8-7-2010

Characterization of Arabidopsis thaliana mutants lacking a jumonji domain containing histone demethylase and a set domain containing histone methyl transferase

Swetha Mamidi Reddy

CHARACTERIZATION OF *ARABIDOPSIS THALIANA* MUTANTS LACKING A JUMONJI DOMAIN CONTAINING HISTONE DEMETHYLASE AND A SET DOMAIN CONTAINING HISTONE METHYL TRANSFERASE

By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Molecular Biology
in the Department of Biochemistry and Molecular biology

Mississippi State, Mississippi

August 2010

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Title of Study: CHARACTERIZATION OF *ARABIDOPSIS THALIANA* MUTANTS LACKING A JUMONJI DOMAIN CONTAINING HISTONE DEMETHYLASE AND A SET DOMIAN CONTAINING HISTONE METHYL TRANSFERASE

Pages in Study: 125

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Condensation of chromatin and alteration of chemical groups in the proteins around which the DNA is wrapped play major role in regulation of transcription. Histones are basic proteins rich in arginine and lysine residues which form the nucleosomal core. Histone modifications like acetylation, methylation, phosphorylation, etc. have broadened the horizon for researchers to study epigenetics more in detail. Recent studies on modifications of histones have revealed many genes responsible for altered phenotypes in plants. Histone methyl transferases and histone demethyl transferases are enzymes which add or remove methyl groups on histone lysine and arginine residues respectively. In this study a jumonji domain containing putative histone demethyltransferase has been shown to be responsible in controlling flowering phenotype in *Arabidopsis thaliana*. The knocked out mutants for this gene (JMJ14) showed an early flowering phenotype along with elevated levels of FT transcript (Flowering locus T, gene responsible for controlling the flowering time in *Arabidopsis thaliana*). We show that methylation was altered on H3K36 in the FT gene in the mutants using ChIP (chromatin

immunoprecipitation) experiments. We also show that the gene was expressed more in reproductive tissue than the vegetative tissue using histochemical GUS experiments.

The possible role of SDG8 gene, a histone methyl transferase in ABA signaling was also studied during the research. A SET domain containing Sdg8 (group 8 methyltransferase) mutant was found to be responsible for ABA signaled altered root growth in *Arabidopsis thaliana*. The cell number and cell size in roots decreased in both meristematic and elongation zones leading to decrease in root size in *sdg8* mutants and number of root hairs increased when treated with Abscisic acid, a plant hormone. An ABA responsive gene, ABR1 was over expressed in these mutants when treated with ABA, but the behavior pattern of the ABR1 gene in the absence of SDG8 gene is different.

In this part of study, as part of an interaction between epigenetics and gene regulation, it was observed that a putative histone demethylase gene, JMJ14 was responsible for regulating the flowering time by controlling the expression of FT and SDG8 played a role in altered root growth in response to ABA in *Arabidopsis thaliana*. Further studies on these genes could lead to generation of commercial crops with phenotypes that would increase the plant productivity and be beneficial agronomically.

DEDICATION

Ganapath, Hanuman & Peddamma Talli

ACKNOWLEDGEMENTS

I would like to thank my Project Supervisor Dr.Jiaxu Li for providing me this opportunity to work on such an interesting project. I owe him my deepest respect and gratitude for being very understating and patient throughout my course. He has not only been a great mentor but also a continuous source of inspiration. I also thank all my committee members, Dr.Zhaohua Peng, Dr.Daniel Peterson, Dr.Edrogan Memili and Dr.Din-Pow Ma for their invaluable suggestions at various crucial points of my project. My sincere appreciation goes to Dr Scott Willard who was always there to help me during the course.

I would like to thank my co lab workers both senior and contemporary. I am grateful to Dr.Geofeng Dong who was a ‘to go’ mentor in the lab during my early days of the course. I would like to thank Dr. Zenglan Wang who was very kind in sharing her work experience and was of great help in various respects. They were both friendly and educating. I would like to thank Jie Song for being such a sweet friend. I can never forget the help she rendered during my stay here both in the lab and beyond.

I appreciate the help of my friends in the department, Ramesh, Raj Kumar, Hana, Victor, Daniel and Udghatri. The discussions with them about experiments and equipment always helped me. I am extremely thankful to Mary, Maria and others from the Department of Biochemistry and Molecular Biology who made all the administrative and other paper works so easy and convenient. I cannot forget QT for all the good words

and big hugs. I acknowledge the help of the staff and friends in the Entomology department for their help with the Confocal Microscopy.

I would like to thank Dr. Mark Lawrence for supporting me as a part time student worker during the last year of the course. This is indeed an invincible support as it helped me continue with the degree. I also thank my co-workers in the MSV's College of Veterinary Medicine for making my life easy, which in fact helped me finish my dissertation work.

I take this opportunity to thank my late grandfather Mamidi Venkat Reddy, it was his encouragement and kind words which made me choose this path. I owe this work in entirely to my parents, Smt Suguna and Sri Jagan Reddy and my siblings Sirisha and Satish for all that I got from them. Sunil, my childhood friend patiently taught me photo shop and helped me in editing pictures, special thanks to him. This dissertation wouldn't have been possible without my friend for life Gokul, who was always there to support me during the rough days at work, with his words of encouragement and backing. I thank all my friends for all their good thoughts and wishes that constantly kept me in good spirits and health.

Finally I thank almighty for his continuous shower of love and blessings.

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CHAPTER I

INTRODUCTION

The discovery of DNA, double helix, precisely the Watson and crick model was a major breakthrough for life sciences research. This not only gave us information about how DNA was packed into the chromosome but paved path for learning the role played by proteins which help in packing the DNA. It is fascinating to learn how a two meter long DNA is packed tightly into the chromosome and how the packing changes with slight chemical modification on the proteins to which DNA is bound with help of ionic interaction. This in turn changes the gene expression and thus the cell function and sometimes alters certain phenotype in some organisms. These modifications are tightly controlled and monitored by the cell and occur when necessary. These modifications do not change the underlying DNA sequence and some of these modifications are stable and occur for many generations. Study of the inherited changes where the underlying DNA sequence is not changed is called as Epigenetics. Epigenetics involves the study of DNA methylation and histone post translational modifications (both the events do not change the DNA sequence).

Histones are the proteins to which the DNA binds during the packing and the N-terminal chains of these histones are subjected to a variety of post translational modifications. These modifications shift the position of histones due to the change in the ionic environment and steric bulk thus exposing or condensing the chromatin and changing the accessibility of the DNA to various transcription factors (Strahl et al., 2000,

Rafew, 2003). Methylation on histones was thought to be a permanent mark till the discovery of the histone demethylases in mammals and yeast (Banneieter et al., 2002). Histone lysine residues are methylated on lysines 4, 27, 36, and 79 of H3 and lysine 20 on H4 to mono, di or tri methylated state. The change in the state of methylation on any lysine residue could lead to gene regulatory effects. The discovery of histone demethylases in animals showed that methylation homeostasis plays an important role in regulating gene expression (Shi et al., 2004, Turner et al., 2000).

The methyl groups on lysine residues of histones can be removed by the action of two types of demethylases. The first group of enzymes is Lysine Specific Demethylases 1 (LSD1) which helps in the demethylation of mono and di methylated lysine residues. They require FAD as co-factor and protonated hydrogen for the reaction, thus they cannot demethylate tri methylated lysine residues. The second group of enzymes is Jumonji (Jmj) C domain containing histone demethyl transferases which help in demethylating mono, di and tri methylated lysine residues. They are metallo enzymes which require Fe (II) and alpha keto glutarate for their action. Studies showed that these proteins are also involved in demethylating arginine residues (Shi et al., 2004).

Abscisic acid (ABA), an isoprenoid plant hormone, helps in regulating many developmental processes. It is often referred to as a stress hormone. In a situation of stress like drought, ABA is produced in roots and alters the osmotic potential in the shoot to close the stomata. ABA also plays an important role in controlling seed germination and bud dormancy. Loss of function mutants of ABA genes showed altered phenotypes in plants reflecting the importance of ABA in various developmental processes. Recent studies indicate that epigenetics also plays role in ABA signaling during stress conditions (Chinnuswamy et al., 2008, Yin et al., 2009). Though a lot of literature is available for

the genes involved in ABA response not much information is known on how epigenetic mechanisms come into picture in ABA signaling pathways in plants.

Arabidopsis thaliana has been used for plant research for a long time and enormous literature is available for the plant. Due to its low DNA content, completely sequenced genome and short life cycle it has evolved as a model organism for plants. Another advantage of *Arabidopsis* is easy transformation and high seed production. Loss-of-function mutants in *Arabidopsis* are generally obtained using *Agrobacterium tumefaciens* mediated plant transformation where a T-DNA is inserted at the gene of interest thus causing mutation in the gene and leading to loss of function of that particular gene. Functions of many genes have been studied and documented for *Arabidopsis* using t-DNA insertion mutants. The β-glucuronidase (GUS) gene isolated from *Escherichia coli* is to date still the most widely used reporter gene in genetically modified plants used to study the localization of the gene (Jefferson et al., 1986).

Although methylation of histones has been widely studied in plants, not much data is available for histone demethyl transferases. In the same way epigenetic control of ABA signaling is also a relatively new field. The main goal of this research was to study and understand the molecular mechanisms underlying plant development under epigenetic control, specially histone lysine methylation and demethylation. For the same I started working on many *Arabidopsis* mutants and finally focused on the mutants which showed interesting and stable phenotype. Using *Arabidopsis* as the model organism, research was carried out to achieve the following objectives:

1. To characterize a *jumonji* domain containing putative histone demethylase.
2. To study the downstream targets in *jumonji* domain containing putative histone demethylase mutants.
3. To investigate the role of the SDG8 methyltransferase in Abscisic acid response in roots.
4. To study the protein interactions of protein kinase (SnRK2.2) using Tandem Affinity Purification in the presence of ABA**.**

CHAPTER II

LITERATURE REVIEW

Arabidopsis Thaliana – Model Plant

Arabidopsis thaliana is the most common model organism used in plant research. It is a small dicotyledonous flowering plant which belongs to the mustard (Cruciferae or Brassicaceae, Figure 2.1) family. It was discovered in the sixteenth century by Johannes Thal (hence, *thaliana*) in the Harz Mountains and was named as *Pilosella siliquosa*. The plant is now commonly called as *Arabidopsis* although it has undergone lot of name changes.

Arabidopsis was used for developmental studies during early 1900s, but as years went by many scientists used this plant for studying plant development, genetics, evolution etc. In 1998 it was designated as a model organism. Though it is not an agronomically significant plant, it plays an important role scientifically due to its small size, rapid life cycle and completely sequenced genome. *Arabidopsis* is a small plant which can be grown in the lab in growth chambers. It grows up to 20-25 cm long and has rosette leaves at the base and cauline leaves on stems. Its flowers are about 3mm in diameter and its fruit is a siliqua which contains about 20 to 30 seeds. *Arabidopsis* has a rapid life cycle; it takes six to eight weeks from seed germination to seed production. It is easy to perform gene transformation in this plant using *Agrobacterium tumefacines* and the plant produces large number of seeds (10000/plant).

Kingdom : Plantae
Order : Brassicales
Family : Brassicaceae
Genus : *Arabidopsis*
Species : *thaliana*

Figure 2.1 *Arabidopsis thaliana*: Model Organism in Plant Research. Discovered by Johannes Thal in the sixteenth century it is a widely used organism in plant research and was designated as model organism in 1998.

Many different ecotypes have been collected and used for experimental analysis, though Columbia and Landsberg ecotypes are the accepted standards for genetic and molecular research (Meinke et al., 1998). With five chromosomes *Arabidopsis* has a small genome size among the plant community and it is the first plant whose genome has been completely sequenced (December 2001). The *Arabidopsis* Information Resource (TAIR) maintains a database of genetic and molecular biology data for *Arabidopsis thaliana.* These genomic resources provide a large amount of data regarding the epigenetic marks which are responsible for various phenotypes in this organism (Zang et al., 2006, Zilberman et al., 2005). The availability of extensive gene data and the easy maintenance of the plant made me the select this plant as my model organism.

DNA packing- Role of Histones

Comparison of the length of the DNA to the size of a cell into which it should be packed is a completely off-scale picture. Typically, billions of base pairs in length are compactly packaged into micron sized cells. This requires a tremendous amount of packaging of DNA. The fact that long polynucleotide chains have to be packed closely might not be an issue if the electrostatic repulsions did not interfere. The strength of the electrostatic repulsions between the phosphate backbone of the DNA increase as the packaging gets tighter. As mentioned earlier, micron order packaging is feasibly only in the presence of packaging molecules that aid in masking the charge repulsions. DNA is packaged with basic proteins to form a nucleo-protein complex called the chromatin. The basic proteins are usually either Histones (in eukaryotes) or histone like basic proteins in prokaryotes. Histones are a family of basic proteins that aid in the convenient packaging of DNA into chromatin in the eukaryotes. Histones are classified into H1, H2A, H2B, H3 and H4 (Holde, 1988).

Histones form a core basic structure around which DNA is wrapped. The core basic structure was found to contain eight sub-units containing a pair each of H2A, H2B, H3 and H4. This central core octamer wraps 1.7 turns of double stranded DNA that approximately includes 146 base pairs. H1 molecules pull in additional 20 base pairs which completes the second turn around the histone group. Thus formed complex structure containing DNA and the histones is regarded as the nucleosome. Nucleosome is considered as the basic repeating unit of chromatin. Not only the authenticity of nucleosome formation but also the structure of the same has been understood quite convincingly. Several elegant experiments have nailed down the presence of nucleosome structure in the chromatin.

Electron Microscopic studies on chromatin showed the presence of 'beads on a string' like arrangement. Enzymatic studies involving careful digestion of non protein bound regions indicated the amount of DNA that associated with the histone core involving in the formation of the nucleosome. Nucleosome, the 'core particle' was also well characterized physically and found to be having a molecular weight of approximately 200 kD. Several physical techniques like Electron microscopy, Neutron scattering and X-ray scattering revealed the physical dimensions of the complex, it was learnt that the complex in general was like disc with a diameter of 110 Å and with a thickness of 55 Å.

The interactions of various proteins involved in the formation of the core complex were studied by means of protein cross linking studies using several cross linking agents. The interaction between H2A-H2B, H2B-H4 and H3-H4 were considered to be relatively stronger in comparison with the interactions between H2A-H3, H2B-H3 and H2A-H4. The understanding of the histone octamer core however could not get any better after the elucidation of the crystal structure of the core octamer. It revealed a tripartite arrangement of the subunits in the octamer. It was realized to be more like a rugby ball rather than a disc with a length of 110 Å and with a diameter of 65 Å. The details learnt about the dimensions of the octamer were quite different from the previous understanding.

The structure of chromatin is quite complex from the nucleosome, since the later is hardly achieved in the cellular dimensions. The nucleosomes are further packaged into higher order structures that are formed by further looping and coiling. There is vague hierarchy that is generally observed in the order of packaging as 30 nm, 300 nm so on

and the chromatin in the chromosome. H1 plays an important role in stabilizing the higher order structures of chromatin.

The other end of the double edged saw is that the DNA which is compactly packed should be simultaneously be accessible to several DNA modifying enzymes like the polymerases. This process of making the chromatin accessible involves modifying the compact complex of DNA-Histones by various processes that primarily involve the modification of the histones. Histones can either be subjected to a variety of modifications like methylation, acetylation, phosphorylation, etc. or can all together be displaced by sophisticated molecular complexes called the Chromatin remodeling complexes. These processes aid in exposing the DNA that is otherwise obscure.

Epigenetics and Histone Modifications

The traits passed on to off springs from the parents or ancestors were thought be the direct result of DNA sequence that followed Mendelian inheritance. But as studies progressed it was discovered that many traits that were inherited did not follow Mendelian inheritance and those traits were caused due to chemical modification on DNA (methylation) and, or post translational modifications on histones. The study of inheritance of traits occurred due to modification without disturbing the underlying DNA sequence is Epigenetics, the term Epigenetics was coined by C. H. Waddington in 1942 (Waddington 1942, Holliday, 1987). As the size of the genome increased form eukaryotes with single cells to humans undoubtedly the complexity of the genome increased with this increased the complexity of modifications that occur of DNA.

Histone tails extend from the compact histone multimer to provide a platform for various post-translational modifications. These modifications affect the ability of histone

to bind DNA and with other histones, which in turn affect gene expression. The modifications include methylation, citrullination, acetylation, phosphorylation, sumoylation, ubiquitination, and ADP-ribosylation (Strahl et al., 2000, Rafew, 2003, Jenumein et al., 2001)

Each modification has a specific role to play. Acetylation helps in the loosening of chromatin and the chromatin is available for the action of transcription factors (Pazin et al., 1997). When histones are methylated they hold DNA together in condensed form and restrict its access to various enzymes. In this way many modifications have various affects as these modifications change the environment around the histones, by either making hydrophobic (methylation) or hydrophilic (phosphorylation) or due to steric bulk.

Recent studies demonstrated that certain histone modifications can actually block or recruit additional histone modifications, for example, methylation in H4 can block acetylation leading to gene silencing. It was also observed that within a histone, the amino acid modified, the type of modification and the degree of modification (e.g. monomethylation, dimethylation or trimethylation) vary depending on the necessity (Trojer et al., 2006). Nucleosome remodeling complexes along with histone modifications form multi subunit complexes that help in remodeling of chromatin and thus regulate gene expression (Strahl et al., 2000).

Histone modifications, on one or more tails, act sequentially or in combination to form a histone code that is read by other proteins to bring about distinct downstream events. The charge distribution on the histone N-terminal tail appears to be a key determinant in the readout of the histone code as exemplified by the differential effects of modification of Lys on enzyme activity. Modifications on histones occur on multiple but specific sites, and it has been suggested that histones can act as signaling platforms, like

transcription activation or repression. With so many possible combinations of modifications that can occur on different sites of histones, it can be proposed that different combinations of histone modifications may result in distinct outcomes in terms of chromatin-regulated functions (Jenuwein et al., 2001).

Histone H3 NH_2-terminus appears to exist in two distinct modification states that are likely to be regulated by a "switch" between Lys 9 methylation and Ser 10 phosphorylation. Lys 9 methylation is inhibited by Ser 10 phosphorylation, but when it is coupled with Lys 14 acetylation it plays an important role in mammalian cells during mitogenic and hormonal stimulation. When Lys 9 is methylated it inhibits Ser 10 phosphorylation, leading to mitotic chromosome dysfunction. Lys 9 methylation by the Histone Methly Transferase is facitilated by deacetylation of Lys 14 (Rae et al., 2000, Nayakama et al., 2002, Melcher et al., 2000, Clayton et al., 2000, Cheung et al.,2000).

For proper functioning of a cell it is important that these modifications should be monitored and controlled with coordination. The promoters of the genes are usually in the nucleosome free regions. Chromatin constrains are altered in response to exogenous cues which create a cell specific or stimulus specific accessible genome to regulate the gene expression. This happens firstly by histone modifiers which covalently alter the N-terminal tails of histones which in turn create or block protein binding sites. Then it's the turn of chromatin remodeling complexes which use ATP and alter the position of nucleosome. This in turn helps in methylation of DNA which interferes with binding of transcription factors and recruits other proteins.

Histone Methyl Transferases (HMTs)

Amino acids residues on proteins that could be subjected to methylation are €-amine of lysine, guanidine group of arginine, imidazole ring of histidine and amide group of glutamine and aspargine. Among these amino acid residues lysine and arginine appear to be the chosen ones on which methylation occur on histone tails. Histone methylation has been critical in regulating gene expression along with DNA methylation. In higher organisms, N terminal tails of histone H3 can be methylated at lysines 4, 27, 36, and 79 (in nucleosomal core) and histone H4 on lysine 20. Modified lysine residues can exist in a mono-, di-, or tri-methylated state (Lachner et al., 2003, Feng et al., 2002, Lacoste et al., 2002, Ng et al., 2002, Leeuwen et al., 2002).

Methylation when occurs on the lysine or arginine residues increases the hydrophobicity and depending on the state of modifications (mono, di or tri methyl states) and the size of the group added steric bulk also plays a role. Hydrophobicity and steric bulk in turn change the position of histone in the nucleosomal core which either expose DNA for the action of transcription factors or condenses the DNA thus repressing the gene. Histone methyltransferases which act on lysine residues are called as histone-lysine N-methyltransferase and those that act on arginine residues as histone-arginine N-methyltransferase. These proteins require the presence of S-adenosylmethonine which is the donor of the methyl group. Methylation reaction is a simple nucleophilic substitution reaction in which hydrogen on the amino group is substituted with a methyl group from S-Adenosylmethionine

The histone-lysine methyltransferases contain a highly conserved SET domain which is necessary to bind to S-Adenosylmethonine during the methylation reaction. The SET domain containing protein is around 130 amino acids long and was found conserved

in three proteins of *Drosophila melanogaster* from where the domain got its name. The three proteins are Suppressor of variegation 3-9 (Su(var)3-9), a modifier of position-effect variegation, Enhancer of Zeste (E(z), a polycomb-group chromatin regulator and trithorax (Trx), a trithorax group chromatin regulator (Tschiersch et al., 1994, Gelbart et al., 1993, Stassen et al., 1995, Jenuwein et al., 1998). Almost all methyl transferases which methylate lysine residues contain SET domain except for lysine 79. The protein involved in the methylation of lysine 79 belongs to DOT1 family which is not structurally related to SET domain proteins.

In mammals around 70 SET domain containing genes have been identified and the function of few has been deciphered. Studies showed that these proteins were highly specific and they would only act on a specific lysine residue to methylate it to a specific state (mono, di or tri methyl state). The SET domain containing proteins have been studied extensively in Drosophila, mammals, yeasts, plants and bacteria and their functions have been documented (Rea et al., 2000). These proteins are divided into seven main families, SET1, SET2, SUV39, EZ, RIZ, SMYD and SUV4-20 (Dillon et al., 2005). SUV 39 family proteins in humans and drosophila were found to methylate lysine 9 on histone H3 (H3K9) (Rea et al., 2000). SET 1 and SET 2 complexes in yeasts were involved in trimethylation of H4K4 and H3K36 respectively and helped in transcription elongation (Ng et al., 2003, Krogan et al., 2003). In mammals a SET2 family member is thought to be involved in methylation of H3K36 and H4K20 (Rayasam et al., 2003).

Twenty nine active genes containging SET domain have been identified in *Arabidopsis* using bioinformatic tools (Baumbusch et al., 2001). In plants the SET domain containing proteins are divided into seven classes based on the homologs they contain. Class 1 includes enhancer of zeste homologs, class 2 includes the ASH1

homologs, class 3 includes the trithorax homologs and related proteins, class 4 includes proteins with a SET and a PHD domain, class 5 includes suppressor of variegation homologs, class 6 includes proteins with an uninterrupted SET domain and class 7 includes RBCMT and other SET related proteins (Ng et al., 2007).

The first identified methyl transferase in plants was E (z) homolog containing protein which is responsible for curly flower phenotype in *Arabidopsis*. The proteins belonging to this family usually act on H3K29 and control the leaf and flower morphology along with flowering time (Katz et al., 2004, Schubert et al., 2006). The ASH1 homologs have an AWS domain in plants. In *Arabidopsis ash2* mutants showed an early flowering phenotype due to decreased H3K36 methylation states (Zhao et al., 2005). ATX1 protein from the trithorax homolog family is involved in floral development by controlling gene expression via H3K4 methylation (Bouveret et al., 2006, Alvarez et al., 2003). Su (Var) homologs are an important class of methyl transferases which act on H3K9. SUVH4 in *Arabidopsis* is extensively studied and its possible role in flowering elucidated. Some studies also show that this class of protein could induce DNA methylation at the target loci (Nakayama et al 2001, Jackson et al 2002). Rubisco subunit methyltransferase (RBCMT) are SET domain containing proteins which act on non histone proteins.

