EXPRESSION ANALYSIS OF FLOWERING SWITCH FACTORS USING CANDIDATE GENE APPROACH IN MANGO

Thesis

Submitted to the Punjab Agricultural University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE
in
BIOTECHNOLOGY
(Minor Subject: Fruit Science)

By

Shilpa
(L-2016-A-174-M)

School of Agricultural Biotechnology
College of Agriculture

LUDHIANA-141 004

2020

CERTIFICATE I

This is to certify that the thesis entitled, "**EXPRESSION ANALYSIS OF FLOWERING SWITCH FACTORS USING CANDIDATE GENE APPROACH IN MANGO**" submitted for the degree of **M.Sc.** in the subject of **Biotechnology** (Minor subject: **Fruit Science**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Shilpa (L-2016-A-174-M)** under my supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged.

_____

(Dr Gurupkar Singh Sidhu)
Major Advisor
Fruit Biotechnologist
School of Agricultural Biotechnology
Punjab Agricultural University
Ludhiana-141 004 (India)

CERTIFICATE II

This is to certify that the thesis entitled, "**EXPRESSION ANALYSIS OF FLOWERING SWITCH FACTORS USING CANDIDATE GENE APPROACH IN MANGO**" submitted by **Shilpa (L-2016-A-174-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.Sc.** in the subject of **Biotechnology** (Minor subject: **Fruit Science**) has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

(Dr. Gurupkar Singh Sidhu)
Major Advisor

(Dr. Manisha Thakur)
External Examiner
Assistant Professor
Department of Biotechnology
Dr. Y.S. Parmar University of Horticulture and Forestry,
Nauni, Solan – 173 230 (HP)

(Dr. Parveen Chhuneja)
Head of the Department

(Dr. Gurinder Kaur Sangha)
Dean, Postgraduate Studies

ACKNOWLEDGEMENT

First and Foremost, I would like to express my sincere gratitude to my advisor ***Dr. Gurupkar Singh Sidhu*** *for the continuous support in my thesis and related research, for his patience, motivation and immense knowledge. His aspiring guidance helped me in all the time of research and writing of this thesis. Without his help and guidance this work would not have seen light of the day.*

I would also like to thank rest of committee members ***Dr. Nav Prem Singh*** *and* ***Dr. Navraj Kaur Sarao*** *for their friendly advice and encouragement. I would also like to thank whole faculty for their comments and hard questions which directed me to widen my research from various perspectives.*

I would also like to extend my thanks to Director, School of Agricultural Biotechnology ***Dr. Parveen Chhuneja*** *who provided me with the facilities being required and conductive conditions for research.*

It is my pleasure to record deep sense of gratitude and sincere thanks to ***Mr. Inderjit Singh Yadav*** *and* ***Dr. Amandeep Mittal*** *for their encouragement, help and affection throughout the study period.*

I would also like to thanks my seniors ***Harmanpreet Kaur*** *and* ***Sumedha****, and my friends* ***Aman, Divya, Nidhi, Nishtha, Pooja, Shweta, Shanu and Yogita*** *for their help, stimulating discussions and sharing their truthful and illuminating views on a number of issues related to research.*

I express my profound gratitude to my parents and for the unceasing encouragement, support and attention throughout my years of study. This accomplishment would not have been possible without my sister and grandparents who directly or indirectly have supported me during this period.

And above us all ***The Almighty*** *for answering my prayers and for providing me the opportunity and giving me the strength to complete my research.*

Dated:

Place: ***(Shilpa)***

Title of the Thesis	**:**	Expression analysis of flowering switch factors using candidate gene approach in mango
Name of the student and Admission No.	**:**	Shilpa L-2016-A-174-M
Major Subject	**:**	Biotechnology
Minor Subject	**:**	Fruit Science
Name and Designation of Major Advisor	**:**	Dr. Gurupkar Singh Sidhu Fruit Biotechnologist
Degree to be Awarded	**:**	M.Sc. (Biotechnology)
Year of Award of Degree	**:**	2020
Total Pages in Thesis	**:**	50 + VITA
Name of the University	**:**	Punjab Agricultural University, Ludhiana – 141 004, Punjab, India

ABSTRACT

Mango (*Mangifera indica* L.) is a major fruit crop of the tropical and subtropical regions of the world. Some of the varieties especially grown in north western India are prone to alternate/biennial/irregular bearing which is controlled genetically. For the investigating this problem, the gene responsible for floral induction i.e. *Flowering Locus T* (*FT*) gene was isolated from the leaf tissues of the two mango varieties, Amrapalli and Dashehari. After confirmation and characterization, gene sequence (approximately 1.5 kb) was isolated. The gene sequence obtained for Amrapalli was 78 base pairs longer than the gene sequence of Dashehari. Blastn analysis of the isolated *FT* gene showed high similarity nucleotide sequence identity from different mango cultivars as well as other perennial fruit trees. The transcript expression of *FT* was evaluated in leaf tissues during early stage (mid-January) *i.e.* before flowering, mid stage; prior to induction of flowering (mid-February) and late stage; prior to flower bud initiation (early-March) using semi quantitative method in two consecutive years i.e. 2017 and 2018. Its expression level was more in mid stage as compared to other two stages. The expression level was more in Amrapalli in the year 2018 due to its regular bearing habit.

Keywords: Mango, Alternate bearing, floral induction, *Flowering Locus* T, Transcript expression, semi quantitative method

Signature of the major advisor **Signature of the student**

ਖੋਜ ਪੱਤਰ ਦਾ ਸਿਰਲੇਖ	:	ਅੰਬ ਵਿੱਚ ਪ੍ਰਤਿਆਸ਼ੀ ਜੀਨ ਵਿਧੀ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਫਲਾਵਰਿੰਗ ਸਵਿੱਚ ਕਾਰਕਾਂ ਦਾ ਐਕਸਪ੍ਰੇਸ਼ਨ ਮੁਲਾਂਕਣ
ਵਿਦਿਆਰਥੀ ਦਾ ਨਾਮ ਅਤੇ ਦਾਖਲਾ ਨੰ.	:	ਸ਼ਿਲਪਾ ਐਲ-2016-ਏ-174-ਐਮ
ਪ੍ਰਮੁੱਖ ਵਿਸ਼ਾ	:	ਬਾਇਓਟੈਕਨੋਲੋਜੀ
ਸਹਿਯੋਗੀ ਵਿਸ਼ਾ	:	ਫ਼ਲ ਵਿਗਿਆਨ
ਮੁੱਖ ਸਲਾਹਕਾਰ ਦਾ ਨਾਮ ਅਤੇ ਅਹੁਦਾ	:	ਡਾ ਗੁਰਉਪਕਾਰ ਸਿੰਘ ਸਿੱਧੂ ਫਰੂਟ ਬਾਇਓਟੈਕਨੋਲੋਜਿਸਟ
ਡਿਗਰੀ	:	ਐਮ.ਐਸ.ਸੀ.
ਡਿਗਰੀ ਮਿਲਣ ਦਾ ਸਾਲ	:	2020
ਖੋਜ ਪੱਤਰ ਵਿੱਚ ਕੁੱਲ ਪੰਨੇ	:	50 + ਵੀਟਾ
ਯੂਨੀਵਰਸਿਟੀ ਦਾ ਨਾਮ	:	ਪੰਜਾਬ ਖੇਤੀਬਾੜੀ ਯੂਨੀਵਰਸਿਟੀ, ਲੁਧਿਆਣਾ - 141 004, ਪੰਜਾਬ, ਭਾਰਤ

ਸਾਰ-ਅੰਸ਼

ਅੰਬ (*ਮੈਂਗੀਫਿਰਾ ਇੰਡੀਕਾ* ਐਲ.) ਉਸ਼ਣਕੱਟੀਬੰਧੀ ਅਤੇ ਉਪ ਉਸ਼ਣਕੱਟੀਬੰਧੀ ਖੇਤਰ ਦਾ ਇੱਕ ਪ੍ਰਮੁੱਖ ਫ਼ਲ ਹੈ। ਇਸ ਸਮੱਸਿਆ ਦਾ ਅਧਿਐਨ ਕਰਨ ਲਈ ਅੰਬ ਦੀਆਂ ਦੋ ਕਿਸਮਾਂ ਅਮਰਾਪਾਲੀ ਅਤੇ ਦਸ਼ਹਿਰੀ ਦੇ ਪੱਤਿਆਂ ਦੀਆਂ ਕੋਸ਼ੀਕਾਵਾਂ ਤੋਂ ਫ਼ੁੱਲਾਂ ਦੇ ਖਿੜਣ ਵਿੱਚ ਸਹਾਈ ਜੀਨ ਭਾਵ *ਫਲਾਵਰਿੰਗ ਲੋਕਸ ਟੀ* (*FT*) ਜੀਨ ਨੂੰ ਨਿਖੇੜਿਆ ਗਿਆ। ਪੁਸ਼ਟੀਕਰਨ ਅਤੇ ਚਿੱਤਰ-ਚਿੱਤਰਣ ਮਗਰੋਂ, ਪੂਰੀ ਲੰਬਾਈ ਵਾਲੇ ਜੀਨ ਅਨੁਕ੍ਰਮ (1.5 kb) ਨੂੰ ਨਿਖੇੜਿਆ ਗਿਆ। ਨਿਖੇੜੇ ਗਏ *FT* ਜੀਨ ਦੇ Blastn ਅਤੇ Blastp ਮੁਲਾਂਕਣ ਨਾਲ ਅੰਬ ਦੀਆਂ ਵੱਖੋ-ਵੱਖਰੀਆਂ ਕਿਸਮਾਂ ਅਤੇ ਹੋਰ ਫ਼ਲਾਂ ਦੇ ਰੁੱਖਾਂ ਤੋਂ ਉੱਚ ਦਰਜੇ ਦੀ ਨਿਉਕਲੀਓਟਾਈਡ ਅਨੁਕ੍ਰਮ ਸਮਰੂਪਤਾ ਦਾ ਪਤਾ ਚਲਿਆ। ਲਗਾਤਾਰ ਦੋ ਸਾਲਾਂ ਭਾਵ 2017 ਅਤੇ 2018 ਵਿੱਚ ਅਰਧ-ਮਾਤਰਾਤਮਕ ਵਿਧੀ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਸ਼ੁਰੂਆਤੀ ਪੜਾਅ (ਮੱਧ-ਜਨਵਰੀ) ਭਾਵ ਫ਼ੁੱਲ ਖਿੜਣ ਤੋਂ ਪਹਿਲਾਂ, ਫ਼ੁੱਲ ਖਿੜਣ ਤੋਂ ਪਹਿਲਾਂ ਦਰਮਿਆਨੇ ਪੜਾਅ (ਮੱਧ-ਫ਼ਰਵਰੀ) ਅਤੇ ਅੰਕੁਰ ਦੇ ਵਿਕਸਤ ਹੋਣ ਪਹਿਲਾਂ ਪਿਛਤੇ ਪੜਾਅ (ਮਾਰਚ ਦੀ ਸ਼ੁਰੂਆਤ) ਵਿੱਚ *FT* ਜੀਨ ਦੇ ਪ੍ਰਤੀਲੇਖ ਐਕਸਪ੍ਰੇਸ਼ਨ ਦਾ ਮੁਲਾਂਕਣ ਕੀਤਾ ਗਿਆ। ਬਾਕੀ ਦੋ ਪੜਾਵਾਂ ਦੇ ਮੁਕਾਬਲੇ ਦਰਮਿਆਨੇ ਪੜਾਅ ਦੌਰਾਨ ਇਸਦਾ ਐਕਸਪ੍ਰੇਸ਼ਨ ਪੱਧਰ ਵਧੇਰੇ ਸੀ। ਲਗਾਤਰ ਫ਼ਲ ਦੇਣ ਦੀ ਸਮਰੱਥਾ ਕਾਰਨ ਸੰਨ 2018 ਵਿੱਚ ਅੰਬ ਦੀ ਕਿਸਮ ਅਮਰਪਾਲੀ ਵਿੱਚ ਵਧੇਰੇ ਐਕਸਪ੍ਰੇਸ਼ਨ ਵੇਖਣ ਨੂੰ ਮਿਲਿਆ।

ਮੁੱਖ ਸ਼ਬਦ: ਅੰਬ, ਵਿਕਲਪਿਕ ਪ੍ਰਭਾਵ, ਫ਼ੁੱਲ ਪੈਣਾ, *ਫਲਾਵਰਿੰਗ ਲੋਕਸ ਟੀ*, ਪ੍ਰਤੀਲਿਪੀ ਐਕਸਪ੍ਰੇਸ਼ਨ, ਅਰਧ-ਮਾਤਰਾਤਮਕ ਵਿਧੀ

ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Mango (*Mangifera indica* L.) is a major fruit crop of tropical as well as subtropical regions of the world. Morphologically, it belongs to the subtype drupe, is single seeded and seed is surrounded by fleshy mesocarp. Its fruit is dicotyledonous in nature belonging to family Anacardiaceae. The centre of origin is Indo-Burmese region. Mango is one of the most important fruit crop of tropical Asia and is grown on commercial scale in more than 87 countries. It currently ranks fifth in total production amongst other major fruit crops worldwide. The total mango production worldwide is approximately 40 million tonnes (Mitra 2016). India holds first rank amongst world's major mango producing countries, contributing 54.2 per cent of the total mango production. Other major mango producing countries are China, Thailand, Indonesia and Philippines. Generally, fresh mango fruit is consumed but a considerable amount is processed and traded internationally as pulp, puree, concentrate and juice.

Mango is commercially the most important fruit crop of India, with more than a thousand varieties known so far. It is rich in vitamin A and C. It contains proteins (0.6%), fair amount of carbohydrates (11.8%), minerals like calcium, phosphorus and iron (0.3%). Mango fruit is utilized at all stages of development. It is aptly acknowledged as the "King of Fruits" in India. This crop accounts for 39.0 per cent of total area under fruit crops in India and 23.0 per cent of total production of these crops. In India the total area under cultivation is 12.2×10^6 hectares. The highest production occurs in the state of Andhra Pradesh producing 2.9×10^6 MT per year with 0.24×10^6 hectares area under mango cultivation (Anonymous 2019a). Uttar Pradesh, Bihar, Karnataka, Odisha, Maharashtra, West Bengal and Tamil Nadu are the other main mango producing states. Most of the Indian varieties possess strong aroma and intense peel coloration, characterized by attractive fragrance, delicious taste, and high nutritional value, owing to high amount of vitamin C, β-carotene and minerals. The income accounted from the export of fresh and processed fruit was 41.5 per cent of the total income from export in mango. Also, India export fresh mango fruits to approximately 50 countries. More than 90 per cent of mango fruit is exported to seven countries *viz.*, UAE, Saudi Arabia, Kuwait, UK, Singapore, Netherlands and Bangladesh. In case of export market, the processed form of mango is highly demanded as compared to fresh mango fruit due to the lesser shelf life of the fruit as well as its availability during single season only.

In Punjab, it is being cultivated on an area of 6896 hectares with an annual production of 116515 metric tonnes (Anonymous 2019b). Horticultural varieties of *M. indica* has

chromosome no. 2n=40 and has a small genome size of 439 Mb (Singh *et al* 2016). However, the yield in India is low as compared to many other countries. The major reasons for its low yield are poor management, inadequate nutrient application, irregular biennial flowering of most of the cultivars grown commercially. The behaviour of irregular bearing is common in many fruit trees like pear, apple, citrus, apricots etc.

In mango, the flower bud differentiation occuring in the shoots is followed by flowering. This differentiation period differs amongst varieties and is also dependant on the climatic conditions of the local area. The mango inflorescence is hermaphrodite in nature and after pollination and fertilization, sets fruit. In case of mature mango trees, there is production of approximately 1000 inflorescence each with 500–6000 flowers. Whereas, the setting of fruit is generally less than 10 per cent and only 0.1–0.25 per cent make upto the harvesting stage. Generally, the fruit development in mango tree constitute of four stages. The first stage persists for 21 days after fruit has been set and leads to abrupt growth of the cells in the immature/juvenile stage. The maximum growth stage is the second stage (21-49 days) that marks in cell enlargement and maturity. The third stage is the maturation and ripening stage of 49-77 days period. The fourth stage is the senescence stage (77^{th} day onwards), also known as post-ripening stage. The growth pattern of the mango appears to follow the form of a simple sigmoid curve whereas other fruits follow double sigmoid curve. The softening and colouring of the flesh starts from the seed to the outward of the fruit; at this stage, the fruit becomes enclosed by a cartilaginous resulting in strong endocarp. These changes are easily visible and can be used as a measure of judging the optimum date of picking fruits for direct consumption and for better storage (Tharanathan *et al* 2006). But the flowering process in mango is exceptionally complex. Normally, it bears heavy crop in one year (on year) and less or no crop in the following year (off year). The term irregular, alternate or biennial bearing implies to the tendency of mango trees to bear a heavy crop in one year and very little or no crop in the following year. Several studies in various plants have shown that the process of development of flowers can be better understood by not only understanding the internal factors such as regulation of multiple genes and their regulation, but also various environmental or external factors such as temperature, vernalization etc. During latest research in plants, the results showed that the *Flowering Locus T* (*FT*) gene is studied widely in promotion of early flowering. Also, its homologous genes such as *CiFT*, *Hd3a*, *PtFT1* and *SFT* have been isolated from different plants such as citrus, rice, poplar and tomato, respectively. It was also observed that if these genes are overexpressed in transgenic plants

then there was promotion of early flowering. The *FT* gene and its homologous genes which were found in different plants are also assumed to play an important role in the same way.

