Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.)

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(2012-11-193)

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by

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2014

DECLARATION

I hereby declare that the thesis entitled **"Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.) "** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Date: 18-09-2014 **(2012-11-193)**

CERTIFICATE

Certified that this thesis, entitled **"Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.)"** is a record of research work done independently by Mr. Hemanth (2012-11-193) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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HEMANTH

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
A_{595}	Absorbance at 595 nm wavelength
AMV-RT	*Avian myeloblastosis virus* reverse transcriptase
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cm	Centimetre
Ct	Threshold cycle
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
F	Forward primer
G	Guanine
g	gram
g	standard acceleration due to gravity at the earth's surface
h	Hour
ha	Hectare
kbp	Kilo basepair
kg	Kilogram

M	Molar
mg	Milligram
min	Minute
ml	Millilitre
m*M*	Millimolar
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometre
^{o}C	Degree celsius
OD	Optical density
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
qRT-PCR	Quantitative reverse transcriptase - polymerase chain reaction
R	Reverse primer
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcription - polymerase chain reaction
s	Second
SAM	Shoot apical meristem
SD	Short day
sp.	Species
spp.	Species (plural)
t	Tonne

T	Thymine
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (hydroxy methyl) aminomethane hydrochloride
U	Enzyme unit
V	Volt
v/v	volume/volume
w/v	weight/volume

INTRODUCTION

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae. It is often called as "King of Spices" and 'Black Gold'. It is a perennial climbing vine grown for its berries which are extensively used as spice and medicine. It is native to humid, tropical evergreen forests of the Western Ghats in India. It is economically one of the most important spice crops in the world (Ravindran *et al*., 2000). It is predicted that global demand for pepper will escalate colossally to about 280,000 t by the year 2020 and that it will further climb to 360,000 t by the year 2050 (Nair, 2011). Though India tops the list among the producers in acreage and production, its productivity is the lowest in the world.

The area under black pepper has been drastically shrinking in India during the last few years. The area under cultivation in Kerala is reduced from 2.02 lakh ha in 2000-01 to 1.54 lakh ha in 2009-10. The productivity has also come down from 376 kg per ha during 1998-99 to 221 kg per ha during 2009-10. Consequently, the production has declined from 60,000 t to 37,899 t during the corresponding period (Anon., 2010). To overcome this, more emphasis has to be given to crop improvement programmes which involve molecular and genetic tools.

Flowering is one of the important processes which decides the yield. A study of the molecular and genetic processes of flower initiation to floral organ development and its interaction with environmental parameters may prove useful for improving and predicting the yield of black pepper. It gains added importance in view of the emerging scenario of global warming and climate change.

Most of the previous work on flowering in *Piper nigrum* has been confined to floral morphology and floral development. However, none of these studies have tracked the molecular and genetic basis of the development of the flowers from floral initiation to organ differentiation and development in black pepper.

To understand the flowering process it is essential to isolate and characterize the genes involved in flowering and to relate their expression to actual morphological development in plants. This could in turn lead to the development of a flowering model which could help to predict the time of flowering and yield more accurately.

The alteration of flowering character by transfer of desired genes regulating flowering, or by over-expression or suppression of such genes through biotechnological methods has been found successful in many plants (Giovannini, 2006). Determination of the molecular pathways leading to formation of flowers in black pepper can aid in the increase of flowers in the plant which in turn produces more number of berries, to produce spikes at any environmental conditions and also to produce superior varieties of black pepper with increased productivity. With this in mind the present study was done in order to identify the flowering genes, specifically floral meristem identity genes, which are essentially involved in conversion of vegetative meristem to floral mersitems.

In model plants such as *Arabidopsis* and *Antirrhinum*, several genes regulating flowering have been uncovered with complete knowledge of their interactions and functions. These genes are found to be highly conserved among several species of monocot and dicot plants.

In *Arabidopsis*, genes *viz.*, *LEAFY* (*LFY*), *APETALA 1* (*AP1*), *FRUITFULL 1* (*FUL1*) and *TERMINAL FLOWER 1* (*TFL1*) were generally regarded as floral meristem identity genes (Weigel *et al*., 1992; Bowman *et al*., 1993; Schultz and Haughn, 1993). Among them the *APETALA 1* and *FRUITFULL 1* were selected to analyze in the present study.

This study includes,

- Designing degenerate primers for the isolation of floral meristem identity gene in black pepper.

- Target isolation and sequencing of floral meristem identity gene using the designed primers through PCR and RT-PCR from black pepper variety Karimunda.
- Sequence analysis of the gene and characterization of expression patterns of the isolated gene through qRT-PCR and RT-PCR in different tissues and at different stages of floral development.
- Histological studies to track floral development in black pepper.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Black pepper belongs to the botanical family Piperaceae, which is a large pantropical family of subclass Dicotyledonae and order Piperales **(**Cronquist, 1978). This family shares close features to the monocotyledonous boundary (Metcalfe and Chalk, 1950) which make their entry into 'paleoherbs''- an assemblage of dicots resembling monocot (Donoghue and Doyle, 1989). The family is represented by over 10 genera (Cronquist, 1981**)** with *Piper* (Linn.) and *Peperomia* (Ruiz and Pav.) being the major ones, each with a minimum of 1,200 species (Callejas, 2001).