Histone Demethyl transferases (HDTs)

Among the histone modifications, histone methylation was thought to be a permanent modification (Bannister et al., 2002) till the histone Demethyl transferases (HDTs) were discovered recently. The existence of Histone Demethyl transferases was proposed in 2002 (Banister et al., 2002) and very soon their existence was confirmed and

the mechanism of action of few was deciphered in a span of 8 years (Shi et al., 2004, Tsukada et al., 2006, Lan et al., 2008, Schneider et al., 2006). Two families of histone demethylating enzymes have been identified in mammals.

Lysine Specific Demethylase 1 (LSD1) is a family of HDTs which contain a SWIRM domain that codes for a chromatin associated protein and an FAD dependent amine oxidase domain. The proteins of this family are involved in demethylation of mono- and di-methylated lysines, specifically on histone 3, lysine's 4 and 9 in yeast. Their mechanism of action is amine oxidation, in which an imine intermediate is formed. Protonated hydrogen and FAD (co factor) are required for this reaction. As protonated hydrogen is required for the demethylation reaction, this protein can only act on di methylated and mono methylated lysine residues. LSD1 proteins are involved in gene repression and activation by forming a complex with other transcriptional regulators and they also help in chromatin organization to higher order. LSDs specific for H3K4Me1 and H3K4Me2 and H3K9Me have been studied in yeast (Shi et al., 2004, Rudolph et al., 2007, Metzger et al., 2005). Genes homologous to human LSD1 in *A. thaliana* control flowering by regulating the expression of FWA & FLC (genes responsible for repression of flowering (Jiang et al., 2007)

The second family of histone demethylases is the Jumonji domain-containing histone demethylase (JmjC). According to the sequence similarity in the JmjC domain, seven sub families of JmjC domain containing proteins have been identified in humans. The sub families include JHDM1, JHDM2, JHDM3, JARID, UTX, PHF2/PHF8 and JmjC only (Klose et al., 2006, Takashi et al., 2006). More than 100 proteins containing JmjC domain have been identified and the functions of few have been studied in different organisms. These proteins act like DNA demethylases (AlkB) where in the methyl group

is converted to hydroxyl group and formaldehyde is released. Thus they can even demethylate tri methylated lysine residues along with di and mono methylated residues. Unlike LSD1 Jumonji domain containing proteins are predicted to be metalloenzymes that regulate chromatin function.

One of the first JmjC domain containing protein to be identified was JHDM1A which belongs to the JHDM1 family. It was observed that the protein extracted from *Hela* Cells acted on H3K36Me2 and H3K36Me1 during *in vitro* analysis (Tsukada et al., 2006). The proteins belonging to family JHMD2/3 help in demethylation of tri and di methylated lysine residues and they did not act on mono methylated lysine residues (Klose et al., 2006, Whetstine et al., 2006). Later studies revealed their catalytic activity was dependent on the presence of JmjC and JmjN domains along with tudor domain (Fodor et al., 2006). Another subfamily called the JARID sub family have a DNA binding ARID domain and studies indicate that the proteins encoded by this family along with other factors were responsible for proliferation of cancer cells (Yamane et al., 2007). Recent discovery of UTX/UTY sub family proteins revealed the role of histone demethylases in cell differentiation in coordination with histone lysine methyl transferases and DNA methylases (Agger et al., 2007, Issaeve et al., 2007, Nottke et al., 2007).

Studies on histone demethylases in humans and yeast showed that the lack of the demethylases lead to abnormal carcinogenesis, spermatogenesis, X-linked mental retardation, etc throwing light on how methylation homeostasis played an important role in controlling important pathways in the cell. This paved a path for plant biologists to study the behavior of histone demethylases in plants and the curiosity lead to identification of 21 *Arabidopsis* and 20 rice JmjC domain containing proteins using

bioinformatic tools (Lu et al., 2008, Hong et al., 2009). Though the histone demethylase genes have been identified using bioinformatic approaches, the functions of most of the genes have not been elucidated. The few genes which have been studied which contain JmjC domain in plants are EFF6 (EARLY FLOWERING 6) and REF6 (RELATIVE OF EARLY FLOWERING 6, a pair of homologous genes which encode nuclear proteins with JmjC domain and zinc-finger domain antagonistically regulate flowering time in *A.thaliana*. MEE27, which is involved in gametophyte development (Pagnussat et al., 2005) and IBM1 (INCREASED EXPRESSION OF BONSAI METHYLATION 1) (Saze et al., 2008) are few other genes in plants which contain JmjC domain. In *Arabidopsis thaliana* most of the JmjC domain containing proteins have not yet been characterized and it would be interesting to see the phenotypic and functional affects of these proteins in *Arabidopsis.*

Protein kinases in Arabidopsis Thaliana

The overall development of any organism depends on availability of resources and biotic and abiotic stress conditions. In eukaryotes the energy sensors are highly conserved and are responsible for proper maintenance of activities occurring in the cell. These sensors are protein kinases and are represented as AMPK (AMP activated protein kinases) in mammals, Snf1 (sucrose non fermentling-1) in yeasts and SnRK 1 (SnF1 related protein kinases) in plants (Hardie & Carling, 1997, Hardie , 2007, Hrabak et al., 2003, Halford and Hey , 2009, Zhang et al 2010). Protein Kinases are enzymes which add a phosphate group to the target protein and trigger a cascade of reactions.

Snf 1 of *Saccharomyces cerevisiae* is a Ser/Tre protein kinase which plays an important role in the diauxic shift to oxidative state from fermentation state in yeast

(Zheng et al., 2010). AMPK of mammals not only controls the whole body energy levels but also act as metabolic regulator during stress conditions when ATP is not properly assimilated (Hardie 2007, Dzamko & Steinberg, 2009). Though most of the protein kinases appear to act similarly in plants, few differences have also been registered. For example receptor kinases in animals phosphorylate only tyrosine (tyr) residues where as in plants they phosphorylate both tyr and ser (serine) residues. Few calcium dependent proteins like calmodulin-dependent protein kinases (CaMKs) and protein kinase C does not seem to play an important role in plants.

In plants a number of protein kinase families have been identified which are not found in animals and yeast. Some of them like CDPK's (Calcium dependent protein kinases), CDPK-related kinases (CRK's) and phosphoenol pyruvate carboxylase kinase related kinases (PEPRK's) are unique to plants. In *A. thaliana* as the genome of the plant is completely known; around 1085 protein kinases have been identified using bioinformatic tools. Around 38 protein kinases related to SNF1 of yeast have been identified in *Arabidopsis thaliana*. Based on sequence similarity and domain structure, the SnRK protein subfamily in plants has been divided into SnRK1, SnRK2 and SnRK3 sub groups. SnRK protein kinases in *Arabidopsis* include AKIN 10 and AKIN 11 which through Pleioltropic Regulatory Locus (PRL 1) control the sucrose, hormone and stress signaling pathways (Nemeth et al., 1998, Bhalerao et al., 1999).

SnRK1 proteins are the largest proteins among the sub groups and are most closely related to the yeast SNF1 and mammalian AMPK protein. They play an important role during environmental and nutritional stress by regulating metabolism. Similar to yeast and mammalian protein kinases, plant SnRK1 proteins interact with plant orthologs of proteins that regulate yeast SNF1 by forming heterotrimeric complexes (Bhalerao et al,

1999, Hardie 2000). The SnRK proteins which are unique to plants fall under SnRK2 and SnRK3 sub groups. Proteins belonging to SnRK2 sub group are about 40 kD in size with a characteristic patch of acidic amino acids at the C-terminal. Around ten SnRK2 genes have been identified in *Arabidopsis thaliana* out of which SnRK2.2, SnRK2.3 and SnRK2.6 have been characterized. In *Arabidopsis* around twenty five SnRKs fall under SnRK3 sub group, which are also known as CBL-interacting protein kinases. This sub group of proteins is involved in stress responses pertaining to salt, sugar and ABA (Gong et al., 2002, Shi et al., 1999).

Flower development pathways in *Arabidopsis Thaliana*

Reproduction is an important process in the life cycle of any organism. In both lower and higher organisms it is a complex process and a number of genes act in coordination to ensure proper completion of this process. In case of plants not only genes but many environmental factors play important role in deciding the time of reproduction and the overall development of the plant. Along with the environmental cues like day length, temperature, humidity, hormones etc. in *Arabidopsis thaliana*, several pathways form a regulatory network to induce flowering. Using loss-of-function mutants many *Arabidopsis* genes involved in flowering have been discovered and the action of proteins encoded by them have been studied and documented for *Arabidopsis thaliana*.

In *A. thaliana* day length plays an important role in deciding the time of flowering, plants when grown in long days (16 hour photo period) flower early compared to plants grown in short day (8 hour photo period). Not only the day length but intensity of light and temperature also affect the flowering time (Davis 2002). Previous studies indicate that CONSTANS (CO) (Lopez et al., 2001, Nagatani et al., 2007) a transcription

factor, is the link between light perception and flowering time. The expression of the gene CO is controlled by circadian clock and this gene up regulates FT (Flowering locus T) which is responsible for flowering (Kardailsky et al., 1999, Kobayashi et al., 1999). GIGANTEA (GI) is another circadian clock regulated gene which encodes a nuclear protein and plays an important role in flowering (Flower et al., 1999, Parl et al., 1999, Haq et al., 2000). SUPPRESSOR OF CONSTANS OVEREXPRESSION (SOC1) is a MADS-box transcription factor which regulates flowering in *Arabidopsis* through gibberellins pathway (Moon et al., 2003).

Autonomous pathways, gibberellin pathway, vernalization pathway and photoperiodic pathways play their respective roles of activating or suppressing genes involved in flowering and thus control the flowering pathway in *Arabidopsis thaliana* (Figure 2.2). Genes involved in these pathways have been extensively studied by using loss-of-function mutants and other methods and enormous data is available regarding these genes. Triggering the expression of a gene in these pathways leads to a cascade of events which in turn affects the growth and flowering pattern of the plant. Some genes involved in the autonomous pathways are FCA, which is activated by FY due to protein interaction (Simpson et at., 2003), FVE and FLD, which are chromatin remodeling proteins (Audtin et al., 2004), FPA, LD, FLK (RNA binding protein), etc. (Sheldon et al., 1999, Simpson et al., 2004, Lim et al., 2004, Jiang et al., 2007).

VRN1, VRN2 and VIN 3 are some important genes involved in vernalization pathway. These genes along with DNA methylation act as negative regulators of FLC gene which a MADS box transcription factor (Michael et al., 1999, Sheldon et al., 1999). FLC is flowering repressor gene and over expression of this gene delays or interferes with the flowering pattern in *A. thaliana*. Expression of FLC was affected by histone

modifications and H2K36 trimethylation increased FLC expression which in turn delayed flowering (Zhao et al., 2005). FLC expression was also regulated by *ARABIDOPSIS* TRITHORAX 1 (ATX1) by H3K4 trimethylation (Pien et al., 2008).

FT gene is responsible for the mobile signal which is necessary for the initiation of inflorescence. It was observed that this signal was produced in the leaf and was transported to the shoot apex via phloem to initiate flowering (Zeevaart et al., 2007 Corbesier et al., 2007 Jaeger and Wigge et al., 2006, Notaguchi et al., 2008, Schwartz et al., 2009). FT gene encodes a 20 kD protein which belong to the family CETS. This family of proteins is homologous to Raf-kinase- inhibitor protein in mammals (Banfield et al., 2000, Kobayashi et al., 1999) which play an important role in protein kinase signaling cascades. Studies indicate that there is a twofold or even higher increase in more than hundred genes in the shoot apex region during flowering (Schmid et al., 2003). FT gene is activated by CONSTANS (CO) gene (Putterill et al., 1995, Valverde et al., 2004) suppressed by FUL (FRUITFUL) and its action is dependent on the expression of flowering time gene FD (Samach et al., 2005). This gene is responsible for the accumulation of SEPALLATA3 (SEP3) and FUL in the leaves. APETALA1 (AP1) an important floral meristem gene was found to be activated by FT along with LEAFY (LFY) another floral meristem gene. LFY in turn is regulated by a nuclear protein encoded by LD (LUMINIDEPENDENS) (Aukerman, et al., 1999, Adrian et al., 2010).

The transition to flowering in *Arabidopsis thaliana* leads to production of cauline leaves. These leaves are relatively smaller than rosette leaves and show some morphological differences. The number of rosette leaves does not increase much in the reproductive phase when compared to the vegetative stage (Hempel and Feldman, 1994,

Poethig, 2003). This could be because the plants main focus when it starts bolting is production of flowers and seeds and the genes which help in this process are activated.

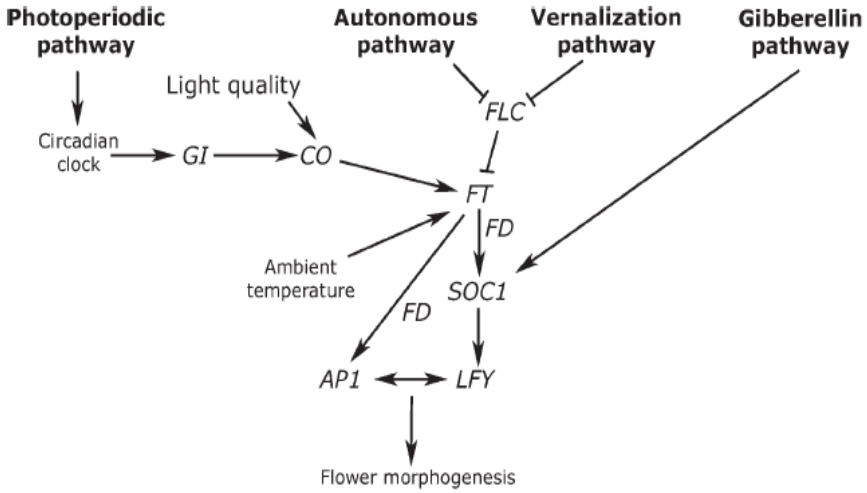


Figure 2.2 Schematic figure showing different pathways involved in Flowering in *Arabidopsis thaliana*. Environmental cues also play a major role in controlling the flowering time. The expression of FT, an important gene in deciding the time of flowering, is controlled mainly by autonomous pathway, though indirectly many genes involved in vernalization pathway and circadian clock affect its expression. The expression of FLC, a negative regulator of flowering, is suppressed by vernalization pathway and autonomous pathway along with FT.

(Source: Corbesier L et al., 2006).

T-DNA insertion mutants – Reverse Genetics

Agrobacterium tumefaciens, a rod shaped gram negative bacterium (family Rizobiaceae) is responsible for the crown gall disease in dicotyledonous plants where the formation of tumors is observed (Chilton et al., 1977). This bacterium has a feature of inserting a small segment of its DNA, known as T-DNA or transfer DNA into the plant cell randomly, which is expressed by the plant cells. This tumor causing bacterium though not beneficial for plants, studies revealed the potential use of this bacterium in

plant transformation. Now-a-days *Agrobacterium* mediated transformation in plants had become a strong tool in generating transgenic plants. But only *A. tumefacians* strains containing a tumor-inducing plasmid (Ti plasmid or pTi) which has all genes necessary for T-DNA integration event can help in the production of transgenic plants.

The Ti plasmid has the virulence region which encodes genes (virA, B, C, D, E, F and G) whose gene products help in infecting the plant and integration of DNA. The bacterium can swim through the soil with the help of flagella produced by vir B, vir A/vir G together produce a protein which is attracted to opines (example: acetosyringone) produced by wounded plant. Once on the plant the bacteria bind to the wall with the help of flagella. This resembles the reproductive conjugation event in bacterium. VirD1 and D2 help in the production of a mobile T-DNA and form an immature T-DNA complex. This complex is transported to the nucleus with the help of vir E, F which play their role of integrating the T-DNA into the plant genome. The plant expresses the gene on the T-DNA causing hormonal imbalance and thus produces tumors (Stachel, 1986, Tzfira et al., 2006, Tzfira et al., 2004, Kahl et al., 1991).

The cDNA libraries or the nucleotide sequence give us information about the genes but they cannot tell us the exact function of the gene product and the phenotypic affects of the gene. To study the effect of a gene, mutation in that gene would be an efficient way; this is nothing but reverse genetics. The presence of T-DNA in the plants can be verified using primer specific for the T-DNA tag. To identify a loss of function mutant the primer specific for the gene along with the T-DNA specific primer are used. As the T-DNA is single stranded, heterozygous lines are obtained due to the recessive nature of the mutation but after few generation homozygous loss of function mutants are obtained (if the gene of interest is not lethal when mutated) which can be used for further

studies. In this way using the T-DNA insertion technique many loss of function mutants have been produced and their gene functions have been revealed (Walden et al., 1990, Sussman et al., 2000, Krysan et al., 1996).

Abscisic Acid- Plant hormone

Abscisic Acid was first studied by Addicott *et al.,* 1967 and the nomenclature that was used was *abscisin II*. Wearing *et al.,* 1971 as a part of their photo periodic studies on woody species identified a compound called *dormin.* It was found that both the compounds found by Addicott and Wearing were similar and a uniform nomenclature was later established. Abscisic Acid (Figure 2.3) is a naturally occurring sequeterpenoid in plants. It is partially synthesized through the mevalonic pathway in plastids. The production of abscisic acid in general is induced by stress. Carotenoids in specific Violaxanthin, is isomerised followed by an oxidation reaction, resulting in the formation of xanthonin that spontaneously gives rise to Abscisic Aldehyde. Furter oxidation of Abscisic Aldehyde gives rise to Abscisic acid.

Abscisic Acid was initially considered as the plant hormone that has accentuating effects on the plant growth. But it has been learnt that ABA serves several other important functions like closing of stomata (protecting cells from dehydration), root growth, seed maturation and dormancy, bud dormancy, abscission, seedling growth, apical bud dominance etc. ABA-deficient mutants revealed the role of ABA as an endogenous anti-transpirant and in embryo and seed dormancy. Hence ABA plays an important role in vivipary.

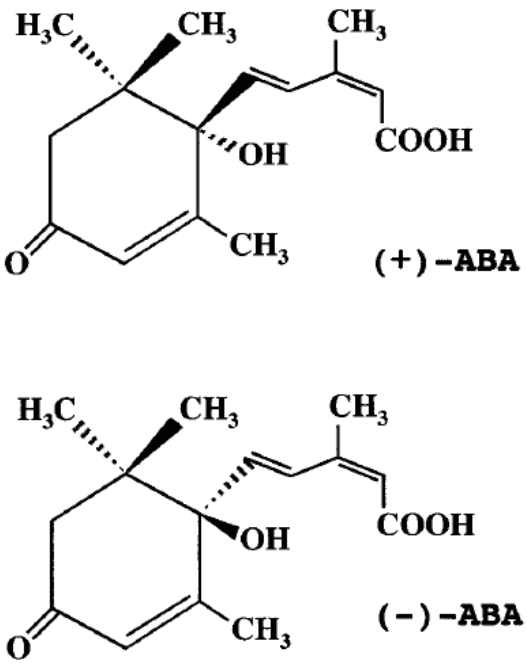


Figure 2.3 Structure of Abscisic Acid.

ABA is a plant hormone that regulates seed dormancy and germination. ABA can exist in two forms, (+)-(S)-ABA and its mirror image (-)-(R)-ABA. The behavior of plants when these stereoisomers are applied exogenously is different.
(Source: Nambara et al., 2002).

ABA signal transduction pathways in *Arabidopsis thaliana*

The change brought about by alteration in gene expression in the presence of ABA is known as ABA gene response. During stress conditions like drought and low temperatures the photosynthetic performance of the plant reduces and the senescence is accelerated. Adaptation to stress by plants has always been under scrutiny as it helps in understanding the pathways that control stress and thus improves the plant productivity. Elucidation of ABA signaling pathways, though difficult (as it involves a cascade of signaling events) it has been a useful tool to understand how plants behave under water stress- a major limiting factor in plant productivity. By studying the ABA mutants some components involved in ABA signaling have been identified. In general the ABA

mutants are divided into three classes, ABA deficient, ABA hypersensitive and ABA insensitive mutants.

The ABA-insensitive genes include ABI1, ABI2, ABI3, ABI4 and ABI5. These mutants (*abi*) are insensitive towards ABA, and exogenous application of ABA does not cause changes in the growth of the mutants (Meyer et al., 1994). ABI1 and ABI2 negatively regulate ABA responses by encoding 2C-type phosphotases (Koornneef et al., 1984, Meyer et al., 1994). The ABI3 encodes seed-specific B1 where as ABI5 encodes basic leucine zipper (bZIP) type transcription factors (Soderman et al., 2000). ABA induced activation of transcription factors, involving many ABA-responsive element-binding factors which are either activated or repressed by phosphorylation. Around nine AREB/ABF type b-ZIP proteins have been identified in *Arabidopsis*. These binding factors include ABF1, ABF2, ABF3, ABF4 and ABI5 which activare expression by binding to ABRE elements (Uno et al., 2000, Choi et al 2000). Usually the proteins encoded by the ABA responsive genes act as protective proteins as they are produced during stress conditions. These proteins were discovered using loss-of-function mutants and other techniques. ABA deficient mutants show a wilty phenotype due to impaired gene expression and ABA induced stomatal closure (Taylor et al., 2000). ABA hypersensitive mutants (*era1*) show enhanced response to ABA. ERA1 encodes farnesyl transferase which reduces ABA sensitivity in seeds and plants. Though plethora of information exists for ABA response a simple ABA signaling pathway has not been defined, indicating an existence of a complex signaling topology.

Tandem Affinity purification

Studying protein-protein interactions and the consequence of those interactions has been an interesting subject and an informative tool. Coupled with bioinformatics this area of research is being used to produce large amount of data in different species. Through these studies not only do we gain knowledge about genome function and evolution but we also learn about the cellular processes. Biochemical techniques like co-immunoprecipitation, bimolecular fluorescence complementation (BiFC), affinity chromatography, yeast two hybrid screens, Tandem affinity purification, Chemical cross linking, Strep-protein interaction, etc. have been used for studying the protein protein interactions. Few biophysical techniques like dual polarization interferometry, static light scattering, and dynamic light scattering, surface plasmon resonance, fluorescence correlation spectrometry, protein-protein docking, etc. have also been used for studying protein-protein interactions.

Among the above mentioned techniques Tandem Affinity Purification (TAP) is a biochemical method for studying protein-protein interactions. It is a two step purification of the protein-protein complex. In this technique the protein of interest is fused with a TAP tag and introduced into the expression system. The TAP tag was discovered in 1990's but has come to be used in early 2000's. TAP tag contains two binding sites, an N-terminal calmodium binding site and a protein A-IgG binding site along with a tobacco etch virus (TEV) protease cleavage site. The levels of expression of the recombinant protein must be the same as it is in the normal case. The fusion protein would be free to interact with other proteins in the cell on one end.

The first step in the purification is retrieving the fusion protein from the cell extracts using affinity selection (IgG beads). In the next step TEV protease cleaves the

IgG tag and releases the protein which is then incubated with calmodium beads in presence of calcium. In this step the protein is further purified with washes to remove the contaminants. The protein complex bound to calmodium beads was then eluted with a EGTA-containing buffer and analyzed using SDS-PAGE. As the procedure includes a two-step affinity purification the chance of contamination is low. TAP helps us in determining the protein partners *in vivo,* usually these partners are unknown. The major disadvantage of this technique is that the TAP tag might interfere with the binding of other proteins to the protein of interest (Puig et al., 2001, Brown et al., 2006).

