conditions leading to floral initiation (Martin et al., 1981). Malmberg & McIndoo (1983) produced tobacco mutant lines that were resistant to SAMDC-inhibitor, methylglyoxal bis-guanylhydrazone (MGBG), which exhibited both altered SAMDC and abnormal floral development. When mutant cell lines high in SAMDC activity were grown into plants, they often produced aberrant flowers.

Slocum & Galston (1984) reported highest ODC activity in the meristematic tissues of tobacco flowers. Later, Malmberg et al. (1985) analyzed the activities of ADC, ODC and SAMDC in various tobacco tissues and reported that the highest activities of ODC and SAMDC were found in the developing flowers. Conjugated polyamines were also elevated during floral initiation. Kaur-

Sawhney et al. (1988) reported that tobacco thin layer tissue cultures programmed to produce vegetative buds could be induced to flowering upon exogenous addition of spermidine. In *Iris hollandica*, spermidine levels were greatly increased and hence their levels were noted as markers during floral induction in the apical bud (Fiala et al., 1988). In *Agrobacterium rhizogenes* transformed tobacco plants, delayed flowering was strongly correlated with a reduction in polyamine accumulation (Tanguy, 1987). Gerats et al. (1988) reported correlations between flower abnormalities and polyamine content in *Petunia hybrida*. In their study, all abnormal flowers contained lower polyamine conjugates. De Cantu & Kandeler (1989) implicated spermidine in later stages of flower development in induced apices of *Spirodela*.

Burtin et al. (1991) reported that conjugated polyamines derived through ODC are required for floral development and initiation and free polyamines derived from ADC are involved in vegetative development. Sun et al. (1991) showed that tobacco lines expressing *Agrabacterium rhizogenes rolA* gene were male sterile and produced abnormal flowers supposedly due to increased polyamine activity. Later, elevated levels of polyamines were shown to be associated with male sterility in tobacco (DeScenzo & Minocha, 1993) and abnormal anther development in transgenic carrot plants (Bastola, 1994). Male and female sterility due to putrescine overproduction was also observed in transgenic mice (Halmekyto et al., 1991; Pietila et al., 1997). Wada et al. (1994) provided the evidence that polyamines, especially putrescine have the ability to induce flowering in *Pharbitis nil* under non-inductive conditions. The presence and relative abundance of putrescine, spermidine and spermine has been

reported in apple pollen during germination (Bagni et al., 1981) and in tobacco pollen (Chibi et al., 1993). Although the above results suggest spermidine involvement in floral stimulus, it seems to vary from species to species. In *Glycine max*, no spermidine involvement was reported during floral induction (Caffaro & Vicente, 1994). Bokern et al. (1995) reported the presence of conjugated polyamines, e.g. trisubstituted hydroxycinnamic acid spermidine, in *Quercus dentata* pollen. In strawberry (*Fragaria* x *ananassa*), it was reported that spermidine was the predominant polyamine in shoot tips during floral initiation (Tarenghi et al., 1995), and the sterile flowers lacked conjugated polyamines. Scaramagli et al. (1999) studied spermidine biosynthesis in different whorls of developing flowers during different stages of floral development in tobacco. Their findings suggest that spermidine is the dominant polyamine over putrescine and spermine in different whorls of tobacco flower.

Applewhite et al. (2000) reported that exogenous addition of spermidine synthase inhibitor cyclohexylamine reduced the spermidine content in Arabidopsis and prevented bolting and flowering whereas plants transferred to an inhibitor-free medium showed restoration of both bolting and flowering. Another study conducted with the same spermidine synthase inhibitors in *Polianthe tuberosa*, applied twice at dosages of 150 or 250 µg per plant at regular interval of 4 days reduced flower primordium counts indicating spermidine role in floral development and initiation. Also, in *Polianthe*, an increase in free cadaverine and spermidine, and a decrease in free putrescine and spermine, was reported during early floral initiation (Huang et al., 2004). These authors reported decreased ADC activity and increased ODC activity

during floral initiation.

Although extensive research has been carried out on their role in various developmental processes in plants, a thorough analysis of expression of polyamine biosynthetic genes or their role in floral development has not been carried out so far. The present study is an attempt to elucidate the role of two key polyamine biosynthetic genes (*ADC2* and *SAMDC1*), and over expression of a mouse (*ODC*) and carrot (*SAMDC*) in specific whorls of Arabidopsis flower during its development. A brief description of flower development and polyamine biosynthetic genes in Arabidopsis is given below:

I.4. Molecular biology of flower development in Arabidopsis

Arabidopsis is a common weed that belongs to the mustard family Brassicacae. Laibach (1943) had proposed it as an ideal organism for research due to several characteristics like, short generation time, lower chromosome number, ease of making crosses, and possibility of mutagenesis. Ever since, it has become the most sought-after plant for various molecular, biochemical and cytogenetics studies. The 2000 Arabidopsis Genome Initiative' achieved the complete genome sequencing of this plant. The 'National Science Foundation Project 2010' aims at identifying the functions of all the 25000 available genes in the genome, elucidating the physiological and developmental functions of each gene in the genome. These two events constitute a major breakthrough towards understanding the functional role of various genes and represent the overall effect that Arabidopsis has had on biological research.

Arabidopsis is a self-fertilizing plant with a short life cycle (48 days) giving rise to 5000-10,000 seeds per plant. The sequenced genome represents five

chromosomes covering 115.4 mega bases of 125 mega base total genome, extending into centromeric regions. About 60% of the plant's genes have homologous counterparts elsewhere within the genome and 14% of the genome is made up of transposable elements. Plastid and mitochondrial genomes are small, and encode a further 79 and 58 proteins, respectively. Most of the genes occur in more than one copy with the total number of unique genes being approximately 12000, which are actually comparable to the 14,000 and 11,000 genes estimated in *Caenorhabditis elegans* and *Drosophila melanogaster.* Other resources like, commercially available microarrays, BAC (Bacterial Artificial Chromosome) and cDNA libraries etc. (Table: 1) make Arabidopsis an ideal material for many molecular, biochemical and cytogenetic studies (www.arabidopsis.org).

In plants, the most critical phase in life cycle is a transition from vegetative phase to reproductive phase. In order to ensure that this change occurs properly in time and space, complete series of gene expression pathways have evolved. Arabidopsis follows the same pattern of development as most other angiosperms (Miksche & Brown, 1965). Flowers develop from a group of undifferentiated cells that grow from the flanks of shoot apical meristem and develop into sepals, petals, stamens and carpels. Flower formation involves a series of several distinct developmental stages that can be classified as floral initiation, floral organ primordial formation and floral organ production. Each of these stages is briefly discussed below.

Floral initiation

There are three types of shoot apical meristems in the plant: 1) vegetative

meristems that produce vegetative parts of the plant like leaves, 2) inflorescence meristems that produce floral meristems and 3) floral meristems that produce flowers. The transition of shoot apical meristem to inflorescence involves two phases that are characterized by indefinite and repetitive patterns of growth and organ formation (Medford et al., 1994). In Arabidopsis after seed germination and growth, on the flanks of the shoot apical meristem, five to eight leaves are produced in a basal rosette arranged in a spiral phyllotaxy. The duration of vegetative phase is controlled by both environmental and endogenous factors. Morphological analysis of the transition to flowering phase from vegetative phase has shown that first primordium that is initiated from the shoot apex after the start of inductive light treatments can adopt a floral fate (Hempel & Feldman, 1994).

The endogenous factors including meristem identity genes, such as, *LEAFY (LFY)*, *APETALA1* (AP1), *CAULIFLOWER (CAL)*, *UNUSUAL FLORAL ORGANS (UFO)*, etc. (Clark et al., 1993) determine flowering time and regulate the conversion of vegetative shoot to the reproductive shoot (Koornneef et al., 1991; Martinez et al., 1994; Simon et al., 1996; Nilsson et al., 1998). The names of these genes reflect the phenotypes of the plants that carry mutations in these genes. These genes are expressed either alone or together controlling the flowering expression. Mutational and gene sequence analyses have provided information on how these genes function and interact to promote flowering. For example, *LFY* and *AP1* act as genetic switches in the choice of floral versus shoot phase during the formation of normal flowers from the shoot apex, thus playing a major role in the floral initiation.

Table 1: Major Arabidopsis databases currently available

Database	Information
AtIDB http://atidb.org	Arabidopsis genome browser
NASC stock http://affymetrix.arabidopsis.info/	Collection of 300,000 accessions of Arabidopsis representing over half a million genotypes
MatDB http://mips.gsf.de/proj/thal/db	MIPS Arabidopsis database is a comprehensive resource to the data of the Arabidopsis genome initiative
MAtDB2 http://mips.gsf.de/proj/thal/db/	A new version of MatDB which includes TIGR and MAtDB annotation.
WatDB http://www.watdb.nl/	A database with curated data on mutants, transgenic lines and genomic position of a transposon.
GeneFarm http://genoplante-info.infobiogen.fr/Genefarm/	A database of structural and functional annotation of Arabidopsis gene and protein families
Phytoprot http://genoplante-info.infobiogen.fr/phytoprot/	A database of clusters of plant proteins (Arabidopis. vs Arabidopis and Arabidopis vs other plants)
GnpMap http://genoplante-info.infobiogen.fr/	Plant genetic map database that stores QTL and traits, loci, markers and maps.
GnpSeq http://genoplante-info.infobiogen.fr/data/gnpSeq/run.php	Plant expressed sequence database
RARGE http://rarge.gsc.riken.jp/cdna/promoter/index.pl	Arabidopsis Promoter database

Through the action of these genes, shoot apical meristem switches to the reproductive phase and becomes an inflorescence.The conversion of the shoot apical meristem into inflorescence meristem is itself under the control of an extensive network of genes that respond to internal as well as external stimuli, e.g. developmental age, photoperiod, vernalization, and plant growth regulators, particularly gibberellins (Alvarez et al., 1992; Blazquez et al., 1997, 1998; Bradley et al., 1997). Studies on transgenic plants that over express certain genes (e.g. *TERMINAL FLOWER - TFL1*) have divided the reproductive growth into two distinct phases based on the activity of the shoot apex: first inflorescence phase or I1, during which the shoot apex produces cauline leaves that enclose secondary flowering shoots called paraclades; and second inflorescence or I2 phase, during which flowers are produced on the flanks of the shoot apical meristem (Ratcliffe et al., 1998). However, in this process of conversion it is important for the shoot meristem to retain its identity from the floral meristem it produces. Hence, during I2, the actions of two complimentary sets of genes (floral meristem identity genes and shoot meristem identity genes), which apparently have opposing functions, maintain this distinction. As mentioned above, floral meristem identity genes (*LFY, AP1, CAL, UFO*) establish floral identity and regulate the flowering time, whereas shoot meristem identity genes such as *TERMINAL FLOWER 1 (TFL1)* prevent conversion of shoot apical meristem from becoming a flower (Ratcliffe et al., 1998).

It was earlier shown that in wild type flowers during inflorescence phase, these two types of genes are expressed in separate domains. *TFL1* is expressed in the center of the apex and the floral meristem identity genes at the periphery (Mandel et al., 1992; Weigel et al., 1992; Kempin et al., 1995). The appropriate

action of these genes is so important that reduction in activity of floral meristem identity genes produces flowers with various shoot like characteristics (Irish & Sussex, 1990; Schultz & Haughn, 1991, 1993; Mandel et al., 1992; Weigel et al., 1992) and vice versa. Inflorescence meristem or flower-bearing meristem produces floral meristem in the axils of both the primary and the secondary meristems.

Production of floral primordia

Floral meristems are small, spherical clusters, which produce four types of lateral organs in concentric rings called whorls. The cells in the floral primordia divide independently and differentiate into respective floral organs at the appropriate places. During flower development, each organ determines its position relative to others and differentiates accordingly. Mutational analyses have shown that more than 80 genes play important roles in regulating this process (Ratcliffe & Riechmann, 2002). Genetic and molecular studies on natural variations in flowering time and development have identified additional genes responsible for flowering based on different active periods in flowering control.

These genes are divided into two groups: floral meristem identity genes and homeotic genes. Meristem identity genes are required for the initiation of the flower and homeotic genes are required to specify the individual organs of the flower and are expressed in the various domains. The ABC model (Figure 2) of homeotic or floral organ identity genes (Table: 2) proposed by Coen and Meyerowitz (1991) has found widespread experimental support both in genetic and molecular studies not only in Arabidopsis but in several other plants as well. This model postulates the activation of three sets of genes called A, B, and C in

different layers of the flower meristem, which interact to determine the formation of four whorls of a typical flower, i.e. the sepals, petals, stamens and carpels. The A-class of genes, *APETALA1-AP1*, and *APETALA2-AP2* specify sepal and petal identities in whorls 1 and 2 (Bowman et al., 1993; Irish & Sussex, 1990; Mandel et al., 1992). The B-class genes, *APETALA3-AP3* and *PISTILLATA-PI* specify petal and stamen identities in whorls 2 and 3 (Bowman et al., 1989; Goto & Meyerowitz, 1994; Jack et al., 1992) and C class of genes *AGAMOUS-AG* confer stamen and carpel identities in whorls 3 and 4 (Bowman et al., 1989; Yanofsky et al., 1990). The identities of the four whorls of a typical flower are determined by the expression of these genes either alone or in combination with other homeotic genes. The activity of C-class of genes specifies the carpels, while its interaction with B-class of genes determines the stamens.

APETALA1 together with *LEAFY* establishes floral meristem identity and its interaction with *AP2* specifies sepal formation while its interaction with *AP2*, *AP3* and *PI* specifies petal formation (Schultz & Haughn, 1991; Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Kempin et al., 1995; Mandel & Yanofsky, 1995; Weigel & Nilsson, 1995). The functions of ABC genes in specifying organ identities in specific domains correspond to their expressions in developing flower respectively (Weigel & Meyerowitz, 1993; Riechmann & Meyerowitz, 1997). The meristem identity gene *LFY* is required for transcriptional activation of all three classes of ABC genes (Weigel & Meyerowitz, 1993). It has been shown that *LFY* transcription factors bind to the sequences of *AP1* and AG in enhancer regions to direct normal levels of expression (Busch et al., 1999; Parcy et al., 1998). *LFY* together with *AP1* is required for *AP3* expression in outer two domains of the

flower (Hill et al., 1998). The ABC model (Coen & Meyerowitz, 1991) also implies that there must be mechanisms to inhibit gene expression. Class A genes can only be expressed in the outer two whorls and not in the inner two whorls. Conversely, class C genes can only be expressed in the inner two whorls and not in the outer two whorls. Class A genes inhibit the expression of class C genes and vice-versa. Similarly, something must prevent the expression of the class B genes in the innermost whorl. All these genes that control flower development in Arabidopsis are classified as MADS-box genes, which contain a highly conserved sequence motif, found in a family of transcription factors (Shore et al., 1995). The name MADS-box has been derived from the first letters of the four transcription factors: MCM1, AGAMOUS, DEFICIENS, and SRF. Each of these contains a 56 amino acid sequence, which is necessary for the protein to bind to the DNA. More than 100 MADS box sequences have been found in eukaryotes (Table: 3). Most MADS domain factors play important roles in developmental processes especially in flowering process and can be referred as "molecular architects" of floral morphogenesis. All MADS-domain proteins have additional domains attached to the C-terminus, in plants it is K-domain (similarity to Keratin), and also have N-terminus extensions.

The precise timings of expression and the effects of ectopic transgenic expression of several of these genes have been studied by *in situ* hybridization of their mRNAs and promoter-reporter (*GUS*) gene expression approaches (Mandel et al., 1992; Weigel et al., 1992; Jack et al., 1994; Jofuku et al., 1994). The specific role(s) of most of these proteins in establishing meristem identity at molecular and cellular levels is unclear. It was reported that the expression of these genes is not

only whorl-specific during early stages of flower development but also continues in the various organs till maturity (Jack et al., 1994; Jofuku et al., 1994). Studies were also conducted using promoter-reporter fusions (e.g. LFY promoter with *GUS* gene) to study the role of gibberellins in flowering and it was shown that gibberellins activate flowering under short day conditions (Blazquez et al., 1998). Using similar approaches (promoter- reporter gene fusion and ectopic transgenic expression) we intend to investigate the role of polyamine biosynthetic genes in the development of flowers in Arabidopsis.

Formation of floral organs

Following the floral initiation, floral meristems produce floral organ primordia, which develop individually into sepals, petals, stamens and carpels. In wild type Arabidopsis, flowers develop in a raceme so that a single stem can have a series of flowers in different stages of development from primordia at the top to the mature fruit near the bottom. Vaughn (1955) and Muller (1961) described the earliest and later stages of flower development in Arabidopsis. Flower development in Arabidopsis has been divided into 12 early stages of development followed by 8 mature stages using a series of landmark events. The early developmental stages are described from floral initiation until the opening of the bud (Table: 4) and the later stages of development are described from the formation of mature flower until the dispersal of seeds. Arabidopsis mature flower is composed of four organs arranged in concentric whorls (Figure 2).

Four sepals occupy the first whorl; inside and alternating to the sepals are four petals occupying the second whorl. Six stamens with two lateral short stamens and four long medial stamens occupy third whorl. The floral meristem

eventually terminates with the formation of two central carpels, which are fused to form a pistil occupying the fourth whorl. The entire structure together referred to as gynoecium is made up of an ovary that contains two chambers separated by a septum. The ovary is topped by the style and papillate stigma. The withering of sepals, petals, foliage, and yellowing of green siliques thus becoming dry and finally resulting in the seed dispersal, characterizes the last stages of development.

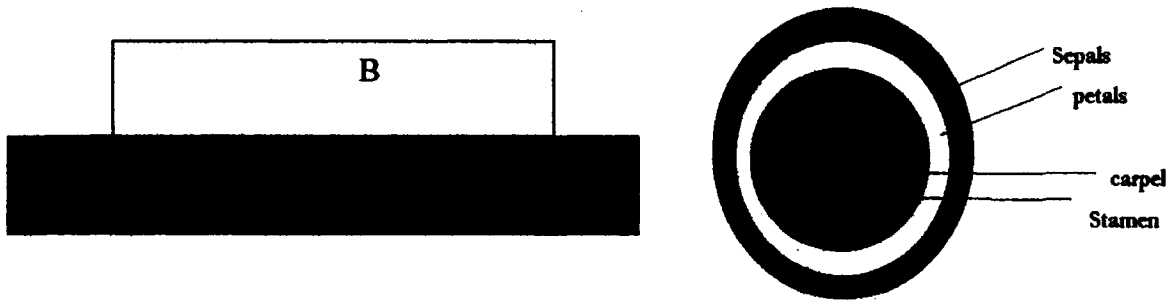


Figure 2: ABC system of flowering

Table 2: ABC floral genes in Arabidopsis

Class	Gene	Organ	Expression
A	APETALA 1	SEPALS	1&2
	APETALA2	PETALS	
B	APETALA 3	PETALS	2&3
	PISTILLATA	STAMENS	
C	AGAMOUS	STAMENS	3&4
		CARPELS	

Table 3: A partial list of MADS box genes found in plants

Number	Gene name	Locus	Species
1	AGL43	At5g58890	*Arabidopsis thaliana*
2	AGL41	At2g26880	*Arabidopsis thaliana*
3	AGL30	At2g03060	*Arabidopsis thaliana*
4	AGL28	At1g01530	*Arabidopsis thaliana*
5	AGL26	At5g26950	*Arabidopsis thaliana*
6	AGL29	At2g34440	*Arabidopsis thaliana*
7	AGL33	At2g26320	*Arabidopsis thaliana*
8	AGL39	At5g27130	*Arabidopsis thaliana*
9	AGL40	At4g36590	*Arabidopsis thaliana*
10	AP1	At1G69120	*Arabidopsis thaliana*
11	AP3	At3G54340	*Arabidopsis thaliana*
12	DEFH125	Y10750	*Antirrhinum majus*
13	DEFH200	X95469	*Antirrhinum majus*
14	DEFH49	X95467	*Antirrhinum majus*
15	FDRMADS5	af141964	*Oryza sativa*
16	FDRMADS6	af139664	*Oryza sativa*
17	FDRMADS7	af139665	*Oryza sativa*
18	GGM11	aj132217	*Gnetum gnemon*
19	GGM9	AJ132215	*Gnetum gnemon*
20	GRCD1	AJ400623	*Gerbera hybrida*
21	HAG1	AF099937	*Hyacinthus orientalis*
22	FBP11	X81852	*Petunia hybrida*
23	FBP1	M91190	*Petunia hybrida*

Table 4: Floral developmental stages of Arabidopsis flower

Stage	Event at the beginning of stage
1	Development of floral buttress
2	Formation of floral primordia
3	Formation of sepal primordia
4	Overlapping of sepals over floral meristem
5	Formation of petal and stamen primordia
6	Covering of the bud by sepals
7	Formation of stamen primordia
8	Appearance of locules in long stamens
9	Petal primordia stalked at base
10	Leveling of petals with short stamens
11	Appearance of stigmatic papillae
12	Leveling of petals with long stamens
13	Anthesis, opening of buds
14	Extension of long anthers above the stigma
15	Extension of stigma over long anthers
16	Withering of sepals and petals
17	Foliage
18	Yellowing of green siliques
19	Separation of valves from dry siliques
20	Falling of seeds

I.5. Polyamines in Arabidopsis

As described earlier, the major polyamine biosynthetic genes are *ADC*, *ODC* and *SAMDC* for not only their presence in almost all organisms but also due to their nature of location in the biosynthetic pathway. Curiously, in Arabidopsis, the synthesis of putrescine is achieved solely by ADC-catalyzed reaction, as no ODC activity or a corresponding *ODC* gene has been found (Hanfrey et al., 2001). The *ADC* gene in Arabidopsis was thought to be duplicated at the origin of the Brassicacae family yielding two paralogues: *ADC1* and *ADC2* (Galloway et al., 1998). *ADC1* is located on chromosome 2 (At2g16500, Accession # NM_127204) and *ADC2* on chromosome 4 (At4g34710, Accession # NM_119637) (Galloway et al., 1998). Amino acid sequences of At ADC1 and At ADC2 proteins show 80% homology.

The analysis of N-terminal sequences suggests that subcellular location of *ADC1* and *ADC2* might be different (Hanfrey et al., 2001). The sequence for ADC1 contains a putative transit peptide suggesting that it may be localized in the chloroplast. Preliminary expression patterns of two paralogues of *ADC* gene were studied by using promoter-reporter fusion system by Hummel et al. (2004). They reported that *ADC* expression is influenced by external factors like temperature. *ADC2* expression was predominant during seed germination and seedling development whereas *ADC1* expression was predominant in root development. Also, under temperate conditions, ADC2 promoter activity was high during germination, root and leaf showed low ADC1 activity. On the contrary, in cold conditions, higher ADC1 promoter activity was reported in roots. Leaves subjected to cold treatment showed decreased ADC2 promoter activity.

Earlier, another study reported *ADC1* expression to be visible in all tissues and *ADC2* in siliques and cauline leaves only (Watson & Malmberg, 1996). Soyka & Heyer (1999) reported that *ADC2* expression is induced upon osmotic stress and wounding. The increased ADC2 enzyme activity was reported in barley plants treated with methyl jasmonate (Walter et al., 2002). Also, similar findings were observed in oil seed rape (Cowley et al., 2005). As with *ADC*, *SAMDC* expression is also highly regulated by a variety of physiological, hormonal and environmental stimuli (Tabor & Tabor, 1984). The *SAMDC* gene family in Arabidopsis was studied in detail by Franceschetti et al. (2001). The two paralogs, *SAMDC1* and *SAMDC2*, differ both in their spatial expression and in their chromosome location. *SAMDC1* is abundant and ubiquitous expressing almost all the times, whereas *SAMDC2* is preferentially expressed in leaves and inflorescences. *SAMDC1* (Accession # NM 111114) is located on chromosome 3 (At3g02470) and *SAMDC2* (Accession # NM 121600) is located on chromosome 5 (At5g15950), respectively. *SAMDC1* and *SAMDC2* share 49% sequence similarity at nucleotide level and 93% similarity at amino acid level.