Flowering is either governed autonomously or by different internal, external and genomic factors as well as the interactions between these factors affecting the formation of flowers in most fruit crops or other perennials. For example, different external factors like temperature, water and photoperiod stress etc. and other internal factors like interaction with other organs, carbon-nitrogen ratio, hormonal level affects the flower formation in various fruit crops. The flowering mechanism was first studied by Sachs in 1865, who did the flowering induction experiment and hence proposed the flowering material concept. His different studies and experiments led to the observation that there is a factor for floral promotion produced first in the leaf region and is transported later to the stems bearing buds capable of flowering during the favourable conditions (Chailakhyan 1936a). The concept of the flowering hormone was then proposed by Chailakhyan (1936b). The various experiments conducted confirmed that the presence of flowering hormone is a common component responsible for floral initiation in different angiosperms (Chailakhyan 1936c). Huang *et al* (2005) put forward a theory associated with the hormone related to flowering that it might have relation with the mRNA. This brought the attention of other scientists towards the related genes and its products. However, Corbesier *et al* (2007) later suggested that mRNA is an intermediary product and not a flowering hormone, while the real flowering hormone should be the end product protein. Two expeiments were conducted to prove this assumption. In the first experiment, protein fused to green fluorescent protein (GFP) to confirm that the protein is transported to a long distance to promote flowering. The second experiment included the grafting of *Arabidopsis* mutants and it also proved that the long distance transport of protein ultimately promotes flowering. During the above performed experiments, the transport of mRNA was not detected. All these observations led to the conclusion that the protein may be the major element of the "Flowering hormone". In case of *Arabidopsis,* favourable environmental conditions *i.e.* long-day photoperiod are required by the protein to move from leaves to the shoot apices and ultimately interact with *Flowering Locus D* (*FD*). The complex formed is *FT-FD* complex and it either directly or indirectly activates the flower-type genes, such as *APETALA1* (*AP1*) genes, *LFY* (*LEAFY*) genes etc. These genes convert the vegetative apices into the inflorescence meristems leading to the initiation of flowering. The protein is also proven to be a part of the phloem transfer signal and earlier has been verified as the homologue of the protein in sap present in phloem of gourds, rice and mustard. Consequently, similar studies showed that during the process of flower initiation,

the signal of floral promotion transfer from leaf region to the apical meristem and it mainly constitutes the protein.

For promotion or inhibiting flower production the plant growth regulating substances in mango have been tested and it was observed that these substances have varying effects, depending on cultivars and the climate. There are different types of plant stress depending either on the low temperature or drought or girdling of branch/trunk, which checks vegetative growth leading to floral induction in mango. Such treatments mark the conductance and gas exchange capacity of the leaves, the significance of which to flowering is, however to be determined. Further researches have unraveled the relationship between carbohydrate level stomata, shoot maturity and the synthesis and nature of the floral stimulus in mango leaves (Iyer and Subramanyam 1993). The maturity of terminal shoots and accumulation of carbohydrate in the shoot apex experimentally indicates that these are somehow associated with the synthesis of floral stimulus, the absence of which can result in lack of flowering or setting of bienniality of bearing in mango. The whole flowering process involves the action of two groups of genes occurring sequentially, *i.e.* the genes those acts as switch between the transition of meristem from vegetative to floral (floral meristem identity genes), and those that further leads to the differentiation of flower and formation of its various parts (organ identity genes).

The regular bearing mainly seems to be associated with Floral Induction (FI) rather than floral organ differentiation, so, it may be hypothesized that in this phenomenon floral meristem identity genes and floral integrator genes are involved. The key genes that regulate the development of flowers have been identified in different model plants including *Antirrhinum majus* and *Arabidopsis thaliana*. These key genes include one of the flowering promoter gene *i.e. FLOWERING LOCUS T* (*FT*), encoding for a protein which is a major component of florigen and other genes *i.e. APETALA1* (*AP1*) and *LEAFY* (*LFY*) genes, which are identified as essential for determining the identity of floral meristem. Other genes such as *TERMINAL FLOWER 1* (*TFL1*), *FLOWERING LOCUS C* (*FLC*), *SHORT VEGETATIVE PHASE* (*SVP*) and *BROTHER OF FT (BFT*), are identified as the repressors of the floral pathway integrators. Even though the process of flowering is fundamentally different between annual and perennial plants but both, the FI and floral organ formation, seems to have genetic similarities among themselves (Tan and Swain 2006). The perennial fruit crops have long gestation periods/cycles and due to which there is less information on the flowering time genes as well as the other genes related to it.

The complex phenomenon of alternate or irregular bearing in mango including other horticultural traits associated has been studied by various research workers throughout the world. The pace of the selection can be increased with the integration of the molecular markers with conventional breeding (Sharma *et al* 2018). Similarly, by molecular mapping, the recovery of hybrids for the complex traits could be improved. But, for the use of molecular markers, indirectly for the selection of the target trait, mapping of the above stated traits with the tightly linked markers is required. In addition, analysis of transcriptome may result in several genes that express differentially, resulting in the fractional identification of mechanisms that convert 'on' into 'off' buds (Wu *et al* 2014). Allele specific expression in heterozygous plants like mango can be observed using RNA-sequence. The data of the hybrids between divergent genotypes can be used to deduce the presence of cis-regulatory variation that effect the expression levels of flowering gene, to find out regulatory regions and their respective functions in mango. Other candidate genes could be recognized in these studies and it was found that the differential expression of these genes could be associated with different parameters such as growth habit and variation in architecture of plants. A comprehensive study is required so that the transcriptional regulation, involvement of regulatory genes switching 'on' and 'off' mechanism and alternative splicing during vegetative to flowering and fruiting transition could be coordinated. To study the regular/alternate bearing behaviour of mango, transcriptome profiling is needed for both types of mRNAs and small regulatory RNAs. On the basis of gene regulation, differential expression analysis was noticed, that is, under the effect of changing physiological situations or different stimulation levels, the expression level gene may increase or decrease. The information generated can be used for mapping the genes responsible of regular bearing and will facilitate molecular breeding in mango.

Recently, in herbaceous plant like *Arabidopsis thaliana*, orthologous of *Flowering Time* gene (*FT* gene) as originators of florigen have been identified. Various studies related to mango reported the presence of Florigen promoter, produced in leaves of mango at levels which are temperature dependant (Zeevart 2006). Hence, intensity of flowering varies from year to year depending upon climatic conditions. Other reason like level of carbohydrate is associated with intensity of flowering. Isolation of these flowering related gene and investigation of their expression at different growth stages will considerably aid in understanding the initiation of flowering in mango.

In perennial trees, the application of *FT* is of specific utilisation as it can enable as well as accelerate new types of breeding and research. Further, the information related to its

application will be useful in selecting suitable recombinants and hybrids in early nursery stage itself. This can significantly be used in better understanding of the problem of long gestation periods and other economic restrictions as well as in overcoming them.

Keeping all the factors in mind, it is of utmost importance to ascertain the expression analysis and regulation of flowering time gene in mango. Therefore, the present investigation was designed with the following objectives:-

1) Identification of haplotypes and cloning of flowering time gene in regular and irregular bearing varieties of mango.
2) Expression analysis of flowering time gene in mango.

CHAPTER II

REVIEW OF LITERATURE

Mango is native to Indo-Burma region and one of the most popular among all tropical fruits. Mango belongs to genus *Mangifera* consisting about thirty species of the tropical fruiting trees in the family Anacardiaceae. In general the production of mango tree is good but some varieties have lower yields and have tendency of agriculturally undesirable problem of alternate bearing because of irregular flowering. A *Flowering Locus T* gene (*MiFT*) has been isolated from mango and it resembles with the *FT* genes of other crops. This *MiFT* gene has a significant role in mango flowering. The review of literature on 'Expression analysis of flowering switch factors using candidate gene approach in mango' has been discussed under following sub headings:

2.1 Genetics of flowering in plants

2.2 Plant growth hormones involved in flowering

2.3 Mechanism of flowering in mango

2.4 Identification of candidate gene for flowering

2.5 Role of *FT* gene in flowering in different crops

2.1 Genetics of flowering in plants

In angiosperms, flowering is a central developmental process for reproductive success and continuity of the species over time. The annual angiosperms (annual plants) complete their life cycle within a year whereas others have a longer reproductive life and is characterized by the generation of new vegetative and flowering shoots every year (perennial plants). Even with these lifespan differences, the fundamental genetics of flower induction and floral organ formation appears to be similar among these plants. Therefore, the knowledge obtained from the study of the flowering mechanism in *Arabidopsis thaliana* can be used to understand similar processes in other plant species in a better way, in particular the perennials, which are generally not suitable for genetic analysis and also have a long generation time. Using *Arabidopsis* as a model, Tan and Swain (2006) briefly discussed how the knowledge on current understanding of the conversion of vegetative phase to reproductive phase and the formation of individual floral organ can be successfully applied for the identification of homologous genes from perennial crops. Although annuals appear to share many similarities with perennials in terms of gene function, they differ in their commitment to flowering. In case of annuals, once they reach the reproductive phase, all meristems convert into either inflorescence or floral meristems. Whereas, in perennials, each meristem has the choice of either producing a reproductive shoot or a vegetative shoot, each year. Citrus

flowering physiology and genetics have been used to highlight the complexity of reproductive development in perennials and to discuss possible future research directions. Flowering is the first stage of sexual reproduction and is of supreme importance in horticulture, agriculture and plant breeding. This change from the vegetative state to the reproductive state is one of the most intense events in the ontogeny of a plant. Flowering in plants leads to the progression of other important events like anthesis, fruit set, fruit development, maturation and ripening. It helps in the crop improvement through genetic recombination and provides for the propagation of the species. Because of its great utility, mango occupies a well-known place amongst the fruit crops grown in India. The habit of flowering and fruiting in mango varies widely due to different reasons including varietal differences and diversified agro-climatic conditions. It is highly cross pollinated crop, contains male and hermaphrodite types of flower. The flowering mechanism of mango is very complex process and many factors including physiological and environmental affects it (Sandip *et al* 2015).

The response of flowering to different environmental factors such as humidity, light intensity, temperature, water availability etc. involves numerous signalling pathways that converge to the regulation of floral meristem identity genes. The first of the genes to be identified were [*LEAFY* (*LFY*) and *APETALA 1* (*AP1*)] because their mutation clearly disturbs the outcome of the production of shoot apical meristem. Other genes are called 'integrators' and they act upstream of *LFY*/*AP1* and their mutations delay flowering severely in different growing conditions. These genes include *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*). The most models proposed four promoting pathways, until recently (Bernier and Perilleux 2005).

2.1.1 Primary promoting pathways

The first signalling pathway in response to Long Durations (LDs) promotes flowering. It includes the genes that encode the photoreceptors phyA, cry2 and the clock components that are needed for a correct circadian time measurement, several clock-associated genes, such as *GI*, and the downstream gene *Constans* (*CO*). The abundance of *CO* depends on photoperiod and only in LDs a large amount of protein coincides with the presence of light, sensed by phyA and cry2. This 'external' coincidence allows *CO* to activate its target *FT*, *SOC1* and *TFL* are the other targets of *CO*.

A second pathway also known as 'vernalization' pathway gets stimulated by a specific duration of cold period conditions and acts through suppression of one of the strong repressor of flowering *i.e. FLC* gene. After optimal vernalization, the expression of *FLC* is eliminated and this repression becomes stable. This pathway also involves the functions of other genes, the *VIN3* and *MAF2* genes, which makes sure that cold periods of inadequate

duration will not cause flowering. Another gene involved is the *VRN* genes, which are necessary for the maintenance of *FLC* repression once warm temperature conditions occur.

A third pathway also known as 'autonomous' because it was initially found that it promote flowering independent of environmental factors. This pathway includes subgroups of independent genes, such as *FCA*/*FY*, *FVE*/*FPA*, *LD* and *FLD*. These encourage flowering and this effect is mainly applied through their common suppressive function on *FLC*. Thus, both the pathways *i.e.* autonomous as well as vernalization pathways cooperate to down-regulate the expression of *FLC*. In contrast, there is a positive regulation between *FLC* and *FRI*: in so-called 'winter' (late-flowering) accessions having an active *FRI* gene, repression of *FLC* by the autonomous pathway is entirely overcome (thus vernalization is absolutely required), whereas 'summer' (early-flowering) accessions with a loss-of-function *FRI* gene are not dependent on vernalization for flowering. FLC-dependent pathways also interact with the photoperiod pathway. In addition, the *VRN1* gene acts in *FLC* independent pathway to positively regulate the downstream gene *FT* (Boss *et al* 2004).

A fourth pathway also known as 'GA promotion' pathway includes genes, such as *GA1*, *GAI*, *RGA*, *FPF1* and *AtMYB33*, having roles in GA biosynthesis or signalling. The physiologically active GAs is applied to promote flowering in *Arabidopsis*, especially in short days (SDs). Their amount increases in the shoots of plants after SDs are transferred to LDs. However, mutations that impair the GA pathway are inhibitory to flowering in plants grown under SDs. This indicates that the importance for flowering of these promotive hormones is greater under unfavourable day length conditions under favourable ones. However, recent evidence links the GA and LD pathways (Tseng *et al* 2004). Early flowering is caused in SDs if mutations at the EBS locus occur; this phenotype requires GA biosynthesis and is due to the fact that EBS normally represses the expression of *FT*.

2.2 Plant growth hormones involved in flowering

In plants, florigen is a mobile flowering signal. It strongly influences the reproduction process in plants and is considered as one of the most significant target in crop improvement. At the molecular level, florigen is considered as highly conserved region and characterised as a protein product encoded by the *FLOWERING LOCUS T* (*FT*) gene in flowering plants. Further light has been shed on the presence of intercellular receptors for florigen, an essential transcriptional complex required for the functioning of florigen and the molecular basis of pleiotropic function of florigen beyond flowering due to the recent advances in cell biology, molecular genetics and structural biology in plants. Moreover, other technologies, such as next generation sequencing and live cell imaging has revealed the detailed distribution of florigen and transcriptional targets of the Florigen Activation Complex (FAC) during early stages of floral transition.

Chailakhyan in 1930s proposed the florigen as the universal and long distance flowering regulator. This leads to the discovery that *FT*-like proteins helped in the prediction for florigen. These are small globular proteins having an approximately 175 amino acids, belonging to phosphatidylethanolamine (PEBP) binding proteins. These are graft transmissible and effectively stimulates the process of flowering in many plants as these have the ability to move to the apex of the shoot. There has been recent identification of the genes that regulate the movement of *FT* protein in *Arabidopsis* and some features of the *Arabidopsis FT* protein that makes it an effective florigen (Putterill *et al* 2013).

The flowering in the plant photoperiod pathway can be stimulated by *FT*. It can also facilitate different types of flowering pathways including vernalization flowering pathways. The products of the *FT* gene and their expressions are documented as essential parts of the flowering hormone. It can also be transported to a long distance in plant, leading to the induction of flowering. In the study by Xu *et al* (2012) in China, there was isolation of several *FT*-like genes and the results of transgenic show that the *FT* gene can be responsible for promoting flowering in plants.

In flowering plants, the differentiation of the shoot apical meristem into a floral meristem is one of the key developmental processes. The regulation of this transition is through the integration of environmental and endogenous stimuli and inovles a complex, hierarchical signalling network. In *Arabidopsis*, the *FLOWERING LOCUS T* (*FT*) protein which is a mobile signal has been recognized as a major component of florigen. It has a central role in mediating the onset of flowering. *FT*-like genes seem to be involved in the regulation of the floral transition in all angiosperms examined till date. The studies on molecular evolution suggest that the development of *FT*-like genes coincides with the evolution of the flowering plants. Hence, the role of *FT* infloral promotion is conserved, but it appears to be restricted to the angiosperms only. *FT*-like proteins are also major regulatory factors in a varied range of developmental processes including vegetative growth, fruit set, stomatal control and tuberization. These multiple roles of *FT*-like proteins resulted from extensive gene duplication events in almost all modern angiosperm lines, followed by neo- or sub-functionalization (Pin and Nilsson 2012).

2.2.1 Flower-promoting material *FT*

2.2.1.1 Flowering pathway and basic functions of *FT* genes

The *FT* gene was isolated in early 1999, but a certain understanding about its homologous gene and its regulatory networks have been built in recent years only. For example, under long-day conditions in *Arabidopsis,* flowering is modulated when the *FT* gene regulation obtains the signal from the photoperiod regulatory centre gene *i.e. CONSTANS*

(*CO*). The *CO* transcription factors may possibly interact with the promoter of *FT* gene. Secondly, through the flowering integration genes, *SUPPRESSOR OF OVEREXPRESSION CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*), which ultimately promotes *Arabidopsis* flowering, the integration of flower induced signals by the *FT* gene from the photoperiod, autonomous and vernalization pathway is promoted (Yoo *et al* 2005). The *FT* gene should therefore be the final target gene of the life cycle.

The early flowering characteristics of transgenic plants with overexpression of the *CO* gene could be eliminated by *FT* gene mutations, while transgenic plants with overexpression of the *FT* gene showed early flowering in both short and long days. *CO* and *FT* are normally expressed completely in vascular bundles, while *GIGANTEA* (*GI*) is expressed in various tissues. *GI* expressed in vascular or mesophyll tissues increases expression of *FT* without up-regulating expression of *CO* under short-day conditions. For example, *GI*s ectopic expression test showed that *GI* can activate *FT* expression under short day conditions in a *CO*-independent manner by binding and weakening several *FT* repressors, such as SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO (TEM1) and TEM2. Hence, the *FT* gene is helpful in promotion of early flowering in plants as well as in regulation of different developmental changes occurring in pods, seeds and other plant parts (Sawa and Kay 2011).

Arabidopsis is a long-day plant. If the plants of *Arabidopsis* are placed suddenly in a short-day environment then there will be minimal change in their growth, but there will be significant reduction in the level of *FT*. In case of poplar tree, the development termination in short-day plants, bud differentiation, initiation of dormancy during vernalisation and other developmental processes are controlled by *FT* homologous genes. In Norway Spruce, *FT* homologous genes regulate differentiation of flower bud, formation of floral structure, and other transition processes. This suggests that among gymnosperms, *FT* behaves in a very conservative manner. The photoperiod pathway as well as the environmental temperature, both regulates *FT*. During low temperature conditions, *Arabidopsis* low temperature signal is synchronised by the pathway of and the development process is induced when there is reduced *Flowering Locus C* (*FLC*) level, which regulates upstream *FT* expression. On the other hand, the "ambient temperature transcriptome" changes due to rise in temperature and this change also leads to an elevation in levels of auxin, increased abundance/activity of Phytochrome Interacting Factor 4 (PIF4) and greater *FT* expression. The temperature and photoperiod can therefore control the *FT* expression independently (Franklin *et al* 2011).