The genus *Piper* is recorded with the greatest diversity in American tropics (700 spp.), followed by South Asia (300 spp.) where the economically important species, *Piper nigrum* L. (black pepper) and *Piper betle* L. (betel leaf) are originated (Jaramillo and Manos, 2001). The Western Ghats of South India and the North-Eastern India are the two hotspots of diversity of the genus *Piper* in India, inhabiting about 110 species (Purseglove *et al*., 1981; Parthasarathy *et al*., 2006).

Black pepper is the most widely used spice crop in the world with the global production of 327,090 t in 2012. The productivity of black pepper in India is very low, producing 43,000 t from 182,000 ha in 2012. Whereas, Vietnam is the leading producer and exporter of black pepper, producing 100,000 t of black pepper from 52,000 ha in the same year (Joseph, 2012).

Black pepper is mostly dioecious in wild form undergoing cross pollination (Krishnamurthi, 1969). However, in cultivated types, the plants are mostly gynomonoecious (i.e. bearing female and bisexual flowers in the same plant) or trimonoecious (i.e. bearing female, male and bisexual flowers in the same plant), and are fertilized by self-pollination (Nair *et al*., 1993; George *et al*., 2005; Thangaselvabai *et al.*, 2008).

The genus *Piper* generally bears the terminal solitary type of inflorescence. Though, rare umbellate inflorescences were reported in *Macropiper* and *Pothomorphe* (Jaramillo and Manos, 2001).

The uncovering of genetic pathways leading to spike formation can be a boon in crop improvement programme, producing more number of spikes per plant. This could be possible initially by identifying the genes associated with flowering i.e. floral initiation genes, floral meristem identity genes, organ identity genes and cadastral genes and by applying the molecular studies in determining these genes and their regulation which could be modified to increase the flowering.

In this chapter, the literature concerned with flowering genes, especially floral meristem identity genes and the genetic pathways regulating them in some important plants like *Arabidopsis thaliana*, *Antirrhinum* and the molecular techniques associated with identifying these genes are presented.

2.1 GENERAL MORPHOLOGY OF BLACK PEPPER PLANT

Black pepper is a perennial climber, climbing on support trees with the help of aerial clinging roots. The climber produces two types of branches, *viz.*, orthotropic branches (main stem) and plagiotropic branches (fruiting stem). The orthotropic branches are straight, upward growing with monopodial growth habit. The nodes are swollen with 10-15 short adventitious roots and a leaf per each node. At the axil of each leaf of the orthotropic branches lies an axillary bud which develops into a plagiotropic branch. The plagiotropic branches are without aerial roots that grow laterally with sympodial growth habit and produce flowers and fruits. As the shoot grows, the terminal bud gets modified into a spike and the growth is continued by the axillary bud (Ravindran *et al*., 2000).

The plant has a broad, shiny green, pointed, petiolate leaves alternately arranged on the stem. The inflorescence is catkin (spike), 7-10 cm long, clustered with sessile small flowers. Between 50 to 150 whitish to yellow-green flowers are produced on a spike. The berry-like fruit is a drupe, each containing a single seed, and when dried, it is called a peppercorn (Chaveerach *et al*., 2006).

2.2 INFLORESCENCE AND FLORAL DEVELOPMENT IN BLACK PEPPER

Detailed study on the development of inflorescence and flowers in black pepper has not been reported till date. The only available information is the studies on inflorescence and flower development by Tucker (1982) in three *Piper* spp. *viz*., *Piper aduncum*, *Piper amalgo* and *Piper marginatum*, and the process of development is believed to be conserved in all other species as well (Ravindran *et al*., 2000).

According to the observations by Tucker (1982), initially the *Piper* inflorescence has a convex apical meristem, subtended by a vegetative leaf and a bract. The early apical meristem is zonate, usually with two tunica layers, a large central initial zone, a peripheral zone and a massive pith rib meristem. The apical meristem of the inflorescence grows extensively in length before any organs form and as it grows in length its apical meristem diminishes. The bracts are initiated close to the apex by periclinal division in the second tunica layer on the flanks of the apical meristem. Each flower arises in the axil of a bract. Cells in the axils remain meristematic and cells in the outer layer divide anticlinally. The next two layers undergo intensive cell divisions, including periclinal divisions before the formation of protuberence. Cells elongate anticlinally in the outer two layers making the protuberance. This enlarges further and differentiates into a flower bud. As the primordium (protuberence) mentioned above grows, stamens are initiated from the two lateral sites. The carpels are initiated by periclinal divisions in the sub surface

layer on the side of the small floral apex. Ovule initiation begins by a periclinal division in the second tunica layer at the centre of the floral apex (Bernier *et al.*, 1993; Bernier *et al.*, 1998).