Arabidopsis *SAMDC* gene was shown to be regulated by polypeptides that are encoded in the 5'UTR.There are two untranslated ORFs in the 5' UTR of *SAMDC1*: tiny uORF, which is 4 amino acid residues and small uORF, which is 50 residues. Although the tiny uORF is not translated, the small uORF is translated and is believed to play an important role in translational repression in response to altering polyamine levels. The expression and activity of *SAMDC* gene have been studied in various plants. The activity of SAMDC was found to be inversely proportional to ethylene production where, decreased SAMDC activity was

shown to enhance the rate of biosynthesis of ethylene (Apelbaum et al., 1985) and inhibition of ethylene production increased the SAMDC activity (Roustan et al., 1992). The plant *SAMDC* genes are mainly expressed in actively dividing tissues, e.g. early stages of potato tuber development (Taylor et al., 1992; Mad-Arif et al., 1994), rapidly growing transformed culture of *Datura* (Michael et al., 1996), and carnation petals (Lee et al., 1997). It was also shown that *SAMDC* expression could be induced by stress and salinity, drought, ozone, and exogenous abscisic acid (Bors et al., 1989; Ye et al., 1997). Li & Chen (2000) studied the expression pattern of *SAMDC* in rice in response to various abiotic factors. They reported a steady accumulation of *SAMDC1* transcripts in shoots in response to stress and drought resistant conditions, an increased *SAMDC1* mRNA accumulation in salt-tolerant variety than in salt-sensitive variety when exposed to salt stress conditions, and varying levels of *SAMDC1* expression when treated with abscisic acid (*SAMDC1* transcript levels were induced with the application of abscisic acid within 3 h and then repressed at 6 h).

Similar results were found in pea plants treated with ozone (Marco & Carrasco, 2002), where *SAMDC1* expression increased in plants exposed to 100 ppb ozone and in leaves of older pea plants exposed to 30 ppb ozone. Many plant *SAMDC* genes have been isolated cloned and characterized. Two cDNAs of *SAMDC* were cloned from carnation petals (Lee at al., 1997). There are two functional copies of *SAMDC* in Arabidopsis as described earlier. No introns in coding regions of plant *SAMDCs* have been found but they are present in the 5' UTRs. In all plant *SAMDC* 5'UTR's, three highly conserved introns are found: first located at the 3' terminus of the small uORF, second located at prior to the tiny

uORF and the third, the least conserved intron located close to the beginning of the transcript. In contrast, many introns in animal *SAMDC* coding regions have been reported, e.g. human (Maric et al., 1992), *Rattus rattus* (Pulkka et al., 1993), *Onchocerca volvulus* (Accession # X95714) and fruit fly (Accession # Y11216), but no introns in the 5' UTR region were located. Also, it was reported that 5' UTRs of mammalian *SAMDCs* contain a single uORF that encodes a hexapeptide MAGDIS (Hill & Morris, 1993). There is also low homology reported between plant and animal *SAMDC* coding sequences (Lee et al., 1997).

There are 3 *spermidine synthase* homologues in Arabidopsis of which 2 have been shown to code for active SPDS enzymes (Hanzawa et al., 2002). The first, named *SPDS1* (Accession # NM124691) is located on chromosome 5 (At1g23820) while the second *SPDS2* (Accession # NM102230) and third *SPDS* gene, *SPDS3* (Accession # NM 105699A) are located on chromosome 1 at loci At1g23820 and At1g70310, respectively. Hanzawa et al. (2002) detected all 3 *SPDS* transcripts in whole seedlings, leaves, stem internodes, roots, inflorescence, and siliques in Arabidopsis using RNA gel blot hybridization. *SPDS1* and *SPDS2* transcripts were present at higher levels in roots than other organs; *SPDS3* expression was most prevalent in stem internodes, flower buds and roots.

Another polyamine biosynthetic gene, *SPMS* is much less studied as compared to *ADC*, *SAMDC* and *SPDS*, and has been found to play a role in internode elongation in Arabidopsis. Only one copy of *SPMS* (Accession # NM121958; At5g19530) has been identified in Arabidopsis. Panicot et al. (2002) have shown that SPMS shares 57% similarity with SPDS1 and SPD2. This is perhaps due to their utilizing similar substrates for aminopropyl transfer. It should be

pointed out that the naming of genes as 1 and 2 (e.g. *SAMDC1* and *SAMDC2*) is based solely on the order of their cloning and not on sequence similarity in different species.

I.6. Transgenic manipulation of polyamines

A common approach for studying the role of polyamines in physiological and developmental processes is by modulating their levels using several different methods like chemical inhibitors, over expression, down regulation and mutational analyses. Of the various methods, transgenic gene expression provides an effective means of both up and down regulating specific steps in a metabolic pathway. All major polyamine genes have been used for transgenic expression. Both homologous and heterologous transgenic systems have been used for genetic manipulation of the polyamine modulation in various species.

Spermidine is produced from putrescine by the enzyme spermidine synthase using dcSAM as a substrate for the aminopropyl moiety (Bagga et al., 1997). The enzyme SAMDC is considered the key enzyme to regulate the production of dcSAM, and, in turn, the production of spermidine and spermine (Bagga et al., 1997). Several studies have reported changes in spermidine and spermine contents by transgenic manipulation of *SAMDC* in various plant tissues. However, in most cases, the changes in the cellular levels of these two polyamines were much lower than those observed for putrescine in *ADC* and *ODC* over expression studies. Noh and Minocha (1993, 1994) used human *SAMDC* under the control of 35S CaMV promoter to modulate spermidine metabolism in tobacco cells and reported a two-to-four fold increase in SAMDC activity with increased spermidine levels and decreased putrescine levels.

However no significant changes were observed in plant growth and morphology.

All the transgenic plants appeared normal and produced fertile flowers. Kumar et al. (1996) reported the transgenic manipulation of *SAMDC* in a homologous system in potato. They modulated the *SAMDC* in both sense and antisense orientation under the control of 35S CaMV promoter. The transgenic plants expressing *SAMDC* sense construct could not be recovered indicating that over expression of *SAMDC* in sense orientation was probably lethal. Transgenic plants expressing *SAMDC* antisense construct however, showed decreased *SAMDC* transcript levels and altered phenotype like stunted growth, short internodes, small and chlorotic leaves. However, over expression of the *SAMDC* gene under the control of tetracycline inducible promoter in sense orientation resulted in higher levels of *SAMDC* activity, and transcript accumulation with an increase in spermidine in all the transgenic lines of potato (Kumar et al., 1996).

Later, Pedros et al. (1999) reported increased *SAMDC* transcript levels, *SAMDC* enzyme activity, and spermidine levels in transgenic potato plants expressing *SAMDC* sense construct under the control of tuber-specific patatin promoter. Morphologically, potato plants produced small sized but larger number of tubers. The transgenic plants expressing antisense *SAMDC* did not show any obvious phenotype changes. Over expression of *Datura stramonium* *SAMDC* cDNA in rice resulted in accumulation of mRNA transcript with an increase in *SAMDC* activity (Thu-Hang et al., 2002). Over expression of Arabidopsis *SAMDC* in tobacco however did not lead to an increase in spermidine, but a decrease of spermine levels was observed (Hanfrey et al.,

2002). All of the transgenic lines showed altered morphological characteristics; viz., stunted growth, short internodal lengths, wrinkled and curled leaves, delayed flowering, reduction in fertility and small petals with intense pigmentation.

In animals, plants and fungi, ODC catalyzes the formation of diamine putrescine directly from ornithine. Hamill et al. (1990) over expressed yeast *ODC* cDNA in tobacco roots to modulate the metabolism of putrescine and nicotine. The transformed root cultures had an accumulation in both putrescine and nicotine. Over expression of mouse *ODC* in *Nicotiana tabacum* produced similar results where increased amounts of putrescine without much effect on spermidine and spermine content were reported (Descenzo & Minocha, 1993). Bastola and Minocha (1995) transformed carrot cells using the mouse *ODC*; transgenic lines not only showed higher levels of putrescine but also showed improved production of somatic embryos in the presence of inhibitory concentrations of 2,4-D. Bhatnagar et al. (2002) have reported on the overproduction of putrescine and its metabolic regulation in poplar (*Populus nigra* x *maximoviczii*) cell suspensions.

I.7. Reporter-promoter fusion analysis

The expression of a gene can be studied using a variety of techniques. The most widely used techniques being microarrays, northern blots, and RT-PCR (Qualitative and Quantitative). All these methods differ in their approaches in the detection of transcript level. DNA microarrays can be used to study expression of thousands of genes simultaneously (Marchal et al., 2002). Northern blot analysis provides a direct method for the comparison of the message transcripts of

specific genes. However, neither the microarrays nor northern blots are often specific to different members of the same gene family, which show high homology and also do not yield information about sub cellular localization of the transcripts. The most sensitive technique for mRNA detection and quantification is RT-PCR. There are two types of RT-PCR: quantitative RT-PCR and qualitative RT-PCR. Qualitative RT-PCR is used to detect the presence of mRNA and quantitative RT-PCR is used to both detect and quantify the mRNA in a given tissue. An alternative approach to study gene expression that completely eliminates RNA isolation methods is promoter-reporter fusion approach. In this procedure, a reporter gene is fused to the promoter region of the gene of interest and then cloned into a plant transformation vector. Upon successful transformation of plant cells, the reporter gene expresses itself under the control of the promoter and thus gives the information on the spatial and temporal expression pattern of that gene as the expression of the reporter gene supposedly mimics the activity of the gene of interest in different tissues.

The promoter-reporter gene techniques offer two advantages: 1) Since, the expression of the reporter gene is driven and controlled by the promoter of specific gene, it provides high degree of specificity of expression of that gene; and 2) the procedure allows study of different members of a gene family that may have a high degree of homology among them. Another advantage being that expression can be studied in vivo in all the tissues of the plant at the same or different developmental stages without cumbersome RNA preparations. In promoter-reporter fusions, the nature of the reporter gene is very important. The gene product should be easily detectable, assayable or be a selectable marker.

Some of the commonly used reporter genes are: *lacZ, β-galactosidase, galactokinase, Green Fluorescent Protein (GFP)* and *β-glucuronidase (GUS)* for which there are easy color assays and photometric assays. One of the most common reporter genes used in reporter-promoter studies is *β-glucuronidase* or *GUS* gene (Novel & Novel, 1976). The product of the *GUS* gene, the GUS enzyme can be assayed quantitatively by fluorometry using the fluorometric substrate: 4-methylumbelliferry-β-D-glucuronide (4MUG) and qualitatively using a colorimetric substrate: 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc) (Jefferson et al., 1987; Martin, 1992).

Reporter-gene system has been widely used for the dissection of gene families (Martin, 2002) to analyze functional and regulatory components of promoters (Sessions et al., 1999), strength of the promoter, the effect of exogenous factors on plant growth and its changing transcript levels. For example, Medina et al. (2001) used GUS reporter system to study the expression of two cold inducible genes: *rci2a* and rci2b in response to development and stress conditions, and found high *GUS* activity under stress conditions than the normal levels. *GUS* gene system was also used to analyze the susceptibility of mutagenized *Arabidopsis* to *Xanthomonas campestris* (Godard et al., 2000). In this study, the growth of pathogenic bacteria expressing the *GUS* gene was traced using histochemical *GUS* staining.

I.8. Tissue-specific promoters

A promoter is the region of DNA (5' to the transcribed sequence) required for the initiation of transcription and to determine the appropriate temporal and spatial expression pattern of the gene. Such regulatory sequences are generally

located upstream (1000 bp or more) from the transcription start site (Dietrich et al., 1992). However, additional regulatory elements can also be found further upstream of 5' sequences or intragenically or downstream of the coding sequence (Dietrich et al., 1992; Zhang et al., 1996). There are different promoter types that can be used for transgenic expression of a desirable gene e.g.: constitutive promoters, inducible promoters, tissue specific promoters, and developmentally regulated and synthetic promoters.

Tissue-specific promoters control gene expression in a tissue dependant manner and according to the developmental stage of the plant. These promoters can also be used to drive the expression of transgenes in tissues where their expression is desired without modifying the rest of the tissues of the plant. Tissue-specific expression is the result of a complex interaction of regulatory molecules and transcription factors in a tissue for gene regulation. Hence promoters from the homologous or closely related species can be used to achieve efficient expression of transgenes in the specific tissues.

In the present study, promoter sequences of genes whose expression pattern during floral development was found to be localized in specific domains in the developing floral meristem were used for up-regulation of polyamine production by over expression of heterologous *SAMDC* and *ODC* genes. In the present project, the promoters selected for use in this study (*AP1* & *AP3*) represent two of the four key genes involved in flower-meristem identity and organ identity as mentioned earlier. These promoters have been well characterized with respect to their spatial and temporal expression pattern during flower development.

APETALA 1 (AP1) belongs to the A class of meristem identity and homeotic genes (Schwarz-Sommer et al., 1990; Coen & Meyerowitz, 1991). The AP1 protein plays a dual role in flower development in that it regulates flower meristem identity and also specifies the organ identity of the sterile whorls of the flower (sepals and petals). Consistent with its meristem identity role, *AP1* RNA begins to accumulate in young flower primordia as they arise on the flanks of the inflorescence meristem (IM) (Mandel et al., 1992). *AP1* is expressed throughout the developing flower from floral stage 1 to floral stage 3 (Mandel et al., 1992); as the development progresses, its expression is limited to outer two whorls, and is diminished in third and fourth whorls, cells that give rise to stamens and carpels. The loss of *AP1* RNA in third and fourth whorls corresponds to the onset of *AGAMOUS (AG)* expression, which is shown to be the negative regulator of *AP1* in these whorls (Drews et al., 1991). *AP1* interacts with *LEAFY* and converts the shoot meristem to reproductive meristem as discussed earlier (Mandel et al., 1992; Yanofsky, 1995).

APETALA 3 (AP3) belongs to the B-class of floral organ identity genes. It specifies petal and stamen identities and is expressed in specific domains of the floral meristem (whorl 2 and 3) that give rise to these organs. In wild type plants, *AP3* transcripts are first detected after the formation of sepal primordia (stage 3), in a meristematic domain that gives rise to petals and stamens (Jack et al., 1992). Also, upon the formation of petal and stamen primordia, *AP3* transcripts are detected throughout tissues of these organs at a high level, together with the integuments of the developing ovules, and its expression continues until the time of fertilization (Jack et al., 1992). Normal expression of *AP3* requires action of

meristem identity genes *LFY* as well as *AP1* (Hill et al., 1998; Tilly et al., 1998), the products of which bind to the promoter region of the *AP3* for its transcriptional activation.

Table 5: List of tissue-specific promoters in Arabidopsis

Promoter Type	Promoter
Root-specific	Pyk10
Gametes-specific	AG
Sepal –specific	AP1
Petal-specific	AP2
Stamen-specific	AP3
Stamen-specific	PI
Pollen-specific	LAT52
Anther-specific	APG
Carpel-specific	AGL5
Ovary Specific	AGL1

LFY is a positive regulator of *AP3* as reduced *AP3* expression was found in strong *lfy* mutants. LFY transcription factor interacts with two cis-acting elements within *AP3* promoter that are required for *AP3* expression and acts through several pathways for regulating *AP3* expression (Lamb et al., 2002). Also mutations in *AP1* reduce *LFY*-dependant induction of *AP3* (Lamb et al., 2002). Weigel & Meyerowitz (1993) reported *AP3* expression in *ap1* mutants during early stages of floral development in sterile whorls while in *lfy ap1* double mutant. *AP3* expression was undetectable during any stage of development. These results suggest that *AP1* together with *LFY* acts as the positive regulator of *AP3* expression (Weigel & Meyerowitz, 1993). Constitutive over expression of *AP3* under the control of a CMV 35S promoter resulted in partial conversion of carpels to stamens suggesting that *AP3* expression is auto regulated (Jack et al., 1994), which might have resulted from the direct binding of these regulatory genes to the *AP3* promoter.

I.9. Background research

In our lab, *GUS* reporter system has been used to study the spatial and temporal expression of polyamine biosynthetic genes *ADC2* and *SAMDC1* in Arabidopsis during various developmental stages (Mitchell, 2004, MS thesis). The first step in this study was isolation of ADC and SAMDC promoter regions and cloning them in-frame with the GUS reporter gene in a binary vector. The plasmids with promoter regions, named, pADC2::*GUS* (Figure 3 A, B) and pSAM1::*GUS* (Figure 4 A, B) were previously constructed by Paul Nuzzi. These plasmids were tested for the transient expression of GUS in poplar cells by biolistic bombardment and found to be functional. Then, the PCR-amplified, cloned

promoter regions of the two active genes of both *ADC2* and *SAMDC1*, recombined with the coding sequence of *GUS* were transferred to pCAMBIA, and then into Arabidopsis by floral dip method (Mitchell, 2004, MS thesis).

In the present study, transgenic plants (T2 stage) for the *ADC2* and *SAMDC1* constructs were used to study the expression of *GUS* gene in different whorls of the developing flower at various stages of flower development. GUS activity was localized histochemically (Jefferson et al., 1987) in different tissues using tissue sections as well as whole organs of the developing flower. The localization of blue color in different cell layers at different stages of flower development was tabulated using qualitative (presence or absence) observations. This information will, hopefully, be useful in targeting the timing and location of over expression and down regulation of these genes in order to characterize their role in flower development. *Daucus carota SAMDC* cDNA sequence (Accession # AF334029) was previously cloned and characterized by Varma (2003) and Chretien (2004) from wild type carrot (*Daucus carota* L., Queen Anne's lace). The cloned sequence has a 466 bp 5' UTR, 1086 bp of open reading frame (ORF) encoding 361 amino acids, and a 209 bp 3' UTR.

The functionality of the cloned gene was determined in *E. coli* (strain HT375, a mutant strain of *E. coli*, which has *indel* mutations in the *SAMDC* gene resulting in the loss of SAMDC activity) using pFLAG-SHIFT expression vector. This transformed bacterial strain has shown 111- folds increase in SAMDC activity indicating that the isolated carrot *SAMDC* ORF coded for a functional enzyme. Later, the PCR amplified and cloned *SAMDC* ORF in pZ(+) cSAMDC (Figure 5A) was transferred into pCAMBIA 1380 (Chretien, 2004; Figure 5B). In order to test the

effects of over expression of the carrot *SAMDC* in tobacco, a constitutive expression vector was created where the PCR amplified, cloned and characterized carrot *SAMDC* ORF recombined with the double CaMV 35S (2×35S) promoter was constructed in pCAMBIA vector (Figure 5C).

Transgenic studies conducted on tobacco, carrot and poplar in our laboratory have shown that polyamine levels can be manipulated by transgenic expression of a mouse *ODC* cDNA (Descenzo & Minocha, 1993; Bastola & Minocha, 1995; Bhatnagar et al., 2002). The results showed that while putrescine production in the transgenic cells was increased, the alternate pathway involving *ADC* was not affected (Anderson et al., 1998; Bhatnagar et al., 2001; 2002). Since Arabidopsis lacks an *ODC* gene, mouse *ODC* expression in Arabidopsis should provide an effective means of modulating polyamine metabolism. As mentioned earlier, although polyamines are ubiquitous, their importance and specific role in floral development have not been demonstrated. Hence, manipulating polyamine genes in shoot apical meristem during floral development and in different whorls of flower will help us to elucidate some of their functions in floral ontogeny.

If polyamines do play a crucial role downstream of floral meristem and organ identity genes, specific changes will be expected in flower development, timing and morphology in response to up-regulation of polyamine metabolism. These studies will strengthen our understanding of the role of polyamine metabolism in flower development. Thus the main hypothesis of my study is that polyamines play a key role in flower development and their manipulation during

flower development will alter the morphology or the timing of flower development.

The specific objectives of my research were:

1. To analyze the spatial and temporal expression patterns of *ADC2* and *SAMDC1* during the development of flowers in Arabidopsis.
2. To study the effects of transgenic manipulation of polyamine metabolism by overexpression of a *SAMDC* and an *ODC* gene under the control of floral tissue-specific promoters AP1 & AP3 on flower development.

CHAPTER II

MATERIALS AND METHODS

II.1. Bacterial culture and growth

Transformed *E. coli* were plated on solid LB (10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl) medium containing 1.5 % Bacto agar and grown overnight at 37°C. The following day, potential colonies harboring desired plasmid were grown in a 3 mL liquid LB medium supplemented with an appropriate antibiotic at a desired concentration and incubated at 37°C for 12-18 h on an orbital shaker at 250 rpm.

II.ii. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed using Ready-to-go PCR beads® (Amersham Pharmacia, Piscataway, NJ) with 10-100 ng of plasmid DNA or 50 ng of genomic DNA. The primers were designed using the program OLIGO primer analysis software and synthesized by IDT, Inc. (Coralville, IA). The optimal temperature for primer pairs was determined by a gradient PCR (Mastercycler Gradient Thermocycler; Eppendorf Scientific, Westbury, NY). All other PCR reactions were performed in a PTC-100 programmable thermocycler (MJ Research Inc. Watertown, MA) with the heated lid. The PCR reactions were set up in a final volume of 25 µL, with 10 pm/µL final concentration each of the primers and an appropriate amount of DNA. The typical PCR reaction profile included an initial denaturation for 2 min at 94°C, followed by 35 cycles of

denaturation for 30 sec at 94°C, appropriate annealing temperature and an elongation for 30 sec at 72°C, and a final elongation at 72°C for 5 min. The reaction mix was held at 4°C until further use.

II.3. PCR®2.1-TOPO® cloning

The PCR product was cloned into TOPO® cloning vector (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. TOPO® cloning reaction was performed with 2 µL of fresh PCR product and incubated at room temperature for 5 min. For electroporation, the reaction was cleaned by organic solvent extraction method using phenol: chloroform and ethanol to remove excess salts and ions, and the DNA was eluted in 10 µl of deionized water. For chemical transformation, 1-4 µL of TOPO® reaction mix was used to transform 50 µL of chemically competent cells; for electroporation, 1 µL of TOPO® reaction mix was used with TOP10 electrocompetent cells. The chemical and electrocompetent cells were prepared as follows:

A 3 mL culture of *E. coli* was grown overnight at 37°C. The following day, this culture was used to inoculate 400 mL LB medium and grown at 37°C until an O.D. of 0.6 was reached. At this stage, cultures were chilled on ice for 10 min and centrifuged at 5000 g at 4°C for 10 min to pellet the bacteria. The resulting pellet was resuspended in 50 mL of 0.1M $MgCl_2$ and centrifuged at 5000 g for 10 min. The supernatant was discarded and the pellet was resuspended in 50 mL of 0.1M $CaCl_2$ and chilled on ice for 10 min. Chilled cells were centrifuged for 10 min at 5000 g and the resulting bacterial pellet was resuspended in 4 mL of 0.1 M $CaCl_2$ +15% glycerol solution. The cells were then aliquoted into 50 µl volumes in 0.5 mL microfuge tubes in cold room, and stored at –80°C until further use.

Bacterial growth for electrocompetent cells was carried out similarly until an O.D. of 0.6, which represents the log phase and is critical for bacteria to be made competent. At this stage, bacteria were placed on ice. The chilled bacterial culture was pelleted at 4000 g for 10 min at 4°C and the resulting pellet was resuspended in 200 mL sterile ice-cold water. The centrifugation and resuspension steps were repeated thrice with a decreased volume of 100 mL ice-cold water to remove excess salts and ions. The resulting pellet was resuspended in 2 mL sterile ice-cold 10% glycerol and aliquoted as 50 µL volume into 0.5 mL tubes each in cold room, and stored at –80°C until further use.

A control plasmid pUC19 was used to check the transformation efficiency of the competent cells. For electroporation, different concentrations (0.1ng, 0.01ng and 0.001ng) of plasmid pUC19 or 1 µL of the purified ligation product or TOPO reaction mix were electroporated into the electrocompetent TOP10 cells. About 1 µL of the plasmid was mixed with 50 µL of the freshly thawed competent cells in a 1 mm gap electroporation cuvette and shocked at 1800 V in a 2510 electroporator (Eppendorf, Westbury, NY). Following electroporation, 250 µL of liquid LB medium was immediately added to the cuvette and mixed gently by pipetting. The culture was transferred to a 1.5 mL microfuge tube and incubated at 37°C for 60 min. If the plasmid used was suitable for blue/white selection, 40 µL of X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 mg/mL)] was evenly spread on 100 x 15 mm plates containing solid LB medium supplemented with appropriate antibiotic and incubated at 37°C for 30 min. After 60 min incubation, 100 µL of the culture was evenly spread on these plates. The remaining bacterial culture was centrifuged at 16,000 g for 30 sec, the pellet resuspended in 50 µL of

LB medium, and spread evenly on the second LB plate. All plates were incubated overnight at 37°C. The following day, selected colonies were used to incubate 3 mL of LB medium and grown overnight at 37°C for plasmid isolation.