CHAPTER III

CHARACTERIZATION OF JUMONJI DOMAIN CONTAINING PUTATIVE HISTONE DEMETHYLASE IN *ARABIDOPSIS THALIANA*

Introduction

The N-terminal tails of histones are subjected to a variety of post translational modifications which are thought to be involved in regulation of many genes in higher organisms. Among these modifications histone methylation has been critical in regulating gene expression along with DNA methylation. Histone H3 can be methylated at lysines 4, 27, 36, and 79 (in nucleosomal core) and histone H4 on lysine 20. Modified lysine residues can exist in a mono-, di-, or tri-methylated state (Lachner et al., 2003, Feng et al., 2002, Lacoste et al., 2002). Histone methylation was thought to be a permanent modification (Bannister et al., 2002) till the histone demethyl transferases were discovered recently. Three types of histone demethylases have been discovered in mammals and yeasts. Peptidyl arginine deiminase (PAD14) is a protein which helps in removing the methyl group on arginine residue by converting it to citrulline (Cuthbert at al., 2004). Though this group of proteins removed the methyl groups the reaction was not a demethylation reaction. The quest for histone demethylases lead to the discovery of LSD1 (Lysine Specific Demethylase 1) and Jumonji (Jmj) C domain containing proteins. LSD1 proteins are FAD (flavin adenine dinucleotide) dependent nuclear amine demethylases which helps in removal of methyl groups from mono and di methylated lysine residues of histones (Shi et al., 2004). JmjC domain containing proteins are metallo

enzymes which can demethylate mono, di and tri methylated lysine residues in the presence of Fe (II) and alpha keto glutarate (Klose et al., 2006).

Studies on histone demethylases in humans and yeast showed that the lack of the demethylases lead to abnormal carcinogenesis, spermatogenesis, X-linked mental retardation, etc. throwing light on how methylation homeostasis played a significant role in controlling important pathways in the cell. This paved a path for plant biologists to study the behavior of histone demethylases in plants and the curiosity lead to identification of 21 *Arabidopsis* and 20 rice JmjC domain containing genes using bioinformatic tools (Lu et al., 2008, Hong et al., 2009). Though the histone demethylase genes have been identified, the functions of most of the genes have not been elucidated. Few genes which have been studied so far which contain JmjC domain in plants are EFF6 (EARLY FLOWERING 6) and REF6 (RELATIVE OF EARLY FLOWERING 6), a pair of homologous genes which encode nuclear proteins with JmjC and zinc-finger domains antagonistically regulate flowering time in *A. thaliana* (Nho et al., 2004). MEE27, which is involved in gametophyte development (Pagnussat et al., 2005) and IBM1 (INCREASED EXPRESSION OF BONSAI METHYLATION 1) (Saze et al., 2008) are few other genes in plants which contain JmjC domain. In the present study we show the possible role of a jumonji domain containing putative histone demethylase in controlling the flowering time in *Arabidopsis thaliana.*

Along with the environmental cues like day length, temperature, humidity, hormones etc. several pathways in *Arabidopsis thaliana* form a regulatory network to induce flowering. Using *Arabidopsis* loss-of-function mutants many genes involved in flowering have been discovered and the actions of proteins encoded by them have been documented. Among these genes FLC (Flowering locus C) which is regulated by

autonomous pathways is thought to be involved in repressing flowering (Zhao et al., 2005) and FT (flowering locus T) regulated by CO (CONSTANS) is involved in controlling the flowering time in *Arabidopsis* (Kardailsky et al., 1999, Kobayashi et al., 1999). Literature indicates that there is a twofold or even higher increase in more than hundred genes in the shoot apex region during flowering (Schmidt et al., 2003). This throws light on how proteins coordinate during the process of bolting and production of flowers. For examples APETALA1 (AP1) an important floral meristem gene was found to be activated by FT along with LEAFY (LFY) another floral meristem gene. Not just CO regulates the expression of FT but a lot of chromatin associated complexes play a major role in regulating FT and thus control the flowering time in *Arabidopsis thaliana* (Farrona et al., 2008, Olmo et al., 2009, Jeong et al., 2009, Adrian et al., 2010). The gene pertaining to FT encodes a 20 kD protein which belong to the family CETS, which help in switching from vegetative to reproductive growth in plants (Pnueli et al., 2001). This family of proteins is homologous to Raf-kinase-inhibitor protein in mammals (Banfield et al., 2000, Kobayashi., et al., 1999) which play an important role in protein kinase signaling cascades in yeast and mammals.

In our present study we show that a Jumonji C domain containing putative histone demethylase is involved in controlling the flowering time in *A. thaliana*. Based on sequence similarity, we have identified a putative Jumonji domain-containing histone demethylase gene in *Arabidopsis* and obtained T-DNA insertion lines for this genes form TAIR. The knocked out mutants (*jmj14-1* and *jmj14-2*) (Figure 3.1) of JMJ14 show an early flowering phenotype and increased mRNA levels of FT gene. A change in H3 methylation pattern was also observed in these mutants at the FT loci indicating the regulatory role of JMJ14 in controlling the flowering time in *A. thaliana*.

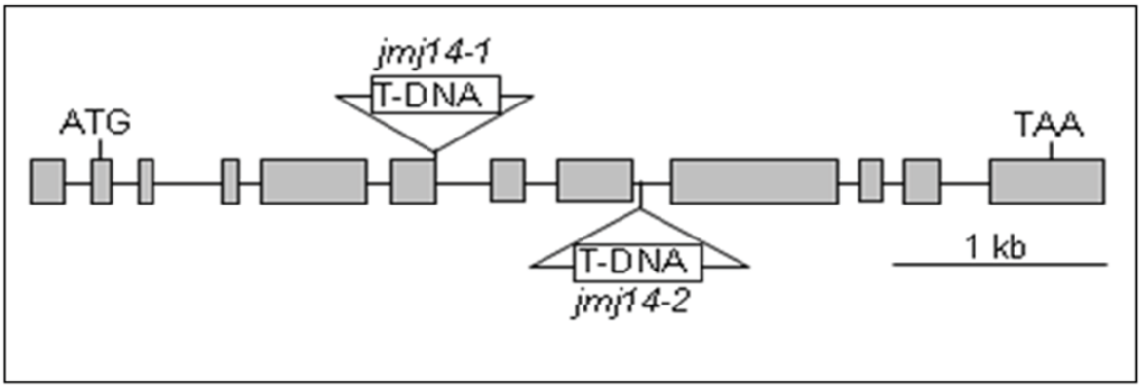


Figure 3.1 T-DNA insertion sites in JMJ14.

Exons and introns are represented by filled boxes and lines, respectively. ATG and TAA indicate the stop and start codon respectively.

Materials and methods

Arabidopsis thaliana Growth conditions

Arabidopsis seeds were sterilized using 70 % ethanol first and then a solution of 50 % bleach and 0.1 % Triton X 100 for 15 minutes. After washing thoroughly with water (4 times) they were transferred onto plastic dishes containging 0.5 X Murashige and Skoog (MS) medium supplemented with sucrose (1 %) and Agar (1 %). After sterilization seeds were kept at 4^{o}C for 48 hours and then were grown in the incubator at 22^{o}C on the solid medium till four leaf stage. They were then transferred to soil (Schultz potting mix) and moved to green house after keeping them at dark for 24 hours where the optimum conditions of temperature, 21 – 25^{o}C and light, 120 – 150 µmol/m^{2}sec were provided. The plants were grown in 14 hour light photoperiod till seeds were obtained.

Isolation of T-DNA insertion mutant lines

Two T-DNA insertion lines SALK-135712 (*jmj14-1*) and SALK- 136058 (*jmj14-2*) were obtained for JMJ14 gene from the SALK collection at the *Arabidopsis* Biological

Resource Centre. PCR genotyping was performed to verify the T-DNA insertion using T-DNA left border primer and JMJ14 gene specific primers. Genomic DNA for PCR genotyping was extracted by homogenizing a rosette leaf in liquid nitrogen and adding the tissue power obtained to the pre warmed (65^{o}C) extraction buffer (2 % w/v PVP-40, 5.0 mM ascorbic acid and 4 mM diethyldithiocarbamic acid along with 10 mg/ml RNase). This solution was heated at 65^{o}C for 5 minutes and to it chloroform and isoamyl alcohol solution (24:1) was added. The upper DNA containing phase was recovered and DNA was precipitated using isopropanol, washed with 70 % ethanol and dried adequately before using for PCR amplification. DNA was amplified using Taq polymerase (Gene script) and the amplified PCR product were electrophoresed on a 1 % agarose gel in 1 X TEV buffer at 100 mA for approximately 30 minutes.

Gene Expression Studies: RT PCR

To confirm that the mutants were knocked out lines we isolated the total RNA using RNeasy Plant Mini Kit (QIAGEN) from the leaves of four week old plants (wild type and mutant lines). RNA from various tissues (leaf, stem, roots, flower and siliuqe) of wild type plants (38 days old) was also extracted using the RNeasy Plant Mini Kit from QIAGEN. The RNA from seeds was obtained by using hot borate method. cDNA was prepared from the RNA extracted using Super Script^{TM} First Strand Synthesis System for RT-PCR (Invitrogen). For this purpose 1 µg of RNA was used and 1 µl of oligo dT and 1 µl of dNTPs were used. The reaction mixture was adjusted to 12 µl with double distilled water. This mixture was heated to 65^{o}C for 5 minutes and chilled on ice for 5 minutes, to this 7 µl of reaction mixture contating 5 X first-starnd buffer, 0.1 M DTT and RNase OUT provided by the manufacturer was added. The mixture was incubated at 42^{o}C for 2

minutes and then 200 units of Super Script II RT were added and gentle mixed by shaking. This was further incubated at 42^{o}C for 50 minutes. The reaction was stopped by incubating at 70^{o}C for 15 minutes. The cDNA otained was used for RT-PCR. For all the polymerase chain reactions Taq polymerase (gene script) was used. Primers used for PCR amplification were 5'GCTTCTTGGAGCTGCTATGG 3' (forward primer) and 5'CAAGAAGCACTACCGTTCTC3' (reverse primer). The amplified product was around 230 base pairs.

Gene Expression Studies: GUS

Construction of JMJ14 :: GUS fusion plasmid: The promoter region for the JMJ14 gene was amplified using polymerase chain reaction using pFU polymeras. Pure genomic DNA from leaves was used. The primers used for amplification were, 5' GCG GCT GCA GAT GAG AAA AAT CTA CAA TAG 3' (forward primer) and 5'CGC AGT GAT CCT TAC AGT GAG ATT AAG TTC AC 3' (reverse primer). The size of the amplified product was 935 base pairs. This promoter sequence was cloned into pCAMBIA1391z plasmid (CAmbia) which has a promoter less version of *gus*A gene. *Pst 1* and *Bam H1* (Promega) restriction sites were incorporated in the 5' end of forward primer and 3' end of reverse primer respectively. The products after digestion were gel purified using QIAGEN gel extraction kit and purified DNA products were ligated using T4 DNA ligase (Invitrogen), following the manufacturer's protocol.

Transformation into *E.coli* Cells

An aliquot of the above ligation mixture was added to the DH10B competent cells of *E.coli* and kept on ice for 30 minutes after mixing gently. After a heat shock at 42^{o}C for 45 seconds LB medium was added to the mixture and the bacteria were grown at 37^{o}C

for 1 hour. The cell mixture was spread on LB/Kanamycin (50 μg/ml) plates. Plasmid isolation was carried out using QIAprep Spin Miniprep Kit (Qaigen) for the colonies obtained after overnight incubation at 37°C. To check for the presence of insert in the recombinant plasmid PCR and digestion reactions were conducted and the products were checked by electrophoresis by 1 % agarose gels.

Thermal Cycle DNA sequencing

The recombinant plasmid obtained was checked for mutations by performing a DNA sequencing using ABI Prism 310 DNA Genetic Analyzer (Perkin Elmer). The recombinant plasmid DNA (200 ng/ul) was first amplified using specific primers for plasmid and the gene. PCR was carried out for 25 cycles using Big Dye terminator ready mix as mentioned in the protocol provided by the manufacturer. The PCR product was precipitated using sodium acetate and ethanol and cleaned using 70 % ethanol. This DNA sample was subjected to denaturation at 95°C for 2 minutes, cooled on ice and then loaded onto an ABI Prism 310 DNA Genetic Analyzer.

Transformation into *Agrobacterium tumefacines* using freeze thaw method

After confirming by DNA sequencing the positive recombinant plasmid was transformed into *Agrobacterium tumefacines*. An aliquot of the plasmid DNA was added to the competent cells of *A. tumefaciens* GV3101 strain and kept on ice for 30 minutes after mixing gently. The cells were frozen using liquid nitrogen for 1 minute and then thawed at 37°C for 3 minutes, 1 ml LB medium was added to the cell mixture and cells were grown at 28°C in dark for 2 hours. The cells were then centrifuged at 8,000 rpm for 1 minute. The resuspended cells were evenly spread on LB/Kanamycin (50 μg/ml)/rifampicin plates. The plates were covered with aluminum foil and placed at 28°C

for 2 to 3 days. Plasmid isolation was carried out using QIAprep Spin Miniprep Kit (Qaigen) for the colonies obtained after overnight incubation at 37^{o}C. To check for the presence of insert in the recombinant plasmid PCR and digestion reactions were carried out and the products were checked by electrophoresis using 1 % agarose gels.

Transformation into *Arabidopsis thaliana*

The positive colonies were grown to an OD600 of 0.6 and cells were pelleted and re dissolved in a freshly prepared solution containing 5 % sucrose and 0.02 % Silwet L-77 to give an OD600 of 0.8-1.0. *Arabidopsis* plants (wild type) were grown to flowering and immature siliques and open flowers were removed by plucking. The unopened flowers were dipped in the above made solution and the whole plant was covered with saran wrap and kept in dark for 24 hours before being transferred to green house. *A.tumefaciens* infection was repeated after 3 to 4 days on new unopened flowers. The transgenic plants were selected using 50 µg/ml Cefotamine and 25 µg/ml Hygromycin antibiotics.

Histochemical GUS Staining

The plants were incubated in 90 % acetone on ice for 30 minutes after harvest and then at room temperature for 20 minutes. They were transferred to cold staining buffer (0.2 % Triton-X 100, 50 mM sodium phosphate buffer, pH 7, 10 mM Potassium ferrocyanide, 10 mM Potassium ferricyanide) and were placed on ice for 5 minutes, to this X-Gluc (5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexyl ammonium salt) was added to a final concentration of 1 mM and was kept on ice for 5 minutes. The samples were infiltrated under vacuum twice for 15 minutes each and transferred to 37^{o}C incubator for overnight incubation. After incubation the samples were put in a solution of

50 % ethanol, 5 % formaldehyde and 10 % acetic acid for 30 minutes to fix the tissue and later transferred to 70 % ethanol to remove the chlorophyll pigments. The GUS images of seedlings were acquired using a Zeiss Stemi SV11 (Apo) light microscope and that of whole plant by a digital (Nikon) camera.

Expression of genes related to flowering and Quantitative Real Time PCR

The total RNA was extracted from 4 week old plants (wild type and mutants) and cDNA was prepared as previously mentioned. RT-PCR was performed using actin primers as conrol. The gene specific primers used for various genes are listed in Table 7.5 in the supplementary data. Quantitative real time PCR was performed on the genes which showed altered expression. Light Cycler 2.0 instrument (Roche) was used for the purpose (Li et al., 2005). Ubiquitin was used as control for the real time PCR.

Leaf Count

Arabidopsis plants were grown as mentioned previously. For long day condition 16hour light photoperiod was maintained and for short day a 10 hour photoperiods was maintained. Leaf count was done at regular intervals to observe the difference at various stages (data not shown). The number leaves at the flowering stage was recorded and p-values were calculated. Figure 3.9 and Tables 7.4, 7.5 (supplementary data) represent the difference in the number of rosette and cauline leaves.

Chromatin immunoprecipitation Assay

Chromatin samples were prepared using rosette leaves from 34 days old plants (wild type and mutants). For DNA and DNA-binding proteins crosslinking 2 grams of rosette leaves were harvested and washed with double distilled water. The washed leaves

were transferred to 37 ml of 1 % formaldehyde and vacuume infilteration was performed at 15-25 ^{o}C for 15 minutes. The crosslinking was stopped using 2.5 M glycine. This tissue was washed and stored at -80 ^{o}C for future purpose. For isolation of chromatin the tissue was grinded made into fine powder using liquid nitrogen and this powder was transferred to 30 ml of extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 0.5% Protease inhibitors (Sigma)). The solution was filtered using Miracloth and then cebtrifuged at 3,000 g for 20 minutes at 4^{o}C. The supernatant was removed and pettet was resuspended in 1 ml of extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 % Triton X-100, 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 0.1% Protease inhibitors (Sigma)). This solution was transferred to eppendorf tubes and centrifuged at 12,000 g for 10 minutes at 4^{o}C. The pellet was resuspened in 300 µl of extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 0.15 % Triton X-100, 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 0.1 % Protease inhibitors (Sigma)). This solution was overlaid on fresh 300 µl extraction buffer 3 in new eppendorf tube. This was centrifuged at 16,000 g for 1 hour at 4^{o}C. The supernatant was discared and pellet was resuspened in 300 µl of nuclei lysis buffer (1 M Tris-HCl, pH 8, 0.5 M EDTA, 20 % SDS, 0.2 M PMSF and 0.1 % Protease inhibitors). The solution was sonicated using Sonic Dismembrator 550 (Fisher Scientific) for 4 times, 10 seconds each time. The solution was then centrifuged at 12,000 g for 10 minutes at 4^{o}C. The supernantat was used for immunoprecipitation. To this supernantat desired antibody was added incubated overnight. The list of antibodies and the primers used are listed in the Table 7.3 in supplementary data. Before this step the solution was precleared using agarose beads and chip dilution buffer (1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8, 167 mM NaCl). After overnight incubation with

antibodies, Agarose beads were added to obtain the bound chromatin. The beads were washed with low salt wash buffer (150 mM NaCl, 0.2 % SDS, 0.5 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8), high salt buffer (500 mM NaCl, 0.2 % SDS, 0.5 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8), Lithium chloride wash buffer (0.25 M LiCl, 0.5 % NP-40, 0.5 % Sodium Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8) and TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The chromatin from the beads was eluted using elution buffer (1 % SDS and 0.1 M $NaHCO_3$) after the washes. To remove the crosslinking the sample were treated with 20 µl of 5 M NaCl and placed in 65^{o}C water bath for 6 hours. To this 10 µl of 10 mM EDTA, 20 µl of 40 mM Tris-HCl, pH 8, 2 µl of 20 µg/500 µl proteinase K was added and incubated at 45^{o}C for 1 hour to degrade the protein. To the DNA extract 520 µl of phenol: chloroform was added, mixed by vortexing for 20 seconds and centrifuged for 5 minutes at room temperature. Top aquous layer was transferred to new tube and the step for repeated two times and then the DNA was washed with chloform to remove traces of phenol. To the DNA solution (aquous layer) 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 µl of glycogen, 10 mg/ml was added and incbaed at 4^{o}C overnight. Then the samples were centrifuged for 30 minutes at room temperature. The DNA pellet obtained was washed with 70 % ethanol and air dried in a hood. To dissolve the pellet 20 µl of sterile distilled water was added and vortexed for 30 minutes (Gendrel et al., 2005). This chromatin was used for PCR reaction. The PCR program starting with 3 minutes at 94^{o}C, followed by 32 cycles of 94^{o}C (20s), 55^{o}C (varying depending on primer Tm) (30s), and 72^{o}C (1 min) and then kept at 16^{o}C. Actin was used as control to adjust the levels of chromatin to be used between the samples. The list of primers and antibodies used of the reaction are provided in the supplementary data (Table 7.4).

Recombinant protein expression and Activity Analysis

The JMJ14 gene was amplified from U24778 construct and cloned into pET28a vector (Novagen) to get a recombinant plasmid. This plasmid was first transformed into *E.coli* DH10-B and then into BL21 cells. The protein expression was induced by adding IPTG (0.8 mM,1 mM &1.2 mM) when OD600 of the cells reached 0.8. Controls were collected before adding IPTG and then 1 ml samples were collected every 1 hour after IPGT induction till 5 hours after protein induction.

The recombinant plasmid was then transformed into yeast and protein was induced using 100 % methanol. Medium used for growing yeast contained 1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0 1.34 % YNB 4 x 10-5% biotin, 1 % glycerol or 0.5 % methanol. The protein was induced using 100 % methanol; methanol was added every 24 hours. The sample was collected at a gap 6, 12, 24, 36, 48, 60, 72 and 96 hours after addition of methanol to see the induction of protein.

Transgenic HA :: JMJ14 plants (in which the JMJ14 gene is over expressed) were produced in the lab.Recombinant vector was constructed using pENTR™ 4 vector provided in the Gateway™ Cloning Technology Kit. The coding sequence of JMJ14 gene was amplified using WT cDNA, the amplified PCR product and the vector were double digested using *Sal I* and *Xho I* and later ligated to give a recombinant plasmid (entry vector). Primers used for amplification of coding sequence are 5' ATT AAG TCG ACA TGG ATC AGC TTG CAT CA TAG 3', forward primer, 5' AAG GCT CGA GTT AAG GAC TTA TCT CCA TCT TAT C 3', backward primer. The size of the amplified product is 2886 base pairs.

A 35S-HA tag containing Gateway-OCS vector, CD3-687 was used as destination vector. Using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen) the recombinant

vector for *Agrobacterium* mediated plant transformation was obtained. This plasmid after confirming the presence of insert via DNA sequence results was grown in *A. tumefaciens* and the plants were infected with this bacterium for t-DNA insertion. The plants with the insertion were checked using 0.2 % BASTA (glufosinate ammonium).

These transgenic plants encode the protein with a HA tag. The levels of expression of the transgene was checked using western blot (anti HA antibody) and protein purification was carried out using affinity chromatography technique. The nuclei from the leaves of the transgenic plants were isolated by using the same procedure as ChIP (till sonication). The extract obtained was centrifuged at 15,000 g for 30 minutes at 4^{o}C. 200 µl of resuspended anti-HA affinity matrix was added into each eppendorf tube containing 1ml of cold buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P40, Protease inhibitor cocktail). Pellet matrix at full speed in a microcentrifuge for 10 seconds to wash the matrix. Now the lysate was added to the matrix and incubated for 2 hours at 4 ^{o}C with shaking. The matrix containging bound protein was obtained after centrifuging for 10 seconds and was washed twice with cold buffer A. To obtain the bound protein 100 µl of elution buffer (20 mM Tris-HCl, 100 mM NaCl) was added to the matrix and gently shaked for 15 minutes at room temperature. This solution was centrifuged for 30 seconds and the supernatant was used for activity analysis after concentrating the eluant using Amicon Ultra-0.5 Fliter Device (Millipore).

Histone demethyltarnsferase Assay

The in vitro demethylase reaction was carried out in demethylase buffer containing 20 mM Tris-HCl pH 8, 150 mM NaCl, 50 µM $(NH_4)_2Fe(SO_4)_2.6H_2O$, 1 mM alpha ketogluatarate acid sodium salt and 2 mM L-Ascorbic acid. To this solution 2 µg of

calf thymus type II-A histione was added along with the purified protein. The control reaction had the demethylase buffer and 2 µg histone and no protein was added. Both the samples were incubated at 37^{o}C for 4 hours. The reaction was stopped by adding 5 X SDS sample buffer and 1 mM EDTA and boiling it for 5 minutes in water bath. The protein was analysed on 5 – 15 % gradient SDS gel.

SDS-PAGE analysis of Recombinant Protein

Polyacrylamide gel electrophoresis in the presence of SDS (sodium dodecyl sulfate) was performed to analyse recombinant proteins obtained from bacteria, yeast and plant. A 10 % (w/v) separating Polyacrylamide gel overlaid with a 4 % (w/v) stacking Polyacrylamide gel was used of this purpose. The resolving gel was prepared by mixing 16 ml of double distilled water, 3.75 ml of 3 M Tris-HCl (pH 8.8), 75 µl of 20 % (w/v) SDS, 10 ml of 30 % N',N'- bis-methylene-acrylamide, 150 µl of freshly prepared 10 % (w/v) ammonium sulphate and 15 µl of TEMED. The solution was mixed thoroughly and poured in between the preassembled gel sandwich sets and was allowed to polymerize for 1 hour at RT. The gel was overlaid with water before polymerization. The water was decanted and 4 % stacking gel solution containing 6.15 ml of double distilled water, 2.5 ml of 0.5 M Tris-HCl (pH 6.8), 50 µl of 20 % (w/v) SDS, 1.34 ml of 30 % N', N'- bis-methylene-acrylamide, 50 µl of freshly prepared 10 % (w/v) ammonium sulphate and 10 µl of TEMED was gently applied on the polymerized resolving gel and a comb was inserted into the stacking gel region without disturbing the resolving gel. It was allowed to polymerize for 1 hour after which the comb was removed and the wells were washed with water.