2.2.1.2. *FT* homologous genes and signal transfer

There has been enormous research about the *FT* genes and its function in recent years which has raised the interest to study *FT* genes in diverse plant species. It has led to the extensive study of role of *FT* homologous genes in homologous as well as heterologous plant systems. *FT* genes have been isolated from many plants, including crop plants, like *Oryza sativa* and *Hordeum vulgare*; fruit plants like *Vitis vinifera* , *Malus domestica* and *Pyrus communis*; vegetable plants, like *Solanum lycopersicum*, *Cucurbita maxima*, *Solanum tuberosum*, *Pisum sativum*, and *Beta vulgaris*; ornamental plants like *Ipomoea nil*, *Chenopodium rubrum*, *Oncidium luridum* and *Helianthus annuus*. The early flowering in tomato, pear, poplar and sunflower might be due to overexpression of *FT* homologous genes and this indicates that the function of *FT* homologous genes is conserved in nature. The *FT* gene is a part of a small gene family of floral regulators. In rice, 13 *FT*-like members were found. *RFT1* is one of their members that lies nearby to *Hd3a*, and its amino acid sequence shows 91 per cent identity with that of *Hd3a*. In barley, three members of *FT* were found. Barley has its adaptive mechanism where it uses combination of different *FT*- like genes to adjust the flowering according to the change in photoperiod. In addition, it has been shown in case of grapes and maize that the transcripts of *FT* homologous genes increases before the induction of flowering. Xi and Yu reported that, in different plants including *Arabidopsis*, various flowering genes and *FT* expresses mainly in the vascular tissues of leaves, whereas in soybeans, *FT* homologous genes express mainly in the petiole region during the period of flowering. Thus, in soybean and other crops, petioles may play key role in the regulation of flowering, and may be the regions of different flowering related genes. There can be transfer of *FT* signal by grafting. In case of tomato and tobacco, early flowering gets promoted if then *FT* homologous gene of tomato *i.e. Single Flower Truss* (*SFT*) is overexpressed (Cao *et al* 2016). There can be early flowering of *SFT* induced by SFT protein, if the scion used to graft onto the rootstock of the *SFT* mutant is from a transgenic plant having constitutively expressed *SFT*. On the other hand, the *SFT* mutant couldn't promote flowering when it is used as a scion so that it can be grafted onto the wild-type rootstock. This indicates that any change in level of expression of *SFT* results in the change of outcomes also. Lin *et al* (2007) detected *FT* protein transfer by using rootstock and scion of different crops and extracted the *FT* protein. Whereas, Zhang *et al* (2010) performed another experiment to study the grafting transfer of the *FT* signal using rootstock of transgenic plants and scion of non-transgenic plants. The results showed that the grafting method of split-connection was not able to induce early flowering in the non-transgenic scion, but after the grafting was done, the rate of

flowering decreased by heat-shock induction in transgenic rootstock as compared to the time when grafting is not done indicating the fact that *FT* signal in rootstock was transferred partially after grafting. From these studies, it can be concluded that there must be transport of *FT* over a distance *i.e.* to the top so as to promote flowering ultimately.

2.3 Mechanism of flowering in mango

A model of mango flowering that was previously presented had three suppositions. The very first was that the gibberellic acid (GA) acts as a floral inhibitor leading to a condition where mature mango trees are unable to flower. The second assumption was about the decreased GA levels beyond a certain threshold leading to the initial floral stage development which may be due the carbohydrate accumulations. The last assumption was that the spray of "flower-inducing" chemicals like ethylene, potassium nitrate or thiourea may lead to initiation of floral bud break stage. The above given model was verified using 13-year old 'Carabao' mango trees in which the application of paclobutrazol (PBZ) per meter canopy diameter was reduced to 1.0 gram so as to artificially reduce gibberellic acid (GA_3) level in the plants. Thereafter, every following month the levels of GA_3, presence of floral initials in the terminal shoots and total non-structural carbohydrates were monitored. After an interval of three months, in case of PBZ-treated trees it was recorded that due to decreased PBZ application, there was a reduction in GA_3 content of shoots by more than 79 per cent as compared to the control. At the same time, 56 per cent of the terminal shoots in PBZ-treated trees had floral initials and none in the case of control trees. By the fourth month, in PBZ-treated trees there was an increase in the shoots with floral initials to 86 per cent while 42 per cent of the control trees also had floral initials just before KNO_3 was sprayed. The development of floral initials was accompanied by a decrease in GA_3 levels and a parallel increase in starch levels in the leaves, stem and apical buds. Induction of flower bud break by 2 per cent KNO_3 resulted in a flower intensity index of 4 (flowers all over the canopy) and longer inflorescences in PBZ-treated trees while control trees exhibited a flower intensity index of 2 (less than 25 per cent of the canopy have flowers). This study results in the certification of the model by the demonstration that the low GA content in the shoots of mango allows total non-structural carbohydrates, primarily starch, resulting in its accumulation in the buds and leaves leading to the early floral initials formation. The floral initials were already present before the application of KNO_3 demonstrating that the bud break induction of quiescent pre-existing floral buds as well as the transformation of vegetative buds to reproductive ones is independent of this chemical (Protacio *et al* 2009).

Flowering in mango is a dynamic physiological event that initiates fruit production. The first event is the initiation, for the mangoes to flower. It coincides with shoot initiation;

induction occurs based on the conditions present at the time of initiation. Studies by Ramirez and Davenport (2010) in Colombia were done with mango trees that support the presence of a Florigenic Promoter (FP) being infinitely produced in leaves of mango and brings flowering. The experiments of translocation concluded that the FP is translocated from leaves to buds through vascular tissue *i.e.* phloem. The interaction between FP and a Vegetative Promoter (VP) leads to flower induction. The FP is translocated approximately 100 cm in semitropical conditions and 52 cm in tropical conditions. It was reported that tip pruning is accurate to coordinate vegetative flush events within the cover and nitrate (KNO_3) to stimulate flowering in adequately matured stems. In semi-tropical conditions, cool temperature is an important measure for floral initiation in mango.

Villalobos *et al* (2012) in Mexico designed two pair of primers from *M. indica* to multiply fragments of *FT* transcripts. Designed primers *i.e. FTf1*/*FTr2* and *FTf2*/*FTr2*, amplified fragments of nearly 210 and 150 bp, respectively, and sequenced using Sanger platform. The sequenced fragments were evaluated and BLAST was used to compare them with *FT* submitted in the NCBI GenBank database. FT transcripts of 207 and 147 bp from *M. indica* presented resemblance with FT of *Populus nigra* (86 and 84 per cent, respectively). *FTf2*/*FTr2* was capable of amplifying 150 bp fragments from *Duranta dombeyana, Gazania linearis,* and *Lantana camara,* which showed major resemblance to FT of *Xanthium strumarium* (82 per cent), *Helianthus annuus* (91 per cent), and *Ficus carica* (79 per cent), respectively. In addition, phylogenetic relationship analysis showed that the fragment of 147 bp from *M. indica* FT transcript has more similarity to those belonging to the subclass Rosidae, while *FT* from *D. dombeyana, G. linearis* and *L. camara* are more related to the subclass Asteridae. They concluded that these primers may be of use in amplification of fragments of *FT* transcript from other angiosperm species for various other applications, like analysis of expression under diverse conditions, isolation of full length *FT* transcripts, etc. This will lead to recommend more accurate models and may be a substitute in regulation of flowering in plants.

In order to develop genomic marker resources for mango, Ravishankar *et al* (2015) used next-generation sequencing technology on the Illumina HiSeq 2000 platform to sequence the genomic DNA and examined this sequence data for microsatellite markers. High-quality raw data was obtained and it was assembled into 198,612 contigs after optimization. From these data, 159,228 scaffolds were generated which covers a genome size of 253.6 Mbp. A total of 106,049 microsatellite repeats were identified from these scaffolds. Primers were designed for 84,118 microsatellites. To determine polymorphism and cross-species amplification, ninety simple sequence repeat (SSR) markers were tested, in 64 mango

cultivars and four *Mangifera* species. Thus, their study revealed 2103 alleles, and 15 to 36 allele numbers per locus. The majority of these markers amplified DNA in related species with a transferability of 94.4–98.8 per cent. This increases the sequence coverage of the mango genome and the number of mango-specific SSR markers which will be useful in diversity, identification, mapping and breeding studies.

Singh *et al* (2016) sequenced the RNA of mango cultivars 'Neelam', 'Dashehari' and their hybrid 'Amrapalli'. The sequencing revealed high level of heterozygosity in 'Amrapalli' over its parents and helped in developing Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP) markers. They sequenced double digested restriction-site-associated genomic DNA (ddRAD) of 84 different mango cultivars and identified 1.67 million high quality SNPs and two major sub-populations. They assembled 323 Mb of the highly heterozygous 'Amrapalli' genome using long sequence reads of PacBio single molecule real time (SMRT) sequencing chemistry and predicted 43,247 protein coding genes. They identified 122,332 SSR loci and development of 8,451 Type1 SSR and 835 ISSR markers in mango genome. Among the published genomes, mango showed highest similarity with sweet orange (*Citrus sinensis*). These genomic resources will lead to the mango varietal improvement for high productivity, disease resistance, superior end use quality etc.

The molecular genetic information on flowering-related genes is available in many horticultural crops but the information on this aspect is not available in case of mango. Vyavahare *et al* (2017) isolated and evaluated three *FT*-like genes *i.e. MiFT1*, *MiFT2* and *MiFT3*, and two *TFL1*-like genes, *MiTFL1* and *MiTFL1a*, in mango cultivar 'Alphonso'. The results revealed that genomic *MiFT1* and *MiFT2* are 2540 and 1509 bp long, while cDNA are 543 and 519 bp, respectively. It also showed that *MiFT1* and *MiFT2*, both, have three introns and four exons; however, interestingly, *MiFT3* has no intron. The expression of *MiFT2* was not detected at any stage, suggesting that this gene may be non-functional. Mango *FT* sequences have showed similarity to *FT*-like genes of the citrus group. On genomic DNA, *MiTFL1* is 1055 bp long, while its cDNA is 528 bp. *MiTFL1a* is 1179 bp on genomic DNA, while its cDNA is 519 bp. The expression analysis of these genes suggested that *MiFT1*, *MiFT3* and both the *TFLs* are the significant regulators of flowering in mango.

Alternate bearing is a common problem in most of the mango cultivars whereas some do not exhibit it. The flowering in mango can be managed by proper management of nutrients, timely pruning and the efficient use of growth regulators, but there is partial information on the flowering in mango on the genetic as well as molecular basis. To ensure profuse and early mango flowering, Paclobutrazol (PBZ) which acts as an inhibitor of gibberellic acid (GA) synthesis, is used. Still, there is no available information on how PBZ

suppresses the flowering or flowering inducing genes of the phosphatidylethanolamine-binding protein (PEBP) family. Krishna *et al* (2017) conducted an experiment where 'Alphonso' (alternate bearing) and 'Ratna' (regular bearing) were treated with Paclobutrazol (PBZ) so as to ensure flowering and Gibberellic acid (GA) to ensure no flowering. They studied the expression of mango *Terminal Flower 1* (*MiTFL1*) and *Flowering Locus T* (*MiFT*) genes in both the cultivars. The results showed that PBZ treated plants flowered but those treated with GA didn't show any flowering. Also in the PBZ-treated plants, *MiFT1* and *MiFT3* expression was higher than the GA-treated plants in both cultivars, consistent with a role in floral induction. The *MiTFL1a* gene seems to be related with suppression of flowering.

2.4 Identification of candidate gene for flowering

Isolation of an unknown sequence related to known sequences is an ideal method for investigation of biological function. PCR methods have succeeded in obtaining the genes that are unknown in a single organism but may be homologous to the sequences of identified genes from different organisms. The isolation of resistance candidate genes in plants has found to be successful by PCR using degenerate primers that are designed from the conserved motifs of the resistance genes of other plants.

Many of the key genes governing flowering pathway integration and flowering time control have been found to be conserved in plant species. For example, FT protein which is coded by *FT* gene has been recently identified as a major component of the mobile flowering hormone, whose function is conserved across the plant kingdom. So, the candidate gene approach could be more useful in identifying the presence of these genes and their functions in different plant species using degenerate primers**.**

2.5 Role of *FT* gene in flowering in different crops

In flowering plants, the major step during development is the transition from vegetative to reproductive phase. At the molecular level, the genes in the *FLOWERING LOCUS T* (*FT*)/*TERMINAL FLOWER 1* (*TFL1*) family, which encode proteins having high similarity to phosphatidyl ethanolamine-binding proteins (PEBP), can function either as flowering promoters or repressors. Li *et al* (2015) isolated six members of the *FT*/*TFL1* family from *Jatropha curcas*, a plant with great potential for many uses including biofuels. All the members of this gene family have a common exon-intron organization. They did sequence comparisons and phylogenetic analysis with the homologous genes from other plant species and grouped Jatropha *FT*/*TFL1* genes into three major subfamilies: one into the *FT*-like, three into the *TFL1*-like, and two into the MOTHER OF *FT* AND *TFL1* (M*FT*)-like subfamilies. The expression analysis of these six genes indicates different expression patterns at the temporal and spatial levels. Their studies show that the Jatropha *FT* homolog (Jc*FT*) is

mainly expressed in the reproductive organs. *JcTFL1a* and *JcTFL1c*, two genes in the *TFL1*-like subfamily, are primarily expressed in the roots of juvenile plants, whereas *JcTFL1b* transcripts are abundantly accumulated in the fruits of the plant. In addition, two *JcMFT* genes are mainly expressed in the fruits. This differential expression of the *FT/TFL1* gene family in Jatropha suggests that this gene family plays manifold roles in plant development and growth.

The earlier studies in different plant species have already shown that the expression of *FLOWERING LOCUS T* (*FT*) and its homologues hasten the beginning of flowering, including poplar (*Populus* spp.). There are recent evidences which show that to what extent *FT* is actually effective in initiating flowering in trees including its effectiveness in poplar. Zhang *et al* (2010) described the results using one *FT* gene from *Arabidopsis* and two from poplar which are all driven by a heat-inducible promoter. They transformed these genes into two poplar genotypes. Depending on the *FT* gene and genetic background, the observations show substantial variation in flowering response. Both heat-induced plants shorter than 30 cm and those taller than that failed to flower. The plants that were exposed to daily heat treatments lasting for 3 weeks produced fewer abnormal flowers than those in heat treatments of shorter durations. The increase in the inductive temperature from 37 °C to 40 °C produced similar benefits. There was induction of flowering in 90 per cent of the transgenic plants by using optimal induction conditions. When *FT* rootstocks were induced with scions without *FT*, blooming was observed only in rootstocks. These results suggest that a considerable amount of genotype or species-specific adaptation is required to develop *FT* into a reliable way to shorten the breeding cycle in poplar.

The adult citrus trees show seasonal periodicity of flowering after being in the juvenile phase for several years. In citrus, presence of continued low temperature exposure is one of the most important environmental signals for initiation of flowering. The expression of genes related to flowering during the annual cycle of flowering and inductive low-temperature treatment in Satsuma mandarin (*Citrus unshiu* Marc.) trees was investigated. Simultaneously, floral induction, which occurs before the period of morphological flower development, was estimated as the number of flowers after the forcing of sprouting by defoliation at 25 ˚C. Citrus expression *FLOWERING LOCUS T* homologues, *i.e.* during the floral induction period, *CiFT* showed seasonal increase and was also induced by an artificial low temperature treatment (15 °C) in which floral induction occurred. Whereas, there was no change in level of *CiFT*'s mRNA when treated with warm temperature (25 °C) conditions for 2.5 months, during this time no floral induction occurred. Any change in the expression level of the homologues of citrus such as *LEAFY*, *APETALA1* and *TERMINAL FLOWER 1* showed no

correlation with floral induction in the field or under artificial low-temperature conditions. The juvenile seedlings of Satsuma mandarin did not flower under artifical low-temperature condition *i.e.* the level of *CiFT*s mRNA were not affected by the treatment of low-temperature as compared to the adult tissues (Nishikawa *et al* 2007). These results concluded in adult trees of Satsuma mandarin, the low temperature promotes floral induction via the activation of *CiFT* transcription but *CiFT* transcription does not respond to low temperature conditions in case of juvenile plants.

In order to identify genetic components in flowering pathways of high bush blueberry (*Vaccinium corymbosum* L.), a transcriptome reference composed of 254,396 transcripts and 179,853 gene contigs was developed by assembly of 72.7 million reads using Trinity. Walwoth *et al* (2016) used the earlier mentioned reference of transcriptome and a query of flowering pathway gene of some plants so as to identify the potential flowering pathway genes/transcripts in blueberry. To analyse the transcriptome of flowering pathway genes which are responsible for overexpression of a blueberry *FLOWERING LOCUS T*-like gene (*VcFT*), they conducted an experiment on leaf tissue samples of transgenic blueberry *cv.* Aurora ('*VcFT*-Aurora'). Sixty-one blueberry transcripts of 40 genes showed high similarities to 33 known flowering-related genes of herbaceous plants, of which 17 down-regulated and 16 up-regulated genes were identified in '*VcFT*-Aurora'. A blueberry *CONSTANS-LIKE 5*-like (*VcCOL5*) gene was down-regulated and associated with five other genes that express differentially in the photoperiod-mediated flowering pathway. Three down-regulated genes, *i.e.*, a *MADS-AFFECTING FLOWERING2*-like gene (*VcMAF2*), a *MADS-AFFECTING FLOWERING5*-like gene (*VcMAF5*), and a *VERNALIZATION1*-like gene (*VcVRN1*), may function as integrators in place of *FLOWERING LOCUS C* (*FLC*) in the vernalization pathway. Because no *CONSTAN1*-like or *FLOWERING LOCUS C*-like genes were found in blueberry, *VcCOL5* and *VcMAF2*/*VcMAF5* or *VRN1* might be the major integrator(s) in the photoperiod and vernalization-mediated flowering pathway, respectively. The major down-stream genes of *VcFT*, *i.e.*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*-like (VcSOC1), *LEAFY*-like (*VcLFY*), *APETALA1*-like (*VcAP1*), *CAULIFLOWER1*-like (V*cCAL1*), and *FRUITFULL*-like (*VcFUL*) genes were present and showed high similarity to their orthologues in herbaceous plants. Moreover, overexpression of *VcFT* promoted expression of all of these *VcFT* downstream genes. These results suggest that *VcFT*s down-stream genes appear conserved in blueberry.