II.4. Plasmid isolation

The plasmid isolation was carried out using the Wizard® Plus Miniprep (Promega, Madison, WI) kit. Three mL of the culture was centrifuged at 16,000 g for 30 sec to pellet the bacterial cells. The pellet was mixed sequentially with 200 µL of Resuspension solution (GET buffer), 200 µL of Lysis solution (0.2M NaOH and 1% SDS) and 200 µL of the Neutralization solution (1.32M potassium acetate, pH 4.8) and centrifuged at 16,000 g for 10 min. The supernatant was carefully collected into a new microfuge tube, treated with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, pH ≥ 8.0), vortexed for 5 sec, and centrifuged at 16,000 g for 2 min. The upper aqueous phase was carefully recovered into a new microfuge tube, mixed with two volumes of 100% ethanol and centrifuged at 16,000 g for 10 min to pellet the plasmid DNA. The supernatant was discarded and the pellet was washed with 500 µL of 70% ethanol to remove excess salts. Following centrifugation at 16,000 g for 5 min, the supernatant was discarded and the pellet was vacuum dried for 5 min. The dried pellet was resuspended in 50 µL of nuclease-free water or TE buffer and stored at –20°C until further use.

II.5. DNA quantification and sequencing

Plasmid DNA was quantified using Hoefer DyNA Quant 200 fluorometer, and the dye Hoechst 33258. DNA was quantified by adding 2 µL of plasmid DNA to the 2 mL dye reagent [0.1 µg /mL H33258 in 1X TNE (0.2 M NaCl, 10 mM Tris-Cl,

1 mM EDTA, pH 7.4)]. Genomic DNA concentration and purity were determined by 260 nm absorbance and 260 nm to 280 nm ratio in a UV spectrophotometer (Hitachi Instruments, San Jose, CA). The DNA to be quantified was diluted in water or TE. The concentration of the DNA (μg/μL) was calculated using the formula:

$$\frac{OD_{260} \times 50\ \mu g/mL \times \chi\ \text{(dilution factor)}}{1000}$$

DNA sequencing was performed at UNH Hubbard Center for Genome Studies using dideoxy chain termination method using Big Dye Terminator, and the sequences were analyzed using ABI PRISM system 310 (PE, Applied Biosystems). Sequences were analyzed with MegAlign (Laser Gene Software, DNAstar, Madison, WI), Chromas 2.13 (Queensland, Australia) and BioEdit Sequence Alignment Editor (North Carolina State University, Raleigh, NC).

II.6. Restriction and purification

For characterization of plasmid by restriction digestion, each reaction was performed in a final volume of 50 μL with appropriate amount of plasmid, 1X restriction enzyme buffer, 1X bovine serum albumin (BSA) and 1-10 units of restriction enzyme. All reaction mixtures were incubated at the appropriate temperature for 2 h. Restriction digestion of plasmid vectors (pCAMBIA-1390 and pCGN1547) was performed in a total volume of 100 μL with 10 μg of vector, final concentrations of 1X restriction enzyme buffer, 1X (BSA) and 2.5U/μg DNA of restriction enzyme. Following incubation at appropriate temperature for 6 h, another 2.5U of restriction enzyme was added and incubated overnight to ensure complete digestion. After 16 h incubation, compatible ends of the vector

were dephosphorylated with 0.3 µL of Calf Intestinal Phosphate (CIP) (New England Biolabs, Ipswich, MA) to prevent self-ligation. The reaction mixture was incubated at 37°C for 20 min for dephosphorylation followed by enzyme inactivation at 65°C for 15 min. After CIP treatment, the vector was purified as follows: the digested vector was brought up to a final volume of 100 µL. To this final volume, 1/10th volume of 3M-potassium acetate was added and this mixture was vortexed for 2 sec. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 16,000 g for 2 min. The upper aqueous layer was carefully collected into a new microfuge tube and 3 volumes of 100% ethanol were added to each volume of aqueous phase. After thorough vortexing, DNA was recovered as a pellet by centrifuging at 16,000 g for 10 min at room temperature. The pellet was washed with 70% ethanol to get rid of excess salts, re-centrifuged briefly, vacuum dried in a Speed-Vac and resuspended in 20 µL of water.

The inserts AP1, carrot *SAMDC* and mouse *ODC* genes were digested similarly except with 10 µg of DNA in a 100 µL total reaction volume and separated on 0.8% low melting agarose at 5V/cm. The separated fragments were excised from the gel using a razor blade and purified using QIAquick gel extraction kit (Quiagen, Valencia, CA) following manufacturers instructions. The purified DNA fragments were eluted in 20 µL of de-ionized water and stored at –20°C until further use.

II.7. Agarose gel electrophoresis

For plasmid and PCR product analysis and separation, agarose gel electrophoresis was performed with 6-8 µL of DNA to which 1X of the loading

buffer (15% w/v ficoll type 400, 0.25% (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol) was added and electrophoresed at 5V/cm on 0.8 % agarose gels in 1X TAE buffer until the loading dye front reached 2/3rd of the gel. Gels were stained in 0.5 µg/mL ethidium bromide for 15 min followed by destaining in water for 10 min. The gel was viewed under UV light using Gel Expert 3.5 program (NucleoTech, San Mateo, CA).

II.8. Ligations

Ligations were performed using T4 DNA ligase (Promega, Wisconsin, Madison). The ligation reaction was set up in water in a final volume of 10 µL with 100 ng of vector DNA and 3 molar excess of the PCR fragment in final concentration of 1X ligase buffer, and 0.2- 1U of the T4 DNA ligase. A negative control was also set up similarly with all the reagents except the PCR product to check the efficiency of digestion and ligation. Both the ligations were incubated at 15°C for 4-18 h or 25°C for 3 h as per manufacturer's instructions.

II.9. *Agrobacterium* transformation

Agrobacterium tumefaciens (strains GV3101 and LBA4404) were made electrocompetent as described above for *E. coli*. Except that the bacteria were grown at 28°C instead of 37°C. For electroporation, 2 µL of the desired plasmid DNA was mixed with 50 µL of electrocompetent cells and shocked at 1800 V as for *E. coli*. The bacteria were incubated at room temperature for 4 h in fresh LB medium and then spread on antibiotic plates, and incubated at 28°C until bacterial growth was observed (2-4 d). The resulting colonies were screened for the presence of desired plasmid by PCR. A small portion of the transformed colony was kept at 100°C for 10 min in 10 µL of water. The resulting lysate was

used as a template in PCR reactions with the appropriate primers for the respective gene (*AP1, cSAMDC & mODC*).

II.10. Arabidopsis growth in pots

Arabidopsis thaliana (ecotype Columbia) plants were grown in pro-mix soil-less medium mixed with perlite in 2:1 ratio at 18 h photoperiod under 80 $\mu Em^{-2}sec^{-1}$ fluorescent light at 21°C. Seeded pots were placed in a flat covered with a plastic dome (to maintain humidity for freshly sown seeds) and placed at 4°C for 2 days (for stratification) in order to allow uniform germination. The dome was slightly opened at the seedling stage to allow aeration for growing seedlings and then finally removed after three-day hardening-off period. The plants were watered on alternate days and using Miracle-Gro (Scotts company) fertilizer twice a week. Plant seedlings transferred from the plate to soil were also grown similarly.

II.11. Arabidopsis transformation by floral dip method

Arabidopsis plants intended for transformation were grown as above except with fewer seeds (10-15) per pot. The plants were grown until the primary bolts appeared. Primary bolts were clipped to allow the proliferation of many secondary bolts. After 4-6 days of clipping, plants were transformed using floral dip method as follows: *Agrobacterium* harboring desired plasmid was grown at 28°C overnight in 4 mL LB medium supplemented with appropriate antibiotic. The following day, the 4 mL culture was used to inoculate 400 mL of LB medium with appropriate antibiotic and grown overnight. Next day, the 400 mL culture was centrifuged at 5000 g for 10 min and the bacterial pellet was resuspended in 5% (w/v) sucrose solution to achieve an OD_{600} = 0.8 with gentle stirring to prevent

settling. Silwet L-77 was added at a final concentration of 0.05% before dipping.

The inflorescences were inverted and dipped in *Agrobacterium* solution for 2-3 seconds with gentle agitation. The dipped plants were placed in a tray laid on their side, covered with a plastic dome for 16-24 h, and kept away from direct sunlight. After 24 h, plants were thoroughly washed with water and returned to growth chamber. The loose bolts were tied up with wax paper and allowed to grow until seeds were produced.

II.12. Seed harvesting and sterilization

The initial transformed seeds (named T1) were collected from each pot and sieved through 40-mesh screen to remove debris. The seeds were transferred to 1.7 mL microfuge tube and subjected to ethanol sterilization. In a sterile hood, seeds were treated with 70 % ethanol and one drop of 10% Triton-X-100 (v/v) and incubated at room temperature for 5 min with gentle agitation every two min. The seeds were then washed twice with 100% alcohol and one drop of 10% Triton-X-100 (v/v) with gentle agitation for 5 min at room temperature. After the final wash, alcohol was completely removed by drying the seeds in laminar flow hood. The dried seeds were plated on an autoclaved germination medium (GM) composed of 4.4 g/L of Murashige and Skoog basal salts (Murashige and Skoog, 1962) (Sigma), 0.5 g/L MES (Sigma), 0.5 g/L sucrose, 0.8 % (w/v) Phytagar and B-5 vitamins (Gamborg et al., 1968) all adjusted to pH 5.7. Germination Medium was allowed to cool down to 50°C and appropriate antibiotic was added at concentrations (hygromycin-20 µg/mL; kanamycin-30 µg/mL) to screen for the transformants. The potential transformants were named according to the treatment (pCAM-AP1::m*ODC*, pCAM-AP3::c*SAMDC* and pot number. For

example pCAM-AP1::mODC-1 corresponds to AP1::mODC transformant from pot number 1 .

II.13. Genomic DNA isolation

Genomic DNA was isolated in different ways depending on the amount of the starting material used. For larger amounts of tissue (1-2 g FW), CTAB method (Murray et al., 1980) was used. The plant tissue (generally leaf), was powdered in liquid nitrogen and added to the appropriate amount of CTAB (Cetyl-trimethyl-ammonium bromide)-β mercaptoethanol buffer (5 mL/g of tissue) that was preheated to 60°C and vortexed briefly. This mixture was incubated at 60°C for 30 min with mixing every 5 min by inversion method. Then, it was treated with 500 µL of chloroform: isoamyl alcohol (24:1) and vortexed thoroughly followed by centrifugation at 16,000 g for 2 min. The upper aqueous layer was carefully collected into a new tube and treated with an equal volume of cold isopropanol and centrifuged at 16,000 g for 15 min to precipitate the DNA. The precipitated DNA was treated with 500 µL TNE buffer, 2 µL of RNAse and incubated at 37°C for 10 min. The resuspended pellet was once again treated with 500 µL of chloroform: isoamyl alcohol (24:1), mixed by inversion, and centrifuged at 16,000 g for 2 min. The upper aqueous layer was extracted and treated with 500 µL of isopropanol and centrifuged at 16,000 g for 10 min. The pellet was washed once with 500 µL of 80% ethanol and resuspended in 50 µL of TE or dH_2O after Vacuum dry.

From smaller amounts of tissue (20 – 40 mg FW) genomic DNA was isolated as follows: a small leaf was quickly frozen in liquid nitrogen and ground by a plastic pestle in a microfuge tube. To this, 500 µL of extraction buffer (0.2 M Tris-

HCl, pH 9.0; 0.4 M LiCl, 25 mM EDTA, 1% SDS) was added and centrifuged at 16,000 g for 10 min. A 350 µL of the supernatant was transferred to a fresh tube and an equal volume of isopropanol was added, mixed by inversion and centrifuged at 16,000 g for 10 min to pellet the DNA. The pellet was washed with 500 µL of 70% ethanol and centrifuged at 16,000 g briefly. The pellet was dried in Speed Vac and resuspended in 100 µL of deionized H_2O or TE buffer and stored at –20°C until further use.

II.14. RNA isolation

For RNA isolation, approximately 20 -100 mg FW of tissue at various stages of development were collected. The tissue was frozen in liquid nitrogen and ground to powder with a mortar and pestle and RNA was extracted using RNeasy Mini kit (Quiagen, Valencia, CA) with few modifications. To the powder, 450 µL of RLT buffer premixed with 10 µL/mL of β-mercapto-ethanol was added and transferred to the QIA-shredder column and centrifuged at 16,000 g for 2 min. The flow-through was transferred to a fresh microfuge tube and half volume of 100% ethanol was added and mixed several times by inversion. The entire mixture was transferred on to the RNeasy column and centrifuged at 16,000 g for 15 sec. The RNeasy mini- column was placed in a fresh microfuge tube and 700 µL of RW1 buffer was added and incubated at room temperature for 5 min. Following centrifugation at 16,000 g speed for 15 sec, mini-column was washed twice with 500 µL of RPE buffer with 3 min incubation each time at room temperature. After final wash, in order to elute the total RNA, 50 µL of RNase-free water was added to the mini-column, incubated at room temperature for 2 min

followed by centrifugation at 16,000 g for 1 min. The isolated RNA was stored at –80°C for further use.

II.15. Polyamine extraction

Tissue samples (100 mg FW) were collected from leaves, floral buds and mature flowers for polyamine extraction. Three replicate samples of each tissue type were collected and placed in 5% (v/v) PCA (perchloric acid) in a ratio of 1:4 or 1:8 (w/v), and frozen at –20°C for at least 4 h. Frozen tissue samples were thawed at room temperature for 1 h. After repeating the freezing and thawing steps thrice, the thawed PCA mix was vortexed at high speed and centrifuged at 10,000 g for 15 min. The PCA-soluble polyamines were dansylated and quantified by HPLC (High Performance Liquid Chromatography) according to the procedure of Minocha et al. (1990, 1994, 1999b). Briefly, 20 µL of 0.1 mM internal standard HD (Heptane Diamine) was added to the desired number of 1.5 mL microfuge tubes. To the tubes containing the standards, 100 µl of a mix of the three polyamines (at 0.004, 0.008, 0.02, 0.04, and 0.08 mM of putrescine, spermidine and spermine) and to the sample tubes, 100 µL of samples (PCA extract) were added. All tubes were vortexed briefly to mix well and centrifuged briefly for 30 sec to ensure that no volume is lost upon opening. Later, to all the sample and standards tubes, 100 µL of saturated sodium carbonate and 100 µL of dansyl chloride (10 mg/ml in acetone) solution were added in the same order and vortexed for 30 sec. All the tubes were incubated at 60°C for 1 h and then centrifuged briefly.

Later, 50 µL of L-alanine solution (100 mg/mL in deionized water) was added to each tube and the mixture was vortexed for 30 sec and incubated at

60°C for 30 min to remove excess dansyl chloride. The tubes were then centrifuged briefly and acetone was evaporated from open tubes in a Speed Vac (Savant Instruments Inc. Holbrook, NY) for 3-4 min. "*Photrex*" grade Toluene (400 µL) was added to each tube, vortexed for 1 min and left at room temperature undisturbed for 5 min to separate organic and aqueous phases. After incubation, all tubes were centrifuged briefly, 200 µL of toluene layer was collected in new microfuge tubes and dried in Speed Vac for 15-25 min, and 1.0 mL of methanol was added to each tube, followed by vortexing and centrifugation for 2 min. A 500 µL aliquot of the each methanol extract was transferred to the auto sampler vials, beginning with the standards first. Residual samples were stored at –20°C. Danylated samples were separated by HPLC (Perkin-Elmer) and quantified by fluorescent detector set at an excitation wavelength of 345 nm and an emission wavelength of 515 nm. Polyamines were calculated as nmol/g FW using Microsoft Excel®.

II.16. GUS staining and analysis

The activity of GUS in different parts of the plant was detected histochemically (Jefferson et al., 1987). Flowers were collected at different stages of development, submerged in GUS stain, vacuum infiltrated for 5 min and incubated overnight at 37°C. The following day, the tissues were destained in 70% ethanol for 12-24 h based on stain intensity. The GUS stained flowers were examined under the microscope and three floral stages (described below) were used for sectioning to analyze further for tissue specificity of GUS activity.

II.17. Trimming and sectioning

The GUS stained samples were embedded in small capsules made of methacrylate resin for sectioning. The GUS stained samples were dehydrated in 90% and 100% ethanol for 10 min each with gentle swirling before embedding. After dehydration, samples were treated with methacrylate and 100% ethanol mixed in 1:1 ratio and incubated on a rotor drum overnight. After overnight incubation, samples were treated with 100% methacrylate for 60 min to get rid of any excess ethanol in the samples. The methacrylate capsules used for embedding were made of methacrylate resin and solution B mixed in 50:1 ratio. The dehydrated tissue samples were carefully placed at the bottom of the methacrylate capsules to facilitate easy trimming and sectioning. The capsules with sample tissue were subjected to vacuum infiltration for 24 to 48 h to allow polymerization, following whichthe capsules were prepared for sectioning by trimming with a razor under dissecting microscope in a pentagonal shape to facilitate easy sectioning. Thin sections (2 µm) were cut using a microtome (JB4, OUPONT Instruments) and photographs of the sections were taken under dissecting microscope with Kodak Digital Camera

II.18. An example of plasmid construction

The plasmid pKY72 with 1.7 Kb AP1 promoter (Mandel et al., 1992; Figure 6B) was obtained from Dr. Elliot Meyerowitz (Caltech, Pasadena, CA). The plasmid, pCAM-AP1 was constructed by cloning the 1.7 Kb *Bam*HI fragment containing AP1 promoter into the *Bam*HI site of binary vector pCAMBIA 1390 (Figure 6A). The pCAMBIA 1390 vector contains a CaMV 35S promoter with duplicated enhancer sequences driving the expression of *hptII* gene encoding

hygromycin resistance in plants, and a termination region of *NOS*. It also contains a *nptII* gene encoding resistance for kanamycin in bacteria. The sense orientation of the AP1 promoter was verified by restriction analysis and sequencing. The resulting plasmid was designated as pCAM-AP1 (Figure 6C).

Table 6: List of the plants used for *GUS* expression analysis.
+ Refers to the plants that were tested positive for the presence of *GUS* gene and *hptII* by PCR

Plant	GUS stain	GUS PCR	hptII PCR
A2-3	+	+	+
A4-2	+	+	+
A5-4	+	+	+
A5-5	+	+	+
A6-1	+	+	+
A7-3	+	+	+
S1-1	+	+	+
S1-2	+	+	+
S3-3	+	+	+
S4-3	+	+	+
S2-1	+	+	+
S8-2	+	+	+

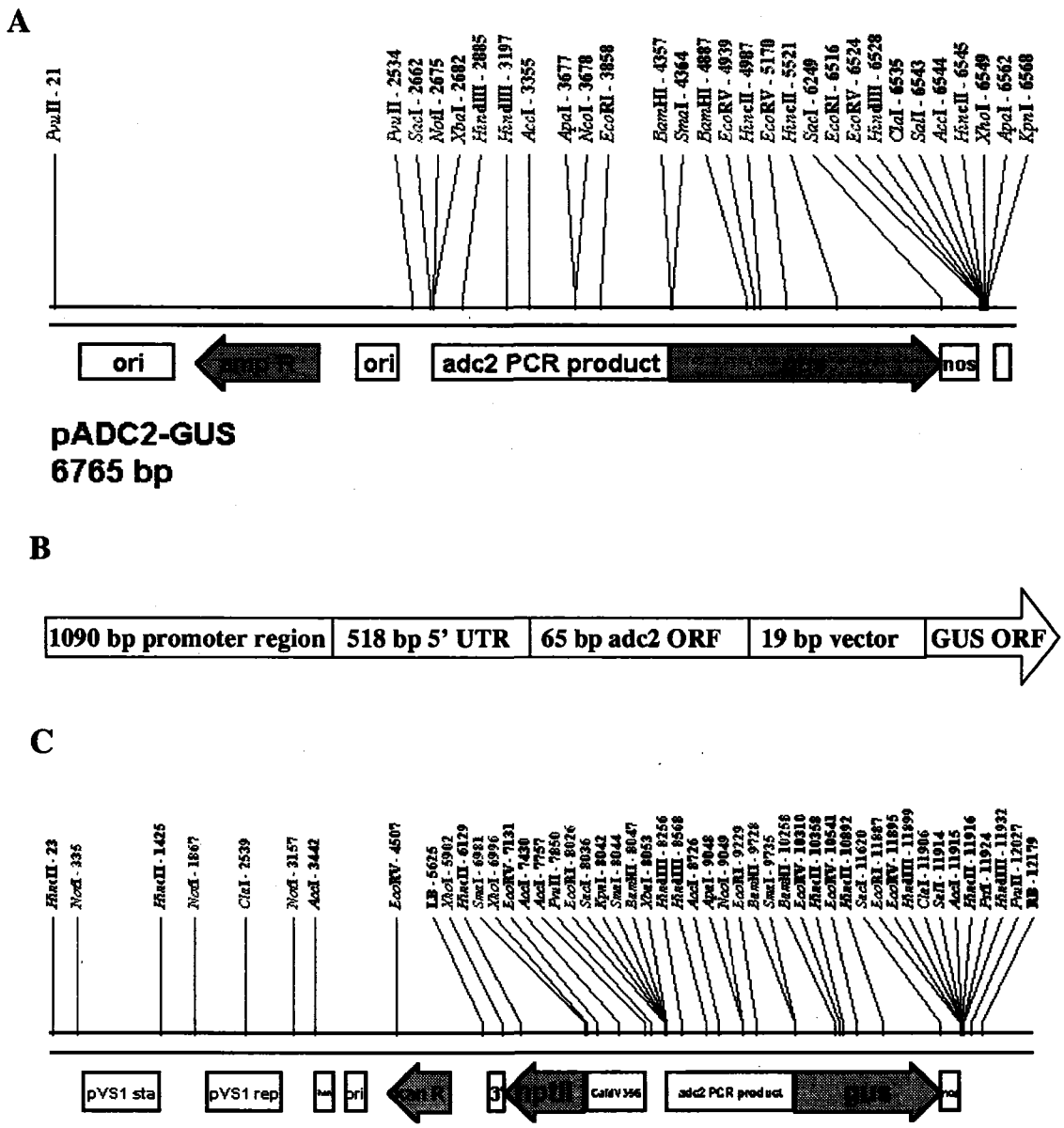


Figure 3: Construction of pCAM-ADC2::*GUS*. A) Linearized map of the pADC2-GUS B) Linearized map showing the fusion junctions between ADC2 promoter and GUS gene. The map contains, 5' UTR, 65 bp SAMDC1 ORF 19 bp vector fused to the GUS ORF C) Linearized map of pCAM-ADC2::*GUS*, ADC2 promoter fused to GUS gene