Protein samples were prepared by adding 5 X sample buffer (50 mM Tris-HCl, pH 7.5, 10 % SDS, 20 % Ficoll type 400, bromophenol blue, 50 mM DTT) to a final concentration of 2 X and denatured by keeping them in boiling water for 5 minutes. The protein samples were loaded into the wells and electrophoresed at 30 mA till the dye reached the bottom of the gel. 1 X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS pH 8.3) was used to run the gel. After electrophoresis the gel was placed in staining solution containing 0.2 % Coomassie blue in 40 % methanol and 10 % acetic acid for 3 hours at room temperature. The gel was distained with 20 % methanol & 10 % acetic acid solution and the bands were analyzed.

Western blot

The proteins separated using SDS-PAGE were transferred on to a PVDF (poly vinylidene difluoride) membrane. The PVDF membrane was first equilibrated using 100 % methanol and then in transfer buffer (25 mM Tris , 192 mM glycine, 20 % methanol pH 8.3) for 15 minutes. Filter paper (whatman 3mm), PVDF membrane and gel were trimmed to the same size and bubbles were avoided when preparing the sandwich of filter paper, membrane and gel. The filter papers (8 layers on each side) were first soaked in transfer buffer and assembled with membrane facing the anode. The proteins were transferred on to the membrane using overnight transfer at 28 Volts. The membrane after transfer was blocked using 5 % BSA solution for 2 hours at room temperature before performing antibody washes.

Results

Phenotype Analysis

To verify the proper T-DNA insertion in the mutant lines, the total RNA was extracted from the leaves of mutants and WT plants and RT-PCR was performed. Both the mutant lines did not show traces of JMJ14 transcript (Figure 3.3A). This JMJ14 loss of function mutant lines when grown under 14 hours photo period showed a stable early flowering phenotype for more than three generations. It was observed that *jmj14-1* started flowering on the 12th day and *jmj14- 2* started flowering on the 14th day where as the WT plants started flowering on the 18th day after transferring to soil (Figure 3.2).

Gene Expression Studies using RT PCR

To investigate the expression of JMJ14 gene in the mutant lines gene expression was checked using RT PCR. It was observed that the *jmj14-1* and *jmj14-2* did not transcribe JMJ14 gene (Figure 3.3 A). To study the localization of gene, the total RNA was extracted form various tissues of WT plants (as mentioned in materials and methods) and RT PCR was performed using gene specific primers for JMJ14. It was observed that the gene was expressed to a greater extent in the reproductive organs like flowers and siliques along with stem but in fewer amounts concentrated in vegetative organs like leaf and root (Figure.3.3 B).

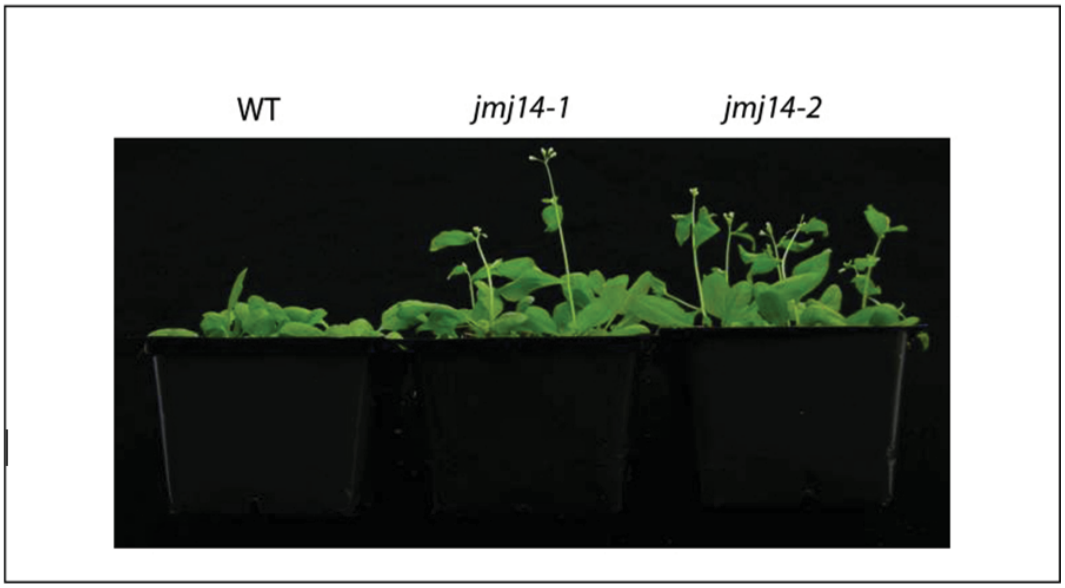


Figure 3.2 Early Flowering Phenotype of *jmj14* mutants.

The seeds were germinated on 0.5x MS media supplemented with 1% sucrose and 1% agar. The seedlings were transferred to soil and grown in green house. The plants showed an early flowering phenotype when grown under 14 hour light period and it was noticed that the mutants *jmj14-1* and *jmj14-2* started flowering on the14th and 12th day respectively when compared to the Wild type plants which started flowering on the 18th day.

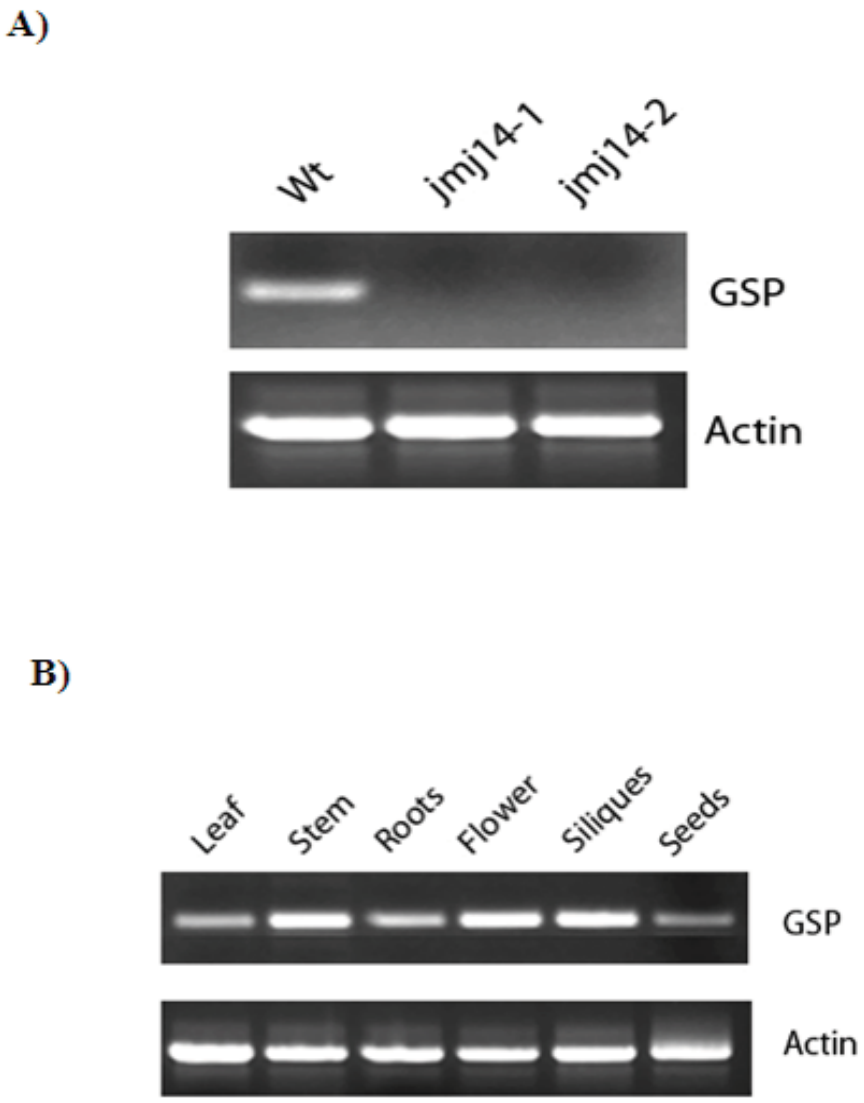


Figure 3.3 Gene Expression Studies in *jmj14* Mutants.

1% agarose gel along with ethidium bromide was used for electrophoresis to observe the bands; GSP indicates gene specific primers for JMJ14. Actin was used as a control to adjust the cDNA input. A) RT-PCR analysis of gene expression in rosette leaves of WT (wild-type), *jmj14-1* and *jmj14-2* mutant plants, mutant plants do not contain traces of detectable JMJ14 gene. B) RT-PCR analysis of gene expression of the JMJ14 gene in wild type plants. The gene expression was studied in Rosette leaves, stem, roots, flower, siliques and seeds using gene specific primers (GSP) for the JMJ14.

GUS reporter aided localization of JMJ14 Gene

To study and investigate the promoter activities of the JMJ14 gene, the 5' flanking region of JMJ14 gene in *Arabidopsis* was amplified from genomic DNA and cloned used recombinant DNA technology. The promoter size of the JMJ14 gene is 914bp. This promoter sequence was fused to the commonly used *E.coli* reporter gene *gusA (uidA)* which encodes beta-Glucuronidase (GUS) reporter enzyme. This gene was introduced into the *Arabidopsis* plant via *A. tumefaciens* mediated plant transformation. The plants were screened using Hygromycin and resistant seedlings were grown for two generations to obtain homozygous lines. Histochemical staining for GUS activity was performed on seedlings of different age, mature plants in vegetative and reproductive phase and floral organs. The blue color observed in the tissues was the result of active GUS expression. Figure 3.4 shows the expression of JMJ14 transgene in various organs of a plant. GUS expression was detected in the stalk, inflorescence stem and young siliques (C, D in the Figure 3.4). The flowers also showed the expression of GUS transgene. Transgenic plants showed stronger promoter activities in reproductive parts in comparison with the vegetative tissues. When seedlings of 5, 7 and 10 days old were subjected to histochemical GUS staining, the GUS expression was detected both in shoot and roots. Histochemical GUS staining was performed on plants before and after bolting. It was observed that as the age of the plant increased, the GUS expression was more concentrated in the stem and that continued till the inflorescence stalk and the color in the leaves in mature plants reduced when compared to the small seedlings. Figure 3.5 shows the histochemical GUS staining in a whole plant just after the start of bolting (A) and seedlings at various ages (B, C, &D).

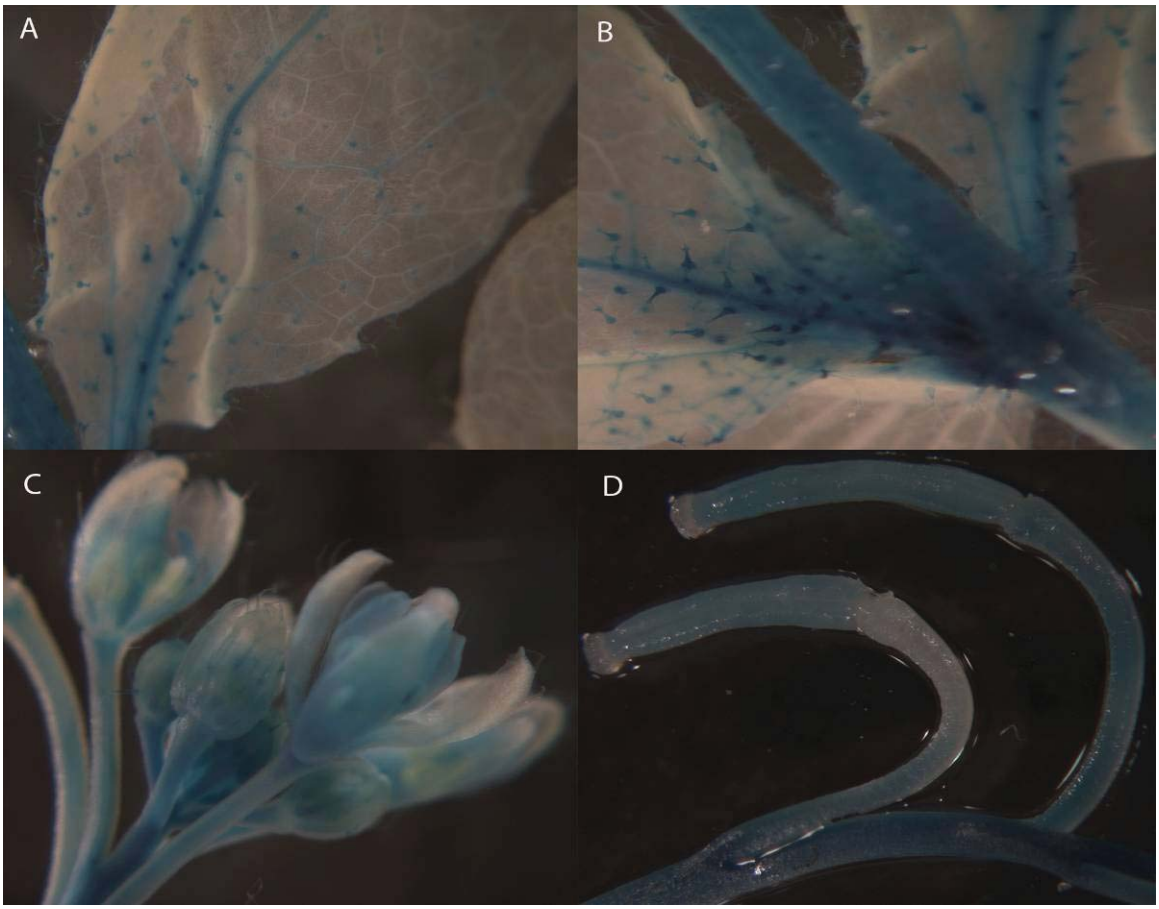


Figure 3.4 Histochemical GUS Staining.

Five week old transgenic GUS::JMJ14 plants were used for this experiment. Images were acquired using Zeiss Stemi SV11 (Apo) light microscope. In consistence with the RT PCR data the gene was expressed more in the reproductive parts (flower, siliques, and stem) and less in the vegetative parts. A) Leaf B) Stem C) Flower D) Silique. All the parts were stained at the same time and a WT control was also used (Data Not Shown)

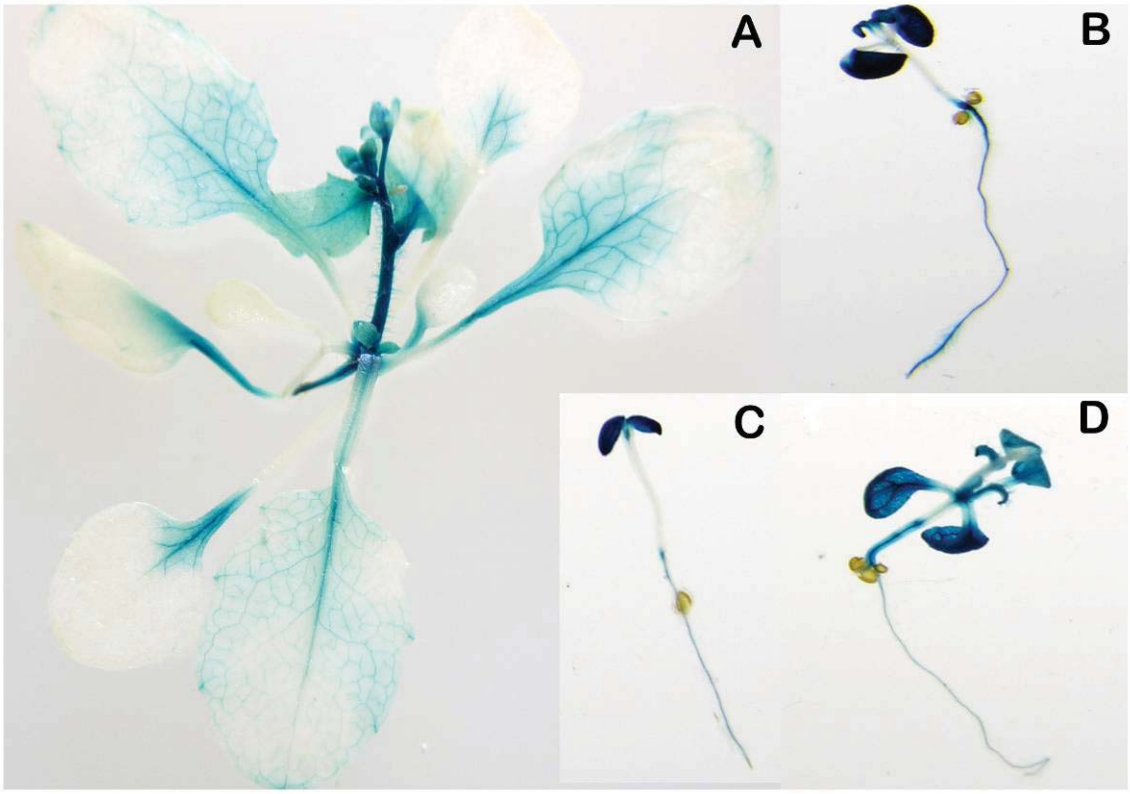


Figure 3.5 JMJ14 Expression in Transgenic GUS :: JMJ14 plants.

Pictures were captured using a Nikon Digital Camera. Pictures show recombinant transgene in transgenic plants form seeding stage to flowering plant. At least four independent transgenic lines were used to obtain the GUS staining data. A) 22 day old plant B) 7 day old seedling at 4 leaf stage C) 5 days old seedling at 2 leaf stage D) 10 days old seedling at 6 leaf stage.

Expression of genes related to flowering in *jmj14* mutants

The early flowering phenotype of the JMJ14 mutants lead us to investigate the expression of few genes that regulate flowering in *A.thaliana* in the mutants. For this purpose total RNA was extracted from 4 week old *jmj14-1* and *jmj14-2* and WT plants and RT PCR was performed using primers specific for the flowering genes. The expression levels of FCA (Flowering Time gene) (Page et al., 1999) , FLC (Flowering Locus C) (Ratcliffe et al.,2001), FLD (Flowering Locus D), FLK (Flowering locus K) (Lim et al 2004), FVE (Kim et al.,2004), FY, FPA, FT (Flowering Locus T), GI

(GIGANTIA), LD (LUMINIDEPENDENS) (Aukerman et al., 1999), SoC (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1) (Moon et al, 2003) were studied. As shown in Figure 3.6 both *jmj14-1* and *jmj14-2* plants showed elevated levels of FT transcript. All the other genes examined did not show much difference in expression. Quantitative real time RT-PCR also showed several fold increase of FT gene in *jmj14-1* and *jmj14-2* mutants (Figure 3.7).

Leaf Count in *jmj14* mutants

When the *jmj14-1* and *jmj14-2* plants started flowering it was observed that the production of rosette leaves decreased and cauline leaves were produced on the stems in both the mutants. As the bolting started early in the *jmj14* mutants the cauline leaves also appeared early. The number of rosette leaves did not increase much indicating that the vegetative growth of the plant reduced as the plants entered into the reproductive stage. The number of rosette leaves in the jmj14 mutant plants after bolting was less compared to the wild type plants and the number of cauline leaves was more in the *jmj14* mutants when compared to the WT plants. Figure 3.9 shows the graphs which clearly indicate the difference in the rosette and cauline leaves when plants were grown in both long day and short day conditions (Tables 7.1 and 7.2 in supplementary data show the recorded leaf count data)

Chromatin Immunoprecipitation

In order to gain insight about the methylation states of lysine residues on H3 at the FT loci we used a chromatin immuno precipitation (ChIP) assay. As shown in Figure 3.12, 3.13, the results indicated that there was indeed a change in the methylation in the *jmj14* mutants on H3K36 in di and tri methylation states. The methylation was not

affected on H3K9 at the FT loci. On H3K36, in the mutants in whom the JMJ14 gene is not transcribed it was observed that the methylation increased in di and tri methylated states at the FT loci but the mono methylated state was not affected.

Activity Assay using recombinant proteins

An attempt was made to produce the recombinant JMJ14 protein in bacteria using pET28a vector. IPTG was added to induce the protein production and the protein was analyzed on an SDS gel (Figure 3.14). The protein production was induced in bacteria but it was observed that as the time increased the bacterial cell number decreased, indicating that the recombinant protein was hindering the growth of bacteria. Thus yeast expression system was used to produce the recombinant protein and protein production was induced by adding 100 % methanol. The recombinant protein in yeast was also not produced in sufficient amounts to perform the in vitro demethylase activity assay. Using transgenic plants which had HA :: JMJ14 gene, we purified the protein and used for activity analysis. A slight change was observed at K36Me2 and K36Me3 was observed (data not shown).

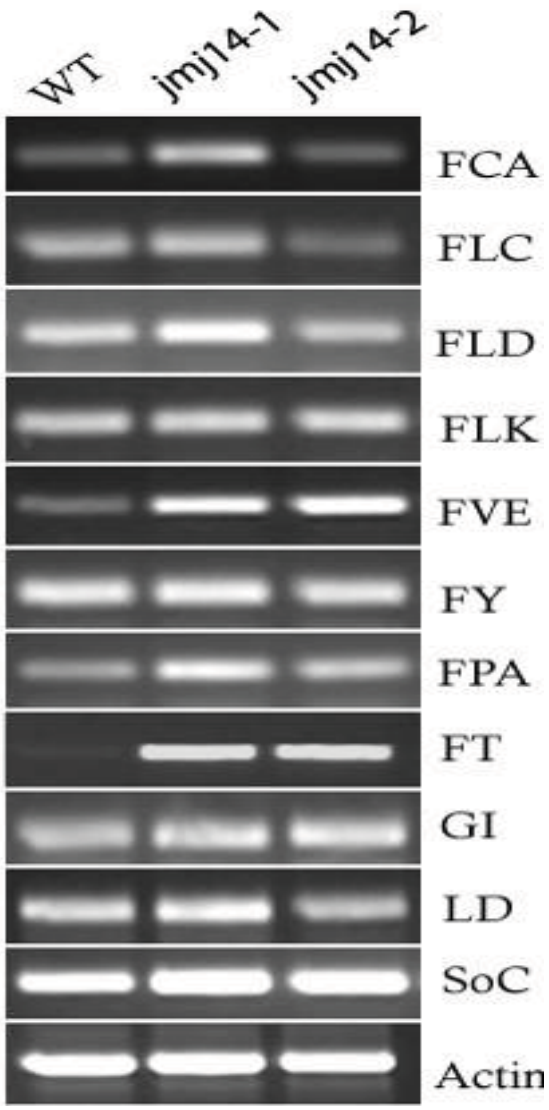


Figure 3.6 Effect of JMJ14 mutation on the expression of genes that regulate flowering in *A. thaliana*. RT-PCR analysis of gene expression in rosette leaves of WT, *jmj14-1* and *jmj14-2* plants. Actin was used as a control to adjust cDNA input.