Cassava is a storage-root crop prominent in tropical regions of the world. The most of the varieties of cassava are late flowering and is non-synchronised leading to sparse flower production. Therefore, flowering becomes an important breeding challenge in cassava. The *FLOWERING LOCUS T* (*FT*) gene is essential for induction of flowering in most of the

angiosperms. The objective of the research work done by Andemeyo *et al* (2017) was the determination of potential roles of the *FT* signalling system in cassava. They transformed *Arabidopsis thaliana FT* gene (*AtFT*) into the cassava cultivar 60444 through Agrobacterium-mediated transformation and found that it overexpressed constitutively. The result showed the overexpression of *FT*, resulting in accelerated flower initiation and associated fork-type branching. This indicated the presence of the necessary signalling factors in cassava that interacted with and responded to the *atFT* gene product. Additionally, overexpression also stimulated lateral branching; the longevity of flower development was extended and the flower production was increased. While in some plants, homologs of *FT* stimulate development of vegetative storage organs; in cassava, *atFT* inhibited the development of storage-root system and decreased root harvest index.

In lettuce, the problem of high temperature-induced bolting exists. This makes it important to find the mechanism that governs the conversion of vegetative phase to reproductive phase. Fukuda *et al* (2011) isolated the *FT* gene (*LSFT*) from lettuce to understand its habit of floral induction. The results of sequence analysis and phylogenetic relationship of *LSFT* showed homology to *FT* gene of *Arabidopsis*, tomato and other species. In transgenic *Arabidopsis*, early flowering was induced by *LSFT*, but it was not completely effective compared to *atFT*. The results suggested that the *LSFT* is a putative *FT* homolog in lettuce and regulates the transition of flowering.

In the case of subtropical fruit tree longan (*Dimocarpus longan*), there has been research on bud development and flower induction due to regulation of plant hormone whereas, knowledge on the genetic pathway is scarce. The orthologous genes from longan (*DlFT*, *DlFT2*, *DlFT1t*, *DlAP1-1*, *DlAP1-2*) were isolated by Winterhagen *et al* (2017). The results of sequence and bioinformatic analyses suggested that these genes had conserved functions within the flowering pathway; however, it was found that *DlFT1* acts as a flowering time promoter whereas *DlFT2* and a truncated *DlFT1t* delayed flowering time in transgenic *Arabidopsis*. In time experiments with chemically induced trees were conducted to investigate the expression pattern of the flowering genes. The reference genes were evaluated and quantitative real-time PCR investigated expression of flowering genes in the longan. The data indicated that *DlFT1* is up-regulated in leaves during the presumed time period of flower induction in agreement with changes of the cytokinin/auxin ratio, which might promote flower development and activate *DlAP1* gene transcription in the bud.

The isolation and characterisation of the two *FLOWERING LOCUS T* (*FT*)-like genes of apple (*Malus* x *domestica* Borkh.), *MdFT1* and *MdFT2*, was done by Kotoda *et al* (2011). They also mapped these genes on distinct linkage groups (LGs) with partial homoeology i. e.

LG 12 and LG 4. It was observed that the expression pattern of *MdFT1* and MdFT2 was different. The expression of *MdFT1* was mainly in apical buds of fruit-bearing shoots in the adult phase and with little expression in the juvenile tissues whereas *MdFT2* expressed mainly in reproductive organs, including flower buds and young fruit. Both the genes induced early flowering in transgenic *Arabidopsis*, which ectopically expressed *MdFT1* or *MdFT2*, flowered earlier than wild-type plants. Likewise, overexpression of *MdFT1* gene resulted precocious flowering in apple. Because of this the expression of other endogenous genes, such as *MdMADS12*, was also altered. These results suggest that if the expression of *MdFT1* gene is altered then it could function to promote flowering and that; at least, other genes may play an important role as well in the regulation of flowering in apple. The juvenile period of fruit trees is long and it prevents efficient breeding as well as early cropping. These findings can be useful information to better understand the molecular mechanism of flowering and to develop different methods to shorten the juvenile period in various fruit trees including apple.

CHAPTER – III

MATERIAL AND METHODS

The present study entitled "Expression analysis of flowering switch factors using candidate gene approach in mango" was conducted at Fruit Molecular Laboratory, School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana. This chapter depicts the material and methods used and followed under the following headings:

3.1 Plant material

3.2 Identification of *Flowering Locus T* gene

3.3 DNA extraction

3.4 Designing of primers

3.5 Sequencing

3.6 RNA isolation

3.7 Semi quantitative PCR

3.1 Plant material

The young leaves of two fruiting mango cultivars *i.e.* Amrapalli and Dashehari were obtained from College Orchard and Fruit Research Farm of Department of Fruit Science and transferred to Fruit Molecular Biology Laboratory, PAU, Ludhiana for isolation of flowering related gene and its expression analysis. Dashehari is a selection, vigorous, irregular bearer and prominent in north-western India. Amrapalli is a hybrid of Dashehari x Neelam. It is dwarf, precocious and regular bearer. The leaves were collected from both cultivars at different time period, labeled properly and were stored at -80 °C for RNA extraction.

3.2 Identification of *Flowering Locus T* gene

For identification of floral integrator gene, *Flowering Locus T* in mango, on the basis of sequences of the gene reported to regulate flowering time and floral pathway integration in several other plant species primers for present study were designed. These primers were analysed for amplification by PCR and semi quantitative PCR in genomic DNA and mRNA, respectively, in both the cultivars.

3.2.1 Primer designing

The primers were designed for gene, namely *FLOWERING LOCUS T* (*FT*) which is reported to function as both flowering time gene and floral pathway integrator in several plant species. Nucleotide sequences of gene in *Arabidopsis thaliana* (accession no. ABA23611) was taken from GenBank database of National Centre for Biotechnology Information (NCBI)

in FASTA format. The primers were designed using PerlPrimer (FT-F1 and FT-R1) for the *FT* gene.

Another set of primers were designed by multiple sequence alignment of amino acid sequences of *FT* gene selected from crops *viz.*, *Arabidopsis thaliana*, *Pyrus pyrifolia*, *Vitis vinifera*, *Populus nigra*, *Fragaria vesca* and *Malus domestica* retrieved from NCBI protein database. These sequences were aligned to find the conserved regions. In *FT* amino acid alignment, four highly conserved regions were identified and two set of primers (FT-F2, FT-R2 and FT-F3, FT-R3) were designed using the nucleotide sequence of the same (Table 1).

Table 1: Sequence of designed degenerate primers

Target gene	Primer name	Primer Sequence (5' to 3')	No. of bases	GC Content (%)	T_m (°C)
FT	FT-F1	AAGCCATAGAGGTCGTGACGTATTGG	26	49.1	59
	FT-R1	GTACTATGGTGTGATCTCCATGACTG	26	51.8	59.4
	FT-F2	TGGTGACATGATATACTCCATGC	23	49.1	57.8
	FT-R2	GGATGTCAGTACGTACCTGTCTTGCCT	27	53.3	62
	FT-F3	CCAGAGAGTTAGTAACGAGTCGTCACTGC	29	52.5	62.3
	FT-R3	TTCTACACTCTGGTTATGGTGG	22	55	62

3.3 Genomic DNA isolation

Genomic DNA isolation was done using DNeasy Plant Mini Kit (Qiagen).

1. The leaf samples were chilled and ground into fine powder in liquid nitrogen using sterile pre-chilled pestle and mortar.
2. 400 µl Buffer AP1 and 4 µl RNase A was added to the eppendorf containing the grounded sample which was vortexed and further put on incubation at 65 °C.
3. During incubation, the eppendorfs were inverted 2-3 times. To the lysate, P3 Buffer (130 µl) was added, mixed and incubation was done on ice for 5 min.
4. Then the lysate was put for centrifugation at 20,000 x g (14,000 rpm) for 5 min. The lysate was then pipetted into a QIAshredder spin column placed in a 2ml collection tube and centrifuged at 20,000 x g for 2 min.

5. The flow-through was shifted into a new tube. The pellet was remain undisturbed. 1.5 volumes of Buffer AW1 were added and then thoroughly mixed by pipetting.
6. From the above, 650 µl was transferred into DNeasy Mini spin column which was placed in a 2 ml collection tube and centrifugation was done for 1 min at 6000 x g (8000 rpm).
7. The flow-through was thrown out.
8. The spin column was then placed into a new 2ml collection tube.
9. 500 µl Buffer AW2 was added and centrifuged for 1 min at 6000 x g. The flow-through was then discarded.
10. Again 500 µl Buffer AW2 was added and centrifuged for 2 min at 20,000 x g. The spin column was then transferred to a new 1.5 ml or 2 ml centrifuge tube.
11. For elution, Buffer AE was added to the lysate and was incubated for 5 min at room temperature. Then it was centrifuged at 6000 x g for 1 min.

3.3.1 Agarose Gel Electrophoresis

To determine the quality and quantity of genomic DNA, 0.8 per cent agarose gel was used. The DNA samples (2.0 µl) were mixed with 8.0 µl of loading dye (6X loading dye: 0.4 per cent bromophenol blue, 0.4 per cent xylene and cyanol, 50 per cent glycerol in sterile water) then loaded in gel placed in electrophoresis buffer (TBE buffer). Then gel was allowed to run for 75 min at 90 Volt/cm and visualized using the gel documentation system (Avegene, Taiwan). The DNA concentration of each sample was determined. Quality of DNA samples was estimated based on whether DNA formed a single high molecular weight band (good quality) or a smear (degraded/ poor quality).

3.3.2 Nano drop quantitative analysis

The DNA quantification was also checked using Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The yield and purity of isolated DNA was also determined at the wavelength of 230 nm for the carbohydrates or polyphenols, 260 nm for the DNA and 280 nm for proteins. Concentration of DNA was recorded as ng/µl while the purity of extracted DNA was calculated as the OD ratio of 260/280. 1.0 µl of DNA sample was used for quantification on NanoDrop.

3.3.3 PCR amplification of genomic DNA with designed primers

The genomic DNA of both the cultivars of mango was amplified using the designed primers for the gene *FT* by the reaction given below (Table 2). Primers used in the first PCR reaction were FT-F3 and FT-R3. The reaction mixture was prepared using 50 ng of genomic DNA of each genotype to make final volume of 20 µl per reaction. The PCR profile included

initial denaturation followed by 30 cycles of denaturation at 94 °C for 1 min, annealing of primers at 50 °C for 45 seconds and extension at 72 °C. The final extension was given at 72 °C for 5min. The amplified products along with 1 kb ladder from 'BioLabs' were separated on 1.0% agarose gel containing 0.5X TBE buffer. The gel was viewed under gel documentation system (Avegene, Taiwan).

Table 2: PCR reaction mixture

Components	Stock	Working
Water	-	11.5 µl
10x Reaction buffer (Tris with 15 mM $MgCl_2$)		2 µl
DNTPs	2.5 mM each	1 µl
Forward primer	10 µM	1 µl
Reverse primer	10 µM	1 µl
Taq Polymerase	$5U\mu l^{-1}$	0.5 µl
PVP	-	0.5 µl
BSA	-	0.5 µl
Template DNA	$50\ ng\mu l^{-1}$	2 µl
Total volume		20 µl

3.3.5 Gel elution and purification using Hiyield Plus™ Gel/PCR DNA mini kit

The desired DNA fragment (1.5 kb) was excised from agrose gel with clean scalpel. Care was taken that no extra agarose was excised along with DNA slice. This excised fragment was transferred to 1.5 ml micro centrifuge tube. The gel/PCR DNA mini kit of Hiyield Plus™ was used to dissolve gel fragment. 500 µl of QDF buffer was added to this sample and incubated at 55-60 °C for 10-15 min with frequent inversion until the gel slice was completely dissolved. The yellow coloured mixture was cooled at room temperature. Then QDF column was placed in a 2ml collection tube and the 800 µl of the mixture was transferred to this column and centrifuged at 14,000 x g for 1 minute, the flow-through was discarded and the QDF column was placed back in 2ml collection tube. To this, 400 µl of W1 buffer was added and centrifuged at 14,000 x g for 1 minute. The flow-through was again discarded. 600 µl of wash buffer was added to the QDF column and kept for 1 min. It was then centrifuged at 14,000 x g for 1 minute and the flow-through was discarded. The QDF column was again centrifuged at 14,000 x g for 3 min to dry the column matrix. The flow-through was the final eluted product, stored at 20 °C, and was checked on 1% gel for confirmation.

3.3.6. Competent cell preparation

E. coli cells treated with cold Calcium chloride ($CaCl_2$) takes up plasmid DNA by transformation. $CaCl_2$ is thought to enhance binding of plasmid DNA to cell surface by affecting plasma membrane permeability.

1. The *E. coli* strain used for the transformation was *E. coli* DH5α. From a freshly streaked LB agar plate, pure culture of *E. coli* strain was inoculated into 10 ml LB broth directly.
2. The primary culture was then incubated at 37 °C overnight with shaking. After incubation, 500 µl of the culture was inoculated into 10 ml LB broth (secondary culture) and allowed to grow for 2-3 h till the OD-600 reached 0.3 to 0.4 (milky appearance).
3. Secondary culture was kept on ice for 20 min with manual shaking.
4. The cells were then harvested after centrifugation of secondary bacterial culture taken in 50ml falcon tubes at 4000 rpm for 10 min at 4 °C.
5. The supernatant was discarded and the pellet formed was then re-suspended in chilled 0.1 M $CaCl_2$ equal to the volume of culture and kept on ice for 45 min.
6. Centrifugation was done at 4000 rpm for 5 min at 4 °C and then pellet was re-suspended in 525µl chilled 0.1M $CaCl_2$ and 225 µl 50 per cent glycerol.
7. 100 µl of aliquots were made from the above and stored at -80 °C.

3.3.7 Ligation of PCR products

The ligation of the PCR products was done using TA Cloning® Kit (Promega). Gel purified PCR product was ligated with vector (pGEM-T Easy plasmid) in 3:1 ratio. The ligation reaction is given in the following table (Table 3). The ligation reaction was incubated overnight at room temperature.

Table 3: Components of the ligation mixture

Components	**Volume**
Fresh PCR product	8 µl
5X T4 DNA Ligase Reaction Buffer	10 µl
pCR®2.1 vector (25 ng μl^{-1})	1 µl
T4 DNA Ligase (5U)	1 µl
Final volume	20 µl

3.3.8 Transformation of ligated products into competent cells

1. The tubes containing ligation reactions were spin briefly and kept on ice.
2. 7 µl of the ligated products was added to the tubes containing 50 µl of competent *E. coli* DH5α cells and gently mix and placed them on ice for 30 min.
3. The cells were then allowed to heat shock at 42 °C for 1 min in waterbath without shaking and immediately kept on ice for 10 min.
4. To each of the tubes containing the cells, 900 µl of LB broth was added. The culture was then incubated for 2-3 h at 37 °C with shaking at 50 rpm in shaker cum incubator.
5. After the incubation, it was pelleted down by centrifugation 5000 rpm for 10 min.
6. Keeping 100 µl of the supernatant, left volume was discarded.
7. Then 80 µl of the transformed product was spread on LB/Amp/IPTG (100 µl)/X-Gal (40 µl) plates using a sterile L- rod.
8. The culture plates were then incubated at 37 °C overnight in shaker cum incubator.
9. Next day, blue-white colonies were observed.
10. The individual white colonies were streaked on LB/Amp plates and then incubated at 37 °C overnight in shaker cum incubator.

3.3.9 Colony PCR

The most efficient method for getting good sequences of the PCR products is Cloning. It is essentially a four-step process including competent cell preparation, ligation of the PCR products, transformation of the ligated products into competent cells (*E. coli* DH5α) and plasmid isolation from transformed colonies.

The positive bacterial colonies were screened through colony PCR using the FT-F3 and FT-R3 primers and PCR conditions as described in section 3.3.3 except the bacterial colony as template and steps 2 to 4 were repeated 35 times. The colony PCR products were separated on agarose gel (1.0 %) and stained with ethidium bromide (0.5 µg/ml). Gel photograph was documented. Positive bacterial clones were separately streaked on separate LB plates containing ampicillin and grown at 37 °C overnight. Then, LB was inoculated with positive bacterial clones at 37 °C for overnight with shaking at 200 rpm. Recombinant plasmid was isolated by QIAprep® Spin Miniprep Kit (QIAGEN, Germany).

3.3.10 Plasmid isolation from transformed colonies

Recombinant plasmid was isolated by QIAprep® Spin Miniprep Kit (QIAGEN, Germany). 5ml of Bacterial starter culture that was grown overnight was pelleted down by centrifuging at 12000 rpm for 1 min. The pelleted bacterial cells were resuspended in buffer P1 (250 µl) and vortexed to mix the pellet properly. After mixing buffer P2 was added and tubes were inverted gently. To this, 350 µl buffer N3 was added and thoroughly mixed by

inverting tube for 4-5 times. The transparent supernatant formed after centrifugation at 13000 rpm for 10 min was then transferred to QIAprep spin column and again centrifuged at 13000 rpm for 1 m and flow through was discarded. 500 µl Buffer PB and 750 µl Buffer PE was used for primary and secondary washing respectively followed by centrifugation. Flow through was discarded and then empty spin was given at 13000 rpm for 1 min. QIAprep column was transferred to clean 1.5 ml microcentrifuge tube. To this tube 35 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was added to center of the column. After incubation for 1 min, centrifugation was carried out at 13000 rpm for 1 min to obtain the recombinant plasmid DNA. Isolated recombinant pGEM-T Easy plasmid was checked on agarose gel (1.0 %) stained with ethidium bromide.

3.4 Sequence analysis of the amplicons

The amplicons produced by the primer pair was eluted from the agarose gel and the isolated recombinant plasmid was outsourced to Eurofins Genomics India Pvt. Ltd., Bengaluru for sequencing. The raw sequences contained the primers and vector sequence on both sides. The vector sequences were removed and sequences were assembled into a contiguous sequence for each primer set. The gene sequences were further analyzed by using BLASTn tool of NCBI (www.ncbi.nlm.nih.gov) to find out the identity and similarity between query sequence and already published *FT* gene sequences. ClustalW (https://www.genome.jp/tools-bin/clustalw) was used for multiple sequence alignment and phylogenetic tree was constructed using the phylip program.