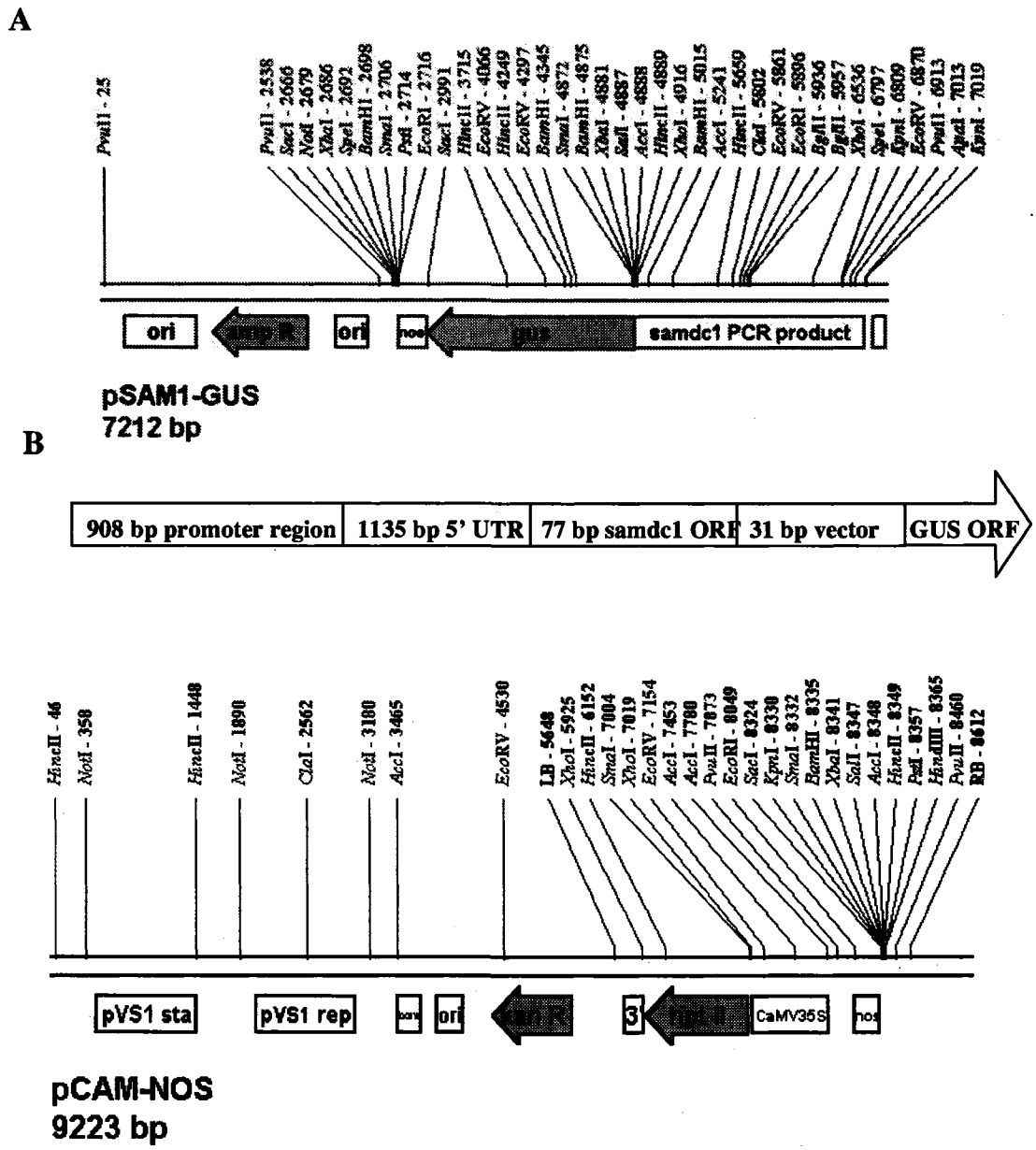


Figure 4: Construction of pCAM-SAM1::*GUS*. A) Linearized map of the pSAM1-GUS B) Linearized map showing the fusion junctions between SAMDC1 promoter and GUS gene. The map contains, 5' UTR, 77 bp SAMDC1 ORF and 31 bp vector fused to the GUS ORF

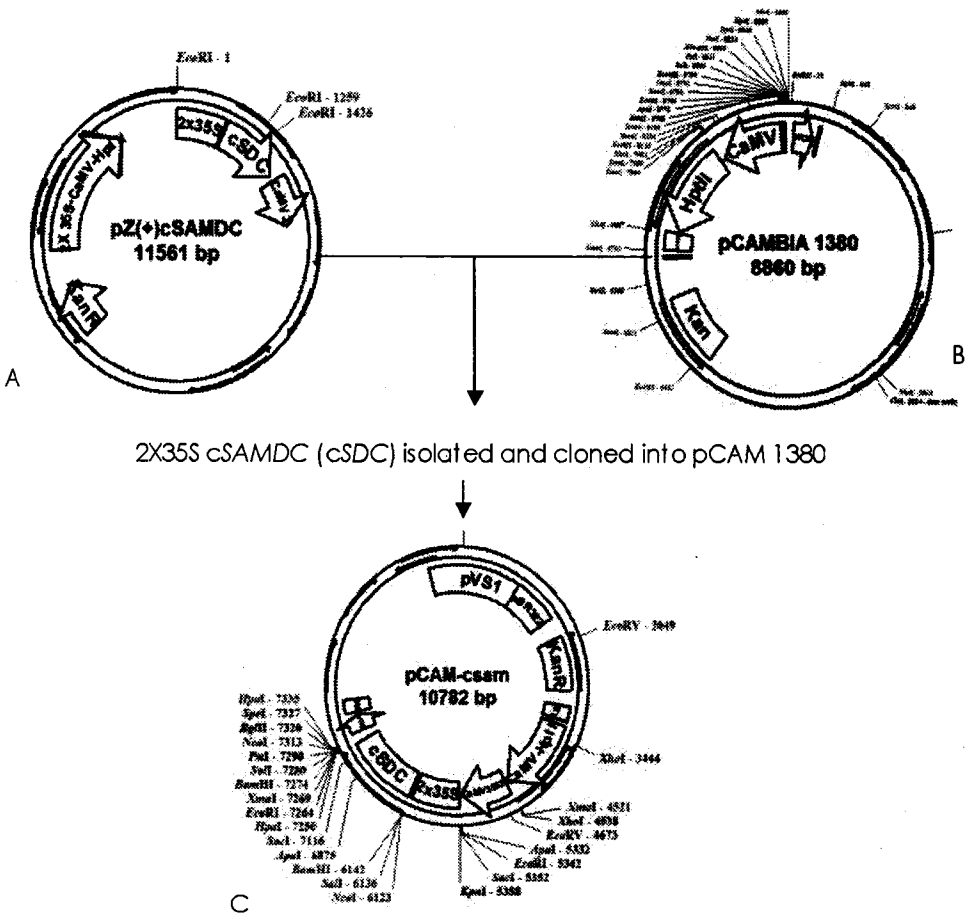


Figure 5: Construction of pCAM-c*SAMDC*. The 2 x 35S::c*SAMDC* from pZ(+)c*SAMDC* (A) was digested and cloned into pCAMBIA 1380 to create pCAM-c*SAMDC* (C).

Table 7: List of primers used for sequencing and PCR

Name	Primer Sequence (5'→3')	Gene
ORFadc2F1	GCGGTTTTTAAACCCCTACC	*adc2*
ORFadc2R1	GCAACAACAAACCACACGAC	*adc2*
ORFadc1F1	TGTTGAAAATTTGAATCCACAAA	*adc1*
ORFadc1R1	CTTAAAGACAAATAAAGAACTAGGATC	*adc1*
Ext-cSAMDC2	TTGTTGCCAGGGTTCTTAAACTGTT	*cSAMDC*
Ext-cSAMDC1	GCATAAACAAATAGGCTGGACTCA	*cSAMDC*
AP1-R1	TATTTATGGAGAGATTTGGG	*AtAP1*
AP1-F1	AGGGGTTTTTTCACACTTG	*AtAP1*
AP1-SR1	CTCTTATTTGGTGTTTTCC	*AtAP1*
AP1-SF1	AGGGGTTTTTTCACACTT	*AtAP1*
CV-mODCF1	ATGAGCAGCTTTACTAAGGA	*mODC*
CV-mODC R1	CTACACATTGATCCTAGCAG	*mODC*
ODC-04-F1	ATGGGCAGCTTTACTAAGGAC	*mODC*
ODC-04-R1	CATGGCTCTGGATCTGTTTCA	*mODC*
mODC-SPR1	CATCTGGACTCCGTTACTG	*mODC*
35DSAMDC-2	GCTTCCTTCAGTCTTGTAAA	*cSAMDC*
35DSAMDC-1	AAAGGTGGGAAAAAGAAGTC	*cSAMDC*
cSAMDC-SPR2	TTCGGTGACTGATAACTCTG	*cSAMDC*
HptII-F	GCTTCTGCGGGCGATTTGTG	*HptII*

List of plasmids used

pCAMBIA 2380: It is a plant expression vector provided to us by Eric Schaller (UNH) with pCAMBIA-1380 as a template. The *hptII* gene encoding hygromycin in 1380 has been replaced with *nptII* gene coding for kanamycin resistance gene and is expressed under the control of 35S promoter in plants. However, an introduced gene requires its own promoter. This plasmid is suitable for *Agrobacterium* mediated transformation (Figure 12B)

pCAMBIA 1390: It is a plant expression vector designed by CAMBIA (Center for the Application of Molecular Biology to International Agriculture – www.cambia.org). It has a *nptII* gene coding for kanamycin resistance in bacteria and *hptII* gene coding for hygromycin resistance under the control of a 35S promoter in plants. An introduced gene requires its own promoter. This plasmid is suitable for *Agrobacterium* mediated transformation (Figure 6A).

pBLUESCRIPT KS+: It is a 2.9 Kb bacterial expression vector with an inducible Lac promoter (Stratagene, La Jolla, CA). The multiple cloning site is capable of blue-white selection. This promoter also permits fusion protein expression with the *ß-galactosidase* gene product. It contains an F1 origin of replication and has ampicillin resistance gene for selection of the transformed bacteria.

pCW122-m*ODC*: It is a 8 Kb plant expression vector containing a mouse *ODC* gene under the control of 2X tandem 35S CaMV promoter, and *nptII* gene also controlled by a CaMV 35S promoter for plant selection (Bhatnagar et al., 2000). Bacteria are selected on ampicillin (Figure 8A).

pD1954: It is a 15 Kb binary vector provided to us by Dr. Tom Jack (Dartmouth College, Hanover, NH) using pCGN1547 as a template. It contains 5' AP3 promoter and 3' terminator sequences with a *Bam*HI clonable site in between for gene of interest. It has COLE1 replication origin in *E. coli* and pRi replication origin in *Agrobacterium*. It has marker genes for resistance to gentamycin in bacteria and *nptII* for resistance to kanamycin in plants (Figure 14B).

PCR 2.1®-TOPO: It is a PCR cloning vector (Invitrogen, Carlsbad, CA) that facilitates easy cloning of PCR products without cumbersome ligation procedures. It has a T7 promoter for sequencing and has lacZ gene under Lac promoter for blue-white screening. It has selection markers for ampicillin and kanamycin in bacteria.

List of plasmids prepared as a part of present study

pCAM-cSAMDC: It is a 10,782 bp plasmid (Chretien, 2004) containing carrot *SAMDC* coding sequence under the control of cauliflower mosaic virus double 35S promoter. It has a *nptII* gene for selection in bacteria and *hptII* gene for selection in plants. It has all major restriction sites for cloning (Figure 5C).

pCAM-AP1: It is a 10,677 bp plasmid containing AP1 promoter in pCAMBIA 1390. The plasmid has all the features of pCAMBIA 1390 (Figure 6C).

pCAM-AP1::mODC: It is a 11,948 bp plasmid contains the moue *ODC* gene under the control of AP1 promoter in pCAMBIA 1390. Like other pCAMBIA vectors, it also has a *nptII* gene for selection in bacteria and *hptII* gene for selection in plants (Figure 8C).

pD1954-AP3::cSAMDC: It is 17 kb binary vector with carrot SAMDC under the control of AP3 promoter. It contains plasmid marker genes encoding resistance to gentamicin in bacteria and *nptII* gene encoding resistance to kanamycin in plants (Figure 14C).

pCAM2380-cSAMDC: It is a 9992 bp plasmid containing carrot SAMDC coding sequence. It has a *nptII* gene for selection in bacteria and *hptII* gene for selection in plants (Figure 12A).

List of bacterial strains used and prepared in the study

TOP10: It is an *E. coli* bacterial strain used as a recipient for transformation with plasmid vectors. It contains a streptomycin resistance gene and supports blue/white selection.

GV3101: The most commonly used strain of *Agrobacterium* carries chromosomal resistance to rifampicin, and has a Ti-plasmid (carrying vir [virulence] genes), which encodes gentamicin resistance. In the present study this strain was used to produce GV3101 (pCAM-AP1::mODC)

LBA4404: A commonly used strain of *Agrobacterium* LBA4404 carry chromosomal resistance to rifampicin, each has a Ti-plasmid (carrying vir [virulence] genes), which encodes streptomycin resistance. In the present study, this strain was used to produce LBA4404 (pAP3::c*SAMDC*).

CHAPTER III

Results

In order to understand the role of polyamines, in flower development, initiation and organ formation, two complementary experimental approaches were used: 1) heterologous expression of a carrot *SAMDC* (c*SAMDC*) and a mouse *ODC* (m*ODC*) gene under the control of Arabidopsis flower-specific promoters in order to manipulate polyamine production and subsequently study their effects in the flower development; and 2) Analyzing the spatial and temporal expression patterns of two native Arabidopsis polyamine biosynthetic genes *ADC2* and *SAMDC1* during flower development and in different whorls of the flower.

III.1. Construction of pAP1::m*ODC* plasmids

As a first step in combining the m*ODC* with the AP1 promoter, the promoter sequence was cloned into pCAMBIA 1390. The construct was named pCAM-AP1. The construction of the pCAM-AP1 is summarized in the Figure 6. AP1 promoter sequence from pKY72 (Figure 6B) was excised as a *Bam*HI fragment, gel purified (Figure 7A) and ligated into *Bam*H1-cut and dephosphorylated pCAMBIA 1390 to create pCAM-AP1. Fifteen colonies of transformed *E. coli* selected on kanamycin were tested for presence of the insert, and only one colony had the insert when digested with *Bam*HI (Figure 7B).

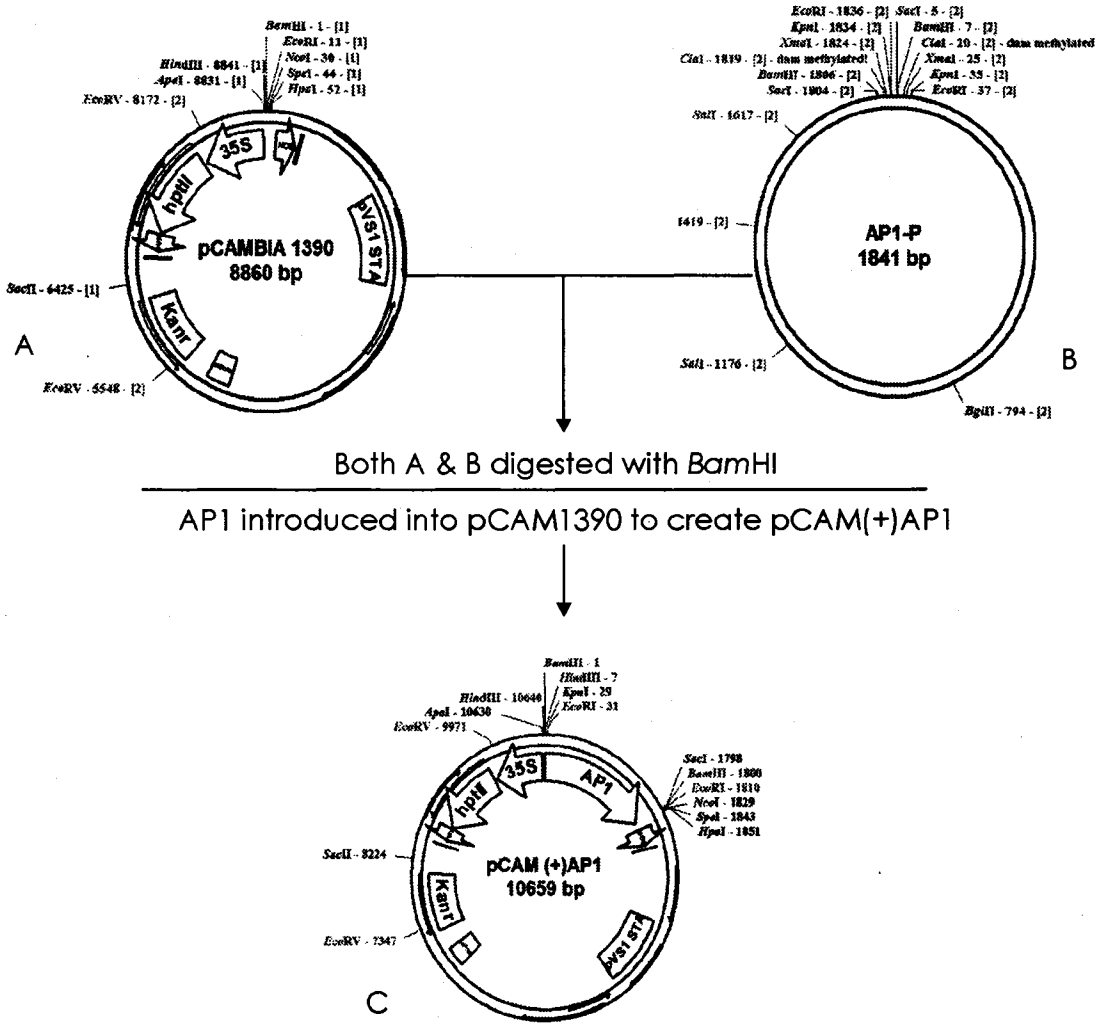


Figure 6: Construction of pCAM-AP1: The vector pCAMBIA 1390 (A) and insert AP1 fragment with *Bam*HI ends (B) are digested with *Bam*HI and ligated to create pCAM (+) AP1 (C)

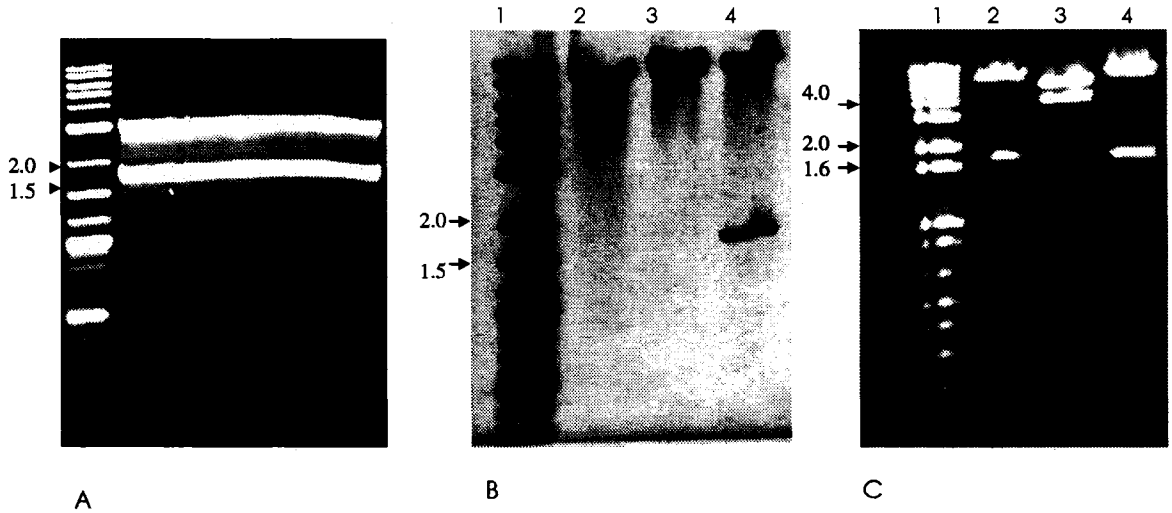


Figure 7: Characterization of pCAM-AP1; A) Plasmid pKY72 digested with *Bam*HI for the isolation of 1.7 kb AP1 fragment, lane 1: 2-dye log ladder; B) Restriction digestion of AP1 promoter in pCAMBIA-1390 with *Bam*HI , lane 1: 2-dye Log ladder, Lane 4: AP1 digested with *Bam*HI; C) Restriction digests of a selected clone of transformed *E. coli* showing the correct size of the insert, lane 1:1 kb ladder; lane 2: pCAM-AP1 digested with *Bam*HI; lane 3: pCAM-AP1digested with *Sac*I/*Sac*II; lane 4: pCAM-AP1digested with *Kpn*I/*Nco*I

In order to check the orientation of the AP1 promoter, the plasmid pCAM-AP1 was digested with *Sac*I/*Sac*II and with *Kpn*I/*Nco*I. The former digest yields two fragments of 4233 bp and 6426 bp if present in the forward orientation. Likewise, when digested with *Kpn*I/*Nco*I, it should yield fragments of 1800 bp and 8859 bp. As seen in Figure 7C, lanes 3 and 4, the band sizes obtained when digested with respective enzymes agree with the predicted sizes, confirming the correct orientation of AP1 in pCAM-AP1. The plasmid with AP1 in correct orientation was named as pCAM (+) AP1.

The plasmid pCAM (+) AP1 was used as a receiver for the m*ODC* coding sequence to create pCAM-AP1::m*ODC*; the steps are summarized in the Figure 8. m*ODC* coding sequence was excised as *Nco*I fragment from pCW122-*mODC* (Figure 9A) and ligated into the dephosphorylated pCAM-AP1 at *Nco*I site. The

resulting recombinant plasmid labeled as pCAM-AP1::m*ODC* was transformed to *E. coli* TOP10 and selected on kanamycin. A total of 10 resistant colonies were digested with *Nco*I to confirm the presence of m*ODC* insert and three were found to be positive (Figure 9B). The orientation of the moue *ODC* insert in

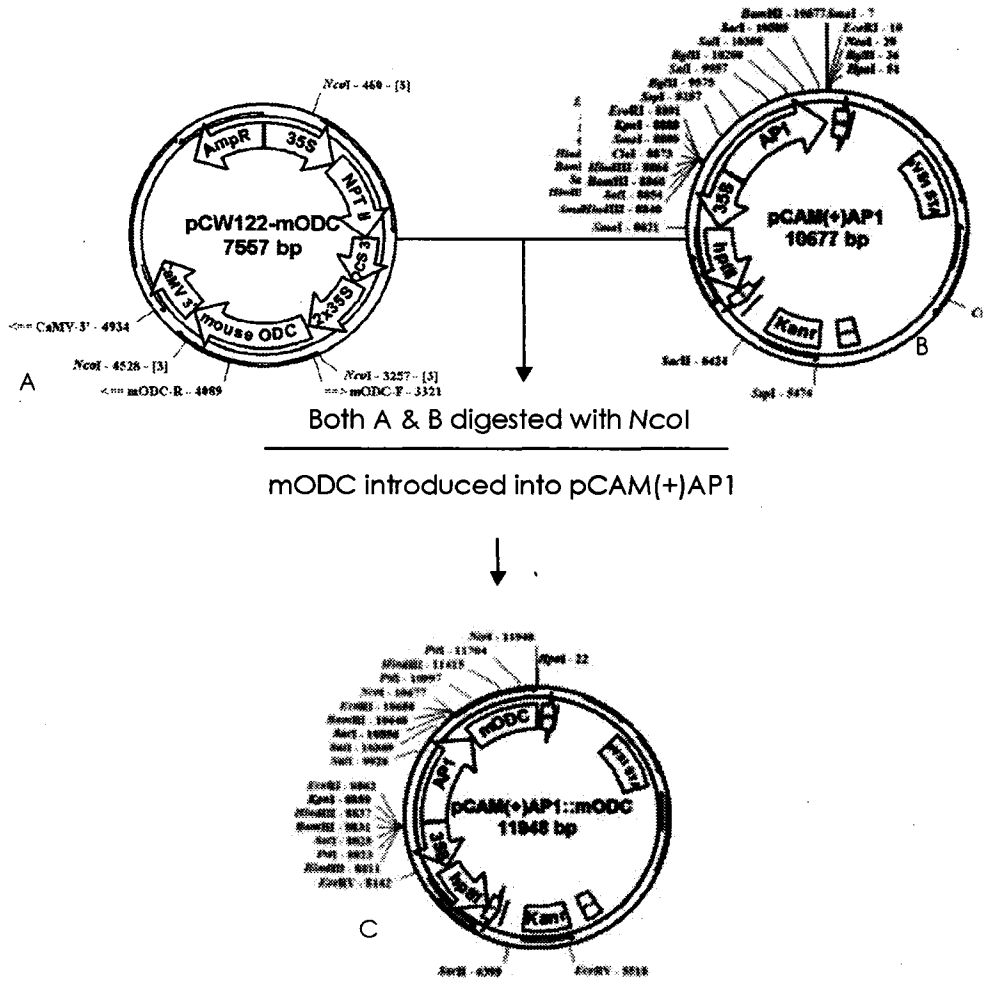


Figure 8: Construction of pCAM-AP1::m*ODC*: The mouse *ODC* from pCW122-m*ODC* (A) was removed as a *Nco*I fragment and ligated into the pCAM-AP1 (B) digested with the same enzyme to create pCAM(+)AP1::m*ODC* (C).