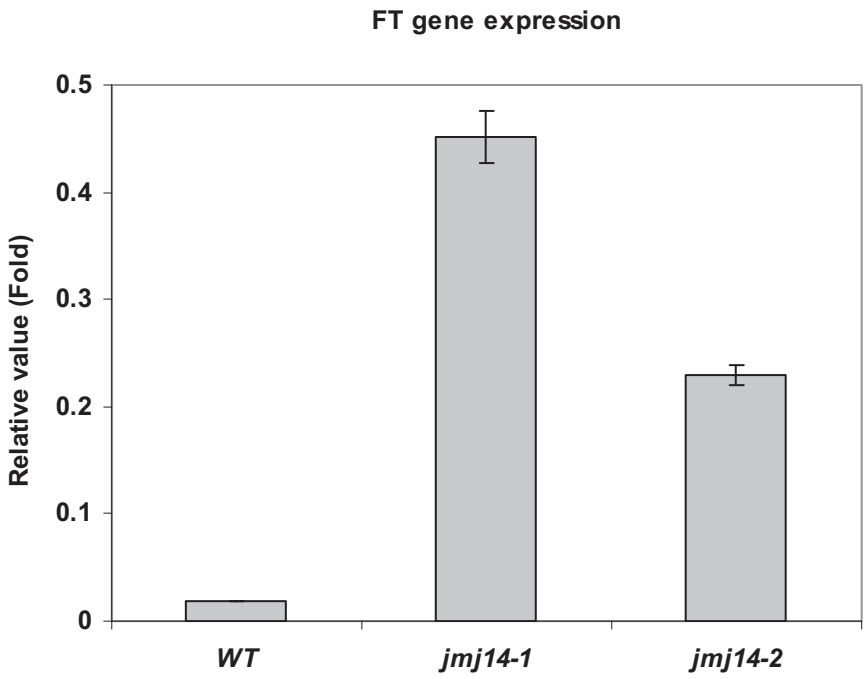


Figure 3.7 Real time PCR quantification of FT transcript in WT, *jmj14-1* and *jmj14-2* plants. Many fold increase was observed in *jmj14-1* and *jmj14-2.*

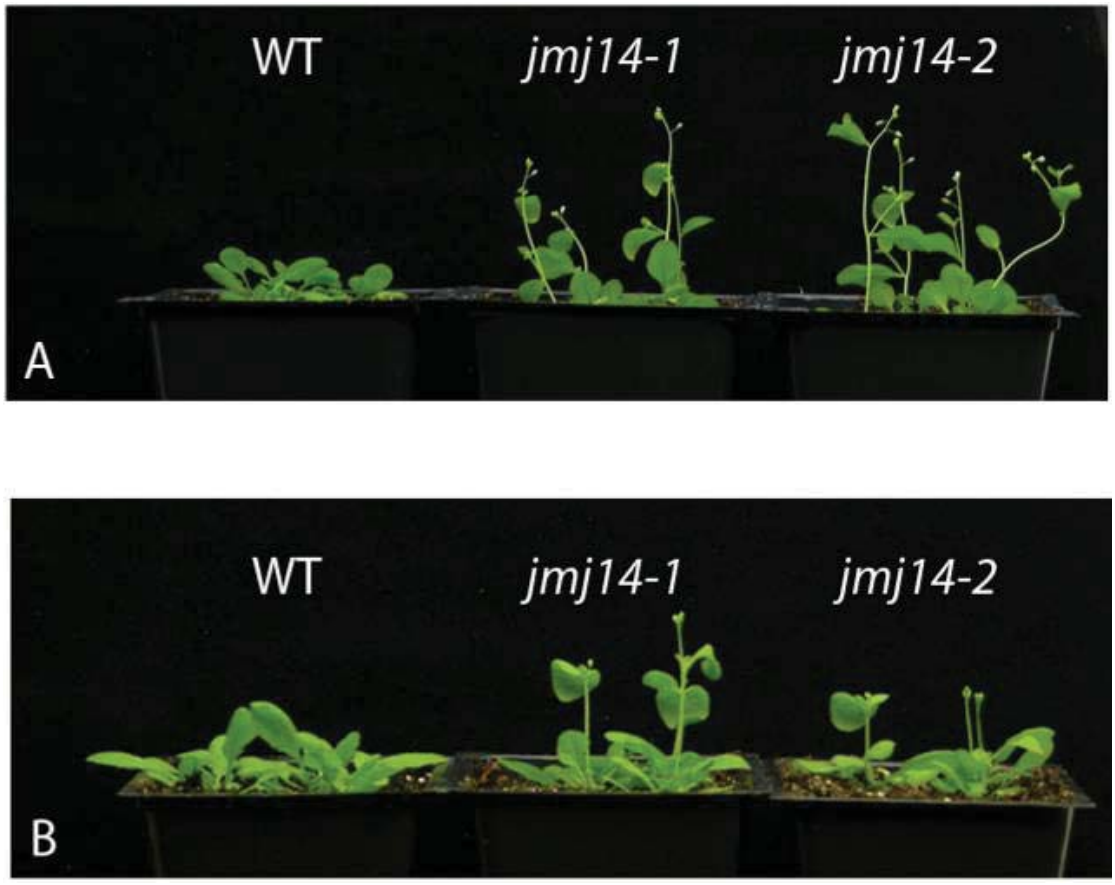
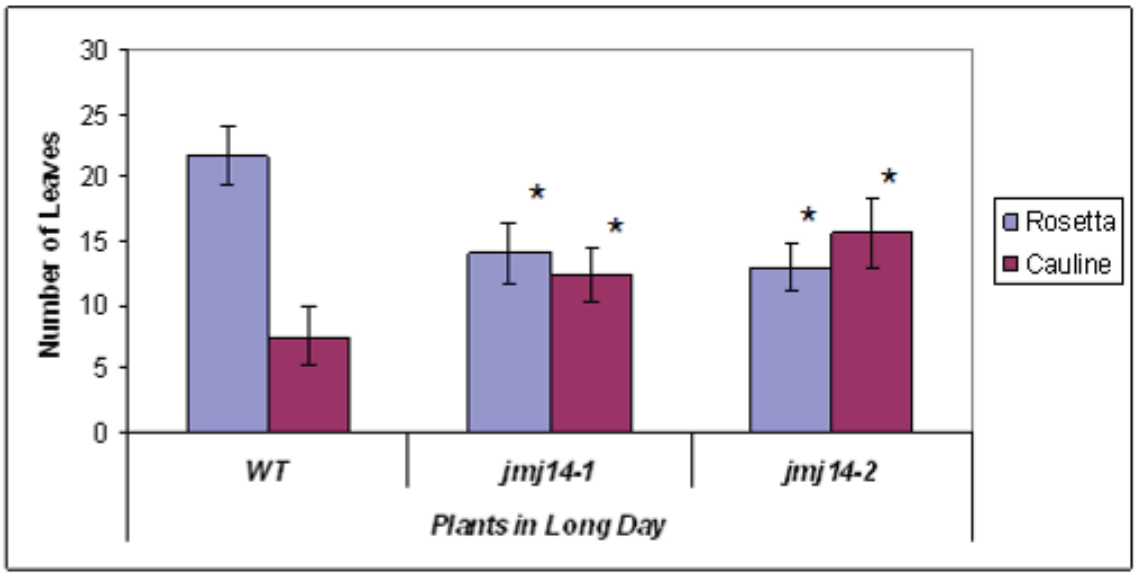


Figure 3.8 Early flowering phenotype in long and short day conditions.

A) Plants grown under long day (16 hour photoperiod) conditions. B) Plants grown in short day conditions (10 hour photoperiod).

A)



B)

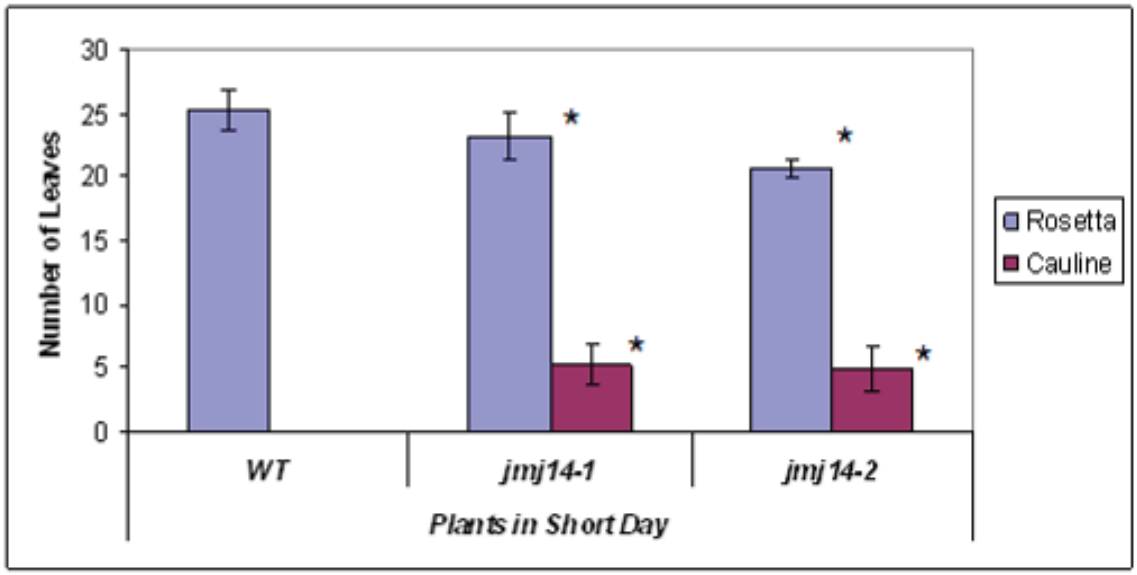


Figure 3.9 Graphs showing number of Rosette and Cauline leaves in *jmj14* mutants.

The average number of Rosette and Cauline leaves during flowering in the WT, *jmj14-1* and *jmj14-2* plants. A) In long day conditions (16 hours of photo period) B) In short day conditions (10 hours photo period). The number of rosette leaves is less in mutant plants as the bolting starts earlier and the number of cauline leaves increases with bolting. All the data are presented as means with standard deviation (n = 15-18; $P < 0.05$ using t-test).* Indicates that the data is highly significant within the group.

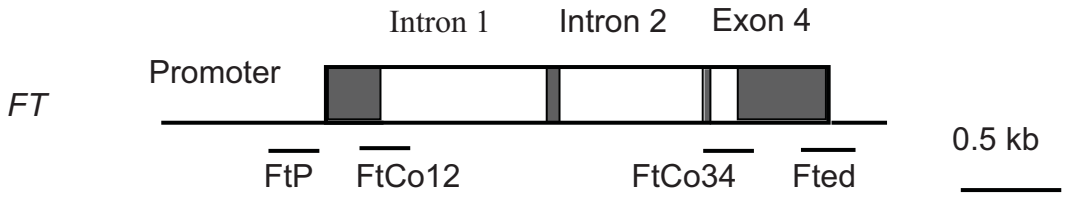


Figure 3.10 Schematic structures of the FT gene.

The filled and open boxes represent exons and introns, respectively. FtP, FtCo12, FtCo34 and Fted represent the regions examined by chromatin.

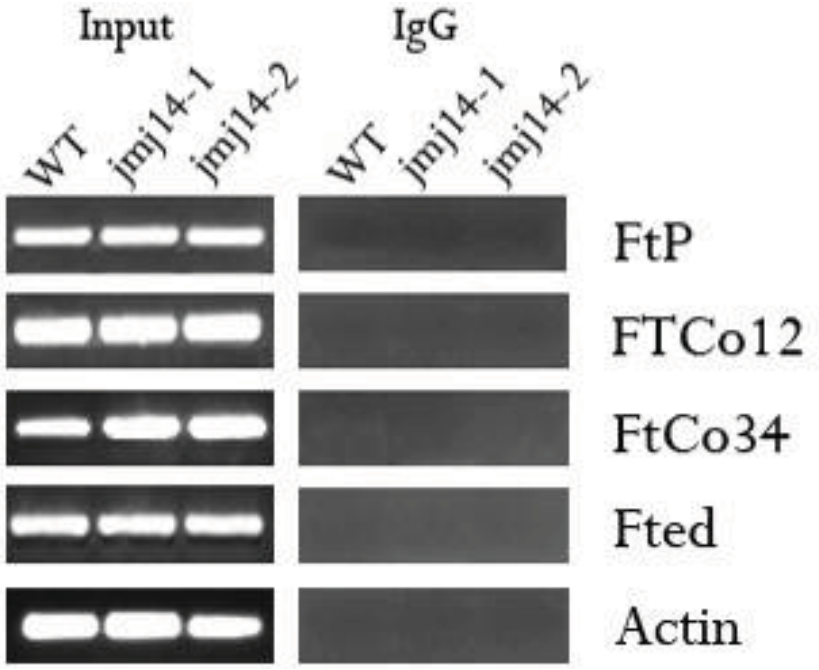


Figure 3.11 Control reactions for ChIP.

Chromatin from WT and *jmj14* mutants was immuno precipitated with methylation state specific antibodies. Immuno precipitated DNA was analyzed by PCR. Chromatin isolated before immunoprecipitation (input) served as positive control. Chromatin precipitated with normal immunoglobulin G (IgG) served as negative control. Actin was used as an internal control. The results were reproducible in two independent experiments.

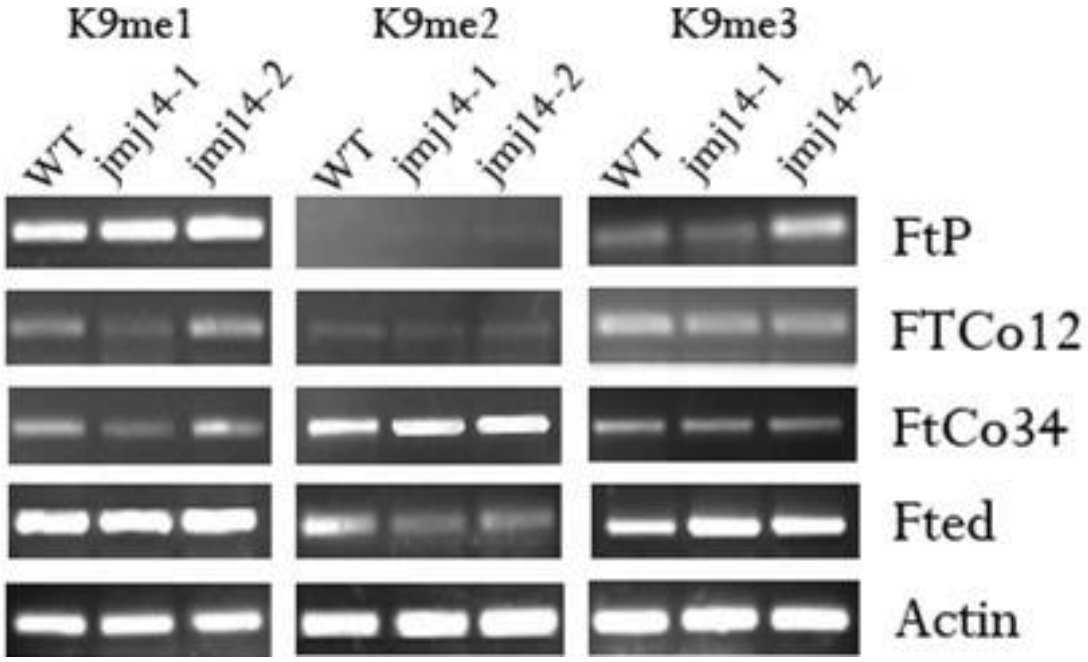


Figure 3.12 ChIP reaction for lysine 9 residue on histone H3.

Chromatin from WT and *jmj14* mutants was immuno precipitated with methylation state specific antibodies for lysines 9 reside on histone H3. Actin was used as an internal control. The results were reproducible in two independent experiments.

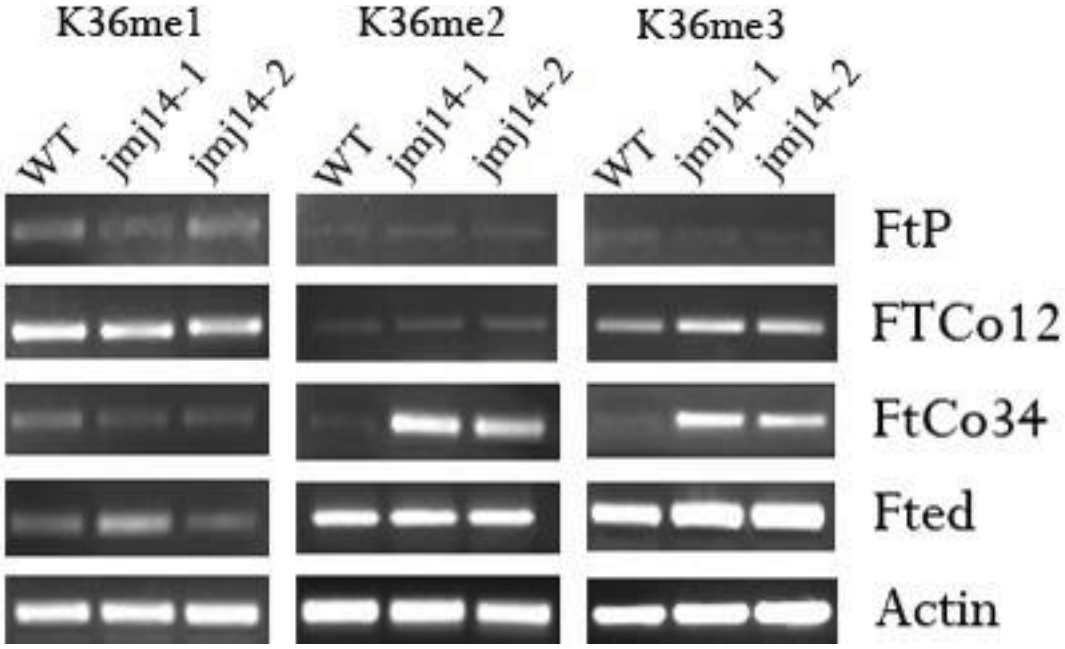


Figure 3.13 ChIP reaction for lysine 36 residue on histone H3.

Chromatin from WT and *jmj14* mutants was immuno precipitated with methylation state specific antibodies for lysine 36 residue on histone H3. Actin was used as an internal control. The results were reproducible in two independent experiments

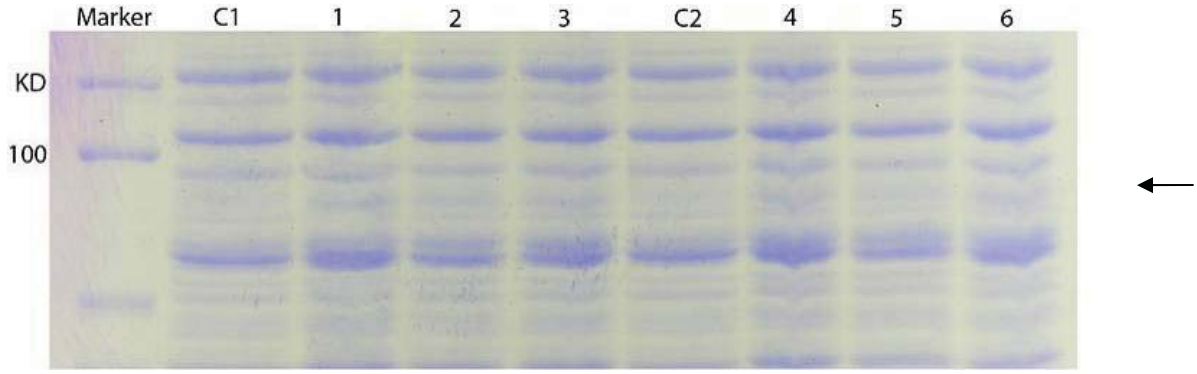


Figure 3.14 Analysis of protein using SDS-PAGE after IPTG induction.

C1 and C2 indicate controls for IPTG induction before addition of IPTG. For samples 1 and 4 the IPTG concentration used was 0.8mM, for sample 2 and 5 the IPTG concentration used was 1mM and for 3 and 6 the IPTG concentration used was 1.2mM. Sample 1- 3 were obtained after 3 hours of IPTG induction and samples 4-6 were obtained after 4 hours of IPTG induction. The induced protein was observed at around 90kd shown by the arrow mark.

Discussion

Flowering and reproduction are important events in plant's life cycle. Research on plants ultimately leads to increase in plant productivity by reducing cost of production so that it could benefit the agriculture industry. Hence studies on mutations which in general increase the production of plants or reduce the cost of production are always important. Early flowering phenotype of *jmj14* mutants fall under such category, which if well studied could be applied to commercial crops.

The mutant lines do not show any traces of JMJ14 transcript indicating that the T-DNA insertion in *jmj14-1* and *jmj14-2* completely knocked out the expression of JMJ14, suggesting both *jmj14-1* and *jmj14-2* are loss-of-function mutants of the JMJ14 gene. As the putative histone demethylase gene is disrupted by the T-DNA, a protein pertaining to this gene is not transcribed in the knocked out mutants. These *jmj14* mutant plants showed an early flowering phenotype, where in *jmj14-1* started flowering on the 14th day and *jmj14*-2 started flowering on the 12th day when compared to the wild type plants which started flowering on the 18th day after transferring to the soil.

After the transition from vegetative stage to reproductive stage the main focus of the plant is to reproduce and proliferate, thus the vegetative growth subsides and the floral organs flourish. For this transition to take place not only changes take place internally (gene activation) but also external factors like light also play an important role by triggering reactions in the plant. By visual inspection the mutants, *jmj14-1* and *jmj14-2* showed an early flowering phenotype, this was even confirmed by counting the number rosette and cauline leaves after bolting in the mutants and the WT plants. It was observed

that in the *jmj14* mutant plants the number of rosette leaves did not increase much after bolting. As the mutants showed an early flowering phenotype the number of cauline leaves was more in the mutants when compared to the wild type plants.

To study the localization of the gene in the wild type plants where the gene is intact and transcribed normally, RT-PCR was used. The RNA was extracted from various tissues like leaves, stem, roots, flower and siliques and the levels of JMJ14 transcript was examined in various tissues. It was observed that expression of the gene was concentrated more in the reproductive organs like flower and siliques and stem and less concentrated in the vegetative organs like leaves and roots. To study the promoter activities of the gene transgenic plants with JMJ14 :: GUS were produced and the localization of GUS expression was histochemically observed. The histochemical GUS assay in *Arabidopsis* tissues clearly shows that the JMJ14 gene is active in young leaves (at the seedling stage) and in the flower tissue. The gene expression is dramatically reduced in the mature leaves. Though the gene expression appeared to be reduced in the in mature leaves high levels of expression were observed in the leaf stalk, stem and inflorescence stalk along with flower and siliques. It could be concluded that the JMJ14 gene is active during the reproductive phase and might not play an important role when the plant is in its vegetative phase.

Previous studies show that when a plant gets ready for bolting there is a two fold increase in the expression of more than 100 genes in the shoot apex. As the *jmj14* mutants were showing an early flowering phenotype we investigated the expression of a few genes which either control flowering or are involved in pathways that control flowering. Using RT-PCR we noticed that the levels of Flowering Locus T (FT) transcript were more in both the mutant lines and this was confirmed using real time

quantitative PCR. All the other genes tested did not show any difference in levels of expression in the mutant and wild type plants. Quantitative real time PCR data indicated an increase in FTgene in both the mutants. The early flowering phenotype appears to be due to over expression of the FT gene in the *jmj14* mutants.

The FT gene is responsible for the mobile signal which necessary for the initiation of inflorescence. It was observed that this signal was produced in the leaf and was transported to the shoot apex via phloem to initiate flowering (Zeevaart et al., 2007 Corbesier et al., 2007 Jaeger and Wigge et al., 2006, Notaguchi et al., 2008, Schwartz et al., 2009). The gene expression studies for JMJ14 and the histochemical GUS assay indicate that the putative histone demethylase gene is expressed in the reproductive organs and stem along with inflorescence stalk indicating the link between the FT and JMJ14 genes.

The ability of *jmj14-1* and *jmj14-2* to flower early indicated that JMJ4 could directly or indirectly interact with the FT loci to modify the chromatin state via histone demethylation. We precluded this as the FT gene was over expressed in the *jmj14* mutants. We investigated this possibility using chromatin immunoprecipitation. Methylation site specific antibodies for lysine 9 and lysine 36 on H3 were used for the purpose. We found that in the coding region of FT gene the methylation (Me2 and Me3) was enriched on lysine 36 of H3 in *jmj14-1* and *jmj14-2* but this was not observed in the wild type plants. This could be due to the absence of the putative histone demethylase gene in the mutant lines linking the action of JMJ14 gene for the expression of FT gene. The altered expression of FT could be due to increased methylation at K36 (Me2 and Me3) on H3.