3.5 RNA isolation

3.5.1 Preparation for RNA isolation

The preparations done before isolation of RNA are quite different than for isolation of DNA. More care is taken during isolation of RNA, the risk of RNAse (catalyses degradation of RNA) contamination is always there, which unlike DNase do not require any co-factor like Mg^{2+} ion for its activity (Reddy *et al* 2015). Therefore, to remove all the contamination by RNAse, all the plasticwares and glasswares were treated with 0.1 % Diethyl pyrocarbonate (DEPC) water .It was left overnight (12 h) at 37 °C. For 0.1% DEPC water preparation, 1 ml of DEPC is dissolved in 1L of distilled water. After mixing thoroughly, it was autoclaved at 20 psi for 30 min.

3.5.2 DEPC water (0.1%) treatment

Plasticwares such as microtubes (1.5 ml and 2 ml) and tips (1 ml, 200 µl and 10 µl) were fully immersed in DEPC water and kept overnight. The pestle and mortars were also immersed in DEPC water overnight. After overnight treatment, the tips and microtubes were

filled in tip boxes and jam jars, respectively. The moisture in tip boxes and jam jars were dried by keeping inside hot air oven at constant temperature (80 °C) for10-12 h. All the material should be autoclaved before use.

3.5.3 Sample collection

The collection of sample for RNA isolation is also a critical step because of human contact, environmental exposure and aqueous solutions are the common sources of RNAse contamination. For sample collection, the material required are gloves, tissue paper, scalpel, liquid nitrogen and RNAse Zap. Fresh gloves should be used to avoid contamination by RNAse. The young and mature leaves from the 10 year old trees of both the varieties were collected at three different stages *i.e.* early stage (mid-January) *i.e.* before flowering, mid stage; prior to induction of flowering (mid-February) and late stage; prior to flower bud initiation (early-March). These samples were transferred immediately to liquid nitrogen and then were stored at -80 °C.

3.5.4 Isolation of RNA

1. To extract RNA for PCR analysis, fresh leaves sample were taken in liquid nitrogen from College Orchard and Fruit Research Farm, Department of Fruit Science for both the cultivars.
2. The frozen sample were ground into a fine powder in liquid nitrogen using DEPC-treated and pre-chilled mortar and pestle.
3. The fine powder was immediately transferred into a centrifuge tube and homogenised by adding appropriate amount of RNA isoplus.
4. The homogenate was kept at room temperature for 5 min at 40 °C. This homogenate was then centrifuged at 12,000 x g force for 5 min at 40 °C.
5. The supernatant was collected and transferred into new centrifuge tube.
6. To this supernatant, 0.2 ml of chloroform per 1.0 ml of RNA isoplus was added.
7. Capped the centrifuge tube and mix until the solution became milky.
8. Kept the solution at room temperature for 5 min.
9. Centrifuged the solution at 12,000 x g force for 15 min at 40 °C.
10. The solution separated into three layers; top layers containing RNA, semisolid middle layer containing DNA and the bottom organic solvent.
11. Transferred the top liquid layer to new centrifuge without touching middle layer.
12. Added 0.5-1.0 ml of isopropanol per 1ml of RNA isoplus used for homogenisation and mixed well. Kept the mixture at room temperature for 10 min.
13. Centrifuged at 12,000 x g for 10 min at 40 °C to precipitate DNA.
14. Carefully removed supernatant without touching the pellet.

15. Washed the RNA with equivalent amount of 75 per cent ethanol and centrifuged at 7500 x g for 5 min at 40 °C.
16. Discarded the supernatant.
17. After the precipitate was air dried, it was dissolved with appropriate amount of RNase-free water.

3.5.4.1 RNA quantification by denaturing agarose gel (1.0 %) electrophoresis

1. The integrity and quantity of RNA was determined on 1.0 % (w/v) denaturing agarose gel.
2. The gel electrophoresis tank, measuring cylinder, combs and mould were cleaned properly with 0.5 % SDS after that with ethanol. Agarose powder (HiMedia) was dissolved in 1X 3-(N-morpholino) propane sulfonic acid (MOPS) buffer (prepared in 0.1 % DEPC water) and 37 % formaldehyde.
3. The mixture was heated till the solution became transparent and clear. It was cooled down to 60 °C with constant stirring followed by addition of ethidium bromide (1.0 µg/ml).
4. This agarose solution was then dispensed into gel mould with combs and allowed to solidify for 40 min.
5. RNA samples were mixed with glycerol (50%) in the ratio 1:1 and then loaded into wells.
6. The gel containing samples was subjected to electrophoresis at voltage (80 V) for about 1 h.
7. RNA under these conditions is fully denatured and migrates according to the $\log_{10}$ of its molecular weight.
8. The gel visualized under UV transilluminator and recorded with gel documentation system (Avegene).

3.5.4.2 Preparation of MOPS buffer

The following reagents (Table 4) were dissolved in 0.1 % DEPC water to prepare 1 Litre MOPS buffer (10X):

Table 4: Composition of MOPS buffer

Component	Amount used (g)
3-(N-morpholino) propane sulfonic acid or MOPS (200 mM)	41.9
Sodium acetate (80 mM)	8.2
EDTA (10 mM)	3.72

Care was taken that the pH of buffer is 7.0, adjusted with concentrated HCl/NaOH.

3.5.5 Synthesis of cDNA using PrimeScrip™ First strand cDNA synthesis kit (TaKaRa)

In a microtube, prepared a reaction containing following reagents (Table 5) followed by incubating at 65 °C for 5 min, then cooled immediately on ice.

Table 5: Reaction mixture for cDNA synthesis

Reagents	Volume
Oligo dT primer (50 µM)	1.0 µl
dNTP mixture (10 mM each)	1.0 µl
Template RNA	5.0 µg
RNase free dH_2O	13.0 µl
Total	20.0 µl

Prepared the reaction mixture in a total volume of 20 µl (Table 6). Mixed the reagents gently. Incubated the reaction mixture at 30 °C for 10 min or at 42 °C for 30-60 min. The enzyme was then inactivated by incubating at 95 °C for 5 min and cooled on ice. The mixture was stored at -20 °C.

Table 6: Reaction mixture for cDNA synthesis

Reagents	Volume
Template RNA Primer Mixture	10 µl
5X PimeScript Buffer	4.0 µl
RNase inhibitor (40 U/µl)	0.5 µl (20 units)
PrimeScript RTase (200 U/µl)	1.0 µl (200 units)
RNase free dH_2O	4.5 µl
Total	20 µl

3.5.5.1 Confirmation of cDNA

The cDNA synthesized was confirmed using *MiEF* gene primers (Forward primer and Reverse primer). The PCR reaction mix (20 µl) contained the cDNA template (2.0 µl), 25 µM $MgCl_2$ (1.5 µl), 5 µM forward and reverse primers (1.0 µl), 10 mM dNTPs (4.0 µl), 5X PCR buffer (4.0 µl), 5 units μl^{-1} *Taq* DNA polymerase (0.5 µl) and sterile water (6.0 µl). The following PCR profile (Table 7) was followed for the amplification of cDNA. The amplicons were visualised on 1.0 % agarose gel and photographed using photogel documentation system (Avegene, Taiwan).

Table 7: PCR profile for cDNA synthesis

Step	Cycling condition	Temperature and time
I	Initial denaturation	94 °C for 4 min
II	Denaturation	94 °C for 1 min
III	Primer annealing	50 °C for 45 sec
IV	Extension	72 °C for 1 min
V	Repeat steps II to IV	30 cycles
VI	Final extension	72 °C for 5 min
VII	Hold	4 °C

3.5.5.2 *FT* gene expression in leaves of mango varieties using semi quantitative method

Total RNA was isolated from leaves of both the mango cultivars *i.e.* Amrapalli and Dashehari at different stages. The early stage is the stage prior to before flowering *i.e.* mid-January, mid stage is prior to induction of flowering *i.e.* mid-February and late stage is prior to flower bud initiation *i.e.* early-March as described earlier in section 3.5.3. The quantification of RNA was performed through agarose gel electrophoresis and NanoDrop as described earlier in section 3.4.4.1. The cDNA was prepared using 1.0 µg of total RNA as described earlier in section 3.4.5. For semi quantitative analysis, equal quantity of cDNA from each of the samples was used. The *MiEF* based gene primers were used as internal control for expression studies (Table 8) (Nakagawa *et al* 2012). Semi quantitative PCR was carried out with *FT* gene specific primer set (FT-F3 and FT-R3). Optimal amplification of *FT* transcript was standardized at exponential phase in PCR. The PCR amplified product was separated on agarose gel (1.0 %). The relative level of *FT* transcript expression was estimated densitometrically in gel documentation system (Avegene, Taiwan).

Table 8: Primer sequence of reference gene for semi quantitative method

Reference gene	Primer	Nucleotide sequence (5'–3')
MiEF	Forward	ACCAGAGATGGGGACAAAGG
	Reverse	TGCTGCTGTAACAAGATGGATG

CHAPTER IV

RESULTS AND DISCUSSION

The study entitled "Expression analysis of flowering switch factors using candidate gene approach in mango" was conducted at Fruit Molecular Laboratory, School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana. The results obtained from the study are discussed in this chapter.

4.1 IDENTIFICATION OF *FT* GENE IN MANGO

4.1.1 Designing of degenerate primers

4.1.2 Total DNA isolation from mango leaves

4.1.3 PCR Analysis of genomic DNA with degenerate primers

4.1.4 Cloning of *FT* DNA in pGEM-T Easy vector

4.1.5 Sequencing of the amplicons produced by degenerate primers

4.2 *IN SILICO* CHARACTERISATION

4.2.1Alignment of the *Flowering Locus T* like gene

4.2.2 Phylogenetic inference

4.3 EXPRESSION OF *FT* GENE IN MANGO

4.3.1 Total RNA isolation from mango varieties at different developmental stages

4.3.2 cDNA synthesis and PCR amplification with gene specific primers

4.3.3 Expression analysis of *FT* gene

4.1 IDENTIFICATION OF *FT* GENE IN MANGO

4.1.1 Designing of Degenerate Primers

Degenerate primers for the floral pathway integrator *FT* gene were designed based on the conserved regions in the multiple aligned nucleotide sequences (Fig. 1) and named as FT-forward (FT – F3) and FT - reverse (FT –R3).

The designed set of primers were checked for different parameters such as primer length, product length, low degeneracy, maximum specificity at the 3' end, before their synthesis. The properties such as annealing temperature, GC-content, potential hairpin formation and 3' complementarity were analysed. The sequences of the resultant primers were then sent to 'Eurofins Genomics India Pvt. Ltd., Bengaluru' for synthesis.

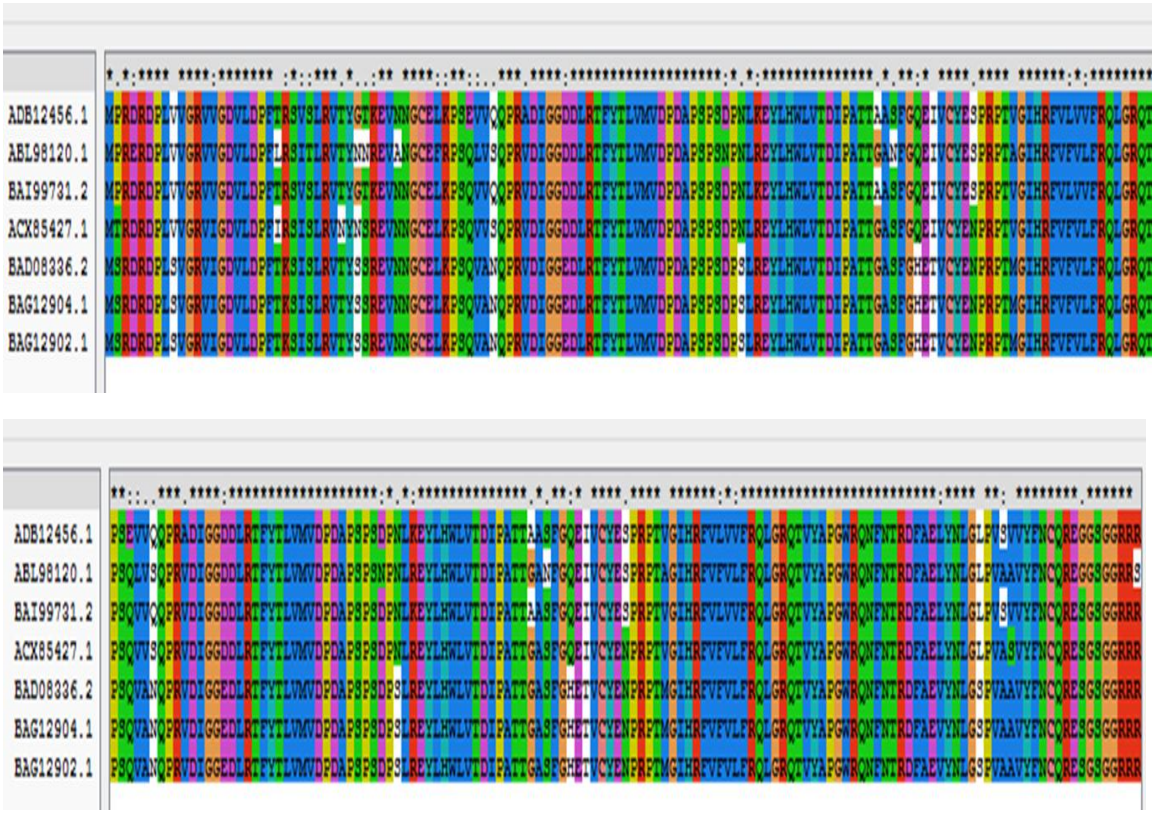


Fig. 4.1: Multiple sequence alignment using Clustalx

4.1.2 Total DNA Isolation

The isolation of genomic DNA was from the leaf samples of both the varieties, Amarpalli and Dashehari (Fig. 4.2), was performed using DNeasy Plant Mini Kit. The DNA quality and quantity in both the varieties was evaluated by resolving on 1.0 % agarose gel and checked on nanodrop spectrophotometer (Thermoscientific Nanodrop^{TM} 1000) (Table 9, Fig. 4.3). The total DNA isolated was stored at -20 °C for further use.

Table 9: Quantification of isolated DNA from mango varieties

S. No.	Sample	Absorbance at 260 nm	Absorbance at 280 nm	Ratio 260 nm/280 nm
1	Amrapalli (A1)	0.11	0.07	1.5
2	Amrapalli (A2)	0.90	0.25	1.52
3	Dashehari (D1)	0.99	0.38	2.06
4	Dashehari (D2)	0.89	0.24	1.45

(A) **(B)**

(C)

Fig. 4.2: Field view of mango cultivars; where, A: Dashehari and B: Amrapalli, C: Flowering and Fruit set at Fruit Research Farm, Punjab Agricultural University, Ludhiana

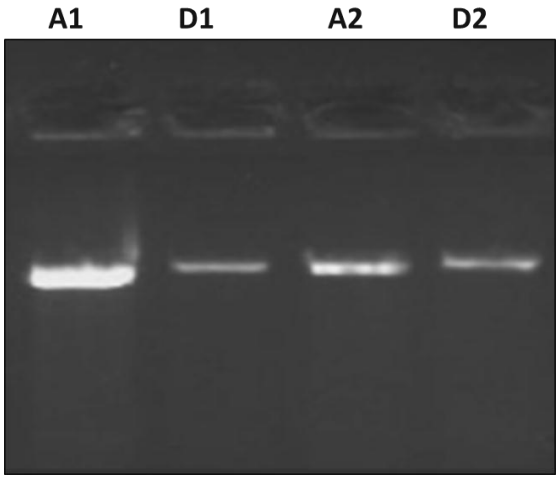


Fig. 4.3: DNA quantification of mango varieties; Where, A1 and A2: Amrapalli, D1 and D2: Dashehari

4.1.3 PCR Analysis of genomic DNA with degenerate primers

Genomic DNA from the leaves was extracted and used in PCR reaction. Designed degenerate primers for *FT* gene produced an amplicon of size ~1500 bp using the FT – F3 and FT – R3 primers. The amplicons were resolved on agarose gel (1.0 %). Lane 2 and 3 shows the PCR products of genomic DNA of leaves from Amrapalli and Dashehari with *FT* primers and Lane 1 shows 1 kb ladder (Fig. 4.4).

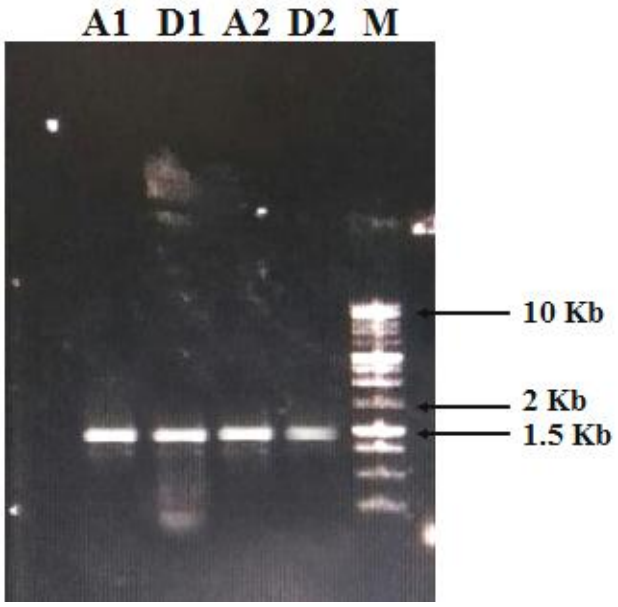


Fig. 4.4: PCR amplification of *FT* from genomic DNA of Mango varieties; Where, M: 1Kb DNA ladder, A1 and A2: Amrapalli, D1and D2: Dashehari DNA amplifications

4.1.4 Cloning of *FT* DNA in pGEM-T Easy vector

The eluted PCR product was ligated into pGEM-T Easy vector in the ratio of 3:1. The pGEM-T Easy vector is also known as TA cloning vector as it contains single 3' Thymidine (T) nucleotide over hangs (Fig. 4.5). The enzyme terminal transferase is responsible for the incorporation of 3' dideoxy Thymidine triphosphate (dTTP) at both the ends which leads to the construction of these overhangs. These 3' Adenine (A) overhangs of the PCR product would match to T overhangs of the vector. By using T4 DNA ligase, the PCR product was ligated into the vector. The ligated product was then transformed into *E. coli* DH5α. Colonies formed were detected on LB agar plate comprising X-gal and IPTG (Fig. 4.6). The plate contains blue-white colonies *i.e.* recombinant as well as non-recombinant plasmids. From this plate, ten white colonies were selected randomly and were inoculated on fresh plates.