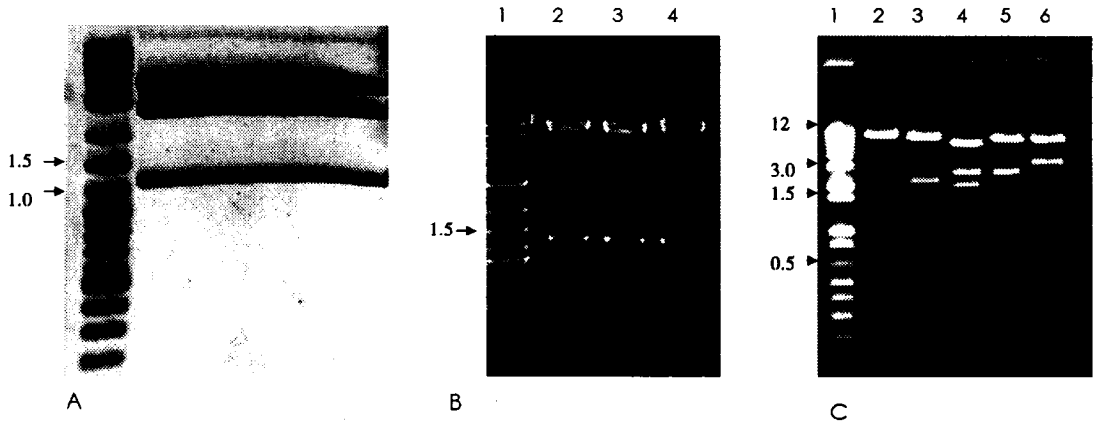


Figure 9: Characterization of pCAM-AP1::mODC; A) Products of pCW122-m*ODC* digested with *Nco*I; B) Confirmation of m*ODC* gene in pCAM-AP1::m*ODC digested with Nco*I; C) Lane 1: ladder, Lane 2: *Nco*I digested fragment, Lane 3: *Pst*I digested fragments, Lane 4: *Sac*II/*Hin*dIII, Lane 5: *Kpn*I/*Hin*dIII, Lane 6: *Kpn*I/*Hpa*I

Start

```
TGCAAACAAG   AGAAACCAGC TTTAGCTTTT CCCTAAAACC ACTCTTACCC
AAATCTCTCC   AAATCTTAAA TCAAACACAA GTCTTTTTAT AAAGGAAAGA
AAGAAAAACT   TTCCTAATTG GTTCATACCA AAGTCTGAGC CGGATCCCCG
GGGAATTCTA   AGAGGAGTCC A***CCATG***GGCA GCTTTACTAA GGACGAGTTT
GACTGCCACA   TCCTTGATGA AGGCTTTACT GCTAAGGACA TTCTGGACCA
AAAAATCAAT   GAAGTCTCTT CCTCTGACGA TAAGGATGCG TTCTATGTTG
CGGACCTCGG   AGACATTCTA AAGAAGCATC TGAGGTGGCT AAAAGCTCTT
CCCCGCGTCA   CTCCCTTTTA CGCAGTCAAG TGTAACGATA GCAGAGCCAT
AGTGAGCACC   CTAGCTGCCA TTGGGACAGG ATTTGACTGT GCAAGCAAGA
CTGAAATACA
```

End

```
AAGTATTACT CATCCAGCAT CTGGGGACCA ACATGTGATG GCCTTGATCG
GATCGTGGAG CGCTGTAACC TGCCTGAAAT GCATGTGGGT GATTGGATGC
TGTTTGAGAA CATGGGTGCA TACACCGTTG CTGCTGCTTC TACTTTCAAT
GGGTTCCAGA GGCCAAACAT CTACTATGTA ATGTCACGGC CAATGTGGCA
ACTCATGAAA CAGATCCAGA G***CCATGG***
```

Figure 10: Sequencing of pAP1::m*ODC* ligation junctions. Grey background indicates AP1 promoter. pCAM1390 sequence with *Nco*I restriction site (start codon) and second *Nco*I restriction site at the end of the sequence are italicized

pCAM-AP1::m*ODC* was confirmed by several restriction digestions (*Pst*I *Sac*II/*Hin*dIII, *Kpn*I/*Hin*dIII and *Kpn*I/*Hpa*I). The plasmid when digested with *Pst*I produced three fragments of 707, 2174, 9067 (Figure 9C, lane 3); with *Kpn*I/*Hin*dIII, it produced fragments of 20, 2604, 2556, 5768 (Figure 9C, lane 4); and with *Kpn*I/*Hpa*I, it produced fragments of 3111 and 8837 bps (Figure 9C, lane 5). All of the digests confirmed the correct orientation of m*ODC* insert in pCAM-AP1::m*ODC*. These clones were then sequenced at the junctions using primers mODC-SPR1 and ODC-04-F1 to confirm the integrity of promoter-gene fusion at these junctions (Figure 10).

III.2. Screening of pCAM-AP1::m*ODC* transformed *Agrobacterium* and Arabidopsis

The plasmid pCAM-AP1::m*ODC* was transformed into *Agrobacterium* GV3101. The transformed *Agrobacterium* colonies were selected on LB medium supplemented with kanamycin. For m*ODC*, five clones were screened by PCR for the presence of the gene using the primers mODC-F and mODC-R (Table 7). Figure 11A shows the amplification of m*ODC* in one of the colonies. The selected colonies were named *Agrobacterium* GV3101 (pCAM-AP1::m*ODC*) and stored in glycerol at –80°C.

Agrobacterium strain GV3101 containing the pCAM-AP1::m*ODC* plasmid was used to transform Arabidopsis. The seeds from each pot were collected, germinated on hygromycin, and the resistant transformants were termed as T1 generation plants. To confirm the presence of the *mODC* and *hptII* genes in transformed plants, PCR was performed on DNA isolated from leaves using *mODC* primers CV-mODCF1 and CV-mODCR1 as well as *hptII* primers (Table 7).

Figure 11B shows the amplification of 750 bp region of mODC and 600 bp region of *hptII*. The plants containing both the genes were considered "transformed" and named according to the generation and pot number; e.g. AP1::m*ODC* T1-1 refers to T1 transgenic line from pot # one. The plants of T2 generation were also screened, grown and labeled similarly, except T1 was replaced with T2 (T2-1).

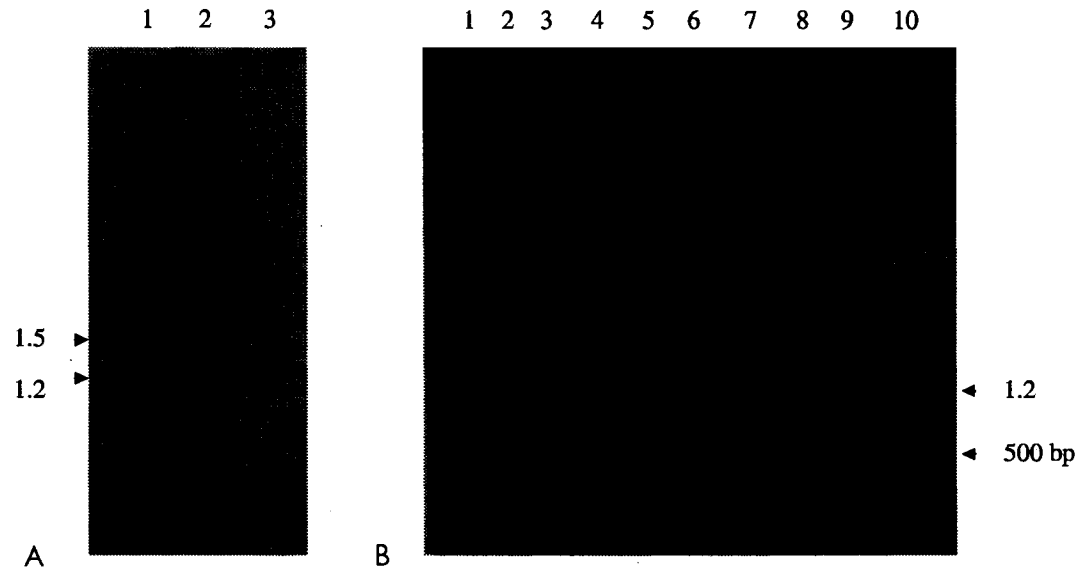


Figure 11: A) Gel photos of m*ODC* in *Agrobacterium* (A) and Arabidopsis (B); A) Lane 1: 2-log dye ladder, Lane 2 represents 1.2 kb m*ODC* and lane 3 represents negative control; B) Arabidopsis. Lanes 1,2,5 mODC and 3, 4, 7 nptII genes, Lanes 8, 9 negative controls of mODC and nptII genes respectively, Lane 10: 2-log dye ladder

III.3. Construction of pAP3::c*SAMDC* plasmids

As a first step, to generate a ranscriptional fusion of tissue specific promoter AP3 (whose expression is spatially restricted to specific domains in the flower), and the Open Reading Frame of c*SAMDC*, c*SAMDC* from pCAM-c*SAMDC* (Figure 5C, Chretien, M.S. thesis, 2003) was transferred into pCAMBIA

2380 (Figure 12B). Although the plasmid pCAM-c*SAMDC* had all the major restriction sites to remove c*SAMDC* coding sequence, most of the restriction enzymes had multiple sites and were created while cloning (e.g. *Bam*HI). Since these sites were never used for ligation, the efficiency of their use was a matter of concern. Hence restriction sites like *Bam*H1 were made "clonable" by ligating *Bam*HI digested c*SAMDC* into binary vector pCAMBIA 2380. The construction of the pCAM2380-c*SAMDC* is summarized in Figure 12. The 1.1 kb c*SAMDC* fragment from pCAM-c*SAM* was digested with *Bam*HI, and gel purified. Parallel to that, vector pCAMBIA 2380 was also digested with *Bam*HI and dephosphorylated. The purified c*SAMDC* fragment was ligated into pCAMBIA 2380 to create pCAM2380-c*SAMDC*.

The resulting recombinant plasmid (pCAM2380-cSAMDC) was transformed into *E. coli* TOP10 and selected on kanamycin. A total of 10 pCAM2380-c*SAMDC* transformed bacterial colonies were selected for plasmid isolation and subsequent analyses. The plasmid from all 10 colonies was digested with *Bam*HI to check for the presence of the insert and eight colonies were found to contain *SAMDC* insert (Figure 13A). The plasmid pCAM2380-c*SAMDC* was digested with *Apa*I to check the orientation of the insert; the correct orientation should yield two fragments of 753 and 9239 bp. Three colonies out of eight were found to contain c*SAMDC* fragment in correct orientation (Figure 13B); lane 3 shows an example of the plasmid from one of the colonies that had c*SAMDC* fragment in correct orientation in pCAMBIA-2380. The plasmid with insert in the correct orientation was named as pCAM2380 (+) c*SAMDC* (Figure 13C) and used as a source for c*SAMDC* to create pD1954-AP3::c*SAMDC* (Figure 14). The plasmid

pD1954 contains 1.1 kb of the AP3 promoter upstream of the transcription start site and 0.6 kb of 3' AP3 coding sequences and 3' NOS termination sequence in pCGN1547 (McBride & Summerfelt, 1990). There is a unique *Bam*HI site between the promoter and the 3' termination sequence for cloning a gene of interest.

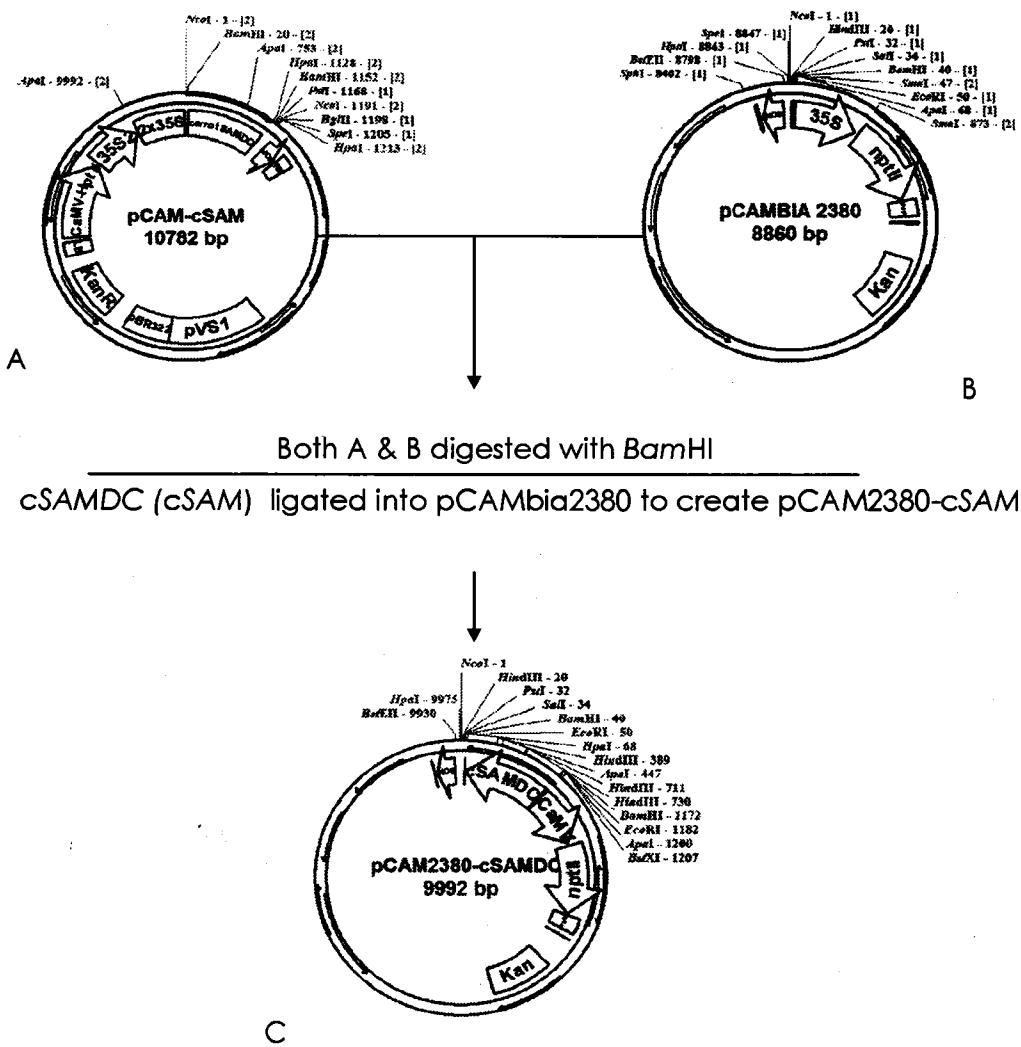


Figure 12: Construction of pCAM2380-c*SAMDC*. The c*SAMDC* from pCAM-cSAM (A) (Chretein, 2003) was isolated as a *Bam*HI fragment and cloned into pCAMBIA 2380 (B) at *Bam*HI site to create pCAM2380-c*SAMDC* (C)

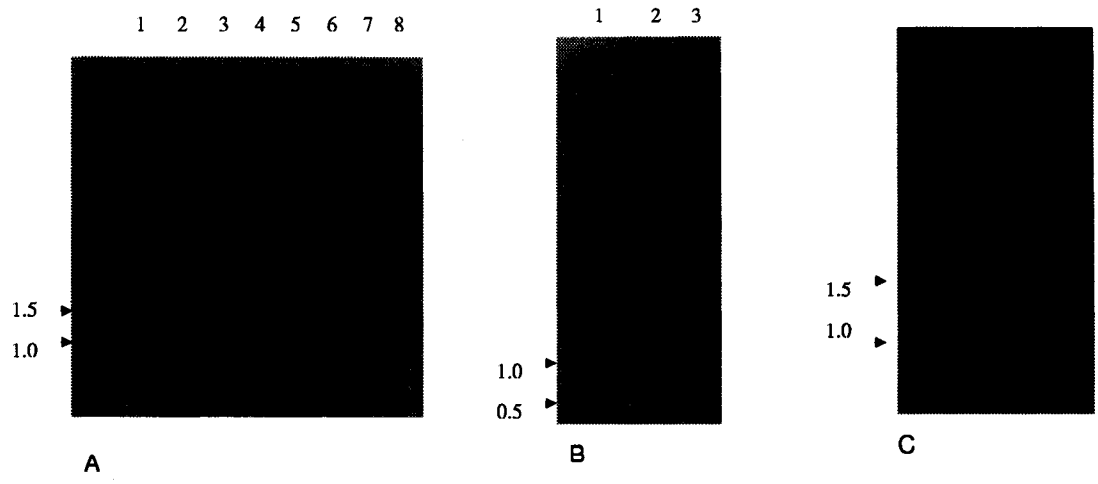


Figure 13: Characterization of pCAM2380-c*SAMDC* plasmid A) lanes 1-8: 1.1 kb cSAMDC *Bam*HI fragment from 8 selected colonies B) lane 3, pCAM2380 (+) cSAM # 10 digested with *Apa*I to confirm positive orientation of c*SAMDC* in pCAM2380. The 753 bp *SAMDC* fragment confirms its positive orientation. C) 1.1 kb c*SAMDC* fragment from pCAM2380- c*SAMDC* used for subsequent ligations. 1 KB ladder was used.

The plasmid pCAM2380 (+) c*SAMDC* was digested with *Bam*HI to excise the *SAMDC* coding sequence (Figure 13C). Likewise, the plasmid pD1954 (Figure 14B) was digested with *Bam*HI, dephosphorylated and ligated with c*SAMDC*; the resulting plasmid was named pAP3::c*SAMDC* (14C). Of the fifteen putatively transformed *E. coli* colonies selected, twelve showed the presence of c*SAMDC* when digested with *Bam*HI (Figure 15). The plasmid pAP3::c*SAMDC* had multiple restriction sites that were not shown on the plasmid map provided. Hence the orientation of the c*SAMDC* insert could not be determined by restriction analysis since too many fragments of unpredicted sizes were produced. Therefore, the orientation of the insert and ligation junctions was confirmed by sequencing the plasmid using the primers Ext-cSAMDC1 and Ext-cSAMDC2 (Table 7); the sequencing results showed that all 10 clones had insert in the correct orientation

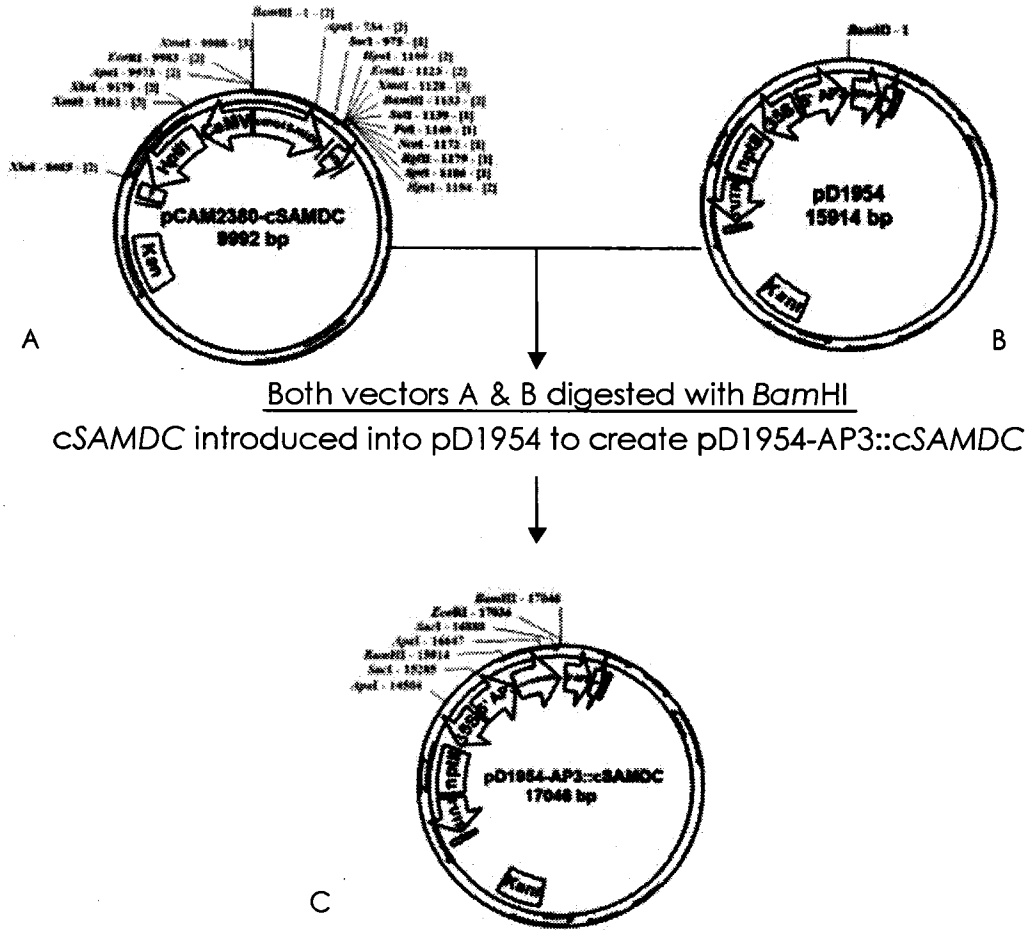


Figure 14: Construction of pAP3::c*SAMDC*(pD1954-AP3::c*SAMDC*): The carrot *SAMDC* was excised from pCAM2380-c*SAMDC* (A) as a *Bam*HI fragment and cloned into pD1954 (B) at the *Bam*HI site to create pAP3::c*SAMDC* (pD1954-AP3::c*SAMDC)* (C).

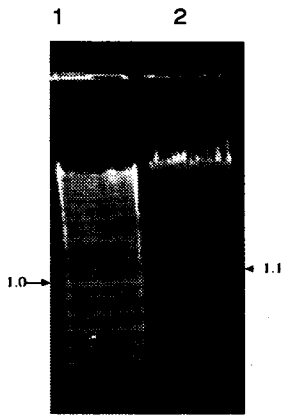


Figure 15: Characterization of pAP3-c*SAMDC* plasmid: carrot SAMDC in pD1954 digested with *Bam*HI

with respect to the promoter (Figure 16).

III.4. Screening of cSAMDC transformed *Agrobacterium* and Arabidopsis

The plasmid pAP3::c*SAMDC* was transformed into *Agrobacterium* LBA4404. The transformed *Agrobacterium* colonies were selected on LB medium supplemented with kanamycin. For c*SAMDC*, five clones (pAP3::c*SAMDC* # 1- 5) were screened for the presence of the gene by PCR with the primers Int-cSAMDC1 and cSAMDC-R (Table 7). Figure 17A shows the presence of c*SAMDC* from *Agrobacterium* DNA. *Agrobacterium* clone # 2 containing pAP3::c*SAMDC* plasmid was used to transform Arabidopsis. The seeds from each pot were harvested and screened on the germination medium supplemented with kanamycin (30 µg/ml). The resistant plants were termed as T1 plants. In order to check for the presence of c*SAMDC* and *nptII* genes in transformed plants, PCR was performed on pAP3::c*SAMDC* plants with c*SAMDC* primers Int-cSAMDC1 and cSAMDC-R and *nptII* primers which amplify 1.1 kb c*SAMDC* gene and 600 bp of *nptII* gene, respectively (Figure 17B). The plants that survived three weeks on kanamycin and showed the presence of the two transgenes were considered to be transformed and named according to the generation and pot number. For example, AP3::c*SAMDC* T1-1 refers to the T1 generation transgenic line from pot # 1. The plants were transferred to soil for subsequent analysis.