Floral bud differentiation studies in black pepper has been done by Nalini (1983). It includes the microscopy study of vegetative bud and flowering bud in black pepper which shows leaf sheath, leaf primodium, shoot primodium, and spike primodium.

Transition from vegetative growth to flowering and its timing is of paramount importance in agriculture, horticulture, and plant breeding because flowering is the initial step of sexual reproduction. This transition is being studied by countless physiologists and have managed to produce an almost unmanageably large amount of information.

Three major theories have put forth to explain the transition to flowering. The "florigen/antiflorigen" concept by Lang (1984) proposes that flowering is controlled by floral promoter which is a universal hormone known as florigen. The "nutrient diversion" hypothesis by Sachs and Hackett (1983) postulates that floral induction depends upon the source/sink relationships i.e. the partitioning of assimilates such that critical areas of the shoot apical meristem (SAM) *viz*., the relatively quiescent central zone receives high concentrations of assimilates under inductive conditions. Finally, the most promising theory is "multifactorial control" which postulates that several chemical assimilates, phytohormones, genetic variation, as well as past and present growing conditions participate in floral induction. Studies conducted in *Sinapis alba* and *Arabidopsis* proved that the control of floral transition is multigenic and multifactorial (Bernier *et al.*, 1993). Hence it can be concluded that the transition of vegetative to reproductive phase is regulated by a complex network of signal pathways and genetic network, which monitors the developmental state of the plant

as well as the environmental factors such as light and temperature (Simpson *et al*., 1999; Giovannini, 2006).

Genetic analysis of 'flowering-time' mutants have identified about 80 genes placed in multiple genetic pathways that control the floral transition in *Arabidopsis*. The photoperiod pathway and the vernalization promotion pathway mediate signals from the environment. The autonomous pathway monitors endogenous cues from the developmental state. Genes involved in gibberellin (GA) biosynthesis and GA signal transduction have been suggested to form a distinct promotion pathway. A number of genetic models have proposed that signals from multiple promotion pathways converge on a central floral repressor (Chen *et al.,* 1997; Koornneef *et al*., 1998) encoded by the *EMBRYONIC FLOWER* genes (Sung *et al.,* 1992; Yang *et al.,* 1995). The inactivation of the floral repressor in turn has been suggested to lead to the activation of the floral meristem identity genes, such as *LEAFY* (*LFY*) or *APETALA 1* (*AP1*), which specify the floral fate of nascent lateral primordia produced by the SAM. An alternative possibility is that different promotion pathways are directly integrated at the promoters of the floral meristem identity genes, such as *LFY* and a class of 'flowering-time' genes that act in parallel with *LFY*. *LFY* plays a central role in the transition from early reproductive to late reproductive phase (Schultz et al., 1991; Huala *et al.,* 1992; Weigel *et al.,* 1992) and also in promoting the floral transition (Weigel *et al.,* 1995; Blázquez *et al.,* 1997).

The multiple genetic pathways that promote the floral transition are directly integrated at the transcriptional regulation of the floral meristem identity gene *LFY* and the 'flowering-time' genes, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*)/*AGAMOUS LIKE* 20 (*AGL20*). There may be extensive crosstalk between the pathways and integrating genes. A study related to the length of the circadian period in null mutants of the floral repressor *FLOWERING*

LOCUS C (FLC) indicated the presence of unexpected crosstalk between pathways (Swarup *et al.*, 1999).

A major quantitative trait locus (QTL) that is responsible for the photoperiod sensitivity of rice (a SD plant) was cloned (Yano *et al*., 2000). This QTL, *Heading-date 1* (*Hd*-1), also known *as photoperiod sensitivity 1* (*Se1*), encodes a *CONSTANS* (*CO*) homolog. It was suggested that *Hd1/Se1* promotes the floral transition in SD conditions and inhibits it in LD conditions (Putterill *et al*., 1995). This is in contrast to *Arabidopsis CO*, which has low levels of expression in non-inductive SD conditions but does not seem to have an inhibitory role (Araki, 2001).

After the formation of floral meristems the flower formation occurs through a series of sequential steps. First, floral meristem fate is specified through the activity of floral meristem identity genes. Second, the floral meristem is patterned into the whorls of organ primordia through the activity of floral organ identity genes. Third, the floral organ identity genes activate downstream effectors that specify the various tissues and cell types that constitute the different floral organ types. Each of these steps is under strict genetic control, and each involves elaborate networks of positive and negative factors that intersect at various levels to regulate floral morphogenesis (Krizek *et al*., 2005).