The colonies having the insert are positive colonies and were futher screened through colony PCR by means of gene specific primers. Of the total ten bacterial colonies, four were observed to be positive (Fig. 4.7). Out of these four colonies, two colonies were chosen for recombinant plasmid isolation. The recombinant pGEM-T Easy vector was then assesed on agarose gel (1.0 %). The recombinant plasmid was then sent for sequencing to Eurofins Genomics India Pvt. Ltd., Bengaluru. After sequence analysis, the produced 1456 bp sequence of *FT* in Dashehari and 1378 bp in Amrapalli was attained as given in Fig. 4.8 (a&b). The gene encoding *FT* of size 1456 bp in Amrapalli and Dashehari (*Mangifera indica*) (KX093179 to KX093244) (Das *et al* 2019) was isolated earlier. In present study, *FT* gene sequence obtained was approximately 120-150 bp larger as compared to the earlier reported gene sequence.

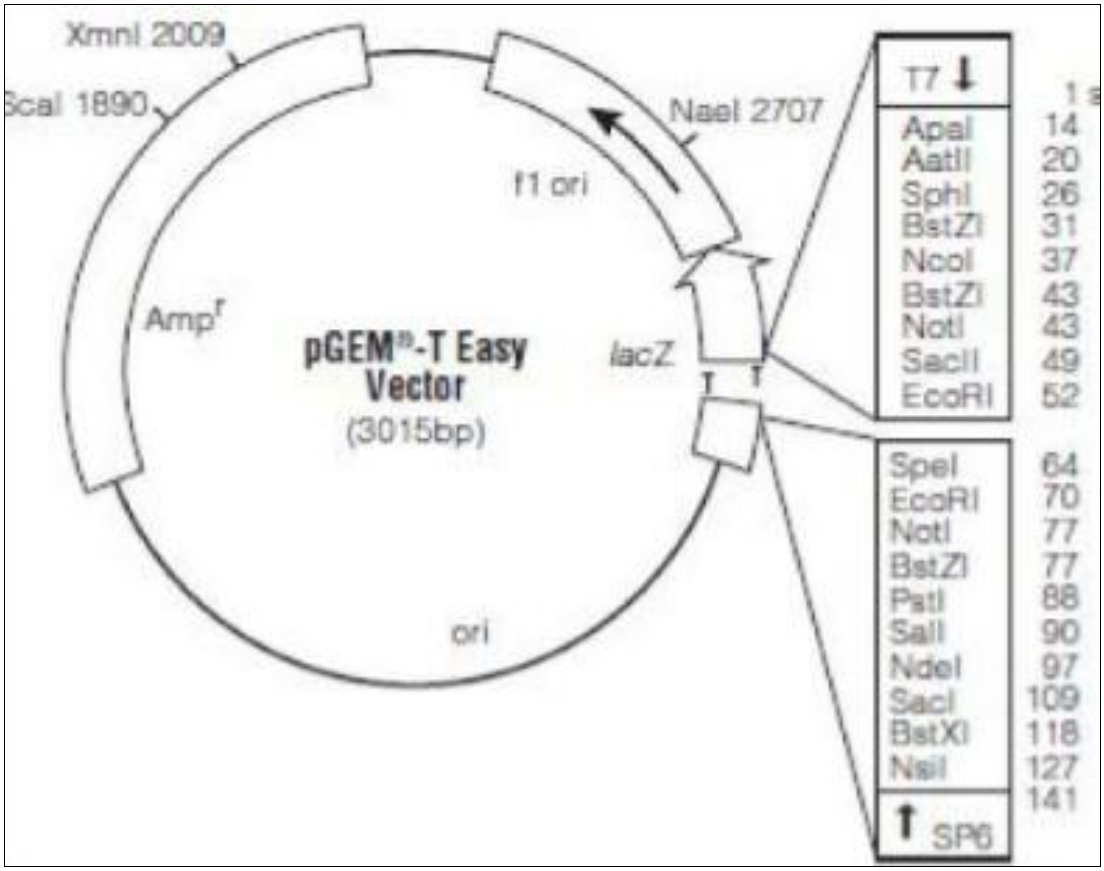


Fig. 4.5: Restriction map of pGEMT Easy vector

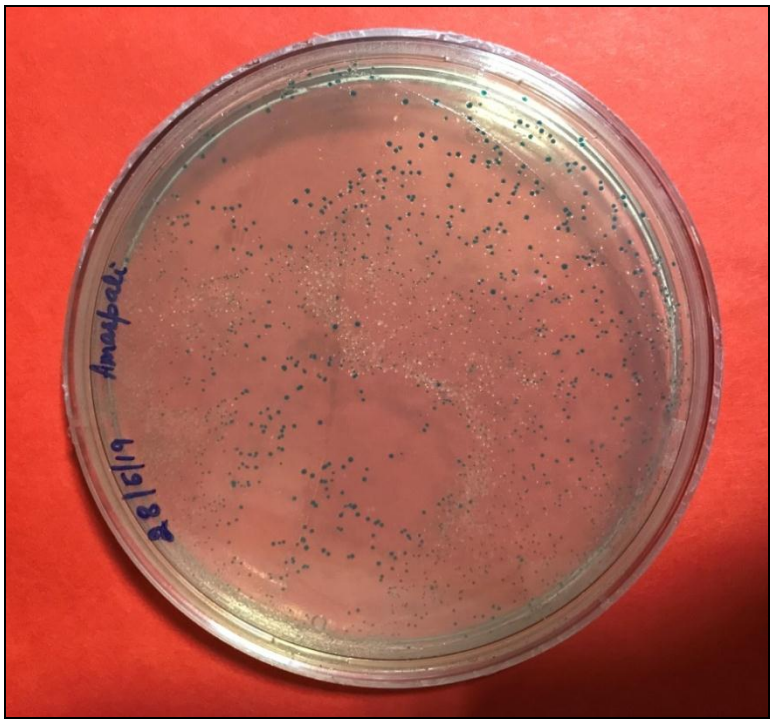


Fig. 4.6: Transformation of ligated product in *E. coli* strain DH5α

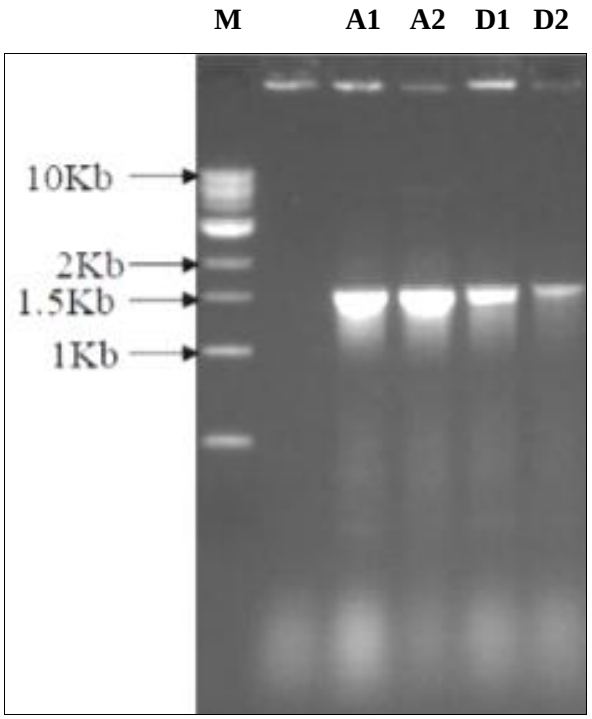


Fig. 4.7: Cloning of *FT* gene into pGEM-T Easy vector through colony PCR; Where, M: 1Kb DNA ladder

Fig. 4.8a: *FT* gene sequence of 1378 bp obtained after sequence analysis in Dashehari

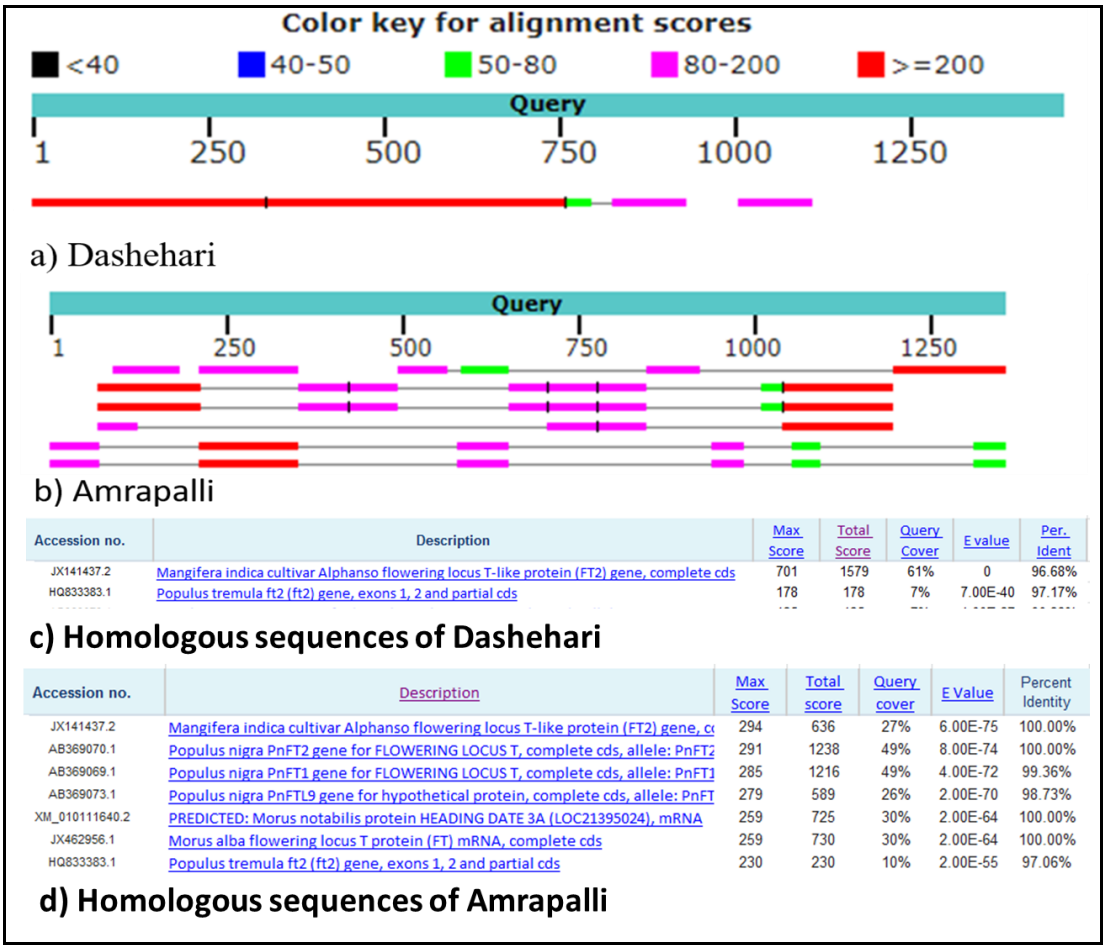
Fig. 4.8b: *FT* gene sequence of 1456 bp obtained after sequence analysis in Amrapalli

4.2 *In silico* characterisation

4.2.1 Sequence analysis of the amplicons

The assembled sequences of the amplicon after sequencing were used for analysis using bioinformatic tools *viz.*, BLAST, NCBI conserved domain search and multiple sequence alignment using ClustalW program and phylogenetic tree construction.

The BLASTn program uses nucleotide databases to search a nucleotide query. The cloned *FT* gene sequences were used as a query in BLASTn program. For Dashehari *FT* gene we identified only single homolog in blast search, covering 61 % of the gene sequence with 98 % identity. The assembled sequene from Amrapalli showed gene coverage of 30% to 49 % with *FT* gene from other species. The sequence identity was 97% sequence identity with *Populus tremula FT2*gene (HQ833383.1), 96.68% with *Mangifera indica* cultivar Alphanso *flowering locus-T like* protein gene (JX141437.2) and 95.74% with *Morus alba flowering locus T* protein (*FT*) (JX462956.1) (Fig. 4.9).



Accession no.	Description	Max Score	Total Score	Query Cover	E value	Per. Ident
JX141437.2	Mangifera indica cultivar Alphanso flowering locus T-like protein (FT2) gene, complete cds	701	1579	61%	0	96.68%
HQ833383.1	Populus tremula ft2 (ft2) gene, exons 1, 2 and partial cds	178	178	7%	7.00E-40	97.17%

c) Homologous sequences of Dashehari

Accession no.	Description	Max Score	Total score	Query cover	E Value	Percent Identity
JX141437.2	Mangifera indica cultivar Alphanso flowering locus T-like protein (FT2) gene, c	294	636	27%	6.00E-75	100.00%
AB369070.1	Populus nigra PnFT2 gene for FLOWERING LOCUS T, complete cds, allele: PnFT2	291	1238	49%	8.00E-74	100.00%
AB369069.1	Populus nigra PnFT1 gene for FLOWERING LOCUS T, complete cds, allele: PnFT1	285	1216	49%	4.00E-72	99.36%
AB369073.1	Populus nigra PnFTL9 gene for hypothetical protein, complete cds, allele: PnFT	279	589	26%	2.00E-70	98.73%
XM_010111640.2	PREDICTED: Morus notabilis protein HEADING DATE 3A (LOC21395024), mRNA	259	725	30%	2.00E-64	100.00%
JX462956.1	Morus alba flowering locus T protein (FT) mRNA, complete cds	259	730	30%	2.00E-64	100.00%
HQ833383.1	Populus tremula ft2 (ft2) gene, exons 1, 2 and partial cds	230	230	10%	2.00E-55	97.06%

d) Homologous sequences of Amrapalli

Fig. 4.9: BLASTn result showing alignment homologs of (a) Dashehari, (b) Amrapalli. Nucleotide sequence identity of isolated *FT* gene sequence (c) Dashehari and (d) Amrapalli with other plant *spp*.

These homologous *FT* gene sequence from other species were used for generation of phylogenetic tree to show the divergence among the sequences. Homologous sequences of two isolated genes were aligned using ClustalW and then phylip was used for constructing the tree. Phylogenetic tree constructed for *FT* sequence of Dashehari (1456 bp), showed that *FT* gene sequence has highest similarity to *Mangifera indica cv.* Alphonso (JX141437.2) (Fig. 4.10)

Similarly, phylogenetic tree was constructed for *FT* sequence of Amrapalli (1378 bp). It showed that *FT* gene sequence has highest similarity to *FT* mRNA of *Mangifera indica cv.* Alphonso (JX141437.2), *Populus nigra* (HQ833383.1), *Pyrus communis*, *Pyrus pyrifolia* followed by *FT* mRNA of *Malus domestica, Populus nigra* (AB369073.1) (Fig. 4.10).

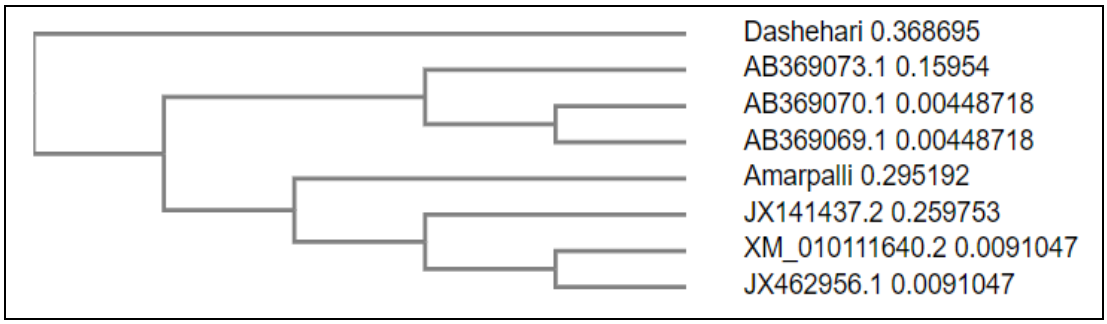
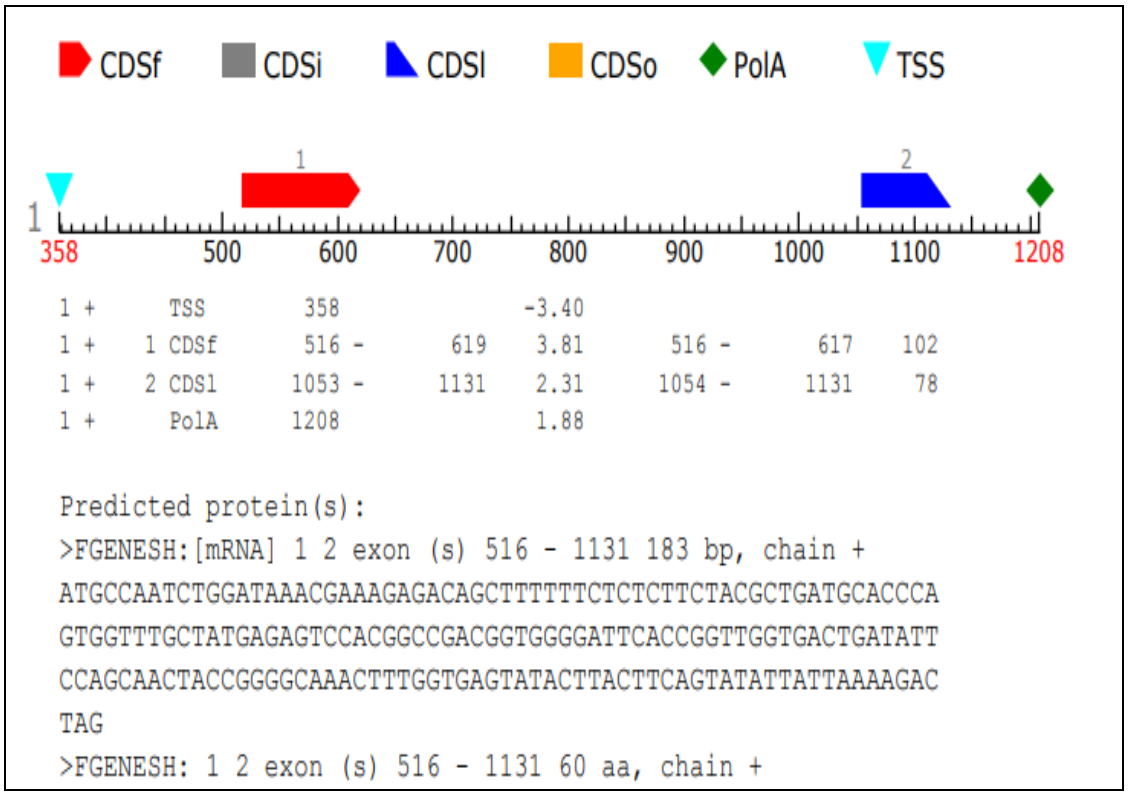