III.5. Transgenic expression of mouse *ODC*

In order to investigate the effects of mODC expression on flowering, T1 generation plants, selected (on hygromycin) were transferred to soil and grown to maturity. The seeds from these plants were again subjected to selection on hygromycin and the resistant seedlings (T2 generation plants) were transferred to

Start

TATCACTTAG TTTTCATCAA CTTCTGAACT TACCTTTCAT GGATTAGGCA
ATACTTTCCA TTTTTAGTAA CTCAAGTGGA CCCTTTACTT CTTCAACTCC
ATCTCTCTCT TTCTATTTCA CTTCTTTCTT CTCATTATAT CTCTTGTCCT
CTCCACCAAC CACCAATGGG TTCGAAATCG ATAAGCTT*GG ATCCAACTAT*
atgTCTTCCG AAGTTTCTGC AATTGGTTTC GAAGGTTTTG AAAAGAGGCT
GGAAATATCA TTTTTCGAGC CAAGTTTCTT TGCTGACACC CTGAAGGAAA
GGGTTTACGT GTCCTCTTCT AAAAACCAAC TCGATGAGTT TTTAGGACCT
GCTGAATGCA CTATTGTTGC GTCATTGTCC NATGAGNATG TTNAACNGAA
GGTTTCANCA TG

End

CAACCTGGGG AGTTCTCTAT AGCTCTTCAA GCTGATATTG CATCTGAACT
ACTCGAAAAG ACCAGCTCTG TACACGTCAA GGGCTATCGT GTGGAAGAGA
AAACTTGTGA AGAGCTCGGA ATGGACGGCT CCATCGTTTA CCAGAAGTTC
GTGAAGACAA CCGAAAGATG TGAATCTCCT CGATCAGTCC TGAAATGCTG
CTGGAAGGAG GAAGAGAAAG AAGAAAAGGA GTATCAATAA ATTACGGGTT
ACCTTCAAGG GCGAATTCCC GG***GGATCC*****GA ATAATTAAAG GCTAAAAGGT**
TTGCTGGTGC CATCATTGTC TATCTAATTA TTTAGTAACT ACTTAAAA

Figure 16: Sequence of the pAP3::cSAMDC ligation junctions. Grey background indicates AP3 promoter. *Bam*HI sequence in pD194 sequence is italicized. Underlined sequence indicates 3' AP3 sequence. Translation Start codon in SAMDC sequence I represented in small letters.

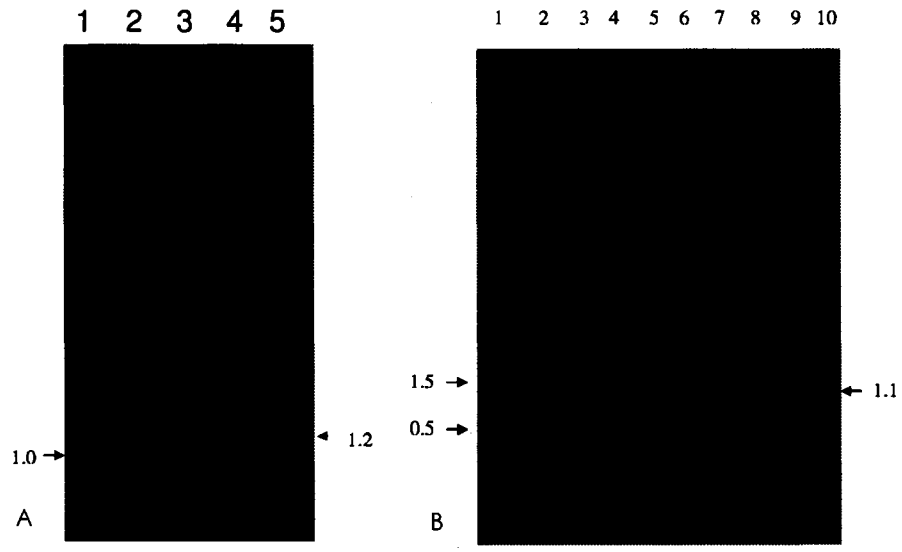


Figure 17: Gel photos of *cSAMDC* in *Agrobacterium* (A) and Arabidopsis (B); A) lanes 3, 4, 5 PCR product of *cSAMDC* in *Agrobacterium* and lane 1 negative control; B) PCR amplification of *SAMDC* and *nptII* from transgenic Arabidopsis: Lane 1: 2-log dye ladder Lanes 2,4,6 nptII gene and 3, 5, 7, 9 *cSAMDC* gene, Lanes 8, 10 negative controls of carrot *SAMDC* and *nptII* genes respectively.

soil and grown to maturity. In the present study, m*ODC* transgenics were studied in both T1 and T2 generations, although detailed analyses were carried out only with T2 plants. Two types of observations were made on mouse *ODC* transgenics: 1) The timing of the development of the whole plant, and 2) abnormalities during floral initiation and in flower morphology.

The most striking phenotype of m*ODC* transgenics was a slow plant growth and delayed flower development. In both T1 and T2 generations, transgenic plants exhibited slow development at early stages compared to the wild type or the c*SAMDC* transgenics (Figure 18). In particular, transgenics that had high polyamine levels had shown delay in the development of seedlings, growth, floral initiation, and opening of the flower buds (Figure 18). These plants showed more abnormalities in vegetative development rather than the phenotype of the flower although sepal and few petal abnormalities were also recorded. Approximately, 100 transgenic plants were observed and greater than 40% of them exhibited delayed plant development, floral initiation, and sepal abnormalities, whereas 15-20% had petal abnormalities. Most plants exhibited delayed development right from the time of floral meristem initiation to seed dispersal (Figure 18A, E & F). In wild type plants, floral meristem initiates around 25 DPG, when 14 rosette leaves are formed. Bolting and flowering are completed between 5 to 13 days after development of the buds. Although most of the m*ODC* transgenics developed the first floral buds around the same time as the wild type plants (i.e. 25 DPG), the buds remained in a closed stage until 48 DPG (Figure 18F). Thus the formation of mature flower was remarkably delayed in

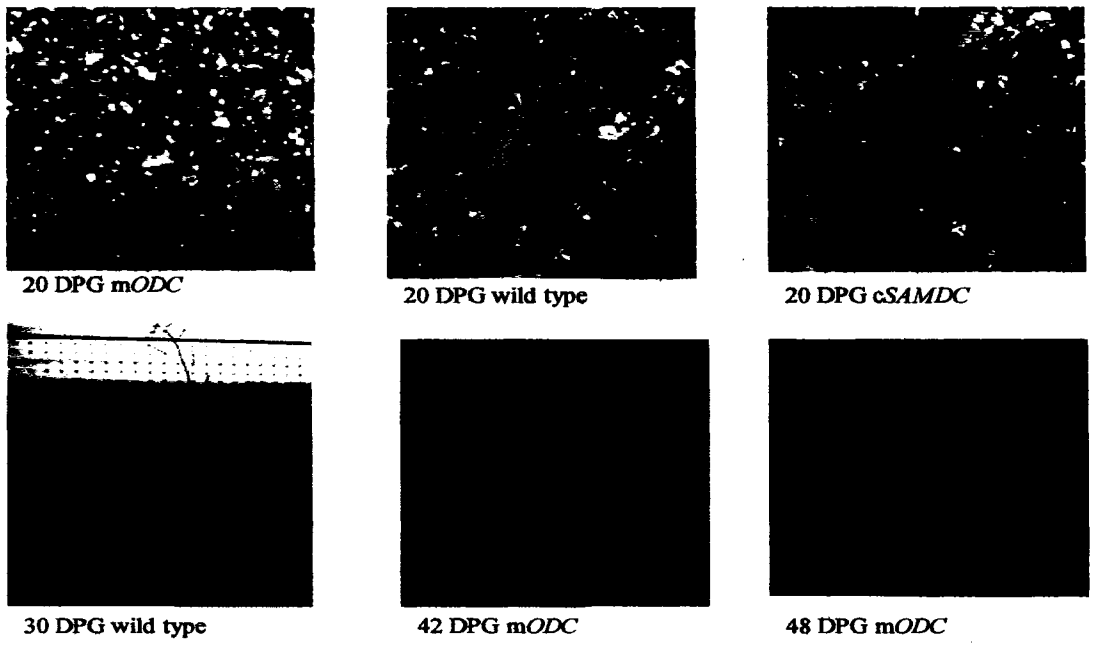


Figure 18: Growth abnormalities in mouse *ODC* (m*ODC*) transgenics. m*ODC* transgenics growth rate was compared to the wild type and carrot *SAMDC* transgenics

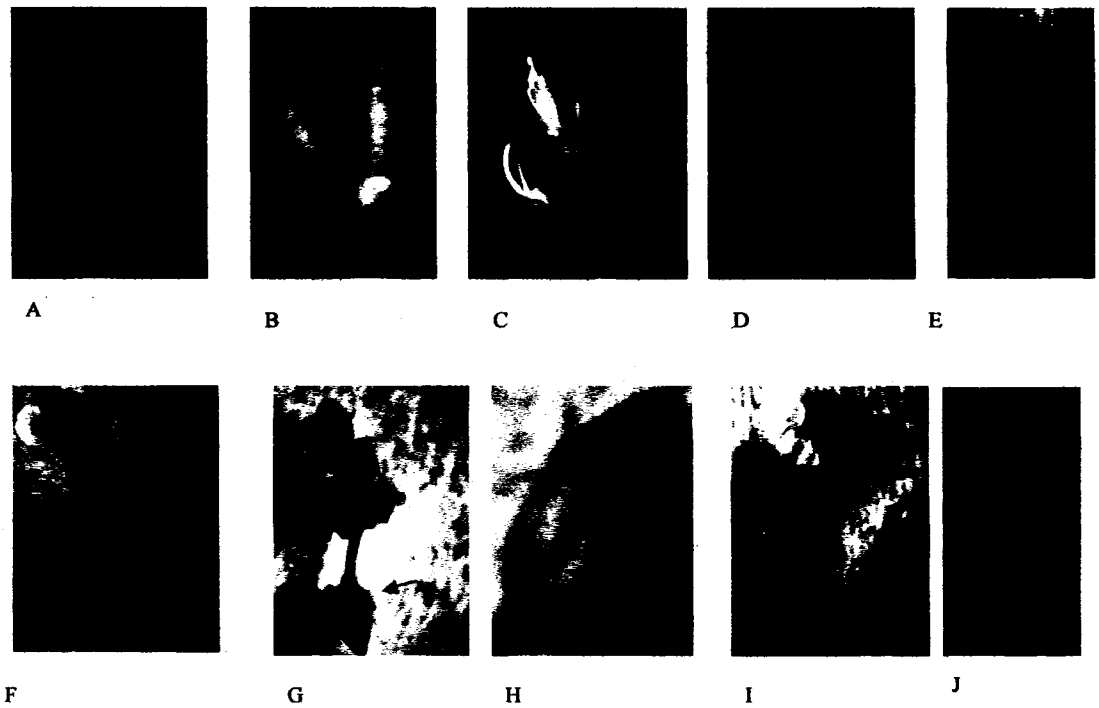
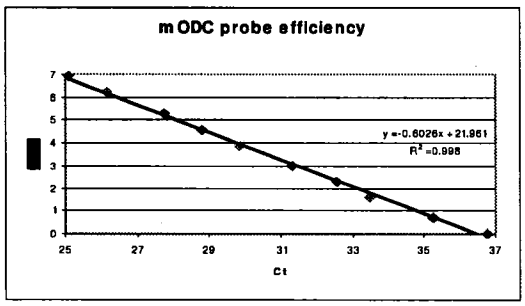


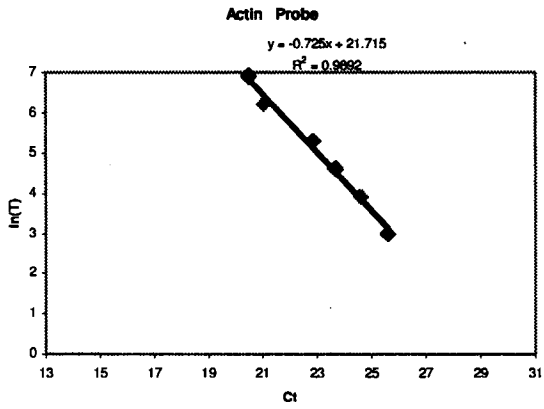
Figure 19: Floral abnormalities in m*ODC* transgenics: A&E) Bract like sepals, absence of petals; B) Bract like sepals; C) absence of sepals and wrinkled petals; D) absence of petals and wrinkled sepals; F & G) 42 DPG un opened buds; I) arrows indicate a space between two adjacent sepals; J) wrinkled sepal and petal

these plants. In few others, first buds appeared on 42 DPG and took another 20 days (i.e. around 60 DPG) to become mature flowers; in contrast, in the wild type plants, senescence had already set in by this time.

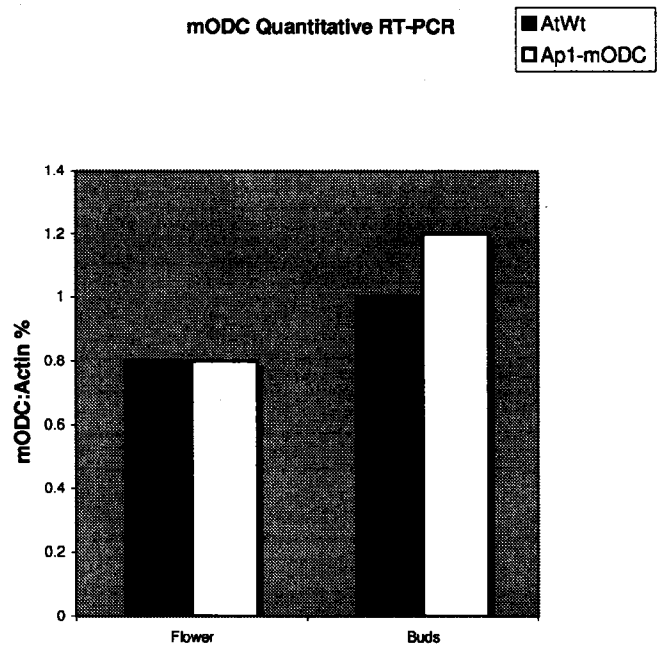
The morphological changes in young buds included lack of petals (Figure 19 A), and in mature flowers, bract like sepals and absence of petals were observed (Figure 19 A, B, E, H). However, once the mature flower had formed, the later stages of flower development, e.g. silique formation and seed dispersal, were not delayed. The m*ODC* expression did not have any effect on the development of fertile organs of the flowers and the siliques, nor did it affect seed formation. In order to find out if the above changes were related to the expression of m*ODC*, two types of analyses were performed: 1) detection and quantification of m*ODC* transcripts by RT-PCR, and 2) changes in the cellular polyamine contents. Mouse *ODC* transcripts were detected by RT-PCR and quantified by Quantitative (Q)RT-PCR; *ACTIN* was used as a control to normalize the data. Qualitative RT-PCR results showed the presence of m*ODC* transcripts in 19 DPG leaves, buds as well as the flowers, whereas siliques did not show positive results for m*ODC* transcript. Additionally, the presence of *AP1* transcripts was analyzed in the same tissues of wild type plants. Buds and flowers showed the presence of *AP1* transcript whereas 19 DPG plants and siliques lacked them. The efficiency of *ACTIN* and m*ODC* primers used in RT-PCR reactions was determined first by performing a dilution series (1, 0.1, 0.01, 0.001, 0.0001, and 0.00001) and plotting the graph of concentration against threshold value (Figures 20 A&B). The gene efficiency was calculated using the equation $E = 10^{-1/slope}$. The ratio of *ODC* to *ACTIN* was calculated using the equation: $T_{ODC}/T_{actin} = E^{Ctactin} / E^{CtODC}$, where T=



A



B



C

Figure 20: QRT-PCR amplification efficiencies for m*ODC* gene products. (A) and *ACTIN* (B) gene products. The threshold cycle (C_t) values (X-axis) are plotted against the log of the diluted concentrations [ln (T) – Y-axis]. C represents the ratios of expression of the m*ODC* and the *ACTIN* genes in flowers and buds

template amount, E= efficiency and C_t= cycle number at threshold. The threshold level was chosen as 0.05 to analyze the results. Using the above conditions, QRT-PCR analysis on m*ODC* expression was found to be equivalent to the *ACTIN* gene expression in the whole flower; however, in the flower buds, m*ODC* expression was greater than that of *ACTIN* (Figure 20 C). No mODC expression was seen in control plants.

III.6. Polyamine analysis in Wt and m*ODC* transgenics

In the wild type as well as m*ODC* plants, polyamine levels were analyzed from 19 DPG whole plants, buds, flowers and the siliques, and also, from the wild type (Wt) plants. As seen in Figure 21, spermidine was the predominant polyamine in all tissues of both transgenic and Wt plants, followed by putrescine and spermine, respectively. Also, it is evident that the vegetative tissues (19 DPG) had considerably less polyamines as compared to the reproductive organs (buds and flowers). Within the two stages of floral development, buds had almost twice the content of both spermidine and putrescine as compared to the mature flowers. It can also be observed that in the wild type flowers, the polyamine levels decreased with the progression of the reproductive stage.

The data in Figure 21 show that m*ODC* transgenics exhibited a significant ($p<0.05$) increase in putrescine as compared to the wild type plants in the vegetative tissues (leaves) at 19 DPG as well as in the buds. Although, an increase in putrescine was seen in the mature flowers of transgenic plants as well, the difference between the transgenics and wild type plants was not statistically significant. There was no increase in putrescine in siliques of transgenics as compared to Wt. The levels of spermidine and spermine in most

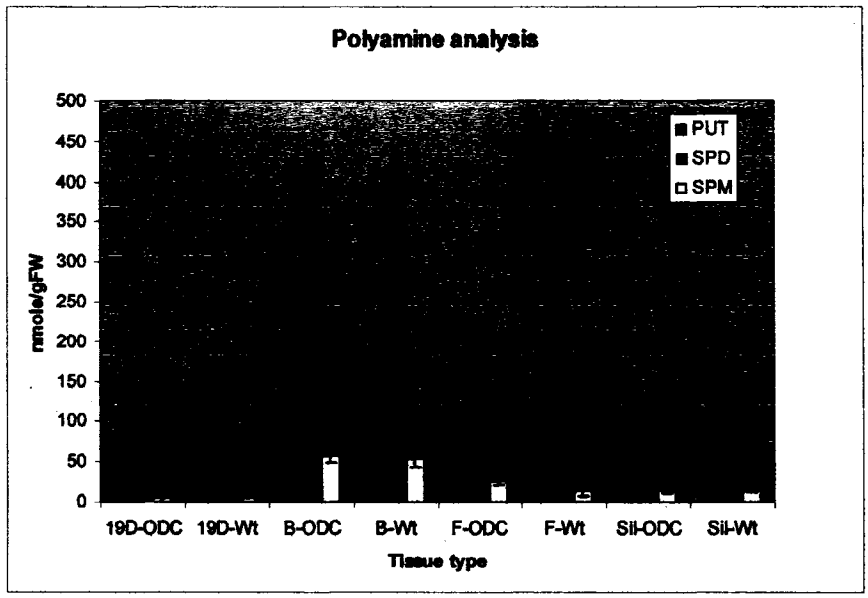


Figure 21: The cellular contents of polyamines in wild type (Wt) and m*ODC*. Each bar is a Mean ± SE of 3 replicates. Astericks in 19D-ODC, B-ODC and F-ODC represent that the values in transgenic cells are significantly different ($p<0.05$) from NT cells at a given time.

tissues were comparable in the two types of plants except in flowers, which exhibited a significant increase in transgenics as compared to Wt. The abnormalities in growth rate observed in transgenics were positively correlated with changes in the cellular contents of polyamines in the flower buds.

III.7. Effects of transgenic expression of c*SAMDC* on floral development

As mentioned earlier, *AP3* belongs to the B-class of floral organ identity genes that specifies petal and stamen identities, and is expressed in specific domains of the floral meristem (whorl 2 and 3) that give rise to these organs. In developing flowers, *AP3* starts to express during floral stage 3 (Smyth et al., 1990; Jack et al., 1992) prior to the initiation of petal and stamen primordia (Jack et al., 1992; Goto & Meyerowitz, 1994). Mutations in *AP3* result in homeotic transformation where petals are replaced by sepal like structures in the second

whorl and stamens are replaced by carpels in the third whorl (Bowman et al., 1989; Hill & Lord, 1989).

In the present study, the T1 seedlings of cSAMDC plants, selected on kanamycin, were transferred to soil for further growth and development. The transgenics were tested for the presence of *cSAMDC* and *nptII* genes by PCR, and found to be positive. At least 12 transgenic lines were monitored at different time points of growth and flowering to look for the phenotypic changes. The results of this study are preliminary since the study was conducted only at T1 stage. Further analysis at T2 stage is required in order to validate the data.

At least 12 transgenic lines were monitored at different time points of growth and flowering to look for the phenotypic changes. The expression of transgenic *cSAMDC* did not affect the normal development of plants and the timing of floral meristem initiation. No abnormal growth or phenotype was observed in vegetative parts of the plant either. More than 100 flowers from 12 transgenics and wild type plants each were examined for stamen and petal abnormalities. The most striking phenotypes observed in the transgenics were a reduction in stamen number (Figure 22 B, E, F, G) and wrinkled petals (Figure 22 D, F, G). A typical wild type Arabidopsis flower has 4 petals and six stamens in whorls 2 and 3, with two lateral stamens and four long stamens. In putative transgenic plants, almost 50% of the flowers exhibited a reduction in stamen number whereas only 10% of the wild type flowers showed similar stamen abnormalities. The most common numbers were five or two stamens (Figure 22), as opposed to the 6 stamens found in Wt flowers. Levin and Meyerowitz (1995)

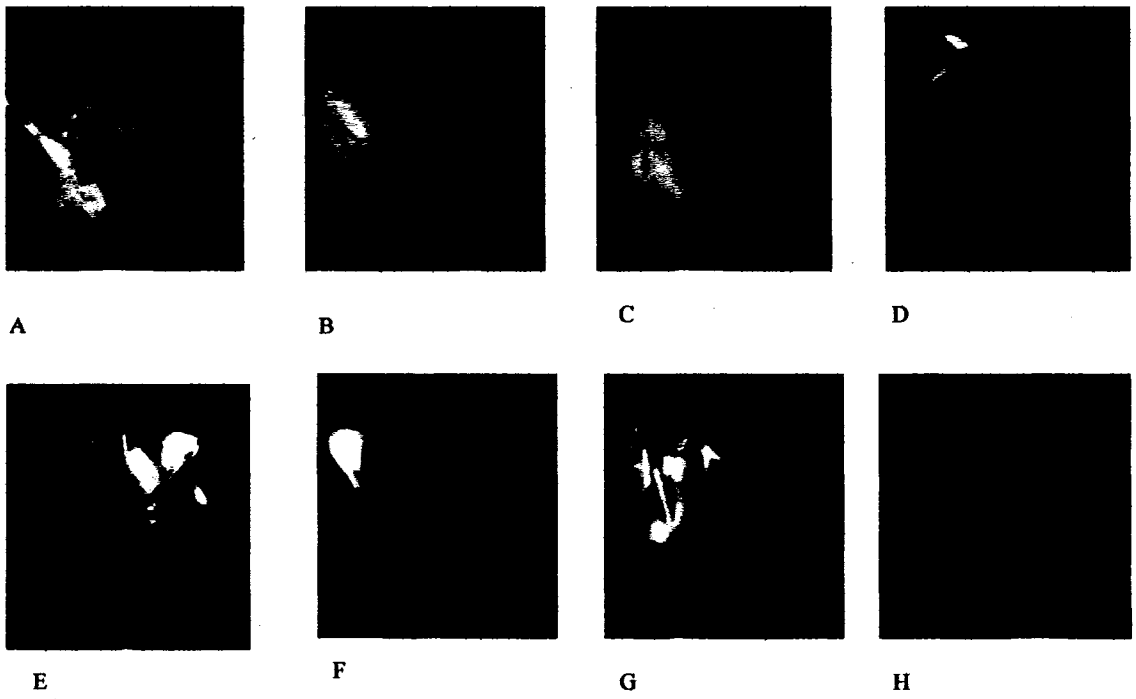


Figure 22: Floral abnormalities in carrot *SAMDC* transgenics. A) absence of petals and reduced stamen number; B &E) presence of 5 stamens and 3 petals; C, D & H) two stamens; absence of petals in C and H; F & G) Reduced petal and stamen number

have reported a reduction in stamen number in mutants over expressing the gene *SUPERMAN*. In their study, twenty flowers out of 112 flowers showed reduction in stamen number. Also, almost 20% of the flowers failed to develop normal petals, the most common alteration being shrunken petals. Some examples of the variation in anthers and petals in c*SAMDC* transgenic plants are shown in Figure 22; e.g. complete lack of petals as well as reduced number of stamens (Figure 22 A and H), five stamens (Figures 22 B, E, F, G), two stamens (Figures 22 C, D) and Wrinkled petals (Figures 22 D, F, G). However, the transgenic expression of c*SAMDC* did not affect other aspects of plant growth and flower development, e.g. floral initiation and ontogeny.