The switch from vegetative to reproductive development is controlled by multiple pathways that respond to different environmental and developmental signals (Simpson and Dean. 2002). These pathways converge on a set of floral pathway integrators that activate floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA 1* (*AP1*) in *Arabidopsis thaliana* and their respective homologues *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*) in *Antirrhinum majus*. The floral meristem identity genes ensure that primordia initiated along the periphery of an inflorescence meristem adopt a flower fate. Mutations in these genes result in a

partial conversion of flowers into shoot-like structures whereas, ectopic expression of these genes is sufficient to convert inflorescence meristems into flowers (Coen *et al.,* 1990; Huijser *et al.,* 1992; Weigel *et al.,* 1992; Mandel *et al*., 1995a; Weigel and Nilsson, 1995). *LFY* and *AP1* encode transcription factors (Riechmann *et al.,* 1996; Parcy *et al.,* 1998) that have partially overlapping roles in specifying a floral meristem fate, as *lfy ap1* double mutants show a more complete conversion of flowers into shoots than either single mutant (Weigel *et al.,* 1992). However, to a large degree *AP1* functions downstream of *LFY* (Liljegren *et al.,* 1999) has been shown to be a direct target of *LFY* activation (Wagner *et al*., 1999; William *et al.,* 2004). Other factors that promote a floral meristem fate include *CAULIFLOWER* (*CAL*), the function of which completely overlaps with that of *AP1*, and *Arabidopsis thaliana UNUSUAL FLORAL ORGANS* (*UFO*) or *Antirrhinum majus FIMBRIATA* (*FIM*).

The principal function of the floral meristem identity genes is to activate a small set of genes that specify floral organ identity. The floral organ identity genes were originally identified in *Arabidopsis thaliana* and *Antirrhinum majus* on the basis of their mutant phenotypes, which featured the homeotic transformation of one floral organ type into another.

Genetic analysis of these *Arabidopsis thaliana* floral homeotic mutants and their counterparts in *Antirrhinum majus* led by Coen and Meyerowitz (1991) to the formulation of the classic ABC model for the specification of floral organ identity. The ABC model postulates that three regulatory gene functions A, B and C, which work in a combinatorial fashion to confer organ identity in each whorl. A function, conferred by the class A homeotic genes *APETALA 1 (AP1)*and *APETALA 2* (*AP2)* in *Arabidopsis thaliana* and the redundant *AP2*-like genes *viz*., *LIPLESS 1* and *2* (*LIP1* and *LIP2*) in *Antirrhinum majus* (Keck et al., 2003), specifies sepal identity in whorl 1. A function combined with B, conferred by the class B genes *APETALA 3* (*AP3)* and *PISITALLA* (*PI)* in *Arabidopsis thaliana* and *DEFICIENS* (*DEF*) and

GLOBOSA (*GLO*) in *Antirrhinum majus*, specifies petal identity in whorl 2. B function combined with C, conferred by the class C gene *AGAMOUS* (*AG*) in *Arabidopsis* and *PLENA* (*PLE*) and *FARINELLI* (*FAR*) in *Antirrhinum majus*, specifies stamen identity in whorl 3. C function alone specifies carpel identity in whorl 4 and also confers floral determinacy.

A second key facet of the ABC model is that A function and C function are mutually antagonistic, such that class C activity expands in class A mutant flowers and *vice versa*. Although the details differ, the basic developmental programme for floral organ patterning that is encapsulated by the ABC model seems to be widely conserved among plant species that have been extensively studied, including tulip (Kanno *et al.,* 2003), petunia (Angenent *et al.,* 1992), primrose (Webster and Gilmartin., 2003) and even plants with less showy flowers such as rice (Nagasawa *et al.,* 2003) and maize (Whipple *et al*., 2004) are also studied. Further studies have led to identification of class E and class F floral homeotic genes (Goto and Bowman, 2001).

2.2.1 Genes Regulating Meristem Identity

The knowledge of molecular and genetic mechanisms leading to flower development is explored in much detail in *Arabidopsis thaliana* and *Antirrhinum majus* and also to some extent in many other plant species. Similar genetic interactions in flower development has been found largely conserved among diverse angiosperms, with key differences often relating to the different inflorescence architecture of each species (Ambrose *et al*., 2000; Ma and dePamphilis., 2000; Ng and Yanofsky., 2001; Benlloch *et al*., 2007).

In *Arabidopsis*, the acquisition of floral meristem identity has been best analyzed by genes, LEAFY (*LFY*), *APETALA 1* (*AP1*), *FRUITFULL 1* (*FUL1*)

and *TERMINAL FLOWER 1* (*TFL1*) (Weigel *et al*., 1992; Bowman *et al*., 1993; Schultz and Haughn, 1993).