Studies on this domain in humans have shown that it is a site specific protein and it acts on the di methylated state of H3K36 indicating that the protein is an active histone demethylating protein. In case of Humans the JmjC domain containing proteins acts in the presence of alpha keto-glutarate and iron (II) to release succinate and formaldehyde, it would be interesting to see if the putative histone demethylase from *Arabidopsis thaliana* also requires the presence of same cofactors or if it acts better in the presence of different cofactors. We can use mass spectrometry to reveal this data using the recombinant protein.

When the JMJ14 gene was over expressed in the WT plants, the phenotype was restored, that is the plants flowered normally clearly showing that JMJ14 plays an important role in controlling the flowering time in *Arabidopsis*. To study if there was any change globally in the methylation states of the histone, the histone extracts from the mutant and WT were analyzed using SDS-PAGE and western blots. The difference in the methylation states were checked using methylation state specific antibodies. It was observed that at global level H3K9Me2 was affected. In summary we demonstrate that the JMJ14 gene directly regulates the flowering time in *Arabidopsis thaliana* by controlling the expression of FT via H3K36 demethylation.

CHAPTER IV

ROLE OF THE SDG8 METHYLTRANSFERASE IN ABSCISIC ACID RESPONSE IN *ARABIDOPSIS THALIANA*

Introduction

Histones, the basic proteins (rich in lysine and arginine) to which DNA binds are subjected to a variety of modifications like serine and threonine phosphorylation, lysine ubiquitination, acetylation and sumoylation, and lysine , arginine methylation and demethylation (Vasquero et al., 2003). Among these modifications, histone methylation has been shown to be critical in regulating gene expression. In higher organisms, histone H3 can be methylated at lysines 4, 9, 27, 36, and 79 and histone H4 on lysine 20. Modified lysine residues can exist in a mono-, di-, or tri-methylated state. When lysine and arginine residues are methylated, they increase the hydrophobicity and also the steric bulk, which disturbs the interaction between histones and DNA and leads to altered gene expression.

Histone methyltransferases include, histone-lysine N-methyltransferase and histone-arginine N-methyltransferase, which catalyzes the transfer of one to three methyl groups from the cofactor S-Adenosylmethionine to lysine and arginine residues of histone proteins. They contain a SET (**S**u (var), **E**nhancer of Zeste, and **T**rithorax) domain and are highly specific for the amino acid residue and the state of modification (except for lysine 79 methyl transferases which belong to DOT1 family). Sdg8 is a SET

domain containing group 8 methyltransferase (Cazzonelli et al., 2009, Cazzonelli et al., 2010)

Flowering locus C (FLC) is an important transcriptional repressor which controls the flowering time in *Arabidopsis thaliana.* The loss of function mutants of *sdg8* showed an early flowering phenotype due to repression of FLC (Zhao et al., 2005). It was observed that in these mutants H3K36Me2 was reduced in the promoter and coding regions of FLC indicating the role of SDG8 in controlling the flowering time by methylation (Zhao et al., 2005). An increased shoot branching phenotype was also observed in the *sdg8* mutants (Dong et al., 2008). Expression of SPS/BUS (Supershoot/ Bushy) and UGT74E2 (UDP-glycosyltransferase 74E2) both genes that regulate shoot branching in *Arabidopsis* was found to be altered. Knockout mutations of the SDG8 gene markedly reduce the global levels of histone H3 trimethylation at lysines 9 and 36 as well as dimethylation at lysine 36. The *sdg8* mutants produce more shoot branches than wild type plants. At SPS locus the H3K9 methylation was unaltered but H3K36 di and tri methylation at the promoter region were reduced. In case of UGT74E2, H3K36Me2, H3K36Me3 and H3K9Me3 were reduced in the *sdg8* plants. These results indicated that SDG8 played an important role in controlling the methylation at lysine 9 and lysine 36 residues of histone H3.

As the shoot phenotype was altered in these mutants, we tried to check if there were any abnormalities in the root phenotype of the sdg8 mutants. In the presence of ABA a plant hormone the root size of the mutants reduced drastically. ABA plays a significant role in regulating developmental and physiological processes in plants. ABA along with calcium dependent protein kinases and phosphotases play an important role in stomatal closure. During stress conditions like drought, ABA response genes help in

accumulation of proline which in turn increases dehydration tolerance. The *sdg8* mutant lines showed an increase in the expression of ABR1 gene. ABR1 is a gene involved in root response to ABA and it encodes an APETALA2 (AP2) domain-containing transcription factor (Pandey et al., 2004). This gene appears to be a negative regulator of ABA, indicating that the presence of this gene will not have no effect on the plants when treated with ABA. The *abr1* mutants show hyper sensitivity towards ABA and other stress conditions.

Materials and Methods

Sdg8 Mutant lines and Growth conditions

Two T-DNA insertion lines for SDG8 (SALK_014569 and SALK_026442) were obtained from the SALK collection at the *Arabidopsis* Biological Resource Center. These mutant lines have been characterized previously (Dong et al., 2008). These mutant seeds were sterilize days mentioned in the previous chapter. The plants which had similar root length were transferred to Petri dishes (with 0.5 X MS, 1 % sucrose and 1 % Agar) containing different concentration of ABA. ABA of 5 μM, 10 μM, and 20 μM final concentrations were used, plate without ABA was used as control. The plants were grown for 7 days till the difference in the root length was evident. The knocked out lines for ABR1 gene was obtained from TAIR. These *abr1* mutant plants were treated the same way as the roots of the *sdg8* mutants and WT plants mentioned above.

WT ***sdg8-4*** ***sdg8-2***

Figure 4.1 Increased shoot branching phenotype of *sdg8* mutants.

WT, *sdg8-2*, and *sdg8-4* plants were grown under a 14 hour light photoperiod for 50 days.
(Source: Dong et al., 2008).

Production of transgenic *Arabidopsis* plants which over express HA-ABR1

Recombinant vector was constructed using pENTR™ 4 vector provided in the Gateway™ Cloning Technology Kit. The coding sequence of ABR1 gene was amplified using WT cDNA, the amplified PCR product and the vector were double digested using *Kpn I* and *Xho I* and later ligated to give a recombinant plasmid (entry vector). Primers used for amplification of ABR1 coding sequence are 5'CCC CGG TAC CGA ATG TGT GTC TTA AAA GTG GC3', forward primer with *Kpn I* digestion site and 5'CCG GCT CGA GTC AGG AGG ATG GAC TAT TAT TG 3', reverse primer with *Xho I* digestion site.

A 35S-HA tag containing Gateway-OCS vector, CD3-687 was used as destination vector. Using Gateway® LR Clonase™ II Enzyme Mix (Invitrogen) the recombinant vector for *Agrobacterium* mediated plant transformation was obtained. This plasmid after confirming the presence of insert via DNA sequence results was grown in *A. tumefaciens* and the plants were infected with this bacterium for T-DNA insertion. The plants with the insertion were checked using 0.2 % BASTA (glufosinate ammonium).

Preparation of Slides

Roots of the plants grown at different ABA concentration were transferred to small Petri dishes containing 0.24 N HCl in 20 % methanol and incubated on a 57°C heat block for 15 minutes. This solution was replaced with 7 % NaOH, in 60 % ethanol for 15 minutes at room temperature. Roots were then rehydrated for 5 minutes each in 40 %, 20 % and 10 % ethanol, and infiltrated for 15 minutes in 5 % ethanol, 25 % glycerol. Roots were mounted in 50 % glycerol on glass microscope slides and cover with a glass slip (Malamy et al., 1997).

Microscopy

The number of cells in the roots and the size of the cells were measured using a Light microscope (Olympus System Microscope). The difference in the cell size in meristematic and elongation zones of the root were observed using Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Microimaging, Inc.). The difference in the number of root hairs on roots in WT and Mutants was observed using Eclipse E600 (Nikon) Binocular Molecular and the pictures obtained were edited with SPOT software.

Results

Reduction of Cell number in the Meristematic zone

When treated with ABA, we noticed that there was a reduction in the size of roots. The wild type plants had longer roots when compared to the *sdg8* mutants. It was necessary to know if the size of the cells was reduced, that is if ABA was causing shrinking of the roots or even the cell number was reduced in the roots. For this we examined the roots under a microscope and documented the number of cells and the size of cells in the meristematic. It was noticed that the number of cells decreased in the meristematic zones. The cell size was reduced in the meristematic zones but in general the cells are very small in the meristematic zone and the difference in the size of the cells was not very clear in the meristematic region. Figure 4.2 shows the reduction in the cell size and cell number in the meristematic zone at various concentration of ABA when compared to the Wild type plants. To statistically prove this the p-values were calculated which showed very low values indicating that the data was highly significant. Graphs were plotted to see the difference (Figure 4.3) in the cell number at various concentration of ABA.

Reduction of Cell size in the elongation zone

After checking the size of cells in the meristematic zones the cell size was checked in the elongation zones. It was observed that the size in the elongation zones was also reduced drastically. Figure 4.4 shows the reduction in the cell size in the elongation at various concentration of ABA when compared to the Wild type plants. When the results were graphed (Figure 4.5) it was observed that the difference in the reduction of cell size was maximum at 5 μM concentration of ABA. At this concentration the ABA

did not have an effect in the wild type plants but the size was reduced in the mutant plants. When the concentration of ABA was increased to 10 μM and then 20 μM, we noticed that the size of the cells in the elongation zone was reduced but difference in the cell size was also noticed in wild type plants. The p-values were less than 0.05 indicating that the difference was statistically significant.

Increase in Number of Root Hairs

When treated with ABA, the *sdg8* knocked out mutants showed a visible difference in the root length. Another abnormal feature which was observed visibly was the number of root hairs. Figure 4.6 clearly shows the difference in the number of root hairs before and after treatment with ABA and at various concentration of ABA. A dramatic increase in the number of root hairs was observed in the mutant plants but this was not observed in wild type plants. The number of root hairs increased with the concentration of ABA.

Screening of ABR1 Knocked out mutants

The ABR1 knocked out mutants show hypersensitivity towards ABA treatment. In the presence of ABR1gene the plants should not be sensitive to ABA. So it was important to see if the same happened when the gene was over expressed in the presence and in the absence of the SDG8 gene to study the interlink between the two gene. For the same the ABR1 knocked out mutants were screened in the presence of ABA and it was noticed that though the plants were sensitive to ABA they were not as sensitive as the *sdg8* mutants. Thus an over expression line of ABR1 gene was prepared using t-DNA insertion technique and the plants are at the screening stage.

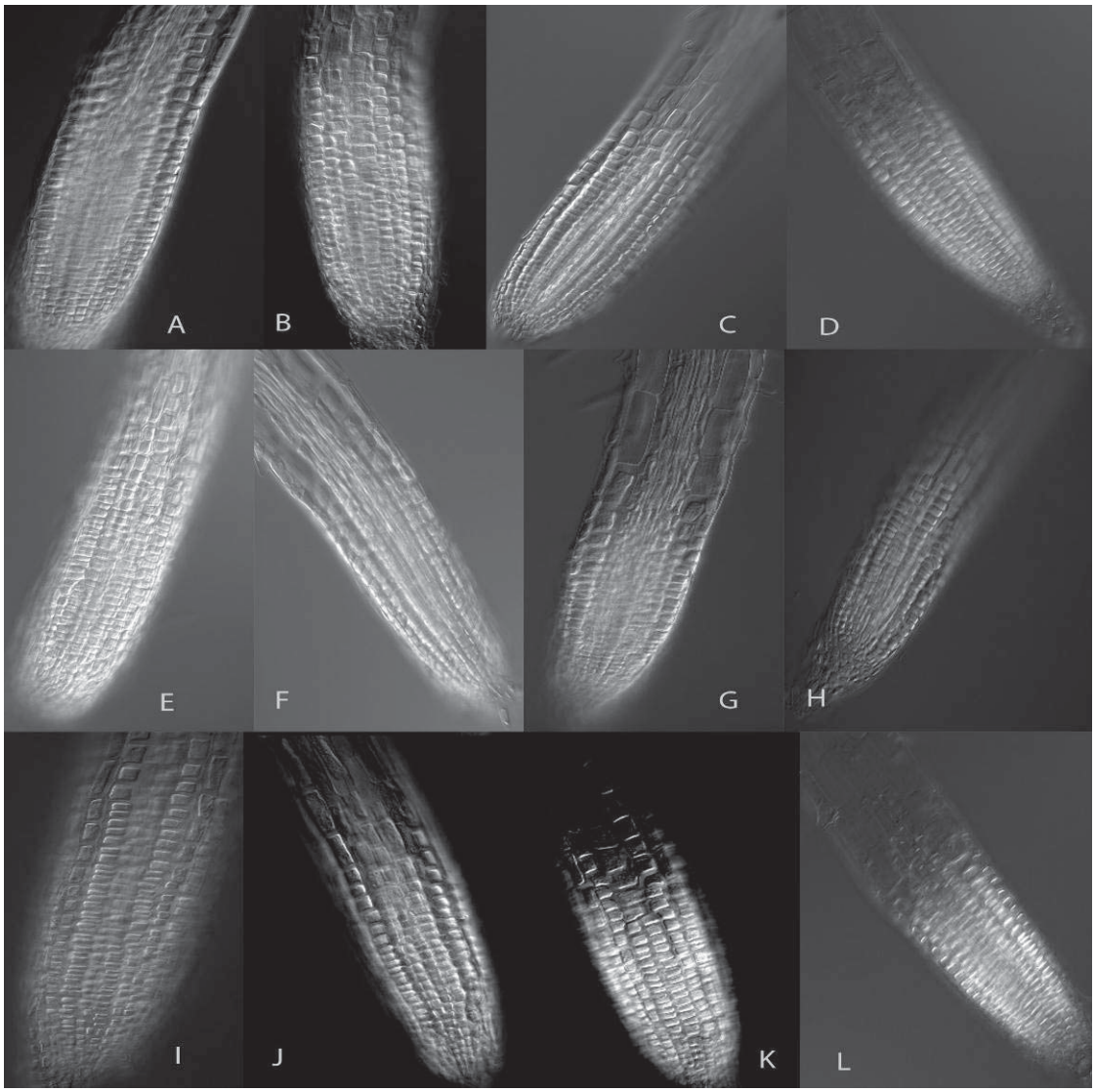


Figure 4.2 Reduction in Cell number and Cell Size in the meristematic zones.

The picture shows roots in WT (A, B, C &D), *sdg8-4* (E, F, G & H) and *sdg8-2*(I, J, K & L) plants. In the figure roots shown in A, E and I were grown without ABA, B, F & J were treated with 5 µM ABA, C, G & K were treated with 10 µM ABA and D, H & L were treated with 20 µM ABA. The size of the cell and number of the cells both reduced in the mutants.

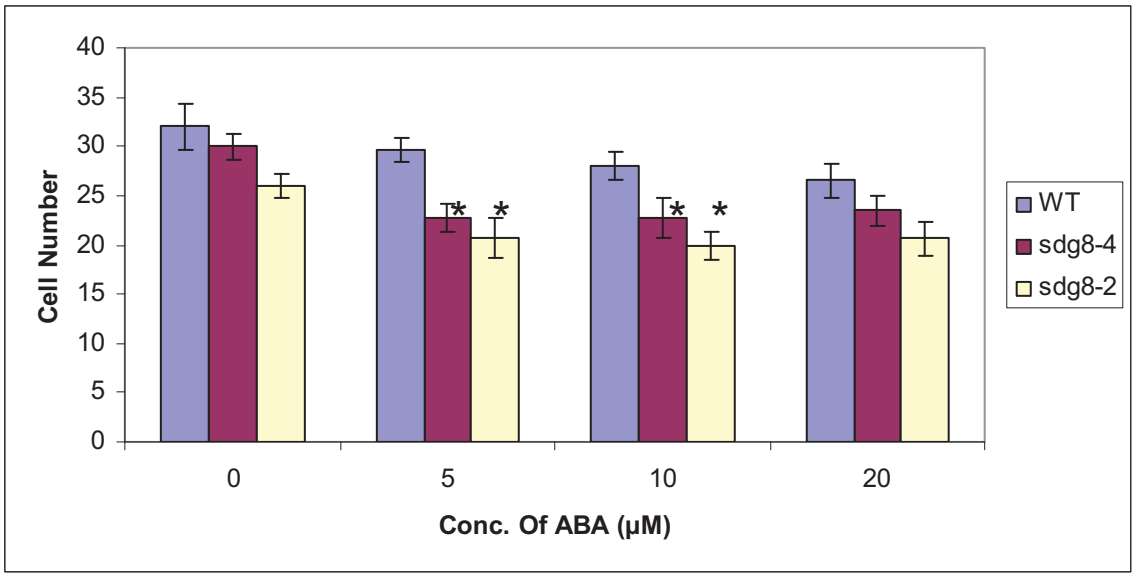


Figure 4.3 Graph showing cell number verses concentration of ABA in the Meristematic zone. 0, 5 10 and 20 µM concentrations of ABA were used to study the effect on the roots. * indicates that the data is highly significant within the group.

Table 4.1 The Cell number in Meristematic zones at various concentrations of ABA. The p-values indicate that the data is highly significant.

Conc. Of ABA(µM)	**WT**		***sdg8-4***			***sdg8-2***		
	Mean	**SD**	**Mean**	**SD**	**P-value**	**Mean**	**SD**	**P-value**
0	32	2.3	30	1.3	0.006675	26	1.25	$1.30x10^{-7}$
5	29.69	1.25	22.75	1.5	$1.46x10^{-11}$	20.69	2	$7.94x10^{-12}$
10	28.08	1.44	22.84	2.03	$1.658x10^{-7}$	19.92	1.44	$2.53x10^{-13}$
20	26.53	1.75	23.53	1.5	$3.322x10^{-5}$	20.66	1.7	$5.65x10^{-5}$

Table 4.2 The size of cells in the meristematic zones of roots. Various concentrations of ABA were used in the study the size of cells in WT, *sdg8-4*and *sdg8-2.*

Conc. Of ABA(µM)	**WT**		***sdg8-1***			***sdg8-2***		
	Mean	**SD**	**Mean**	**SD**	**P-value**	**Mean**	**SD**	**P-value**
0	7.8125	0.4985	6.4583	0.7216	0	6.6145	0.5627	0
5	7.9326	0.4694	7.4038	0.3466	0.0043	5.9134	0.4851	$1.4x10^{-10}$
10	6.5384	0.6046	6.7788	0.5616	0.3801	6.1057	0.3744	0.0502
20	6.375	0.6764	6.8750	0.5282	0.0406	5.5416	0.6190	0.0018

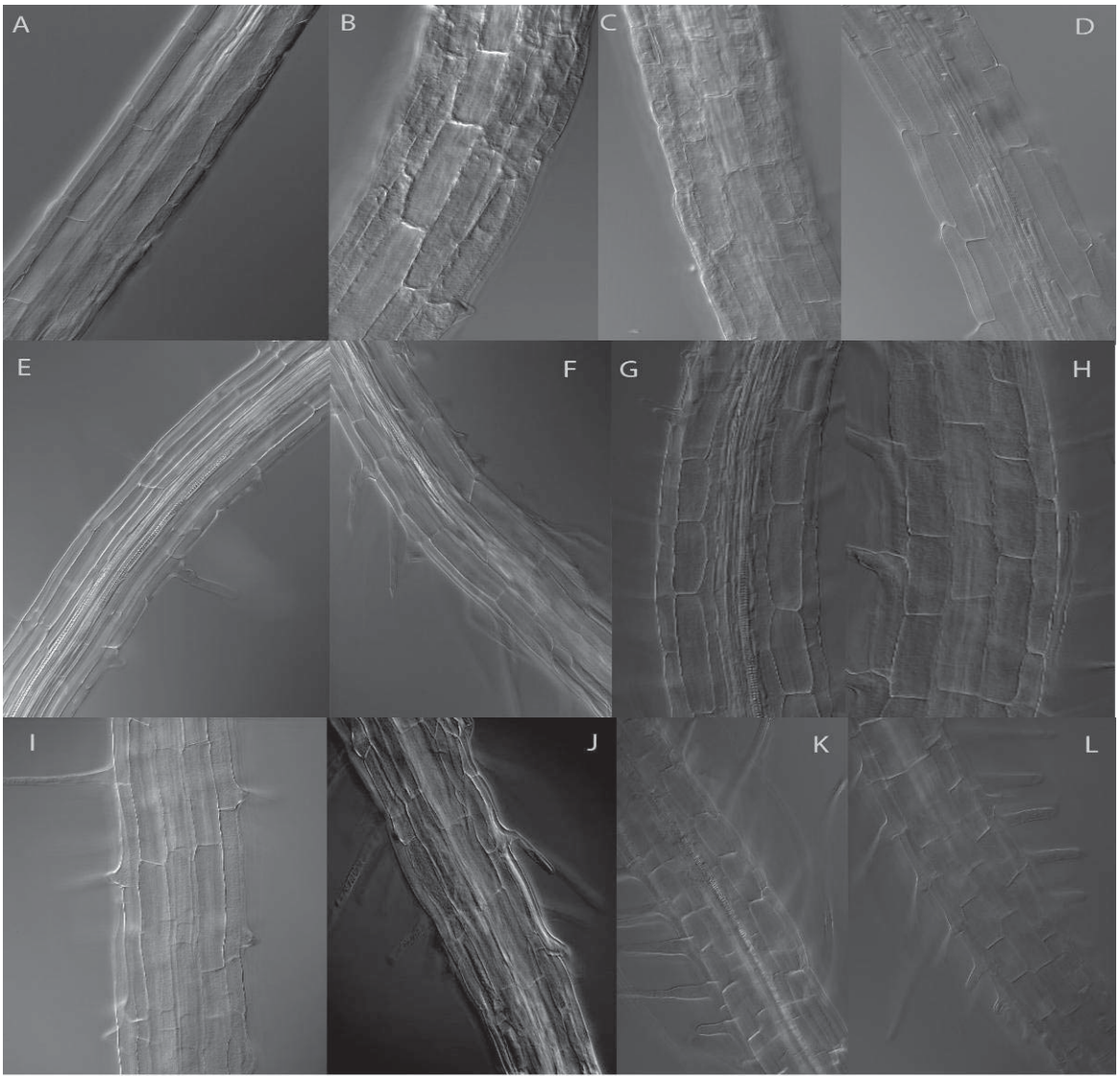


Figure 4.4 Reduction in Cell Size in elongation zones of the roots.

The picture shows roots of WT (A, B, C &D), *sdg8-4* (E, F, G & H) and *sdg8-2*(I, J, K & L) plants. In the figure roots shown in A, E and I were grown without ABA, B, F & J were treated with 5 μM ABA, C, G & K were treated with 10 μM ABA and D, H& L were treated with 20 μM ABA. The size of the cell and number of the cells both reduced in the mutants.