Fig . 4.10: Phlyogenetic tree drawn using ClustalW

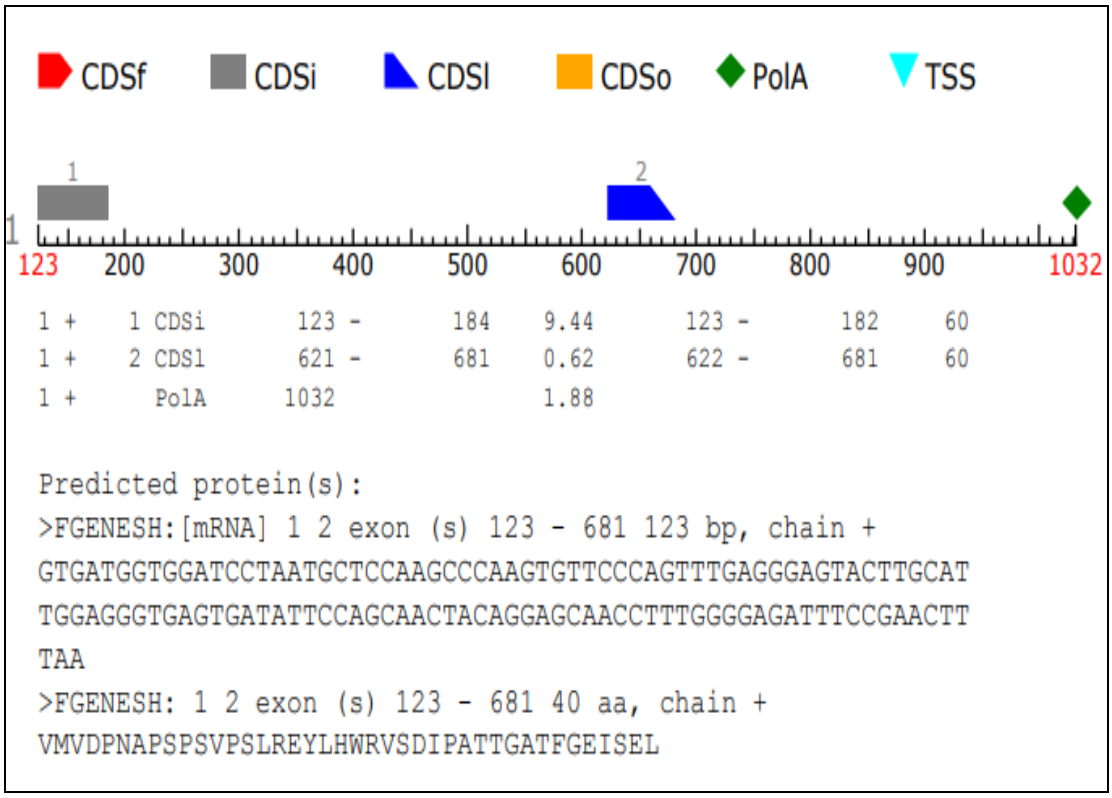
The gene model or gene structure prediction was done using FGENESH tool. It showed that the sequence of both the varieties consists of two exons (Fig. 4.11).




```
1 +      TSS        358                -3.40
1 +    1 CDSf       516 -       619    3.81        516 -       617    102
1 +    2 CDSl      1053 -      1131    2.31       1054 -      1131     78
1 +      PolA      1208                1.88

Predicted protein(s):
>FGENESH:[mRNA] 1 2 exon (s) 516 - 1131 183 bp, chain +
ATGCCAATCTGGATAAACGAAAGAGACAGCTTTTTTCTCTCTTCTACGCTGATGCACCCA
GTGGTTTGCTATGAGAGTCCACGGCCGACGGTGGGGATTCACCGGTTGGTGACTGATATT
CCAGCAACTACCGGGGCAAACTTTGGTGAGTATACTTACTTCAGTATATTATTAAAAGAC
TAG
>FGENESH: 1 2 exon (s) 516 - 1131 60 aa, chain +
```

A




```
1 +    1 CDSi       123 -       184    9.44        123 -       182     60
1 +    2 CDSl       621 -       681    0.62        622 -       681     60
1 +      PolA      1032                1.88

Predicted protein(s):
>FGENESH:[mRNA] 1 2 exon (s) 123 - 681 123 bp, chain +
GTGATGGTGGATCCTAATGCTCCAAGCCCAAGTGTTCCCAGTTTGAGGGAGTACTTGCAT
TGGAGGGTGAGTGATATTCCAGCAACTACAGGAGCAACCTTTGGGGAGATTTCCGAACTT
TAA
>FGENESH: 1 2 exon (s) 123 - 681 40 aa, chain +
VMVDPNAPSPSVPSLREYLHWRVSDIPATTGATFGEISEL
```

B

Fig. 4.11: Gene structure prediction of (A) Amrapalli and (B) Dashehari using FGENESH tool

4.3. Expression of *FT* gene in mango

4.3.1 Total RNA isolation and cDNA synthesis from mango varieties at different developmental stages

Young and mature leaves of both the mango cultivars were used for total RNA isolation at three different developmental stages *i.e.* early stage, before flowering (mid-January), mid stage, prior to induction of flowering (mid-February) and late stage, prior to flower bud initiation (early-March) using RNAisoplus. The total RNA quantity and quality was assesed on nanodrop and in 1.0 % agarose gel (Fig. 4.12).

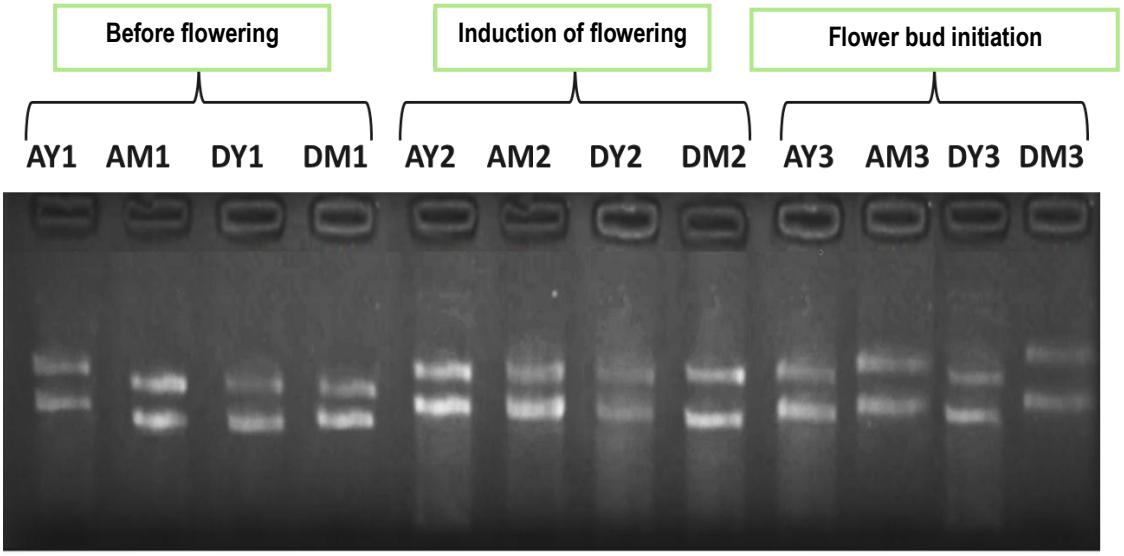


Fig . 4.12: RNA quantification on gel; Where AY1, AY2, AY3: Young leaf samples of Amrapalli, AM1, AM2, AM3: Mature leaf samples of Amrapalli; DY1, DY2, DY3: Young leaf samples of Dashehari, DM1, DM2, DM3: Mature leaf samples of Dashehari, at three different stages

From each sample, total RNA (5 µg) was used for cDNA synthesis using PrimeScript™ First strand cDNA synthesis kit (TaKaRa**).** The cDNA integrity was verified by PCR using *Mangifera indica Elongation Factor* (*MiEF*) gene primers as internal control. The genes that are used as internal control must have a consistent expression level, free of the developmental stages and uneffective by external treatments or conditions. Even though, other genes for example 18S rRNA and actin gene primers have been already stated as stable internal control in different mango cultivars but *MiEF* is the most preferred (Nakagawa *et* al 2012). This *MiEF* gene primer was chosen as internal control for semi quantitative PCR due to its greater abundance. The amplicons were resolved on 1.0 % agarose gel and amplicon corresponding to approx 500 bp was amplified in all the samples that revealed the synthesis of cDNA in all samples (Fig. 4.13).

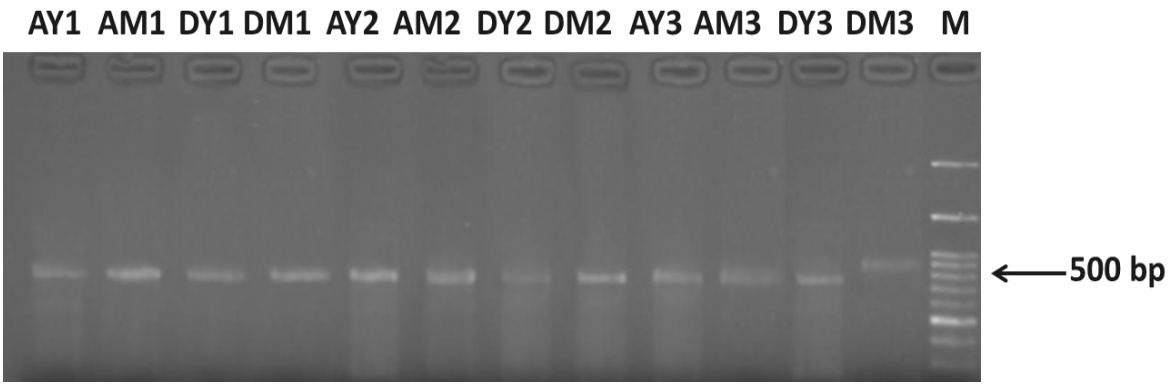
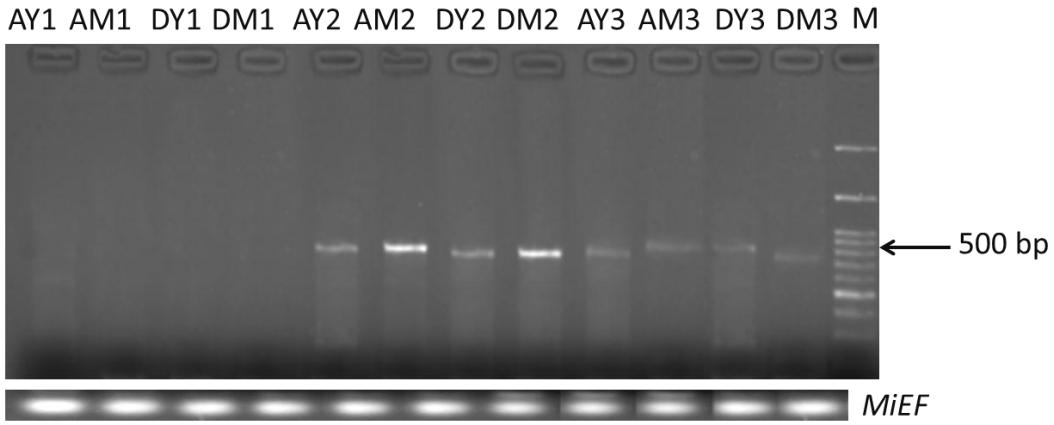


Fig. 4.13: cDNA confirmation of samples of young and mature leaf of Amrapalli and Dashehari .Where AY1, AY2, AY3: Young leaf samples of Amrapalli, AM1, AM2, AM3: Mature leaf samples of Amrapalli; DY1, DY2, DY3: Young leaf samples of Dashehari, DM1, DM2, DM3: Mature leaf samples of Dashehari, at three different stages

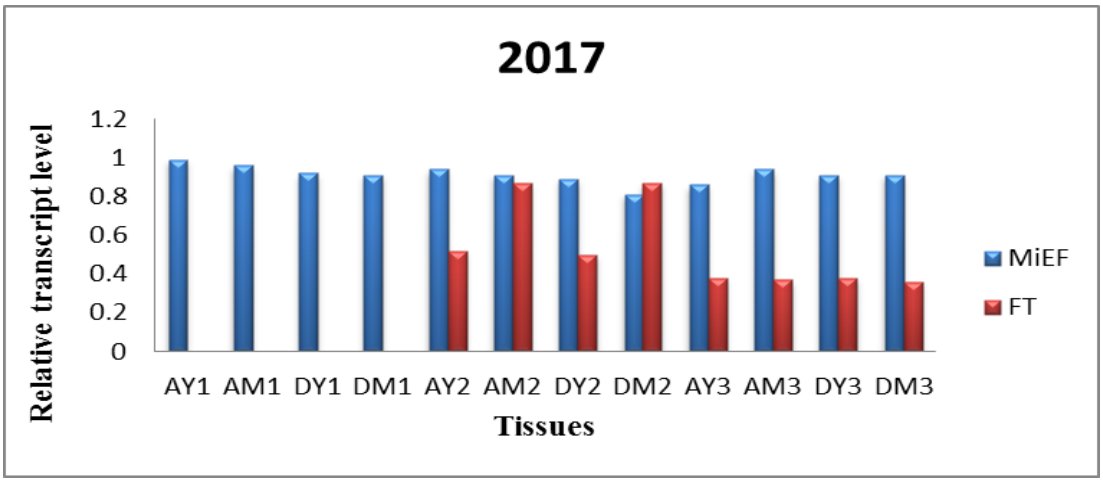
4.3.3 PCR amplification of *FT* gene using gene specific primers

The *FT* gene expression at three different developmental stages *viz.* early stage (mid-January) before flowering, mid stage, prior to induction of flowering (mid-February) and late stage, prior to flower bud initiation (early-March) was then confirmed by amplifying the cDNA using gene specific primers *i.e.* FT-F3 and FT-R3 (Table 1) and resolving the amplicons on agarose gel. The results obtained revealed the expression of approx. 500 bp *FT* gene in leaf samples indicating the presence of *FT* gene. This is in agreement with the result given by Carmona *et al* (2017) that *FT* sequence isolated till now are in the range 500 to 550 bp.

From the expression data obtained from semi quantitative method, it was concluded that in the case of year 2017 the expression of *FT* was more in mid stage as compared to other two stages in both the mango varieties. The early stage showed no expression and the late stage showed very less expression. This is in accordance with the result given by Vyavahare *et al* (2017) in cultivar Alphonso. The expression of *FT* increases in the mid stage *i.e.* during the time of induction of flowering and gradually decreases in the late stage that marks the onset of floral bud initiation. This is in accordance with the results given by Zhou *et al* (2017) in *Phalaenopsis* hybrid that the transcripts of *FT* are accumulated in the leaves and acts as florigen that induces flowering. This indicates that during floral induction the *FT* gene expression is more in leaves and decreases gradually as florigen gets transported from leaves to the flower buds. It was also observed that the expression was more in young or apical leaves during flowering than the mature leaf samples (Das *et al* 2019) (Fig. 4.14).



A

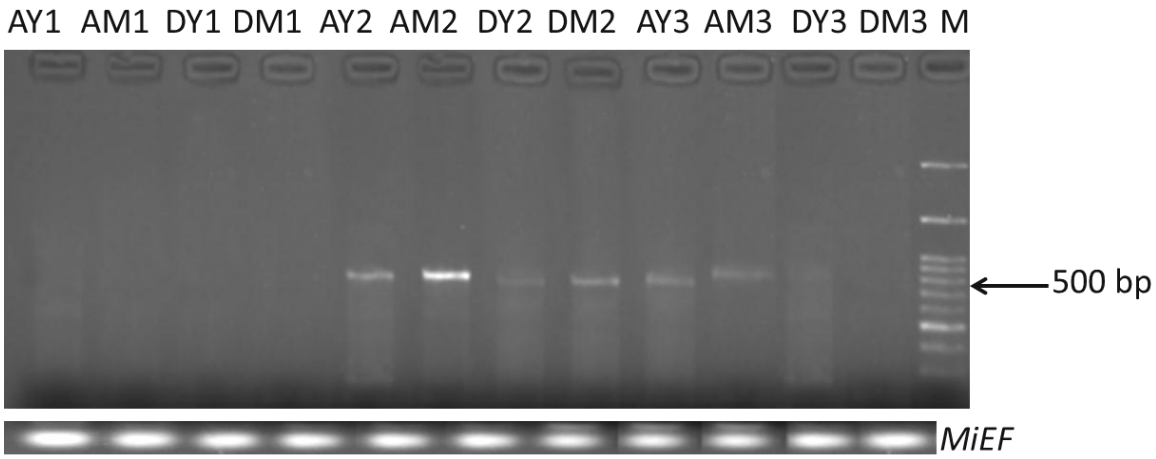


B

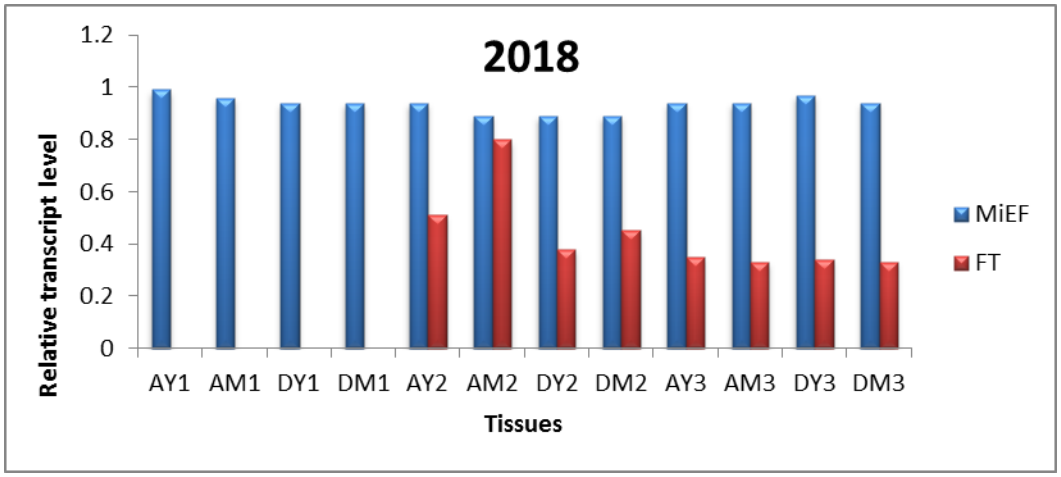
Fig. 4.14: (A) *FT* gene expression monitored in young and mature leaf in 2017. Gel picture in lower panel shows the expression of *MiEF* as an internal control (B) Bar diagram signifies the comparative levels of transcript expression

Similarly in the year 2018, the expression level of *FT* gene was more in the samples of mid stage as compared to no expression in samples of early and very less expression in samples of late stage. But it was observed that the expression level was different among both the varieties *i.e.* it was more in Amrapalli than Dashehari. This in accordance to the results given by Das *et al* (2019) in Amrapalli that being a regular bearer, it maintains an optimum concentration of carbohydrate each year whereas in case of Dashehari cultivar the carbohydrate concentration cannot be maintained every year. The concentration of carbohydrate in alternate bearer *i.e.* in Dashehari varies from "on" year to "off" year. During "off" year, the carbohydrate concentration remains consistent in regular bearer *i.e.* Amrapalli (Fig. 4.15).