Study in *Arabidopsis* with 'loss of function' mutants, in which flowers are replaced by structures intermediate between floral and vegetative shoots. These mutants revealed the existence of master regulators that control the entire floral initiation process (FLIP). Five *Arabidopsis* FLIP regulatory genes have been identified by mutation and cloned, *viz*., *LEAFY* (*LFY*), *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), *APETALA2* (*AP2*) and *UNUSUAL FLORAL ORGANS* (*UFO*) (Ma. 1998; Mandel *et al*., 1992). Two of the FLIP genes, *LFY* and *AP1*, are considered to play a primary role in initiating the floral program. 'Loss of function' mutations in either gene results in a strong floral to vegetative homeotic transformation phenotypes. Plants with 'loss of function' mutations in both genes fail to produce shoots with floral characteristics (Irish and Sussex, 1990; Haula and Sussex, 1992; Weigel *et al.,* 1992; Bowman *et al*., 1993; Schultz and Haughn, 1993). Ectopic expression studies by Mandel and Yanofsky (1995a) also shows that *LFY* and *AP1* are master regulators that mark primordial meristematic cells for a floral fate. Evidence from mutant phenotypic analysis and expression studies indicate that an important function of *LFY*, *AP1* and *CAL* function (*AP1/CAL*) is to up-regulate each other in the floral primordium.

LFY is expressed before other floral initiation process (FLIP) genes. Early in floral meristem identification other FLIP genes are activated independent of *LFY*, but their expression is relatively weak. One role of *LFY* is to enhance the activation of *AP1*, *CAL* and *AG* at this stage of development. In turn *AP1* and *CAL* enhance expression of *LFY*. Once expressed, the FLIP genes are required to direct different aspects of floral development. For example, *LFY* is required for petal and stamen development and plays a role in the activation of *AG*, while *AP1* is required for sepal and petal development and indirectly for stamen and carpel development by

activating the organ identity gene, *AG*. *AG* maintains meristem identity in the center of the floral primordium as well as promoting stamen and carpel development (Pidkowich *et al.,* 1999).

FRUITFULL is a floral meristem identity gene which is a third *AP1*-like gene, originally called *AGAMOUS LIKE 8* (*AGL8*) and exists in the *Arabidopsis* genome (Mandel and Yanofsky, 1995b). Initial functional analysis of this gene revealed a role in fruit development and the gene was renamed *FRUITFULL* (*FUL*) (Gu *et al*., 1998). Mutations in the *FUL* gene affect the development of the valve, replum and style. It was noted, however, that in addition to its later expression, *FUL* is expressed early in the SAM and is upregulated on induction of flowering. Consistent with this expression pattern, *ful* mutants flower slightly later than wild type and have abnormal cauline leaves (Gu *et al*., 1998; Ferra´ndiz *et al*., 2000). Although *FUL* is expressed before both *AP1* and *CAL* and plays a role in the induction of flowering, it took extensive and careful genetic analysis to reveal its true position in the network of genes that promote floral meristem identity. There is no change in the expression of *LFY* in *ful* mutants, indicating that the delayed flowering in these mutants is not a consequence of reduced *LFY*. This indicates that *FUL* is not involved in the activation of *LFY*. However, *LFY* expression, which is already reduced in *ap1 cal* mutants, is even further reduced in *ap1 cal ful* triple mutants. Therefore *FUL* is capable of activating *LFY* expression in the absence of *AP1* and *CAL*. This probably occurs because *AP1* and *CAL* repress *FUL* in the floral primordia of wild-type plants and *FUL* becomes ectopically expressed there in *ap1* or *ap1 cal* mutants. It is therefore not clear whether the ability of *FUL* to activate *LFY* plays a significant role in wild-type plants where *AP1* and *CAL* would exclude *FUL* from the floral meristems. The *ap1 cal ful* triple mutants produce a proliferation of shoots bearing cauline leaves and axillary cauliflower-like meristems and never flower under normal growth conditions, although flowers can still form under extreme conditions. The *ful* mutation does not enhance *ap1* and *ful cal* double mutants look like *ful* single

mutants. Double mutants between *lfy* and *ful* flower later than either single mutant, showing that both genes act independently on flowering time. To test whether the reduction in *LFY* expression was responsible for the lack of flowering observed in *ap1 cal ful* triple mutants, the triple mutant was crossed with plants constitutively expressing *LFY*. These plants produced *ap1 cal*-like flowers showing that *LFY* overexpression can compensate for the loss of *AP1 CAL* and *FUL*. However, although enforced *LFY* expression causes the non-flowering a*p1 cal ful* triple mutant to flower, flowering time varies in the different mutant combinations. *LFY* expression, though reduced, still plays a role in *ap1 cal ful* triple mutants. This was demonstrated by making the *lfy ap1 cal ful* quadruple mutant. This mutant looks similar to *lfy ap1* double mutants, showing that neither *FUL* nor *CAL* contributes to floral meristem identity in the absence of *LFY* and *AP1*. In contrast to *ap1 cal ful* triple mutants, the *lfy ap1 cal ful* quadruple mutant does not have leafy cauliflowers, confirming that *LFY* is necessary for the proliferation of meristems observed in the triple mutant. Unexpectedly, the *lfy ap1 cal ful* quadruple mutant, although seriously impaired in its ability to flower, is more likely to flower eventually than the *ap1 cal ful* triple mutant. This is probably because the triple mutant, even though it expresses *LFY* at a low level, becomes blocked at the stage of proliferation of multiple inflorescence meristems. The quadruple mutant does not express *LFY* and so does not produce a proliferation of inflorescence meristems and eventually produces some inflorescence-like flowers. The ability of *lfy ap1 cal ful* quadruple mutants to flower suggests that all of the floral meristem promoting factors are not identified (Ferra´ndiz *et al.,* 2000). In fact, Parenicova *et al.* (2003) showed two further MADS-box genes in the *AP1/CAL/FUL* clade (*AGL79* and *AGL12*) that might be good candidates for meristem identity (Davies, 2006).