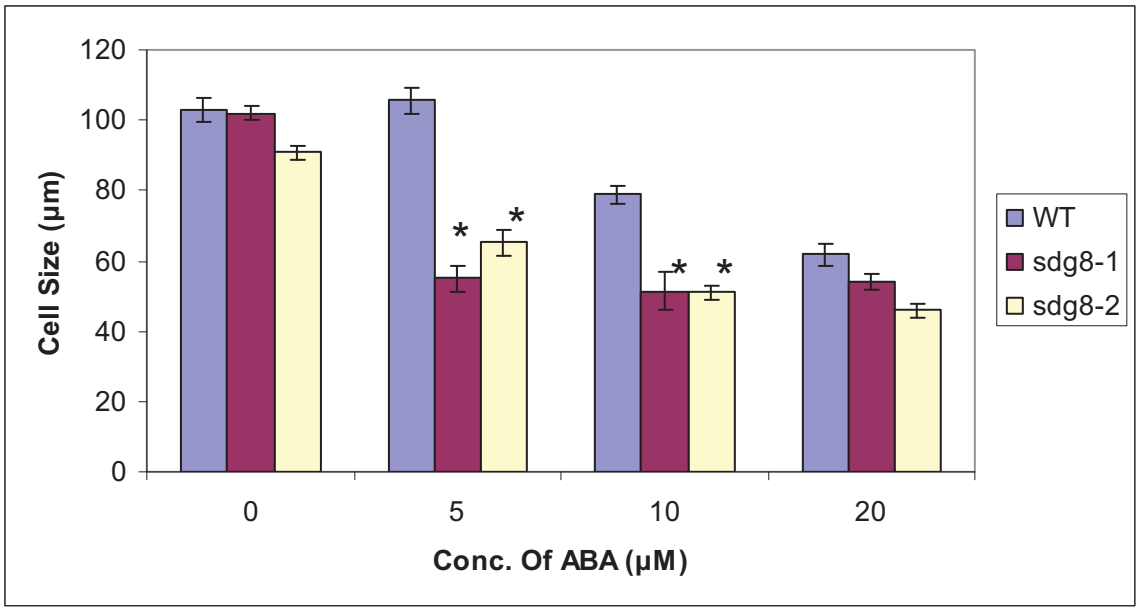


Figure 4.5 Graph showing cell size verses concentration of ABA in the Elongation zone. The difference in size was high at 5 and 10 µM concentration of ABA. * indicates that the data is highly significant within the group.

Table 4.3 The size of cells in the elongation zones of roots. Various concentrations of ABA were used in the study the size in WT, *sdg8-4*and *sdg8-2.* The P-values were <0.05 indicating the data was highly significant.

Conc. Of ABA(uM)	**WT**		***sdg8-4***			***sdg8-2***		
	Mean	**SD**	**Mean**	**SD**	**P-value**	**Mean**	**SD**	**P-value**
0	102.916	3.657	102.031	2.1996	0	90.729	1.764	0
5	105.673	3.6216	55.096	3.696	$4.45x10^{-22}$	65.192	3.7184	$4.4x10^{-07}$
10	78.8125	2.4375	51.346	5.4117	$6.29x10^{-12}$	51.009	1.8317	$1.43x10^{-15}$
20	61.81	3.179	54.1667	2.147	$1.15x10^{-05}$	45.982	2.0172	$5.21x10^{-11}$

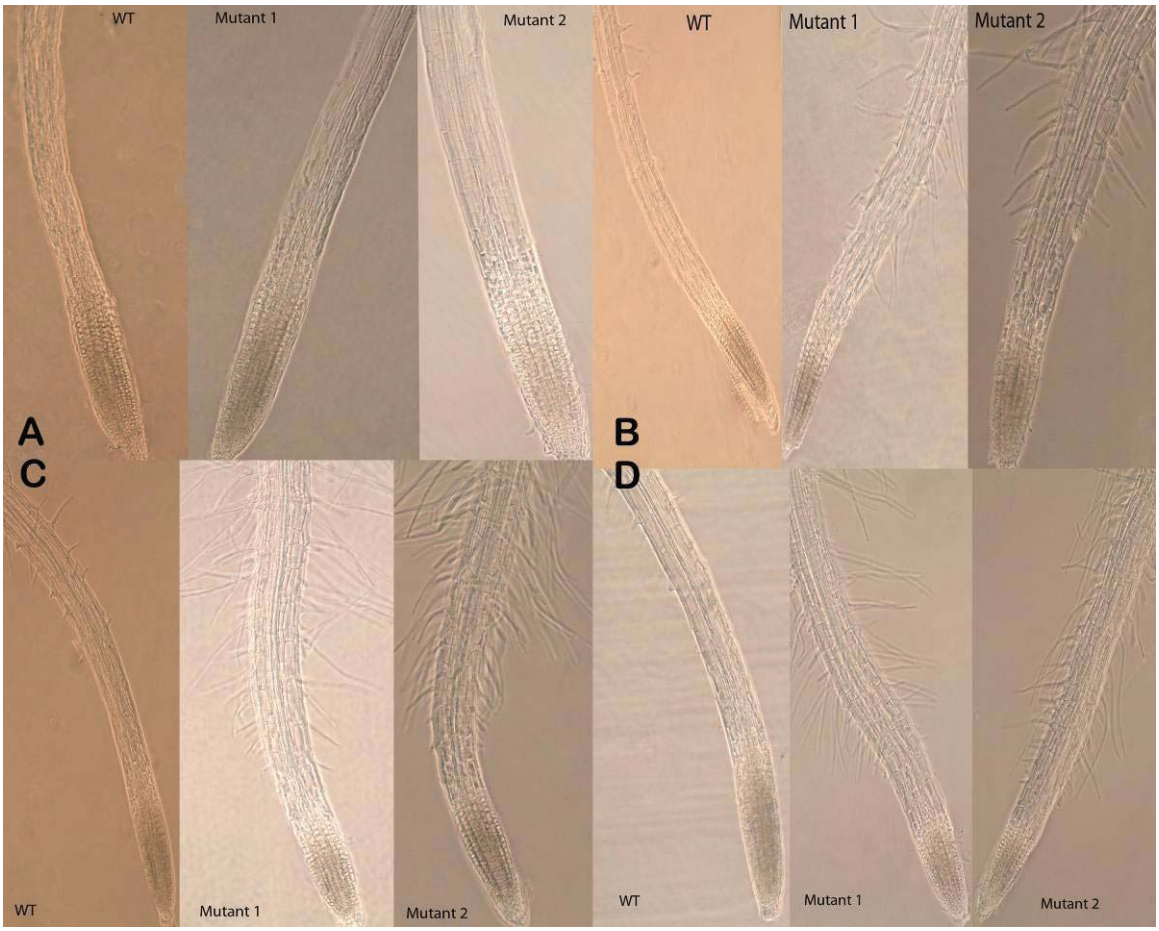


Figure 4.6 Increase in number of root hairs in the *sdg8* mutant plants.

Mutant 1 indicates *sdg8-4* and mutant 2 indicates *sdg8-2*. Roots shown in A were not subjected to ABA treatment and served as control. Roots in B were treated with 5 µM concentration of ABA; roots in C were treated with 10 µM concentration of ABA and D with 20 µM concentration of ABA respectively. An increase in the number of root hairs was observed in plants treated with ABA.

Discussion

Plants have an amazing feature of synthesizing the required carbohydrates using sunlight, water and minerals via photosynthesis. Leaves with the help of chlorophyll pigments trap the sunlight which they use in the production of carbohydrates from raw materials (water and minerals) provided by roots. Roots thus play an important role in providing the plant with adequate amounts of water and minerals to ensure proper growth

of plant. The *sdg8* mutants when treated with ABA showed a remarkable decrease in the overall size of the root compared to the roots of the wild type plants which were treated similarly. To investigate if ABA was causing the reduction in the size of the root by shrinking the cells or by reducing the number of the cells, we examined the roots under microscope. It was observed that the cell size and number were altered in the meristematic zone, and the cell size reduced dramatically in the zone of elongation.

The root in general is divided into the meristematic zone, the zone of elongation and the zone of maturation. The meristematic zone of root is responsible for the making new cells and adding cells to the elongation zone to increase the length of the roots. It is in this zone that the roots cells form undifferentiated cells and enter the zone of elongation where their size increases dramatically (150 fold). The cells in the zone of elongation are easily recognized from the meristematic zone as they are much bigger in size and their vacuoles are filled with water. This zone is responsible for the actual elongation of the root where in cells absorb water and nutrients from soil. When cells in the elongation zone stop growing the root size automatically is affected. The zone of maturation has cells which are differentiated into epidermis, cortex or vascular tissue.

Usually during water stress, to save the plant from dehydration the roots produce ABA which through a cascade of events causes closure of stomata to reduce transpiration. When ABA is applied exogenously a similar affect is observed as plant sensors danger. In the case of *sdg8* mutants when the roots were treated with ABA, it was observed that the size of the roots was dramatically reduced. But the application of ABA did not cause drastic affect in fully grown plants that is in leaves. In the mutants it was noticed that not only did the cell size and number reduced but the number of root hairs also increased when compared to the wild type control when treated with ABA.

The cell number in the meristematic zone decreased remarkably but the cell size though decreased it was not as apparent as in the elongation zone. The number of cells decreased as the concentration of ABA increased from 5 μM to 20 μM. A slight decrease in the number was also observed for the wild type plants but the change was drastic in *sdg8* of mutants, indicating the ABA was causing more changes in the mutant plants. The meristematic zone contains cells usually in undifferentiated state. When the size of the cells in this region was examined under the microscope it was observed that the cell size though changed, the difference was not distinct. However the size difference in the elongation zone was very clear. The cells in the elongation zone showed a significant change in size at 5 μM concentration of ABA. When the concentration of ABA was increased beyond 5 μM the change was still observed that but this time the wild type plants also showed some difference indicating the high stress levels.

The number of root hairs increased with increase in the concentration of ABA indicating that the stress levels play a role in deciding the phenotype. The increase in the number of root hairs could be the mechanism of the plant to combat stress. The important function of root hairs is to increase the surface area of the roots to absorb nutrients and water. By increasing the number of root hairs the plant would get sufficient supply of nutrients even though the size of the main root is reduced.

Previous Studies indicate that the methylation on lysine 9 and lysine 36 residues was disturbed in *sgd8* mutants and this was having a drastic affect on the phenotype of the plant. Just a small change in the methylation was causing a change in gene expression and thus leading to altered phenotype. In the case of *sdg8* mutants the flowering phenotype and shoot phenotype was well studied and the literature is available. The mutant (*sdg8*) plants have overall developmental defects throwing light

on the possible altered gene expression of many genes and above all the interrelation, interaction and coordination of these genes involved in the overall development of the plant. It looks like the presence and absence of the SDG8 gene is having an effect on the expression of the ABR1 gene and thus showing an altered phenotype in roots. When *abr1* plants were treated with ABA like the mutant plants, it was observed that though the plants were sensitive to ABA, they were not as sensitive as the *sdg8* mutants. Once we study the phenotype of transgenic plants where in ABR1 gene is over expressed in the presence of SDG8 gene, the effect of SDG8 gene on ABR1 gene would be clear.

CHAPTER V

TANDEM AFFINITY PURIFICATION OF PROTEIN KINASE IN *ARABIDOPSIS THALIANA*

Introduction

Phosphorylation and dephosphorylation reactions medicated by protein kinases have been under scrutiny for many years. In higher organisms protein kinases regulate key aspects of cellular functions like metabolism, cell division and nerve impulses. Protein kinases encoded by *Arabidopsis* are more in number when compared to yeast (Zheng et al., 2010) and some kinases are unique to plants. Discovery of ABA activated protein kinases lead to the better understanding of behavior of plants during stress. The genes activated by ABA usually produce protective proteins that are required to combat stress or produce transcription factors that regulate expression of other genes.

The yeast SNF1 related proteins in plant belong to the subfamily SnRK which are activated by ABA. The SnRK proteins based on the similarity in sequence and domain structure are further divided into three sub groups, SnRK1, SnRK2 and SnRK3. All proteins belonging to this sub family co ordinate to protect the plant form different kinds of stress conditions. SnRK1 proteins are the largest among the sub families and contain three proteins in their subfamily. SnRK2 and SnRK3 sub groups of proteins are unique to *Arabidopsis*, and are slightly different from the yeast and mammalian proteins. SnRK2 comprises of 10 proteins and SnRK3 comprises of 25 proteins in plants (Boudscq et al., 2004, Umezawa et al., 2004 Zheng et al., 2010).

The ABA activated gene regulation of SnRK2 proteins was first reported in wheat. In wheat PKABA-1, a SNRK2 related protein, is induced by ABA and helps in phosphorylation of TaABF. Another SnRK2 related kinase in *Vicia faca*, AAPK is activated by ABA and is involved in stomatal closure. The SnRK2 kinases are further divided into two groups, SnRK2a and SnRK2b. SnRK 2.2, SnRK2.3, SnRK2.6, SnRK7 and SnRK 8 belong to SnRK2a and SnRK 1, SnRK4, SnRK5, SnRK9 and SnRK10 belong to SnRK2b (Zheng et al., 2010, Umezawa et al., 2004). An interesting feature of this group of protein is that ABA can only activate kinases of SnRK2a but not the kinases of SnRK2b, indicating the functional difference between the two groups. This functional difference was not only between the groups of proteins but it was also among the group members. Snf1 related protein kinases SnRK 2.2, SnRK2.3 and SnRK2.6 which show sequence similarity in amino acids are ABA activated kinases which belong to group SnRK2a. SnRK2.2 and SnRK2.3 are involved in regulating seed germination, dormancy and seedling growth but are not involved in stomatal closure like the SnRK2.6 (Boudsocq et al., 2004, Fijji et al., 2007, Yoshida et al., 2002). Studies indicate that there is a functional redundancy between SnRK2.2 and SnRK2.3 and thus mutations did not show clear response to ABA, but double mutant (*snrk2.2/2.3*) is insensitive to ABA, indicating their involvement in ABA response.

Studying the protein-protein interactions has been useful in proving valuable information about the relations existing between the gene products. Coimmuno precipitation and yeast two hybrid systems were generally used to study the protein-protein interactions. We used a relatively new method of Tandem Affinity Purification to purify SnRK2.2 an ABA activated protein kinase and study its interaction with other proteins in the presence and absence of ABA.

Materials and Methods

Arabidopsis Callus induction

Arabidopsis thaliana seeds of the mutant plant (SnRK2.2) were sterilized and placed on B5 Basal medium. The medium also contained 2 % sucrose, 0.9 % agar and 0.05 % MES salts. pH was adjusted to 5.7 and 2 mg/L of 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and 1 mg/L of Kinetin were added to the medium. After sterilization seeds were kept in 4^{o}C for 48 hours in dark and then were grown in 22^{o}C incubator for callus induction. After 3 weeks the small leaf and root parts were removed, callus was separated into small pieces and transferred to new plates for further growth.

Production of Suspension Cells

The callus was checked for the presence of transgene using western blot and only positive lines were used to start the production of suspension cells. Liquid basal medium supplemented with 0.05 % MES salts, 2 % sucrose and 2, 4 D (2 mg/ml) and 2 % N-Z Amine A with pH 5.7 was used. The callus from the plates was transferred into the medium and grown in a shaker at 250 rpm in dark. The medium was changed every week and suspension cells were harvested at regular intervals of two months. To see the effect of ABA on binding, 5 hours before harvesting the suspension cells ABA to a final concentration of 10 uM was added and the same amount of ethanol was added to the control. The harvested suspension cells were stored at -80 ^{0} C (Mathur et al., Encina et al., 2001).

Tandem Affinity Purification (TAP)

TAP was performed using suspension cells from the transgenic *A.thaliana* lines (in which TAP tag was attached to SnRK2.2). Approximately 25 grams fresh weight of the suspension cells were grinded in liquid nitrogen and the homogenate was transferred to 30 ml of cold extraction buffer (25 mM Tris HCl pH 8, 150 mM NaCl, 15 mM $MgCl_2$, 2.5 M EDTA, 5 mM EGTA, 10 % Glycerol, 0.1 % Nonidet P-40, final pH 8, 10 mM beta mercaptoethanol, 40 mM beta glycerophosphate, 1 mM NaVO4, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 1 mM benzamide) (Zhao et al., 2008, Puig et al., 2001). The solution was mixed and kept on ice for 30 minutes. The extract was centrifuged at 1,450 g for 10 minutes at 4^{o}C after filtering through Miracloth. The supernatant was transferred to equilibrated IgG Sepharose ^{TM} Agarose beads (GE Health Care) and incubated with rotation for 2 hours at 4^{o}C. IgG protein extract was transferred to a Poly-Prep Chromatography Column (0.8 x 4cm, Bio-Rad) and was allowed to drain by gravity. After draining 10 ml extraction buffer without protease inhibitors was added (twice) and was allowed to drain to remove nonspecific binding. After the washes 10 ml TEV cleavage buffer (10 mM Tris –HCl, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT) was added to the column and was allowed to drain. After this the column was closed from bottom, 1 ml of TEV buffer and 100 units of TEV enzyme (AcTEV Protease, Invitrogen) was added to the column. The column was closed from top and the incubated overnight at 4^{o}C with rotation. The flow through was collected and the column was washed with 200 µl of TEV cleavage buffer and this was also collected.

To the elute 3 volumes of calmodium binding buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM Mg-acetate, 2 mM $CaCl_2$,0.1 % NP-40, 1 mM imidazole, 10 mM beta mercaptoethanol) was added and the calmodium beads (Calmodium affinity resin,

Stratagene) which were previously equilibrated. This mixture was incubated for 1 hour at 4^{o}C with rotation. The flow through was collected after one hour and care was taken not to dry the top surface of the beads. The beads were washed 3 times with 6 ml of Calmodium binding buffer without disturbing the column and beads. After the washes the protein was eluted with 1 ml calmodium elution buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM Mg-acetate, 20 mM EGTA, 0.1 % NP-40, 1 mM imidazole, 10 mM beta mercaptoethanol) as 5 fractions and stored at -20^{0}C.

TCA precipitation of proteins

Protein samples were precipitated using Cold TCA (final concentration 20 %). The samples were kept on ice for 20 minutes after adding TCA before centrifuging for 20 minutes at 4^{o}C. TCA supernatant was removed carefully without disturbing the pellet; cold acetone was added to the samples and centrifuged for 30 minutes at 4^{o}C. Acetone was discarded and pellet was air dried. The protein pellet obtained was dissolved in SDS-gel sample buffer containing 8 M urea and heated for 5 minutes before spinning down and loading onto the gel. An 8 % SDS gel was to load the samples.

Silver Staining

The gel was first soaked in the fixing solution (50 % methanol, 10 % acetic acid) for 30 minutes and in a solution of 5 % methanol and 1 % acetic for 15 minutes. The gel was washed thoroughly with milli-Q water 3 times for 5 minutes each time. Then the gel was sensitized by incubating in a freshly prepared 0.02 % sodium thiosulphate solution for 90 seconds. The gel was then washed with water 3 times for 30 seconds each time before $AgNO_3$ treatment. The gel was placed in dark in 0.2 % $AgNO_3$ solution for 30 minutes. Before developing the gel it was washed with water 3 times for 1 minute each

time. A fresh 100 ml solution of 6 % Na_2CO_3 (6 g), 37 % formaldehyde (50 µl) and $Na_2S_2O_3$ (2 ml form the sensitizing solution) was prepared and the gel was soaked in this solution till adequate band intensities developed. The developing was stopped by using 6 % acetic acid solution and the gel was stores at 4^{o}C in 1 % acetic acid.

Preparation of Samples for Mass Spectrometry

The gel band was cut into 1 mm pieces using a razor and pieces were covered with HPLC grade acetonitrile for 10 minutes at RT after washing with distilled water. Acetonitrile was completely removed and ice cold trypsin buffer solution containing 50 mM ammonium bicarbonate and 12.2 ng/µL tripsin (Promega) was added to cover all gel pieces in the tube and incubated on ice bath for 45 minutes to allow trypsin to enter the gel pieces. Then the trypsin containing buffer was removed and 50 mM ammonium bicarbonate buffer was added to cover all the pieces and was incubated over night at 37^{o}C. The ammonium bicarbonate solution was removed and saved in a different tube. Fresh 20 mM ammonium bicarbonate solution was added and incubated at 37^{o}C for 45 minutes. This solution was removed and added to the saved solution. Finally a solution containging 5 % formic acid, 50 % acetonitrile was added to the gel pieces and incubated at 37^{o}C for 20 minutes before spinning for a minute and collecting the supernatant. The solution from the samples was saved and the last two steps were repeated twice. After pooling all supernatants the samples were vacuum dried till completely dried and sent for mass spectrometric analysis.

Results and Discussion

Analysis of Mass Spectroscopy data

Five protein samples from the gel, (Figure 5.1) were sent for mass spectrometric analysis, and protein was our interest, SnRK2.2 was found in one of the sample B.The TAP reaction was successful and suggested a few protein targets for SnRK2.2. But it was also noticed that there were many contaminants and metabolic proteins which were not of interest during mass spectrometric analysis. Further studies would hopefully reveal the exact targets of SnRK2.2 with more accuracy.

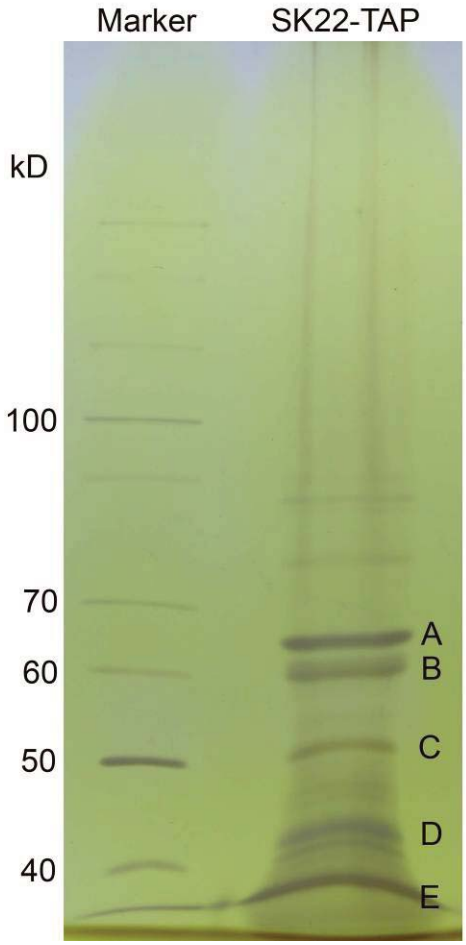


Figure 5.1 SDS-PAGE analysis of the Purified protein after the TAP reaction.

The protein bands appeared after silver staining. A, B, C, D & E are the protein bands which were analyzed using mass spectrometry. The SnRK2.2 protein was observed in band B in the mass spectrometry result.

CHAPTER VI

SUMMERY

Study of histone modifications and DNA methylation patters and the outcome of these modifications, in short Epigenetics has been an interesting area of research for many years. DNA methylation and nucleosome histone post translational modifications drive the gene expression in cells and thus control phenotypes in plants. Though enormous literature has been acquired over the years about epigenetic mechanisms, what leads to the formation of epigenetic code and how this code is passed on to off springs has not been clearly understood. Knowing just about one epigenetic modification might not give us enough information about the altered biological activity. But this one modification could affect the expression of one or many genes or could lead to another modification at different location and cause altered gene expression. This altered gene expression could possibly cause abnormal phenotype.

It is fascinating to learn how plants adapt to stress. In *Arabidopsis* when plants are subjected to stress not only does the overall development of the plant is affected but reproduction takes a back seat. For example during vernalization, when seeds are subjected to low temperatures, a MADS-box protein of FLOLWERING LOCUS C (FLC) is repressed till the transition to flowering. Though many genes that are altered during stress have been studied, the exact mechanism of inheritance of the ‘stress memory’ in plants is not clearly understood (Dennis et al., 2007).

Two genes, JMJ14, a jumonji domain containing putative histone demethylase gene and SDG8, a (SET domain containing group 8) histone lysine methyl transferase in *Arabidopsis thaliana* have been studied as a part of this dissertation. The JMJ14 gene is responsible for controlling the flowering time by regulating the expression of FT gene (Flowering Locus T). Previous studies on SDG8 revealed its role in controlling the flowering time (by repressing Flowering Locus C) and shoot branching by altering expression of genes that control shoot brancing. Here we show that SDG8 could be responsible for controlling the root length and root hair number by interfering with ABA signaling genes.

Jumonji Domain containing putative histone demethylase in *Arabidopsis thaliana*

Transition from vegetative phase to reproductive phase in plants is an important event that leads to production of seeds and fruits. Reproduction in any organism is very complex and involves many pathways that govern the growth of cell; these pathways coordinate to ensure the successful production of healthy off springs. In plants this reproductive process is not only controlled by regulatory pathways occurring in the cell but also depend on environmental cues like light, temperature, water etc. Both external and internal factors co ordinate and perform their respective roles in deciding the time for phase (vegetative to reproductive) transition and the subsequent production of seeds and fruits.