These results also conclude that there is presence of coding and non-coding regions in the *FT* gene of mango. As the primers used for amplification were same for both the genomic DNA as well as for RNA but the amplified region was of different size in both the cases. This suggests that the amplified genomic DNA contained coding (exons) and non-coding (introns) regions, whereas, the amplified RNA was had coding regions (exons) only.



A



B

Fig. 4.15: (A) *FT* gene expression monitored in young leaf and mature leaf samples in 2018. Gel picture (lower panel) shows the expression of *MiEF* as an internal control (B) Bar diagram denotes the relative levels of transcript expression

CHAPTER V

SUMMARY

Mango (*Mangifera indica* L.) is a major fruit crop of both the tropical as well as subtropical regions of the world. In Punjab, it is being cultivated on an area of 6896 hectares with an annual production of 116515 metric tonnes (Anonymous 2019b). Still, in India the yields are low as compared to other countries. There are numerous reasons behind it including poor management practices, inadequate nutrient application and irregular/biennial flowering of most of the cultivars grown commercially. The flowering process in mango is exceptionally complex. Normally, mango trees bear heavy crop load in one year considered as "on year" and less or no crop in the subsequent year or "off year". The term irregular, alternate or biennial bearing implies to the tendency of mango trees to bear a heavy crop in one year and very little or no crop in the following year. The different studies have revealed that the process of development of floral parts in plants is controlled by the regulation of numerous genes included under internal factors as well as by various environmental or external factors. Specifically talking about mango, in subtropical conditions cool temperature induces flower induction. Under tropical conditions, there are different external factors responsible. This suggests that there are different flowering pathways for different crop species.

The present investigation was mainly focused on three experiments *i.e.* first, identification of *FT* gene; second, *in silico* characterisation of the isolated sequence from two mango cultivars *i.e.* Amrapalli and Dashehari and third, expression analysis of this *FT* gene in leaf tissues at different developmental stages using semi-quantitative method.

The total DNA was isolated from young and mature leaf tissues of Amrapalli and Dashehari. The degenerate pair of primers FT- F3 and FT-R3 was designed using the already present nucleotide sequence of the *FT* of different crops from NCBI. These primers were used for PCR analysis of isolated genomic DNA. The amplicons of 1.5 kb was observed after PCR analysis and were eluted and purified for cloning into pGEM-T easy vector for its sequence confirmation. The sequence of 1456 bp *FT* gene was confirmed in Dashehari and 1378 bp in Amrapalli. The gene encoding *FT* of size 1456 bp in Amrapalli and Dashehari (*Mangifera indica*) (KX093179 to KX093244) was isolated earlier. Our *FT* gene sequence is approximately 120-150 bp larger as compared to the earlier reported.

Based on BLASTn analysis, this sequence showed 96.68% homology with *Mangifera indica cv.* Alphonso *Flowering locus-T like* protein gene (JX141437.2). The pylogenetic analysis also showed the relation of these similar sequences. ClustalW program was used to create phylogenetic tree and to show which genes were related to the nucleotide query.

Phylogenetic tree constructed for *FT* sequence of Amrapalli (1378 bp), showed that *FT* gene sequence has highest similarity to *FT* mRNA of *Mangifera indica cv.* Alphonso (JX141437.2), *Populus nigra* (HQ833383.1), *Pyrus communis*, *Pyrus pyrifolia* followed by *FT* mRNA of *Malus domestica*, *Populus nigra* (AB369073.1).

Total RNA was isolated from the young and mature leaf tissues of both the varieties at three different developmental stages *i.e.* early stage; prior to before flowering (mid-January), mid stage; prior to induction of flowering (mid-February) and late stage; prior to flower bud initiation (early-March) and cDNA was synthesised. The integrity of cDNA was verified by PCR using *MiEF* gene primers as internal control. PCR amplifications of *FT* gene using gene specific primer from cDNA resulted in the amplicon size of approx. 500 bp revealing that the gene is having introns and exons These results also conclude that there is presence of coding and non-coding regions in the gene of mango *FT*. As the primers used for amplification were same for both the genomic DNA as well as for RNA but the amplified region was of different size in both the cases. This suggests that the amplified genomic DNA contained coding (exons) and non-coding (introns) regions, whereas, the amplified RNA was having coding regions (exons) only.

The transcript expression of *FT* was evaluated in mature and young leaf of both the varieties at different developmental stages using semi quantitative PCR analysis. From the expression data obtained from semi quantitative method, it was concluded that in case of year 2017 the expression of *FT* was more in mid stage as compared to other two stages. The early stage showed no expression and the late stage showed very less or no expression. This is in accordance with the earlier research in Alphonso, another mango variety. The expression of *FT* increases in the mid stage *i.e.* during the time of induction of flowering and gradually decreases in the late stage that marks the onset of floral bud initiation. This indicates that during floral induction the *FT* gene expression is more in leaves and decreases gradually as florigen gets transported from leaves to the flower buds. It was also observed that the expression was more in young or apical leaves during flowering than the mature leaf samples.

Similarly in the year 2018, the expression level of *FT* gene was highest in the samples of mid stage as compared to the no expression in samples of early and very less expression in samples of late stage. But, it was observed that the expression level was different among both the varieties *i.e.* it was more in Amrapalli than Dashehari. This may be due to the off year of Dashehari, making it alternate bearer.

REFERENCES

Andemeyo O S, Chavarriaga P, Tohme J, Fregene M, Davis S J and Setter T L (2017) Overexpression of *Arabidopsis FLOWERING LOCUS T (FT)* gene improves floral development in cassava (*Manihot esculenta* Crantz). *PLoS One* **12(7)**: 28-32.

Anonymous (2019a) Area and production of different fruits in India. Website: *www.nhb.gov.in*

Anonymous (2019b) Area and production of fruit crops in Punjab, Directorate of Horticulture, Punjab.

Bernier G and Perilleux C (2005) A physiological overview of the genetics of flowering time control. *Pl Biotech J* **3**: 3-16.

Boss P K, Bastow R M, Mylne J S and Dean C (2004) Multiple pathways in the decision to flower: enabling, promoting and resetting. *Pl Cell* **16**: 18-31.

Cao K, Cui L, Zhou X, Ye L, Zou Z and Deng S (2016) Four tomato *FLOWERING LOCUS T*-Like proteins act antagonistically to regulate floral initiation. *Front Pl Sci* **112**: 22-38.

Carmona M J, Calonje M and Martínez-Zapater J M (2017) The FT/TFL1 gene family in grapevine. *Pl Mol Bio* **63(5)**: 637-50.

Chailakhyan M K (1936a) On the mechanism of photoperiodic reaction. *Dokl Akad Nauk SSSR* **1**: 89-93.

Chailakhyan M K (1936b) On the hormonal theory of plant development. *Dokl Akad Nauk SSSR* **3**: 442-47.

Chailakhyan M K (1936c) New facts for hormonal theory of plant developent. *Dokl Akad Nauk SSSR* **4**: 79-83.

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C and Coupland G (2007) *FT* protein movement contributes to long-distance signalling in floral induction of *Arabidopsis*. *Sci* **316**: 1030-33.

Das A, Geetha G A, Ravishankar K V, Shivashankara K S, Roy T K, Dinesh M R (2019) Interrelations of growth regulators, carbohydrates and expression of flowering genes (*FT*, *LFY*, *AP1*) in leaf and shoot apex of regular and alternate bearing mango (*Mangifera indica* L.) cultivars during flowering. *Sci Hort* **253**: 263-69.

Franklin K , Lee S H, Patel D, Kumar S V, Spartz A K, Gu C, Ye S, Yu P, Breen G and Cohen J D (2011) Phytochrome-interacting factor 4 (PIF4) regulates auxin biosynthesis at high temperature. *Proc Natl Acad Sci* **108**: 231-35.

Fukuda M, Matsuo S, Kikuchi K, Kawazu Y, Fujjiyama R and Honda I (2011) Isolation and functional characterisation of the *FLOWERING LOCUS T* homolog, the *LsFT* gene, in lettuce. *J Pl Physio* **168(13)**: 1602-07.

Huang T, Bohlenius H and Eriksson S (2005) The mRNA of the *Arabidopsis* gene *FT* moves from leaf to shoot apex and induces flowering. *Sci* **309**: 1694–96.

Iyer C P A and Subramanyam M D (1993) Improvement of mango. In: Chadha K L and Pareek O P (ed) *Advances in Horticulture*, Vol 1 Malhotra Publishing, New Delhi.

Krishna B, Vyavahare S N, Chaudhari R S, Subramaniam V R and Sane P V (2017) Roles of *Flowering Locus T* (*FT*) and *Terminal Flower 1* (*TFL1*) in flowering of mango. *Acta Horti*. doi 10.17660/ActaHortic.2017.1183.17.

Kotoda N, Hayashi H, Suzuki M, Igarashi M, Hatsuyama Y, Kidou S, Igasaki T, Nishiguchi M, Yano K, Shimizu T, Takahashi S, Iwanami H, Moria S and Abe K (2011) Molecular characterisation of *FLOWERING LOCUS T*- like genes in apple (*Malus x domestica* Borkh.). *Pl Cell Physiol* **51(4)**: 565-75.

Li C, Luo L, Fu Q, Niu L and Xu Z (2015) Identication and characterisation of *FT/TFL1* gene family in the biofuel plant *Jatropha curcsas*. *Pl Mol Biol Rep* **33:** 326-33.

Lin M, Belanger H and Lee Y (2007) *Flowering Locus T* protein may act as the long-distance florigenic signal in the Cucurbits. *Pl Cell* **19**: 1488–1506.

Mitra S K (2016) Mango production in the world, present situation and future prospect. *Acta Hort* **1111**: 287-96.

Nakagawa M, Honsho C, Shinya K and Utsunomiya N (2012) Isolation and expression analysis of Flowering locus T-like and gibberellin metabolism genes in biennial bearing mango trees. *Sci Hort* **139**: 108-17.

Nishikawa F, Endo T, Shimada T, Fujii H, Shimizu T, Omura M and Ikoma Y (2007) Increased *CiFT* abundance in the stem correlates with floral induction by low temperature in Satsuma mandarin (*Citrus unshiu* Marc.). *J Exp Bot* **58**: 3915-27.

Pin P A and Nilsson O (2012) The multifaceted roles of *FLOWERING LOCUS T* in plant development. *Pl Cel Env* **35**: 1742-55.

Protacio C M, Quinto J E, Serrano E P, Marquez I P and Rodriguez F M (2009) Unravelling the mechanism of mango flowering. *Acta Hort* **820**: 259-70.

Putterill J, Zhang L, Yeoh C C, Balcerowicz M, Jaudal M and Gasic E V (2013) *FT* genes and regulation of flowering. *Func Pl Biol* **40**: 1199-207.

Ramirez F and Davenport L T (2010) Mango (*Mangifera indica* L.) flowering physiology. *Sci Hort* **126**: 65-72

Ravishankar V K, Bommisetty P, Bajpai A, Srivastava N, Mani H B, Vasugi C, Rajan S and Dinesh M R (2015) Genetic diversity and population structure of mango (*Mangifera indica*) cultivars assessed by microsatellite markers. *Tree* **29**: 775-83.

Reddy S, Sharma R R, Barthakur S and Srivastva M (2015) An efficient and rapid method for the isolation of RNA from different recalcitrant tissues of mango. *J Hort Sci Biotech* **90**: 285–90.

Sachs J (1865) Wirkung des lichtes auf die blutenbilding verinttlung der laubbatter. *Bot Zeit* Pp. 117-21.

Sandip M, Makwana A N, Warad A V and Nawadw B D (2015) Physiology of Flowering – The case of Mango. *Int J App Res* **1(11)**: 1008-12.

Sharma N, Singh S K, Mahato A K, Ravishankar H, Dubey A K and Singh N K (2018) Physiological and molecular basis of alternate bearing in perennial fruit crops. *Sci Hort* **243**: 214-25.

Singh N K, Mahato A K, Jayaswal P K, Singh A, Singh S, Singh N, Rai V, Mithra A S V, Gaikwad K, Sharma N, Lal S, Srivastava M, Prakash J, Kalidindi U, Singh S K, Singh A K, Khan K, Mishra K, Rajan S, Bajpai A, Sandhya B S, Nischita P, Ravishankar K V, Dinesh M R, Kumar N, Jaiswal S, Iquebal M A, Kumar D, Rai A and Sharma T R (2016) Origin, diversity and genome sequence of mango (*Mangifera indica* L.). *Ind J Hist Sci* **51**: 355-68.

Sawa M and Kay S A (2011) *GIGANTEA* directly activates *Floweing Locus T* in *Arabidopsis thaliana*. *Proc Natl Acad Sci* **108**: 698-703.

Tan F and Swain S M (2006) Genetics of flower initiation and development in annual and perennial plants. *Physio Pl* **128**: 8-17.

Tharanathan R N, Yashoda H M and Prabha T N (2006) Mango (*Mangifera indica* L.), "The king of fruits" – An overview. *Food Rev Int* **22**: 95-123.

Tseng T S, Salome P A, McClung C R and Olszewski N E (2004) *SPINDLY* and *GIGNTEA* interact and act in *Arabidopsis thaliana* pathways involved in light responses, flowering and rhythms in cotyledon movements. *Pl Cell* **16**: 1550-63.

Villalobos S, Cota F, Folter S and Cabriales J (2012) Primers to amplify flowering locus T (*FT*) transcript in mango and their potential use in other angiosperms. *Pl Omic J* **5**: 453-57.

Vyavahare S N, Krishna B, Joshi S S, Chaudhari S S, Subramaniam V R AND Sane P V (2017) Characterisation of *Flowering Locus T* (*FT*) and *Terminal Flower 1* (*TFL1*) genes. *Acta Hort* **1183**: 113-24.

Walwoth A E, Chai B and Song G (2016) Transcript profile of flowering regulatory genes *Vc-FT*-overexpressing blueberry plants. *PLoS one* **11(6)**: 1-16.

Winterhagen P, Tiyayon P, Hegele M and Wunsche J N (2017) Expression of flowering genes in the subtropical fruit tree *Dimocarpus longan*. *Pl Physio Biochem* **71**: 184-90.

Wu H, Jia H, Ma X, Wang S, Yao Q, Xu W, Zhou Y, Gao Z and Zhan R (2014) Transcriptome and proteome analysis in mango (*Mangifera indica* L.) fruits. *J prot* **105**: 19-30.

Xu F, Rong X, Huang X and Cheng S (2012) Recent advances of *Flowering Locus T* in higher plants. *Int J Mol Sci* **13**: 3773-81.

Yoo S K, Chung K S, Kim J, Lee J H, Hong S M, Yoo S J and Ahn J H (2005) *CONSTANS* activates *Suppresor of Overexpression of Constans 1* through *Floweing Locus T* to promote flowering in Arabidopsis. *Pl Physiol* **139**: 770-78.

Zeevart J A D (2006) Florigen coming of age after 70 years. *Pl cell* **18**:1783-89.

Zhang T, Ying Z and Davenport L N (2010) Isolation and characterisation of a *CONSTANS*-like gene in mango. *Pl Bio* **65**: 3395-404.

Zhou S, Jiang L, Guan S, Gao Y, Gao Q, Wang G and Duan K (2017) Expression profiles of five *FT*-like genes and functional analysis of *PhFT*-1 in a *Phalaenopsis* hybrid. *Elect J Biotech* **31**: 75-83.

VITA

Name of the Student	**:**	Shilpa
Father's name	**:**	Mr. Ghanshyam
Mother's name	**:**	Mrs. Sumna Devi
Nationality	**:**	Indian
Date of birth	**:**	27-06-1994
Permanent Address	**:**	Village Khas Narwana, PO Yol Cantt Tehsil Dharmshala, District Kangra, Himachal Pradesh – 176 052

EDUCATIONAL QUALIFICATIONS

Bachelor's degree	**:**	B.Sc. Agri. (Hons.)
University and year of award	**:**	CSK HPKV, Palampur 2016
OCPA	**:**	7.53/10.00
Master's degree	**:**	M.Sc. Biotechnology
University and year of Award	**:**	Punjab Agricultural University, Ludhiana 2020
OCPA	**:**	7.40/10.00
Title of Master's Thesis	**:**	Expression analysis of flowering switch factors using candidate gene approach in mango
Awards/Distinctions/ Fellowships	**:**	JRF - National Talent Fellowship by ICAR