Investigating the homologues of these *Arabidopsis* genes in other plant species has unravelled the functional conservation and divergence of their counterparts in governing the specification of floral meristems (Chang *et al*., 2009).

2.3 IDENTIFICATION OF CANDIDATE GENE

Isolation of an unknown sequence related to known sequences is a powerful method for investigating biological function. PCR methods have succeeded in obtaining the genes that are unknown in one organism but may be homologous to the sequences of known genes from different organisms (Rose *et al*., 1998).

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 (Aarts *et al*., 1998; Shen *et al*., 1998). Deng and Davis (2001) identified the gene associated with the color of strawberry using degenerate primers made from the conserved regions of genomic DNA, cDNA and protein sequences of the candidate genes.

Shen *et al*. (1993) reported the identification of a cytochrome P450 related gene by RT-PCR using degenerate primers designed based on relatively conserved regions of the proteins of P450 gene families.

Many of the genes governing inflorescence architecture have been found to be conserved in plant species (Ambrose *et al*., 2000; Ma and dePamphilis, 2000; Ng and Yanofsky, 2001; Benlloch *et al*., 2007). Candidate gene approach could be advantageous in exploring the presence of these genes and their functions in different plant species using degenerate primers.

2.3.1 Degenerate Primer

A nucleotide sequence is called degenerate if one or more of its positions can be occupied by one of several possible nucleotides. The degeneracy of a sequence is the number of different sequences that it represents (Kwok *et al*., 1994).

Unlike non-degenerate primers which amplify the target region from different members only if the sequences of a pair of primers are strongly conserved in their genomes, the degenerate primers are useful in amplifying homologous genes with weakly conserved sequences among different organisms. These are designed based on multiple known sequence data of related and already sequenced gene homologs, and hence can be used to discover new homologous genes in other species (Aarts *et al*., 1998; Shen *et al*., 1998; Deng and Davis, 2001; Lang and Orgogozo, 2011). Although one can often do this manually for well conserved sequences, computational methods (e.g., HYDEN, SCPrimer, and iCODEHOP) are available to systematically look for conserved sections in the sequence and to design primers (Brand, 2011).

Highly degenerate primers are preferred to match a large number of known genes offering good chance to detect new related ones. However, primers with low degeneracy avoid the probability of amplifying non-related sequences (Linhart and Shamir, 2005).

2.3.2 Primer Designing

Primers function in pairs, the so-called forward primer and the reverse primer. The primer pairs are chosen such that they will be extended towards each other to cover the given target region (Kampke *et al*., 2001).

Proper primer designing is important for applications in PCR, DNA sequencing, and hybridization. The specificity of primers to avoid mispriming, and the efficiency of primers to be able to amplify a product exponentially are the two main goals to be balanced while designing a primer (Dieffenbach *et al*., 1993). Usually primer of 20-24 bases and GC content between 45-60 per cent with T_m of 52-58 °C works best in most applications. Within a primer pair, the GC content and T_m

should be well matched. An annealing temperature is generally calculated as 5°C lower than the estimated T_m (Dieffenbach *et al*., 1993; Abd-Elsalam, 2003).

To avoid mispriming, primers should not be very sticky (high G/C content) on their 3' ends. However, a "G" or "C" is desirable at the 3' end of primers since this will reduce "breathing" and thereby increase yield (Sheffield *et al*., 1989; Abd-Elsalam, 2003).

When designing primers, it is important to have a minimum of intra- molecular or inter-molecular homology to inhibit the formation of either hairpins or primer dimerization (Abd-Elsalam, 2003).

The calculation of T_m can be performed in several ways. The one given by Suggs *et al*. (1981) as $T_m = 2 (A+T) + 4 x (G + C)$ is popular for its simplicity and roughly accurate prediction of T_m. Whereas, T_m calculation based on nearest neighbor thermodynamic parameters appears to be slightly more accurate (Breslauer *et al*., 1986; Freier *et al*., 1986).

Generally the primers are designed to produce the PCR products of 150-750 bp (Dieffenbach *et al*., 1993).

The use of degenerate oligonucleotide primers covering all possible combinations for the bases, as well as the use of inosine to replace the base corresponding to the third or variable position of certain amino acid codons, have been successful for cDNA cloning and for detection of sequences with possible variations (Lin, 1992).