In order to check if the c*SAMDC* was being actually expressed in the transgenic plants and if the expression was tissue specific, total RNA isolated from the buds and flowers was tested for the presence of c*SAMDC* transcripts by RT-PCR. Simultaneously, RNA was also isolated from the leaves, buds, flowers and siliques of wild type plants for the analysis of *AP3* expression. *AP3* expression analysis in wild type was important for two reasons: to confirm the tissue specific expression of *AP3*, and to serve as a negative control for c*SAMDC* expression. In transgenics, *AP3* expression was not detected in leaves and siliques whereas buds and flowers did show its expression (data not shown). As mentioned earlier, in wild type plants RT-PCR was performed on RNA from the leaves and siliques; no c*SAMDC* expression was detected in these tissues indicating the specific domain expression of the c*SAMDC* gene under tissue specific *AP3* promoter. For *SAMDC* transgenics, cellular polyamine concentrations in the buds, flowers and siliques were compared.

III.8. Polyamine analysis in Wt and c*SAMDC* trangenics

The data presented in Figure 23 show that spermidine was the predominant polyamine found in flowers at all three stages of development. The total amounts of polyamines were the highest in the buds followed by the open flowers and the siliques. Putrescine was the next in quantity, with spermine being the lowest in amount. Putrescine levels were also the highest in buds and the lowest in siliques. Compared to the Wt plants, a decrease in all three polyamines was observed in the transgenics. The spermidine content in c*SAMDC*-transgenic buds (226.69 ± 16.5) was about half of that in the wild type plants (414.3 ± 34.9). In transgenic flowers (170.3 ± 46.5), spermidine was about one-third of that

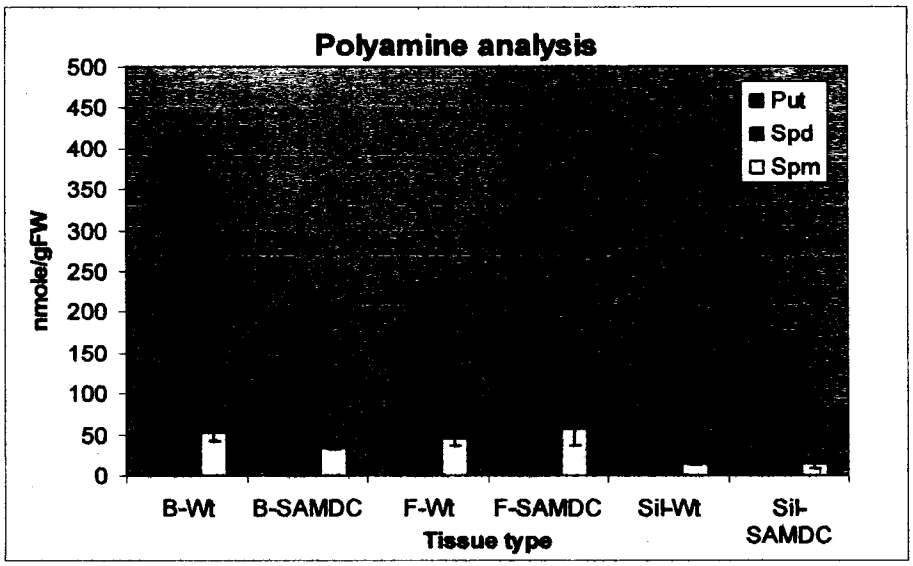


Figure 23: The cellular contents of polyamines in wild type (Wt) and *cSAMDC*. Each bar is a Mean ± SE of 3 replicates. Astericks in B-SAMDC, F-SAMDC and Sil-SAMDC represent that the values in for transgenics cells are significantly different ($p<0.05$) from NT cells at a given time

observed in WT plants (170.3 ± 46.5),and in siliques (127.3 ± 31.8), almost equal amounts of spermidine were observed (122.4 ± 11.07) in the two types of plants. The differences observed in spermidine levels of transgenic buds and flowers were significant ($p<0.05$) at a given time. The differences in spermidine between the three stages of development that were examined were greater in the wild type than the *cSAMDC* transgenics; e.g., buds had 1.5-fold higher putrescine and slightly higher spermidine than flowers in contrast to the wild type, which had almost 2-fold increase in putrescine and spermidine levels. A possible reason for the decrease in spermidine in the transgenics as compared to the wild type plants could be that *cSAMDC* may have caused a co-suppression of the native *SAMDC*.

Sequence analysis of the ORF regions of *cSAMDC* with *AtSAMDC1* and *AtSAMDC2* shows a high degree (66%) of sequence identity at nucleotide levels

(Figure 24). This high degree of sequence identity could actually results in interference with the expression of the native gene. Further analysis of this situation is needed to draw specific conclusions.

III.9. Expression profiles of *AtADC2::GUS* and *AtSAMDC1::GUS*

The temporal and spatial expression patterns of *At*ADC2::*GUS* and *At*SAMDC1::*GUS* were analyzed during flower development and in different whorls of the mature flower. As mentioned earlier (Introduction), transgenic Arabidopsis plants containing At*ADC2*::*GUS* and At*SAMDC*1::*GUS* constructs had been produced and used to analyze the expression of GUS gene during almost the entire life of the plant by Mitchell (2003). In my study, I used the same seeds with a focus on the expression of these two genes as revealed by GUS activity (determined by the presence of blue color) under the two specific promoters during flower development and in different whorls of the mature plant. Approximately 300 T_2 seeds from five ADC2::*GUS* and five SAMDC1::*GUS* transformed lines each (Table 6) were selected on antibiotic and grown to maturity. For each transgenic line, 5 buds or flowers were subjected to GUS staining. The first five stages of flower development were not easy to recognize due to the small size of buds; hence, these stages were not analyzed in detail. Preliminary analysis further showed that GUS activity was visible only from stage 10 onwards; therefore, stages 6 to 10 were also not analyzed in most cases although samples for these stages were collected.

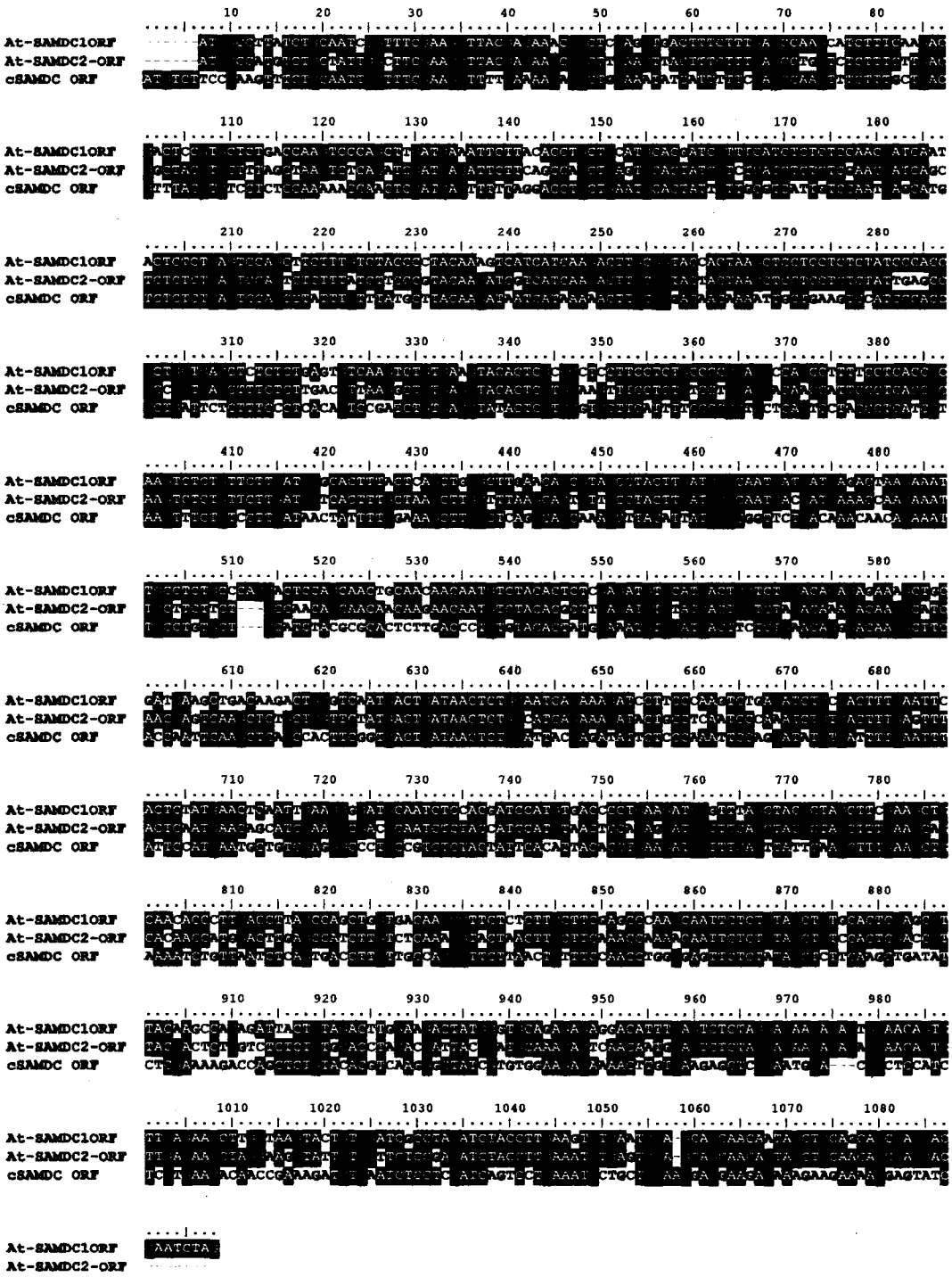


Figure 24: Nucleotide alignment of the carrot and Arabidopsis *SAMDC* ORFs

Subsequent samples for GUS activity analysis were collected at stages 11, 12, 14, 15, mature flower and siliques (Tables 8-13). After staining and de-staining, the floral buds were tabulated as the number of parts stained at a given stage of development as well as the intensity of staining in a given number of flowers rather than the percentage of flowers that showed GUS staining. For example, sepals at stage 11 stained at low intensity, but the same sepals at stage 12 showed stronger staining (Figure 27). Thus, if calculated on percentage basis, both stages of sepals would be counted as stained, but differences in the intensity of staining will not be apparent from these data.

Based on the intensity, it appeared that, *SAMDC* was more prominently expressed in sepals at later stages of development than at early stages. Having said that, the buds at stages 6 to 10 from SAMDC1::*GUS* transgenics did not shown any staining in sepals (Figure 25, Table 8). Stage 6 is characterized by the covering of organ primordia by sepals (Figure 25A). The first signs of GUS activity, although weak, in sepals of SAMDC1::*GUS* transgenics were visible at stage 10 (Figure 25B & 26A, Table 9); by stage 12 staining was quite dark, and the trend of strong staining continued in the sepals until the mature flower stage. Like the sepals, the petals and stamens also did not show any GUS activity until stage 10. At stage 11, while the petals still remained unstained, the stamens showed light staining (Figure 26A). In the gynoecium, appearance of stigmatic papillae occurs at stage 11, and distinct GUS staining was observed in these structures from this stage onwards. GUS activity became stronger as the gynoecium developed with the upper part of the style showing more activity than the lower parts (Figure 26B, Table 9).

Arabidopsis floral buds open (floral stage 12) when petals reach the height of the four long stamens; at this stage the flower is termed mature. At this stage, SAMDC1::*GUS* transformants exhibited high levels of GUS activity in sepals, anthers and the stigma. Greater than 90% of sepals showed high GUS activity at this stage. Like previous time points, GUS activity remained low in petals of SAMDC1::*GUS* transgenics, although veins of petals showed moderate levels of GUS expression in about 30% of the plants. Neither the petals from different flowers of the same plant nor from different plants showed any *GUS* staining at any stage of development. Strong *GUS* staining in stamens was observed at stage 12 (Figure 27, Table: 10); likewise the stigma stained heavily like at previous times.

The next two stages at which observations were made were stage 14, which is characterized by the extension of long anthers over stigma (Figure 28A), and stage 15 (Figure 28B), which is characterized by the extension of the stigma over the four long anthers. At these stages, the trend of high staining in sepals, anthers and gynoecium and low staining in petals continued (Figure 28 A and B, Table 11). In the mature flower, the entire sepal was uniformly stained; its veins and mid rib were all equally blue. Among the fertile whorls, anther filaments were divided into three parts for recording the data on GUS staining: upper and lower parts were lightly stained, whereas, middle region had strong staining (Figure 29; Table: 12). When the anther lobes in mature flower were dissected, it was seen that both the anther walls as well as the pollen inside were blue (data not presented). In the gynoecium, stigma had high levels of *GUS* staining throughout its development whereas upper and lower regions of style had lighter *GUS*

staining. Also, moderate levels of *GUS* staining were seen in pistils and veins of the pistils. The base of the gynoecium that attaches to the plant (i.e. the receptacle) as well as parts of the pedicel had high levels of GUS activity in more than 60% of the plants (Figures 28 & 29, Tables: 11-12). Figure 30 represents the individual whorls of a mature flower exhibiting *GUS* staining.

The final organs that were evaluated for the presence of *GUS* activity were siliques (Figure 31; Table: 13). Both the young and the mature siliques were subdivided into septum, valves, and seeds for observation. Septum and valves were further divided into upper, middle and lower regions for data recording. All three regions of the septum showed low to moderate levels of GUS activity. In the valves, upper and basal regions had higher GUS staining whereas middle valve region almost lacked the blue color. Seeds lacked GUS activity.

The overall pattern of the GUS staining in *ADC2* transformants was moderate to very low. The GUS activity was altogether absent in the sepals, petals, stamens, locules, style, anthers, anther filaments, and siliques at any stage of flower development (Figures 32-36; Tables: 14-18). However, low levels of GUS activity were seen in the stigma (≤ 40%), receptacle and the pedicel. Neither young nor the mature siliques of ADC2::*GUS* showed any GUS activity. The GUS activity was altogether absent in all regions of septum and valves. Seeds and pedicel also had almost zero GUS activity.

A

B

Figure 25: GUS analysis in SAMDC1 transgenics at floral stage 6 (A) and 10 (B)

Table 8: GUS activity in SAMDC1 transgenics at floral stage 6 (A) and 10 (B)

Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	5	+
Petal	5	0	N/A
Stamen	20	0	N/A
Pedicel	5	0	N/A

A

B

Figure 26: GUS analysis in SAMDC1 transgenics at floral stage 11 (A & B)

Table 9: GUS activity in SAMDC1 transgenics at floral stage 11 (A & B)

Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	15	+
Petals	20	0	N/A
Stamens	20	0	N/A
Stigma	5	5	N/A
Pedicel	5	0	N/A
Receptacle	5	5	++

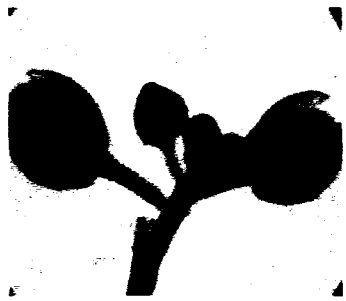


Figure 27: GUS analysis in SAMDC1 transgenics at floral stage 12

Table 10: GUS activity in SAMDC1 transgenics at floral stage 12

Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	15	+++
Petal	5	0	N/A
Stamen	20	0	N/A
Stigma	5	5	+++
Pedicel	5	0	N/A

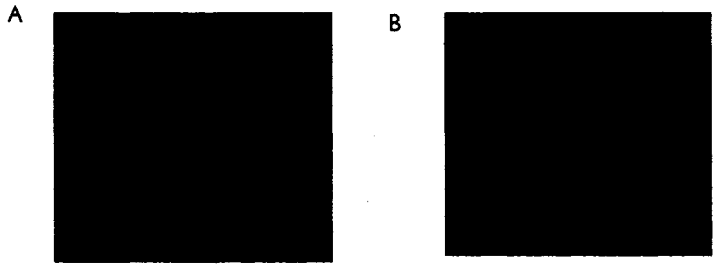


Figure 28: GUS analysis in SAMDC transgenics at floral stage 14 (A) & 15(B)

Table 11: GUS activity in SAMDC transgenics at floral stage 14 (A) &15 (B)

Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	20	++++
Petal	20	0	0
Anthers	20	20	++++
Stigma	5	5	++++
Pedicel	5	0	0
Receptacle	5	5	++

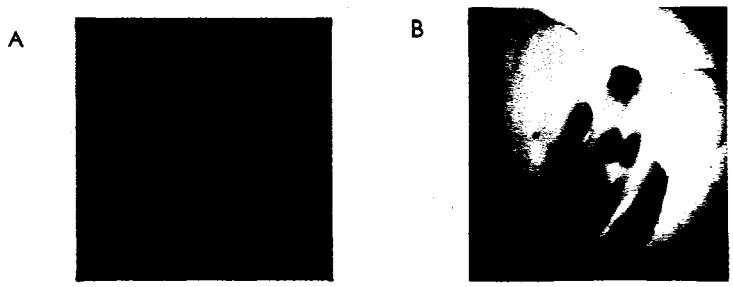


Figure 29: GUS analysis in SAMDC1 transgenics of mature flower (A & B)

Table 12: GUS activity in SAMDC1 transgenics of mature flower (A & B)

Organ Type	Organ Number	GUS Stain	Stain Intensity
Sepals	20	20	++++
Petal primordial	20	0	N/A
Anthers	20	20	++++
Stigma	5	5	++++
Pedicel	5	0	N/A
Receptacle	5	5	++

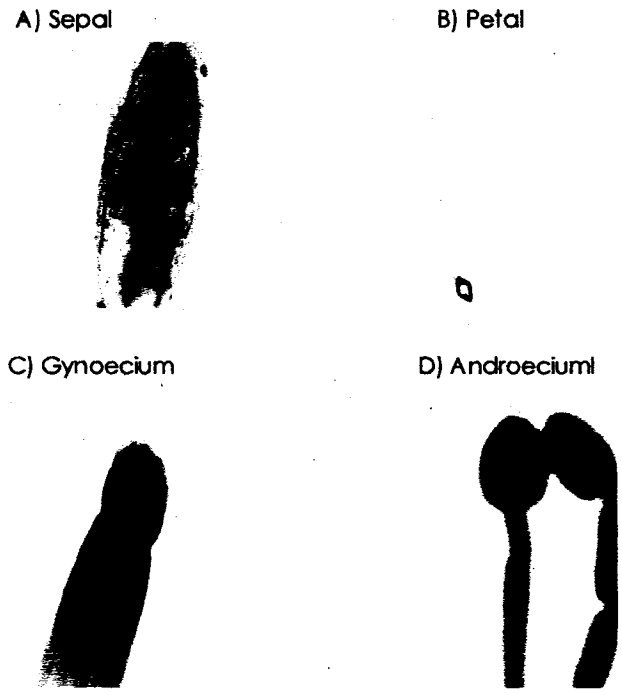
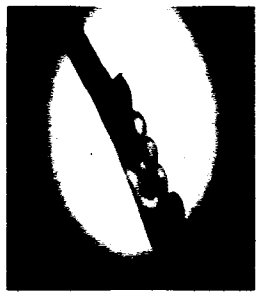


Figure: 30 GUS analysis in SAMDC1 transgenics in different floral organs

A



B

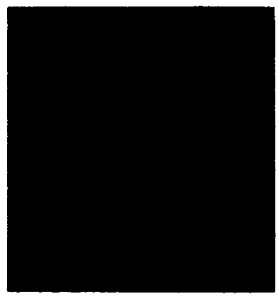
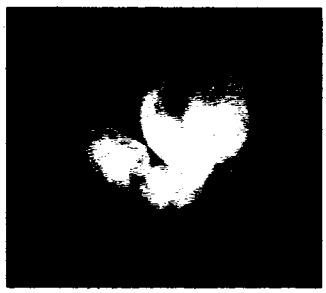


Figure 31: GUS analysis in siliques (A) and seeds (B) of SAMDC1 transgenics

Table 13: GUS activity in siliques (A) and seeds (B) of SAMDC1 transgenics

Organ	Organ Number	GUS stain	GUS intensity
Septum	5	0	0
Valve	5	2	+
Seeds	5	0	0
Pedicel	5	0	0

A



B

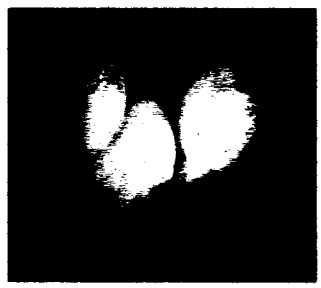


Figure 32: GUS analysis in floral stage 6 (A & B) of ADC2 transgenics

Table 14: GUS activity in floral stages 6 (A & B) of ADC2 transgenics

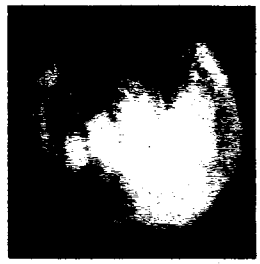
Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	0	0
Petal	20	0	0
Stamen	20	0	0
Stigma	5	0	0
Pedicel	5	0	0
Receptacle	5	0	0

Figure 33: GUS analysis in floral stage 10 of ADC2 transgenics

Table 15: GUS activity in floral stage 10 of ADC2 transgenics

Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	0	N/A
Petal	20	0	N/A
Stamen	20	0	N/A
Stigma	5	0	N/A
Pedicel	5	0	N/A
Receptacle	5	0	N/A

A



B

Figure 34: GUS analysis in floral stage 15 of ADC2 transgenics

Table 16: GUS activity in floral stage 15 of ADC2 transgenics

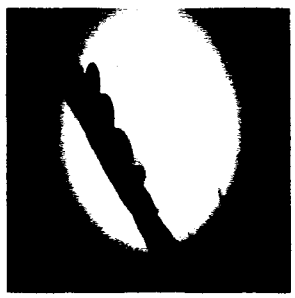
Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	0	0
Petal	20	0	0
Stamens	20	0	0
Stigma	5	0	0
Pedicel	5	0	0
Receptacle	5	0	0

Figure 35: GUS analysis in different whorls of ADC2 transgenics

Table 17: GUS activity in different whorls of ADC2 transgenics

Organ Type	Organ Number	GUS stain	Stain Intensity
Sepals	20	0	0
Petals	20	0	0
Anthers	20	0	0
Stigma	5	0	0
Pedicel	5	0	0
Receptacle	5	0	0

A



B

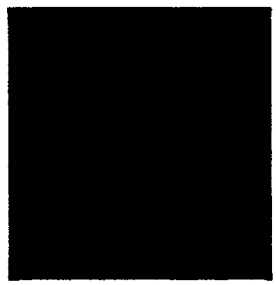


Figure 36: GUS analysis in siliques (A) and seeds (B) of ADC2 transgenics

Table 18: GUS activity in siliques (A) and seeds (B) of ADC2 transgenics

Organ	Organ Number	GUS stain	GUS intensity
Septum	5	0	0
Valve	5	2	+
Seeds	5	0	0
Pedicel	5	0	0

CHAPTER IV

DISCUSSION

It is well established that polyamines are involved in a diverse range of plant growth and developmental processes, including an important role in stress responses of plants. The main goal of my project was to establish if polyamines play a critical role in the development of flower in Arabidopsis. Two complementary approaches were taken to first establish the pattern of expression of two key polyamine biosynthetic genes during flower development, and second, to modulate the biosynthesis of polyamines in the floral apex during flower development and study its effects on flower development and morphology. The first approach involved the use of a well-established technique of 'promoter-reporter gene fusion' for the analysis of gene expression, and the second involved the transgenic expression of a c*SAMDC* and a m*ODC* gene under the control of two promoters that are known to be specific for expression in tissues involved in flower development. The results from the two approaches are discussed here:

IV.1. The tissue-specific c*SAMDC* and m*ODC* expression

In order to study the effects of transgenic manipulation of polyamines during flower development, it is essential that their expression be modulated in a developmental and tissue-specific manner; otherwise the effects on the vegetative development could complicate the analysis of results on flowering.