When JMJ14 gene was completely knocked out from the *Arabidopsis thaliana* plants via T-DNA insertion, *jmj14* mutants flowered early when compared to wild type plants. To investigate the reason for this early flowering phenotype, we performed localization studies of the gene using RT PCR and histochemical GUS. RT-PCR results

indicated that the gene was highly expressed in reproductive organs like flowers and siliques but was expressed to a lower extent in vegetative organs like leaf and roots. These results were in consistence with the results obtained from histochemical GUS assay where in the blue color (which indicated the activity of the JMJ14 promoter) was more concentrated in the floral parts like flower, inflorescence stalk, siliques and stem and less concentrated in leaves. These results suggested the possible role of JMJ14 in the reproductive phase of the plant.

When plants enter the reproductive phase usually the vegetative growth reduces as the plant concentrates on reproduction. For this phase transition to occur as mentioned earlier many factors play a role. In *Arabidopsis* light plays major role in controlling the flowering time. To study the effect of light on the phenotype of plant the wild type plants and mutant lines were grown in long day and short day conditions. The mutant plants in both long day and short day conditions showed an early flowering phenotype. When the *Arabidopsis* plant starts flowering, it starts producing cauline leaves. A leaf count in both conditions was performed and results indicated that the mutant lines had more cauline leaves than the wild type plants. This further confirmed the early flowering phenotype in the mutants.

Early flowering phenotype in the mutants indicated a possible change in the expression of genes involved in flowering. To study the expression of important genes that regulate flowering in *Arabidopsis* RT PCR was used. It was observed that Flowering Locus T (FT), a gene responsible for controlling the flowering time in *Arabidopsis* was expressed to greater extent in the stem and reproductive organs of mutants when compared to that of wild type plants. FT gene is an important gene that controls flowering time in *Arabidopsis.* It is produced in the leaves but its site of action is shoot apex, it acts

as mobile signal which is transported via phloem to the shoot apex where it initiates inflorescence.

The localization of JMJ14 gene in the floral tissue and the elevated levels of FT gene in the mutants indicated a link between JMJ14 gene and flowering time in Arabidopsis. We precluded that JMJ14, via demethylation must be controlling the expression of FT gene which is responsible for deciding the flowering time in Arabidopsis. To see if there was any change in the methylation status at the FT loci, we performed ChIP assay. The resultes showed elevated H3K36 di and tri methylation states in the coding region of FT gene in the mutants. As the JMJ14 gene was disrupted in the mutants, the demethylase protein was not encoded in the plants, thus an altered methylation state was observed in the mutants. JMJ14 homolog in humans and yeast is highly conserved and the demethylase was specific to H3K36 (Tsukada et al, 2006), similar results were indicated by ChIP assay but we can further confirm this after we get the data from activity analysis. Not only this data but when the JMJ14 gene was over expressed in the mutant lines it was observed that the plant flowered normally indicating the role of JMJ14 gene in controlling the flowering phenotype in *Arabidopsis*. In summary we show that the JMJ14 gene directly regulates the flowering time in *Arabidopsis thaliana* by controlling the expression of FT via H3K36 demethylation. Figure 6.1 shows the role of JMJ14 in deciding the flowering time in *Arabidopsis thaliana.*

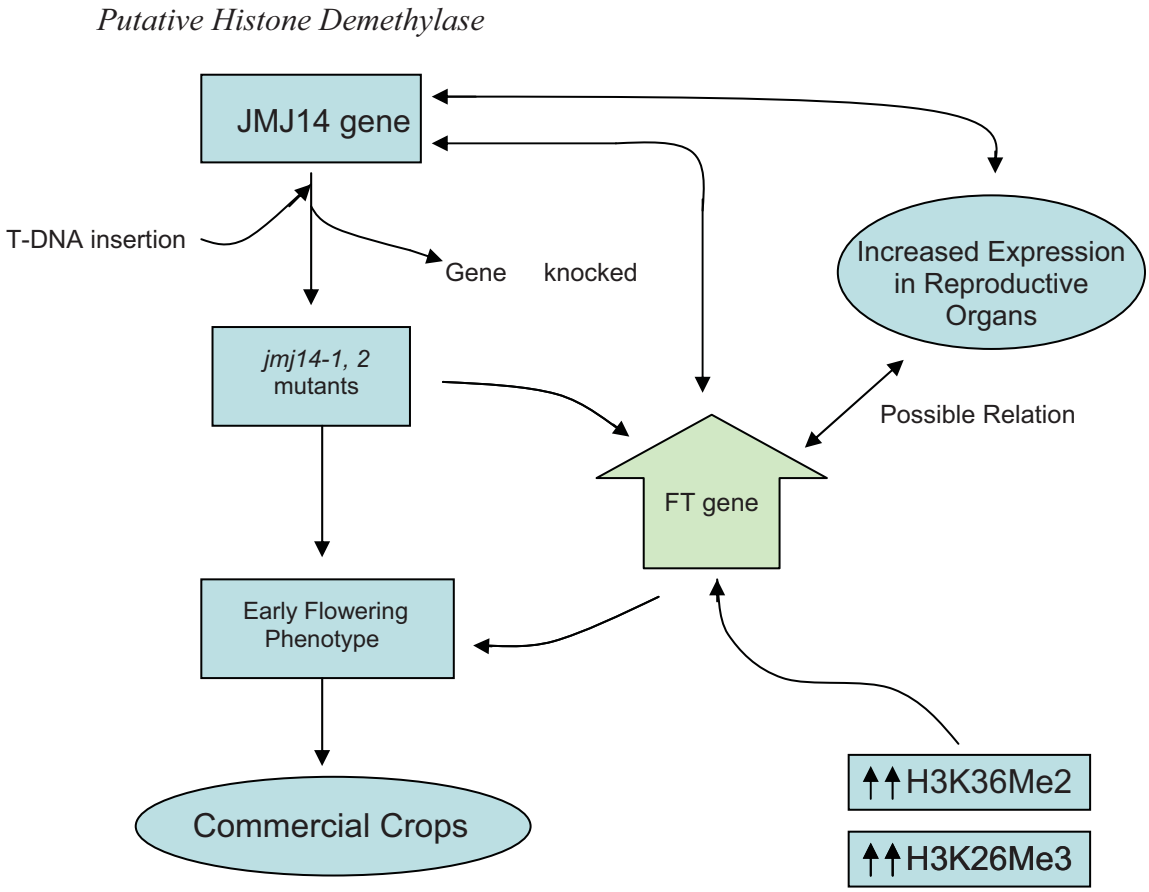


Figure 6.1 Schematic Presentation of possible role JMJ14 gene in controlling flowering time in *Arabidopsis thaliana*

SET domain containing Histone Lysine methyl transferase in *Arabidopsis thaliana*

Stress in any form could reduce the crop yield and quality. Primary stress factors like drought salt and temperature when combined with secondary stress factors like hormones and metabolites could lead to change in gene expression. This change in gene expression usually is via epigenetic mechanisms. These changes could be heritable (non Mendelian inheritance) or non-heritable. The heritable changes are usually stable and are passed on to future generations where as the non heritable changes are plants mechanism to combat stress. ABA, a plant hormone not only plays a major role in seed development and dormancy but also helps the plants combat stress during unfavorable conditions.

Studies indicate change at gene level when plants are treated with ABA. Studying these changes during stress would give us information about what happens in a plant internally, that is at gene level when they are subjected to stress.

SDG8 gene is a well studied gene, the knocked out mutants of this gene showed altered phenotypes like early flowering and excessive shoot branching which have been documented previously. The phrase 'out of sight, out of mind', suits perfectly to the root phenotype of *sdg8* mutants. *sdg8* mutants showed a decrease in root length when treated with ABA. Not only was the root length decreased but the number of root hairs in the mutants increased with increase in stress conditions (that is increase in ABA concentration). This showed how well the plant adapted to stress. By increasing the number of root hairs though the root length was reduced in the plant the nutrient supply was not reduced as the root hairs played their role.

The decrease in the root length and increase in the number of root hairs was visible, but the actual reason behind the altered phenotype was not clear. To investigate this, roots were examined under a microscope which revealed that both the size of cells and the number of cells was altered. Gene expression studies revealed an altered expression of an ABA responsive gene ABR1. This gene is a negative regulator of ABA, but in this case it looked like the SDG8 was having a major role in deciding the phenotype of the root. More studies on the transgenic plants with ABR 1 over expression would reveal the role played by SDG8 gene during ABA response. Figure 6.2 shows the role of SDG8 in controlling various phenotypes in plants.

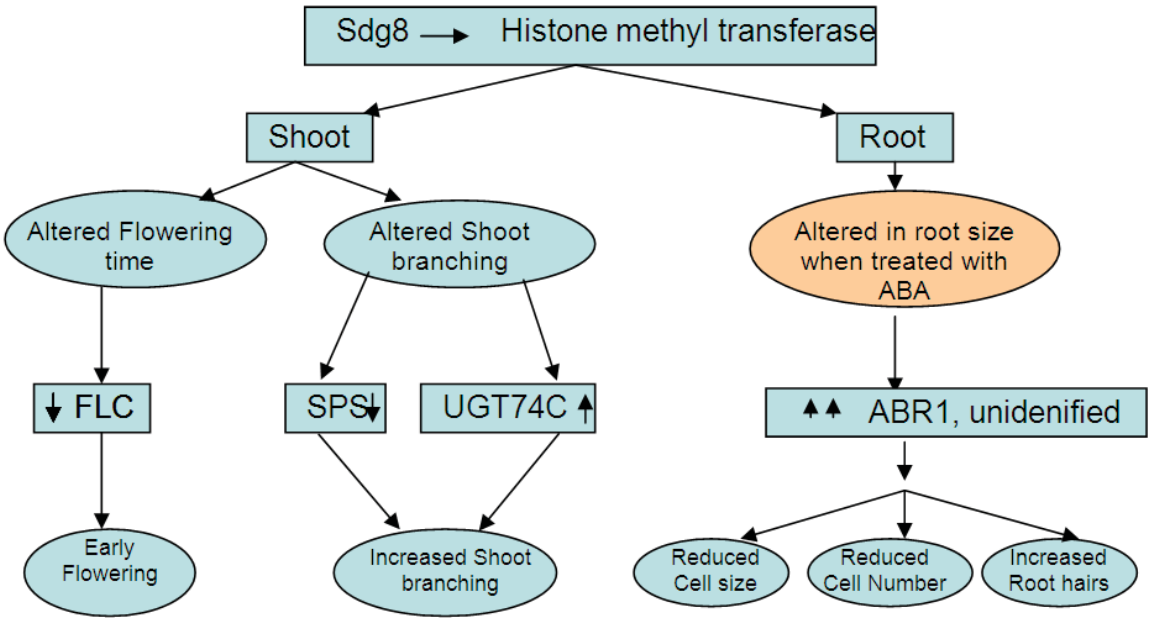


Figure 6.2 Schematic presentation of the effect of SDG8 mutation on the shoot and root phenotype in *Arabidopsis thaliana.*

Further investigation on these genes could lead to elucidation of mechanism of action of these proteins in plants and more downstream targets of these proteins. Insights gained through this research might one day lead to the production of commercial plants with early flowering phenotype which would reduce the effective cost of production and thus benefit agriculture industry.

CHAPTER VII

SUPPLEMENTARY DATA

Table 7.1 Number of Rosette and Cauline leaves in long day conditions. WT, *jmj14-2* and *jmj14-2* plants when grown under long day conditions, n = 15-18. Age of plants =25.

Plant Number	**Wild Type**		***jmj14-1***		***jmj14-2***	
	Rosette	**Cauline**	**Rosette**	**Cauline**	**Rosette**	**Cauline**
1	24	8	16	13	16	21
2	19	11	14	14	13	19
3	19	10	19	11	12	17
4	19	6	17	15	16	15
5	21	5	13	10	12	16
6	23	6	14	15	16	14
7	20	9	12	10	11	15
8	24	9	12	13	11	16
9	20	6	19	12	14	11
10	22	6	12	17	12	13
11	23	7	12	12	14	18
12	25	4	15	10	11	18
13	19	10	13	11	11	14
14	24	9	12	10	13	13
15	20	6	15	15	14	14
16	19	11	12	10	0	0
17	25	4	13	13	0	0
18	24	11	12	12	0	0
Mean	21.7	7.5	14	12.34	13	15.6
SD	2.23	2.33	2.38	2.15	1.89	2.64
P-value	0	0	3.137×10^{-06}	4.445×10^{-07}	8.112×10^{-07}	0.002336

Table 7.2 Number of Rosette and Cauline leaves in short day conditions.WT; *jmj14-1* and *jmj14-2* were grown under short day conditions, 10hrs of photoperiod. n = 13, Age of plants =25 days.

Plant Number	Wild Type		*jmj14-1*		*jmj14-2*	
	Rosette	**Cauline**	**Rosette**	**Cauline**	**Rosette**	**Cauline**
1	25	0	25	3	22	6
2	25	0	21	7	21	5
3	27	0	26	5	21	5
4	25	0	20	6	20	9
5	22	0	26	8	20	5
6	25	0	23	6	21	2
7	27	0	24	3	21	7
8	27	0	24	3	20	4
9	27	0	23	6	20	4
10	24	0	23	4	20	5
11	25	0	22	7	21	4
12	23	0	21	5	21	3
13	26	0	24	5	20	5
Mean	25.6667	0	24	5.25	20.6363	4.9230
SD	1.1547	0	2.6457	1.7122	0.6742	1.7541
P-value	0	0	0.0067	4.91×10^{-13}	9.043×10^{-10}	3.107×10^{-11}

Table 7.3 List of antibodies used for Chromatin immuno precipitation.

S.no	Name of Antibody	Source
1	Anti-H3-monomethyl-lysine 9 (H3K9me1)	Abcam
2	Anti-H3-dimethyl-lysine 9 (H3K9me2)	Abcam
3	Anti-H3-trimethyl-lysine 9 (H3K9me3)	Abcam
4	Anti-H3-monomethyl-lysine 36 (H3K36me1)	Abcam
5	Anti-H3-dimethyl-lysine 36 (H3K36me2)	Upstate
6	Anti-H3-trimethyl-lysine 36 (H3K36me3)	Abcam

Table 7.4 List of primers used for chromatin immuno precipitation

Name (amplicon size)	Forward primer	Backward primer
FTp (250)	GTGGCTACCAAGTGGGAGAT	TCATAGGCATGAACCCTCTAC
FTCo12 (250)	CCTTCTTATAGTAAGCAGAGTTGTG	AAGAAGGTGTAAGAAGCACTGAAGA
FTCo34 (261)	TTGGTGACTGATATCCCTGCT	TACACTGTTTGCCTGCCAAG
Fted (281)	CGAGTAACGAACGGTGATGA	AATGATCGACTTGGATATTATCAG
ACTIN (150)	CGTTTCGCTTTCCTTAGTGTTAGC	AGCGAACGGATCTAGAGACTCACCTG

Table 7.5 List of Primers used for Gene related to flowering in *A.thaliana*.

Gene Acession number (Name, amplicon size)	Forward Primer(5' to 3')	Reverse Primer (5' to 3')
AT4G16280 (FCA, 229)	TTC GTC CAA TGG GTC CTA AC	AAG CTA CCA TGG TCT GAG AT
AT5G10140 (FLC, 155)	AGC CAA GAA GAC CGA ACT CA	TTT GTC CAG CAG GTG ACA TC
AT3G10390 (FLD, 176)	TGG AAT TTG GGA GTT TTT GC	TTG CTG CTG GTT GAA ATG AG
AT3G04610 (FLK, 180)	CAG GGG AGT ACC TGG TGA AA	TAA ACT GAG CCG TGT GTT GC
AT2G19520 (FVE, 241)	CAT CAC AAC GAT TGG GAC AG	AAC CTT CGT GAC AGG GTT TG
AT5G13480 (FY, 160)	AAG GTG GCA TGA ATC CTC AG	TGG AAA GGG TTG TTG TAG CC
AT2G43410 (FPA, 154)	ATG GGT GTC GAT GAG AGG TC	CAA TCC CCT TTC CCA TAG GT
AT1G65480 (FT, 219)	CTG GAA CAA CCT TTG GCA AT	AGC CAC TCT CCC TCT GAC AA
AT1G22770 (GI, 176)	GCA TCT AGT TGC TGG CCT TC	CTC GAA GGA GTT CCA CAA GC
AT4GO2560 (LD, 238)	GTC AAT GGA CCT TTG GCT GT	CGT CAA GTT CCA TTC CTG GT
AT2G45660 (SoC1, 201)	AAT TCG CCA GCT CCA ATA TG	CCT CGA TTG AGC ATG TTC CT

Table 7.6 Number of cells in the Meristematic zone at 0 and 5µM concentration of ABA.

Plant Number	**0µm ABA**			**5µM ABA**		
	WT	***sgd8-4***	***sdg8-2***	**WT**	***sdg8-4***	***sdg8-3***
1	31	29	24	29	25	20
2	32	28	23	31	23	20
3	30	29	27	29	21	18
4	35	29	27	29	21	20
5	33	31	26	31	22	21
6	32	30	27	31	23	18
7	37	31	27	30	21	23
8	28	32	26	28	22	21
9	34	31	27	29	24	20
10	33	31	26	32	22	19
11	34	32	26	29	22	24
12	34	31	26	30	26	24
13	0	0	0	28	24	21
Mean	32.75	30.3333	26	29.6923	22.7692	20.6923
SD	2.3788	1.3026	1.2792	1.2506	1.5892	1.9741
P- value	0	0.00667	$1.3x10^{-07}$	0	$1.47x10^{-11}$	$7.94x10^{-12}$

Table 7.7 Number of cells in the Meristematic zone at 10 and 20µM concentration of ABA.

Plant Numbr	10µM ABA			20µABA		
	WT	sdg8-4	sdg8-2	WT	sdg8-4	sdg8-2
1	29	20	18	26	21	19
2	27	24	19	24	22	23
3	29	27	20	25	23	18
4	28	25	18	26	22	20
5	29	21	20	26	23	18
6	31	22	18	29	23	19
7	27	21	20	28	24	20
8	29	22	20	29	24	22
9	28	21	21	27	26	23
10	28	22	23	28	25	21
11	28	25	21	27	22	22
12	25	24	21	27	26	20
13	27	23	20	28	23	20
14	0	0	0	24	26	23
15	0	0	0	24	23	22
Mean	28.0769	22.8461	19.9230	26.5333	23.5333	20.6666
SD	1.4411	2.0349	1.4411	1.7265	1.5976	1.7593
P-Value	0	$1.66x10^{-07}$	$2.54x10^{-13}$	0	$3.32x10^{-05}$	$5.65x10^{-10}$

Table 7.8 Cell size in the Meristematic and Elongation zones at 0μM concentration of ABA.

Plant Number	Meristematic Zone			Elongation Zone		
	WT	***sdg8-1***	***sdg8-2***	**WT**	***sdg8-1***	***sdg8-2***
1	7.5	7.5	5.625	93.75	100.625	87.5
2	8.125	8.125	6.25	101.875	106.875	91.875
3	8.125	6.875	6.25	102.5	106.25	93.125
4	8.125	6.25	6.25	108.75	101.875	91.875
5	8.125	6.25	6.875	100.625	100.625	92.5
6	8.125	6.25	6.875	103.125	100.625	88.125
7	6.875	6.25	6.25	103.125	100.625	89.375
8	6.875	6.25	6.25	103.75	101.875	90.625
9	8.125	6.25	6.875	101.875	100.625	91.875
10	7.5	6.25	7.5	103.75	101.875	89.375
11	8.125	5.625	6.875	105	101.875	91.25
12	8.125	5.625	7.5	106.875	100.625	91.25
Mean	7.8125	6.4583	6.6145	102.9167	102.0313	90.7291
SD	0.4985	0.7216	0.5627	3.6573	2.1996	1.7644

Table 7.9 Cell size in the Meristematic and Elongation zones at 5μM concentration of ABA.

Plant Number	Meristematic Zone			Elongation zone		
	WT	***sdg8-4***	***sdg8-2***	**WT**	***sdg8-1***	***sdg8-2***
1	8.125	7.5	6.25	103.125	51.25	63.75
2	6.875	7.5	5	103.125	57.5	67.5
3	7.5	7.5	5.625	102.5	61.25	68.75
4	8.125	7.5	5.625	105	52.5	66.25
5	8.125	7.5	5	102.5	61.25	58.125
6	8.125	7.5	5.625	106.875	56.25	66.25
7	8.125	8.125	6.25	100.625	53.75	65.625
8	8.125	7.5	6.25	102.5	56.25	64.375
9	8.75	6.875	6.25	108.75	51.25	57.5
10	8.125	7.5	6.25	107.5	58.75	66.875
11	7.5	7.5	6.25	109.375	52.5	66.25
12	8.125	6.875	6.25	109.375	51.25	65.625
13	7.5	6.875	6.25	112.5	52.5	70.625
Mean	7.9326	7.4038	5.9134	105.6731	55.0961	65.1923
SD	0.4694	0.3468	0.4851	3.6216	3.6961	3.7184
P value	0	0.0043	$1.4x10^{-10}$	0	$4.45x10^{-22}$	$4.4x10^{-07}$

Table 7.10 Cell size in the Meristematic and Elongation zones at 10μM concentration of ABA.

Plant Number	Meristematic Zone			Elongation Zone		
	WT	***sdg8-4***	***sdg8-2***	**WT**	***sdg8-1***	***sdg8-2***
1	6.875	7.5	6.25	76.25	43.75	53.75
2	7.5	6.875	5.625	76.875	52.5	49.375
3	7.5	7.5	6.25	78.125	36.25	48.75
4	6.875	6.25	6.25	76.25	52.5	49.375
5	6.25	6.25	6.25	78.125	55	50.625
6	6.875	6.25	6.875	78.75	52.5	50
7	6.25	6.875	6.25	80	53.75	52.5
8	6.25	7.5	6.25	78.75	55	51.25
9	6.25	7.5	6.25	80.625	52.5	50
10	6.875	6.25	5.625	84.375	52.5	50.625
11	5.625	6.25	5.625	0	55	50
12	6.25	6.25	6.25	0	51.25	51.875
13	5.625	6.875	5.625	0	55	55
Mean	6.5384	6.7788	6.1057	78.8125	51.3461	51.0096
SD	0.6046	0.5616	0.3744	2.4375	5.4117	1.8317
P value	0	0.3801	0.0502	0	6.29×10^{-12}	1.43×10^{-15}

Table 7.11 Cell size in the Meristematic and Elongation zones at 20μM concentration of ABA.

Plant Number	Meristematic Zone			Elongation Zone		
	WT	***sdg8-4***	***sdg8-2***	**WT**	***sdg8-1***	***sdg8-2***
1	6.875	7.5	5	65	58.75	46.25
2	6.25	7.5	5	57.5	56.25	40
3	6.875	7.5	6.25	61.25	51.25	47.5
4	7.5	7.5	5.625	63.75	55	47.8125
5	7.5	6.875	6.25	55.625	53.75	45.625
6	6.875	6.875	6.25	63.125	57.5	46.25
7	6.875	6.875	6.25	64.375	55	46.5625
8	5.625	6.25	6.25	63.125	51.25	47.5
9	5.625	6.25	5	64.375	52.5	46.25
10	6.25	6.25	5	60	53.75	46.25
11	5.625	7.5	6.25	0	52.5	45
12	5.625	6.25	5	0	55	44.0625
13	6.25	6.25	5	0	53.75	47.1875
14	6.25	6.875	5	0	52.5	47.5
15	5.625	6.875	5	0	53.75	0
Mean	6.3750	6.8750	5.5416	61.8125	54.1666	45.9821
SD	0.6764	0.5282	0.6190	3.17938	2.1478	2.01726
P value	0	0.04067	0.0018	0	$1.15x10^{-05}$	$5.21x10^{-11}$

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