2.3.3 PCR for Candidate Gene Identification

PCR is a scientific technique in molecular biology for exponential amplification of a particular fragment of DNA (Joshi and Deshpande, 2010). A specific region of

the DNA can be picked up by using the primer pairs flanking the region of interest. Quality of DNA act as a main factor for the successful amplification of DNA fragments by PCR (Finkeldey *et al*., 2010).

2.3.3.1 DNA Isolation

Numerous DNA extraction methods in plants have been reported, but none with universality (Varma *et al*., 2007). This could be primarily due to the variations in the composition of the primary and secondary metabolites among the plants or even among the different tissues of the same plant, which makes the isolation process needful to be adjusted as plant specific or tissue specific (Sangwan *et al*., 1998; Dhanya and Sasikumar, 2010).

DNA extraction is difficult in the plants rich in polyphenols and polysaccharides, which binds to nucleic acids during DNA isolation and interfere with the isolation process (Puchooa and Khoyratty, 2004; Mishra *et al*., 2008). Polyphenol oxidation and co-precipitation causes the browning of the DNA (Varma *et al*., 2007; Mishra *et al*., 2008). The presence of polysaccharides in DNA will make it viscous causing difficulty in loading (Sharma *et al*., 2000; Sablok *et al*., 2009). These metabolites have also been reported to interfere with the activity of several biological enzymes like polymerases, ligases and restriction endonucleases (Prittila *et al*., 2001; Diadema *et al*., 2003; Karaca *et al*., 2005; Varma *et al*., 2007; Moyo *et al*., 2008; Singh and Kumar, 2010; Sahu *et al*., 2012).

Increasing the concentrations of NaCl and CTAB can remove polysaccharides during DNA extraction (Syamkumar *et al*., 2005; Sahu *et al*., 2012). Adding high concentrations of PVP and ß-mercaptoethanol is helpful to remove tannins and other polyphenolics from the tissues (Warude *et al*., 2003).

2.3.3.2 Polymerase Chain Reaction (PCR)

The PCR involves enzymatic synthesis of a specific DNA segment, generating thousands to millions of copies (Joshi and Deshpande, 2010). In the presence of dNTPs, a thermostable DNA polymerase uses each oligonucleotide primer to synthesize a copy of the adjacent DNA strand. Each newly synthesized strand then provides a new template for synthesis from the opposite primer (Fox and Parslow, 1988).

The three major steps *viz.,* denaturation, annealing and extension are involved in the PCR technique. DNA is initially denatured at high temperatures (from 90-97 ^{o}C). The annealing phase is the most important and occurs at 50-60 °C for 1-2 minutes. The extension of the primers by DNA polymerase occurs at 72 °C for 2-5 min. The time for last step depends both on the DNA polymerase itself and on the length of the DNA fragment to be amplified. As a rule of-thumb, 1 min is allowed for the synthesis of 1 kbp fragment. After the last cycle, samples are usually incubated at 72 °C for 5 min to fill in the protruding ends of newly synthesized PCR products. The three-stages are repeated 25–40 times in a typical PCR procedure (Joshi and Deshpande, 2010).

PCR is commonly carried out in a reaction volume of 10-200 µl and the size of PCR products or amplicons ranges from 100–3000 bp in length (Cheng *et al*., 1994). The method relies on the ability of DNA polymerase enzyme to remain stable at high temperatures. The most commonly used enzyme is Taq DNA polymerase, has a 5'-3' nuclease activity but lacks a 3'-5' proofreading exonuclease activity. This makes its use less advisable when fidelity is the main consideration. Instead, the enzymes with proofreading exonucleases such as Vent or Pfu are favoured (Cline *et al.*, 1996). The K_m value of different enzymes varies, hence reaction conditions must be reoptimised every time a different enzyme is used (Bustin, 2000).

Mg^{2+} affects enzyme activity, it increases T_m of double-stranded DNA and forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes. Therefore, high concentrations of dNTPs interfere with polymerase activity and affect primer annealing by reducing free Mg^{2+} (Eckert and Kunkel, 1991). Cobb and Clarkson (1994) described an easy and effective method to optimize the components of PCR for successful amplification. A master mix containing all of the reactants except the Taq polymerase can be made to minimize pipetting variables (Roux, 1995).

Various additives such as dimethyl sulfoxide (DMSO) (2-5%), polyethylene glycol (PEG 6000) (5-15%), glycerol (5-20%), non-ionic detergents and formamide (5%) can be incorporated into the reaction to increase the specificity of reaction (Pomp and Medrano, 1991; Newton and Graham, 1994)

2.3.4 RT-PCR for Candidate Gene Identification

RT-PCR (Reverse transcription - PCR) is an *in vitro* method for enzymatically amplifying defined sequences of RNA (Rappolee *et al.*, 1988). It is the most sensitive method for the detection of low-abundance mRNA, often obtained from limited tissue samples (Bustin, 2000).