Further useful information can be derived from the domain-specific expression of the transgene if a promoter that shows domain-specificity is used. This design of the experiment involving tissue-specific transgene expression offers two advantages: first, the gene under study can be manipulated only in specific domains of interest, i.e., spatially restricted manner; and second, since the expression is developmentally as well as spatially regulated, the effects of over expression of the transgene might not be lethal to the whole plant during development. Therefore, tissue-specific expression studies of transgenes have become a powerful tool for molecular genetic studies in plants (Osterwalder et al., 2001).

In Arabidopsis, in order to identify the genes responsible for flowering, tissue specific expression of floral genes has been carried out. While several studies on flower development in Arabidopsis have employed floral whorl specific promoter in order to establish the role of a specific gene during development, none of the studies on polyamine biosynthetic genes have employed floral-specific promoters to delineate their role in flower development (Martin et al., 1981; Malmberg & McIndoo, 1983; Slocum & Galston, 1984; Malmberg et al., 1985; Fiala et al., 1988; DeScenzo & Minocha, 1993; Caffaro & Vicente, 1994). The present study is an attempt to modulate the metabolism of polyamines under the control of floral specific promoters AP1 and AP3.

IV.2. Effects of m*ODC* expression on flower development

Arabidopsis follows the same pattern of floral development as most other angiosperms (Miksche & Brown, 1965); the process involves three distinct stages namely, floral initiation, floral organ primordia formation, and floral organ

production. Flowers develop from a group of undifferentiated cells that grow from the flanks of shoot apical meristem. The initial steps of floral development involve two classes of regulatory genes: meristem identity genes and homeotic genes. Meristem identity genes are required for the initiation of the flower and additional homeotic genes are required to specify the individual organs of the flower and are expressed in the various domains. The transition of shoot apical meristem to inflorescence involves two phases that are characterized by indefinite and repetitive patterns of growth and organ formation (Medford et al., 1994).

Several studies have shown that the conversion of shoot apical meristem to floral meristem is under the control of several external factors like photoperiod (Alvarez et al., 1992; Blazquez et al., 1997, 1998; Bradley et al., 1997) and an interaction of several genes is involved in regulating this process (Ratcliffe & Riechmann, 2002). Genetic and molecular studies on natural variations in flowering time and development have identified other genes responsible for flowering; these genes have been divided into two groups: floral meristem identity genes and floral organ identity genes. Interestingly, the A class gene, *AP1*, is both a meristem identity gene and a floral organ identity gene. Consistent with its meristematic activity role, *AP1* together with LEAFY, acts to initiate the floral meristem; and consistent with its organ identity role, it specifies the formation of sepals and petal in specific domains of the flower (whorls 1 & 2). It was shown that *AP1* transcripts are present throughout the young floral primordia; their expression being more localized to outer two whorls at later developmental stages (Mandel et al., 1992). Due to the dual role of AP1 in floral

development, it was used to direct the expression of a mODC in order to manipulate putrescine and investigate its effects on floral development. A DNA fragment of *AP1* (Accession # NM 10086) containing the putative promoter region was previously isolated by Mandel et al. (1992). The cloned promoter region of *AP1* has been used to demonstrate the temporal and spatial expression of *AP1* gene in Arabidopsis; and also to study the effects of the ectopic expression of the floral genes of various other functions. Krizek et al. (1999) using AP1 promoter deduced the phenotypes of plants expressing the chimeric MADS box genes (*AP1, AP3* and *AG*) ectopically. Yun et al. (2002) expressed the floral gene *SUPERMAN*; the flower with AP1::*SUP* had conversion of petals to sepals and suppressed stamen development. *AP1* is known to be involved in the activation of B class genes (Ng & Yanofsky, 2001).

In order to study the effects of transgenic manipulation of putrescine in the developing floral meristem, mODC was expressed under the control of the AP1 promoter. All eukaryotes synthesize putrescine directly from ornithine via the activity of ODC; whereas plants have an additional, indirect pathway from arginine for the synthesis of putrescine. Arabidopsis is the only plant known that apparently does not show ODC activity nor has a functional *ODC* gene (Hanfrey et al., 2001). Therefore, Arabidopsis is totally reliant on the ADC pathway for the synthesis of putrescine. Hence in the present study, a heterologous mODC gene was introduced into Arabidopsis, in order to study if the introduced mODC would increases putrescine levels and cause alterations in either the timing of flower development or the morphology of the flower. The T1 plants as well as T2 plants that had undergone an additional round of selection were monitored for growth

and flower development; both generations showed similar results. This mODC gene has been used earlier to effectively modulate putrescine contents of tobacco and poplar cells (DeScenzo et al., 1993; Bhatnagar et al., 2001, 2002)

The transgenic expression of *mODC* was confirmed in the T1 and T2 plants by RTPCR and cellular contents of polyamines were analyzed in various tissues. Some of the tissues in the flower had higher polyamines than the controls. This expression caused severe effects both in terms of initiation of the floral meristem and in the formation of sepals. In wild type plants, vegetative meristem converts to floral meristem and this is evident by the formation of buds on the axils of the inflorescence meristem. The inflorescence meristem gets converted to the floral meristem through the process of bolting. The floral meristems appear on the flanks of inflorescence meristem. In wild type plants, the formation of buds and their development occurs on the floral meristem on the 26th DPG; which was the case in my study also. The transition of vegetative meristem to the floral meristem was considerably delayed in greater than 40 % of the mouse *ODC* transgenics. All the transgenics showed delayed bolting or few never bolted. Thus floral meristem initiation was not noticed or it was delayed in most of the transgenics. Few transgenics bolted late and all the downstream processes like bud formation, opening of the bud were also considerably delayed.

Several studies have reported the role of polyamines in floral initiation and emergence (Cabanne et al., 1981; Malmberg et al., 1983, 1985; Sawhney et al., et al., 1988; Tarenghi & Tanguy, 1995). A recent study has shown that over expression of *ADC2* results in polyamine accumulation, which in turn affects the gibberellins metabolism leading to dwarfism and late flowering in transgenics

due to increased putrescine levels. When GA3 was applied to such plants, they regained flowering (Alcazar et al., 2005). The delayed development exhibited by mouse *ODC* transgenics could be due to the high polyamine content in early stages of development (19DPG). This high polyamine level might have delayed the process of conversion of shoot apical meristem to the inflorescence meristem in a way confirming the results of Alcazar et al. (2005). We did not test the effects of GA in the transformed plants.

The increase in polyamine levels in entire plant at 19DPG is something that was not expected since the mouse *ODC* was expressed under the control of a developmentally regulated AP1 promoter. Although increased putrescine levels did coincide with m*ODC* transcript levels at 19DPG; Wild type did not show any AP1 transcripts in 19DPG. Chou et al. (2001) introduced AP1::*GUS* into *emf-fwa* and *emf-ft* double mutants of Arabidopsis in order to delineate the mechanisms involved in regulating the initiation of floral development. They observed strong AP1 expression in *emf-1* single mutants in shoot meristems just after 5DPG whereas double mutants did not show AP1 expression. Hence, although native AP1 is not expressed in vegetative parts of the plant, its mutant forms might have an ectopic expression, which might hold true for the present observation in 19DPG transgenics. However, further studies on determining the ODC and ADC activities should give us more definitive answer to this observation. Even if we assume AP1 promoter to be leaky, expression analysis on native AP1 in 19 DPG NT did not show any transcripts thus confirming temporally regulated expression of the native *AP1* gene. A striking phenotype observed in mouse *ODC* transgenics was a delay in both the formation of buds and opening of buds. In

NT plants, buds were visible around 26 DPG and took anywhere from 5-13 days to become a mature flower. (www.arabidopsis.org). Surprisingly, mouse *ODC* transgenics not only showed buds much later i.e., at 42 DPG but also had delay in their opening and becoming a mature flower. A few of the transgenics had taken almost 20 days in the opening of the buds and a few never opened. The reason for late opening could be due to high putrescine levels in transgenic buds as compared to NT. The increased putrescine levels might have resulted in bract like sepals and lack of petals due to which some buds could never open. Mutational analysis on *ap1* mutants has shown a similar phenotype where first whorl sepals were transformed into bract-like organs and petals were absent (Bowman et al., 1993).

Once the transgenic m*ODC* buds opened, no abnormalities were found in stamen and carpel development. Likewise, after the flowers were formed, no abnormality were detected in siliques and seeds either. Quantitative PCR showed higher *ODC* transcript levels in buds as compared to mature flowers (Figure 20).The putrescine levels were highest in floral buds and flowers compared to vegetative parts (Figure 21). The putrescine levels did not increase in siliques in transgenics as compared to NT plants.

IV.3. Characterization of the plant *SAMDC* gene sequences

The *SAMDC* gene sequences from various plant species have been well characterized. All known plant *SAMDC* genes have relatively long 5'UTRs, from 363 bp in *Nicotiana tabacum* (Accession # AF033100) to 614 bp in Arabidopsis (Accession # NM_1111114). The 5'UTRs of plant *SAMDCs* possess within them regulatory elements like the uORFs with internal ribosome entry sites (Meijer &

Thomas, 2002; Wilkie et al., 2003). Even in nematodes, like *C. elegans*, and in mammals, *SAMDC* genes contain a long 5'UTR of more than 450 nucleotides. Of the two ORFs in the 5' UTR of plants, small uORF for all known plant *SAMDC* genes is highly conserved and encodes a polypeptide of 49-54 amino acids. The size of the tiny uORFs is about five to six amino acids (Kwak & Lee, 2001); this ORF is supposedly not translated. The 5'UTR in *SAMDC* is thought to be involved in regulation of gene expression; especially the presence of regulatory elements results in the reduction of the translation initiation efficiency of the main *SAMDC* gene (Hanfrey et al., 2003).

The amino acid sequences of *SAMDCs* are also highly conserved and posses some unique features. For example, the proenzyme-processing cleavage site (YVLSE↓SS), putative PEST sequences (Rogers et al., 1986), and several glutamate residues at key sites are found in both plant and animal *SAMDCs* (Schroder & Schroder, 1995; Lee et al., 1997a). In fact, PEST sequences are found to be located in all major polyamine biosynthetic genes (*ODC*, *SAMDC* and *ADC*). These PEST sequences are believed to be responsible for their short half-life ranging from 20-120 min (White & Morris, 1989).

IV.4. Effects of *cSAMDC* expression on flower development

As described earlier, Arabidopsis flower consists of four whorls of organs whose structure and development depend on a mutual interaction of three classes of the floral homeotic genes called A, B and C. The A class genes *LEAFY (LFY)* and *APETALA1 (AP1)* are meristem identity genes which are important for the transition of the vegetative meristem to the reproductive meristem and establish the floral meristem identity (Irish & Sussex, 1990; Weigel et al., 1992;

Bowman et al., 1993; Mandel & Yanofsky, 1995; Weigel & Nilsson, 1995). Besides playing a role in establishing floral meristem identity, AP1 also plays a major role in the establishment of sepal and petal primordia (Irish & Sussex, 1990; Mandel et al., 1992; Bowman et al., 1993). The B class genes APETALA3 and PISTILLATA specify petal and stamen identities (Bowman et al., 1989; Jack et al., 1992; Goto & Meyerowitz, 1994) in Arabidopsis. A single C class gene AGAMOUS (AG) affects stamen and carpel identities in whorls 3 and 4 (Bowman et al., 1989; Yanofsky et al., 1990). Several studies show that functions of the ABC class of genes are mutual and spatially regulated to the specific domains of their expression during flower development (Weigel & Meyerowitz, 1993; Riechmann & Meyerowitz, 1997).

In order to investigate the role of spermidine in the development of petals and stamens, tissue specific AP3 promoter was used to direct the expression of c*SAMDC* in specific domains that give rise to these organs. The promoter used here was obtained from Thomas Jack (Dartmouth College, Hanover, NH). The AP3 promoter was shown to contain distinct *cis*-acting elements that are required for the differential spatial and temporal aspects of AP3 expression (Hill et al., 1998; Tilly et al., 1998). *LFY* and *AP1* are required for the early activation of the AP3 promoter (Lamb et al., 2002). The two *cis*-acting elements as defined by Lamb et al. (2002) constitute proximal early element (PEE) and distal early element (DEE), and are required for AP3 expression during early stages of floral development (stages 3-5). Several studies have confirmed the role of *AP3* in establishing petal and stamen identities using promoter-reporter fusion approach and other forms of transgenic expression. Ectopic expression of *AP3* under the

control of 35S promoter resulted in the formation of two outer whorls of petals and inner whorls of stamens (Jack et al., 1994; Krizek & Meyerowitz, 1996).

Hill et al., (1998) fused different regions of 1.7 kb AP3 promoter region to *GUS* in order to establish the expression pattern of the promoter in Arabidopsis. They proposed that there are specific domains in the AP3 promoter, which confer tissue-specific expression patterns in petals and stamens whereas other domains confer temporal control. AP3 expression is initiated by the activity of meristem identity genes *LFY* and *AP1* (Weigel & Meyerowitz, 1993) whereas expression maintenance is autoregulated. For example, AP3 is actively expressed in stages 2 and 3 whereas its expression is not detected in stage 6 after the emergence of the petal and stamen primordia (Jack et al., 1994). The 1.1 kb putative promoter region along with the 0.6 kb of the 3' end of *AP3* sequences and a NOS termination sequence was used as a starting point for my study. The entire cassette 5'AP3-3'AP3-NOS (pD1954) has a *Bam*HI site between 5' AP3 and 3' AP3 for the cloning gene of interest. The c*SAMDC* (cloned earlier in our lab and shown to produce a functional enzyme – Chretien, 2003) was cloned into pD1954 at *Bam*HI site to create pD1954-AP3::c*SAMDC*. The pD1954-AP3::c*SAMDC* was then used directly for transgenic expression. The putative transformed plants selected on GM medium supplemented with kanamycin (T1 generation) were tested for the presence of *SAMDC* and *NPTII*, and used directly for developmental studies. There was not enough time to produce the T2 plants.

Ten transgenic lines were monitored at different stages of plant growth and flowering to look for phenotypic changes. Although no change in terms of the normal development of plant and the timing of floral meristem initiation were

observed, several abnormalities in terms of stamen and petal formation were evident. The wild type Arabidopsis flower typically has 4 petals and 6 stamens arranged in whorls 2 and 3. In several transgenics, a deviation in terms of stamen number was observed in that more than 50% of the putative transgenic flowers had five stamens instead of six; a few had only 2 stamens. Although, a small percentage of wild type plants also exhibited some variation in stamen numbers, the percentage seen in transgenics is much higher than that observed in the wild type. Also, many of the flowers failed to develop normal petals and some flowers had shrunken petals.

The *cSAMDC* transcripts were detected in all the putative transformants that showed abnormal floral phenotype. Polyamine analysis in the buds of transgenics revealed lower spermidine levels than the buds of wild type flowers; the mature flowers also had lower spermidine in the transgenics. Increased polyamine levels in buds compared to flowers in both wild type and transgenics suggests that polyamines are present at elevated levels during early stages of floral development than later stages, and these higher levels might be necessary for normal flower development. Polyamine levels were similar in the siliques of the transgenic and the wild type plants, and their growth was normal. The stage at which a decrease in polyamines was seen in the transgenics is consistent with the timing of the expression of *AP3*. Analysis of tissue-specificity of the expression of *cSAMDC* (by the RT-PCR) showed no expression in vegetative tissues like the leaf. Highest levels of polyamines found in the buds as compared to the mature flowers is similar to observations of Rastogi & Sawhney (1990) in tomato; they reported highest SAMDC activity in developing stamens as compared to other

organs of the flower in both a mutant line and the wild type flowers, and also an abnormal stamen development in the mutants. Several other studies have reported increased spermidine levels during floral initiation and subsequent growth (Aribaud & Tanguy, 1994; 2004; Sood & Kumar, 2004; Huang et al., 2004), which is in line with high spermidine levels seen here.

At this stage, it is premature to suggest a causative effect of the decreased spermidine with changes in stamen number in the transgenic plants. First of all we used only T1 generation of plants, and the study must be confirmed with T2 generation. Second, the observed changes in spermidine were opposite to the expectation; i.e. a decrease in spermidine and not an increase was observed. This reduction might have occurred probably due to a potential co-suppression of the native *SAMDC* by c*SAMDC* since the two genes show a high degree of sequence identity (66%) at the nucleotide level. The higher levels of polyamines required for stamen and petal formation may have been compromised leading to abnormalities in these two whorls. Further evidence is needed to confirm if co-suppression is actually occurring and it affects actual SAMDC activity, and in turn, polyamine levels. However, the high percentage of abnormal flowers observed in this study does provide a strong reason to do further analysis of T2 plants, which should broaden our understanding of the role of polyamines in flower development. Thus, the present study together with the previous studies has shown that floral abnormalities in transgenic plants can be correlated to the changing polyamine levels due to the transgenic manipulation of polyamine biosynthetic genes.

IV.5. The promoter-reporter gene system

The promoter-reporter gene fusion system is a common method used for the analysis of promoter activity for specific genes (Beckwith et al., 1970; Cox et al., 1985; Jefferson et al., 1986; Doerner et al., 1990). Although several methods for the analysis of gene expression in different tissues (e.g. RT-PCR, northern blots, microarrays) are available, promoter-reporter system provides some unique advantages as it offers more accuracy in terms of cell/tissue-specific gene expression, and can be used for various tissues at the same time without cumbersome RNA preparations. In this approach, a reporter gene (whose expression is easy to detect and quantify) is fused to the promoter of the gene of interest or the entire gene and introduced into the plant cells as a transgene. The expression of the reporter gene supposedly mimics the information on the spatial and temporal expression pattern of the gene of interest in different tissues of the plant. The nature of the reporter gene plays an important role in these studies. The product of the reporter gene must be easily detectable and assayable. Many reporter genes like *GFP*, *GUS*, and *lacZ* have been used for such studies. The biggest advantage of this approach is that a distinction in the expression of closely related members of the same gene family that have high sequence homology can be made.

This approach is useful for studying gene expression in the whole plant under constitutive promoter; in specific tissues under tissue-specific promoters, and under specific conditions with inducible promoters. In most studies, the reporter gene expression was found to mimic native gene expression. The most common reporter gene used in such studies has been the *GUS* gene (Novel &

Novel, 1976; Blanco., 1980). The *GUS* gene reporter system was used to study the regulatory elements in the promoter of Arabidopsis *MERISTEM LAYER1* (*atml1*) by Sessions et al., (1999). Chiou et al. (2002) used *GUS* reporter system to study the influence of the environment on the *nor-1* gene expression, which encodes a ketoreductase involved in aflatoxin biosynthesis in *Aspergillus*. Basu et al., (2003) have used the GUS system to analyze the relative activities of four different promoters in bent grass (*Agrostis palustris*) in different tissues and developmental stages of the plant. The four promoters used in this study (potato-ubiquitin 3, corn-ubiquitin, rice-ubiquitin and CaMV 35S) were fused to *GUS* and introduced into the plant cells. The relative expression of the *GUS* under the control of the four promoters was used as an indication of their efficiency.

In the present study, the expression of two key regulatory genes in polyamine biosynthesis, namely *AtADC2* and *AtSAMDC1*, was studied during different stages of flower development. The construct used in the present study *ADC2*::*GUS*-NOS contained the 1090 bp sequence located upstream of the transcription initiation site, 518 bp 5'UTR and the first 65 bp of the ORF of Arabidopsis *ADC2*. The cloned *ADC2* and *SAMDC1* promoter regions were found to contain the regulatory elements and other general key features of a typical promoter (Mitchell, 2004).

IV.6. Expression of AtADC2::*GUS* and AtSAMDC1::*GUS*

In our laboratory, we have produced transgenic Arabidopsis plants in which constructs with the promoter regions of *ADC2* and *SAMDC1* fused to the *GUS* gene have been introduced (Mitchell, 2004) in order to follow the expression of these genes during the entire life of the plant. Detailed analyses of vegetative

tissues of transgenic plants have shown that the expression of ADC2 is rather weak and sporadic; the *SAMDC1* expression, on the other hand, is strong and ubiquitous. My observations generally agree with the observations of Mitchell (2004). In ADC2::*GUS* transgenic plants, GUS expression in the vegetative parts of the plant was high during early stages of the plant development especially in roots with progressive decrease as the plant growth progressed. In mature plants, *ADC2* expression was almost absent in most tissues. With respect to the flowers, GUS activity was detected in only in a few parts of the developing flower; in most tissues no staining was seen at stages 6, 10, 11, 12, 14, 15, the mature flower and the siliques.

Since *ADC2* expression was not widely distributed, and putrescine has been shown to be present and plays a role in flower development, it can be argued that putrescine production in the flowers is largely controlled by ADC1. Soyka & Heyer (1999) and Perez-Amador et al. (2002) have shown that while *ADC1* expression is ubiquitous in Arabidopsis, *ADC2* expression is sporadic and occurs only in few tissues like siliques, cauline leaves and under abiotic stress conditions. Scott Sanders (unpublished) in our laboratory has confirmed these observations by following the expression of *GUS* in plants containing the ADC1::*GUS* transgene. The present study extends the results of our earlier studies in that it involves the reproductive structures of the plant. DNA sequence analysis of the two ADC genes reveals 51% identity at nucleotide level and 88 % similarity at amino acid level (Mitchell, 2004). Although proteins are highly homologous, regulatory sequences (the promoter regions) of the two genes only show 47% identity with each other, which might explain their differential expression.

A recent study has shown that over expression of *ADC2* results in GA deficiency eventually leading to dwarfism and late flowering in transgenics (Alcazar et al., 2005). Another important observation in this study was that the putrescine content in the wild type plants was the highest in the floral buds and flowers as compared to the vegetative parts. A similar trend was observed earlier in *Xanthium strumarium* (Hamasaki et al., 1990) where abundant polyamines in flower buds were reported. Although the promoter efficiency is important in driving a gene expression, GUS staining seen in vegetative parts of the plant does show that the isolated and cloned *ADC2* promoter is active for driving the *GUS* expression. A possible explanation for low GUS activity is probably due to the differential gene expression of *ADC* paralogues as they differ in their sub cellular localization.

Unlike *ADC2* expression, *SAMDC1* expression as revealed by GUS activity was strong and ubiquitous in most vegetative parts of the plant at both early and late stages of development, again confirming the earlier observations of Mitchell (2004). The GUS expression was observed in seedlings soon after emergence from the seed coat and continued through 20 DPG. In mature plant, staining for GUS was observed in both rosette and cauline leaves, and roots. However, early stages (1-5) of floral buds were not analyzed due to small size. By stage six, GUS staining was apparent in the sepals and the anthers. At stage 10 and above, anthers and anther filament in the buds started to show strong GUS activity and this trend continued in mature flower.

Gynoecium showed *GUS* expression right from the early stages of development (stage 10) and continued in mature flowers. In order to see if GUS

expression was uniformly distributed in organs that stained intensely dark or was limited to a few tissues from where it diffused to others, several tissues (sepals, anthers, and stigma) were sectioned and examined under the microscope; almost all cells in the stained region showed internal GUS activity. GUS expression was also detected in siliques especially at the tips and in the seeds.

As with *ADC*, Arabidopsis contains at least *SAMDC* genes (Franceschetti et al., 2001). Preliminary expression analyses of two *SAMDCs* revealed *SAMDC2* expression to be abundant and ubiquitous particularly in the leaves and inflorescence (Franceschetti et al., 2001). Mitchell (2004) also saw almost ubiquitous expression of *SAMDC1* in Arabidopsis. The high level of *SAMDC1* expression seen here is consistent with the earlier studies that show both abundant and ubiquitous expression of this gene.

In conclusion: 1) the heterologous expression of m*ODC* under the control of tissue specific promoter AP1 resulted in increased putrescine accumulation with no concomitant changes in spermidine and spermine levels; 2) Transgenic expression of c*SAMDC* under AP3 promoter resulted in a significant decrease of spermidine levels in buds; 3) the expression pattern of *SAMDC1* was strong and ubiquitous whereas *ADC2* expression was weak and sporadic in various floral organs during different developmental stages. The results obtained in this study are consistent with the published literature and further analyses of the polyamine biosynthetic enzyme activities in transgenic plants and effects of the transgenic expression of other genes in the pathways will reveal specific information on the developmental role of polyamines.

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