RT-PCR can be used to compare the levels of mRNAs in different sample populations, to characterise patterns of mRNA expression, to discriminate between closely related mRNAs and to analyse RNA structure (Bustin, 2000). It also circumvent time-consuming and technically demanding cloning steps and generate reagents, such as full-length complementary DNA (cDNA) inserts for cloning (Borson *et al*., 1992), or arbitrarily primed enhanced sequence tag cDNA libraries (Neto *et al*., 1997).

However, this complex technique sometimes faces substantial problems (Wang and Brown, 1999) and different factors must be well optimized. The RNA must be of highest quality and free of DNA.

2.3.4.1 RNA Isolation

RNA isolation requires special care and precautions as it is highly susceptible to degradation (Kojima and Ozawa, 2002; Buckingham and Flaws, 2007). The biggest problem encountered in RNA extraction usually originates from the initial sampling and extraction protocols, and from handling (MacRae, 2007).

Isolation of high quality RNA is difficult from tissues containing high amounts of polyphenols, polysaccharides and other secondary metabolites (Azevedo *et al*., 2003; Mattheus *et al*., 2003; Sharma *et al*., 2003). These contaminants tend to co-precipitate with the RNA in the presence of alcohol, leading to erroneous estimations of RNA quantity, and interfere with reverse transcription and PCR (Koonjul *et al*., 1999; Salzman *et al*., 1999; Singh *et al*., 2003).

RNase is heat-stable and refolds following heat denaturation. They are difficult to inactivate as they do not require cofactors. Strong denaturant agents has always been used in intact RNA isolation to inhibit endogenous RNases. The most common isolation methods can be divided into two classes, *viz.* utilizing guanidinium thiocyanate and utilizing phenol and SDS (Doyle, 1996).

A guanidinium based salt is a strong protein denaturant and inhibitor of RNase. Therefore, it is an ingredient of choice in most of the RNA isolation systems. However, in some studies, the presence of secondary metabolites has been found to interfere with extraction of RNA when extracted with guanidinium salts (Bugos *et al*., 1995; Ding *et al*., 2008; Wang *et al*., 2008; Ghawana *et al*., 2011).

2.3.4.2 RT-PCR (Reverse Transcription – Polymerase Chain Reaction)

As RNA cannot serve as a template for PCR, so the first step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. The complete RT-PCR process involves the use of dedicated RNA- and DNA-dependent DNA polymerases, either in separate ('two-enzyme/two-tube') or in single ('two-enzyme/one-tube') reactions. Separation of the RT and PCR steps has the advantage of generating a stable cDNA pool that can be stored virtually indefinitely. Alternatively, a single polymerase able to function both as an RNA and DNA-dependent DNA polymerase such as *Thermus thermophilus* (Tth) polymerase can be used in a 'one-enzyme/one-tube' reaction to minimise the risk of contamination (Myers and Gelfand, 1991; Bustin, 2000). The assay with Tth polymerase uses bicine buffers containing Mn^{2+} ions that are compatible with both RT and subsequent PCR (Chiocchia and Smith, 1997).

RT-PCR is a complex assay and all physical and chemical components of the reaction are interdependent. They must be considered carefully when optimizing the specificity, sensitivity, reproducibility or fidelity of the reaction (Bustin, 2000). The secondary structures formed by RNA transcripts also affects the ability of the reverse transcriptase enzyme to generate cDNA (Buell *et al*., 1978).

The two commonly used RT enzymes are avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). AMV-RT is more robust than MMLV-RT, retains significant polymerisation activity up to 55^{o}C and can help eliminate problems associated with RNA secondary structure (Brooks *et al*., 1995; Freeman *et al*., 1996). In contrast, MMLV-RT has significantly less RNAse H activity than AMV-RT (Gerard *et al*., 1997) which make it a better choice for the amplification of full-length cDNA molecules (Bustin, 2000).

The RT step can be primed using specific primers, random hexamers or oligo-dT primers. The use of mRNA-specific primers decreases background priming, whereas the use of random and oligo-dT primers maximises the number of mRNA molecules that can be analyzed from a small sample of RNA (Zhang and Byrne, 1999). cDNA synthesis using oligo-dT is more specific to mRNA than random priming, as it will not transcribe rRNA. However, since oligo-dT priming requires very high-quality RNA that is of full length, it is not a good choice for transcribing RNA that is likely to be fragmented (Bustin and Nolan, 2004).

2.3.4.3 qRT-PCR (Quantitative Reverse Transcriptase -PCR)

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Using PCR, specific sequences within a DNA or cDNA template can be copied, or "amplified", many thousand to a million fold.

In traditional (endpoint) PCR, detection and quantitation of the amplified sequence are performed at the end of the reaction after the last PCR cycle, and involve post-PCR analysis such as gel electrophoresis and image analysis. In real-time quantitative reverse transcriptase PCR (qRT-PCR), the amount of PCR product is measured at each cycle. This ability to monitor the reaction during its exponential phase enables users to determine the initial amount of target with great precision. PCR theoretically amplifies DNA exponentially, doubling the number of molecules present with each amplification cycle. The number of cycles and the amount of PCR end-product can theoretically be used to calculate the initial quantity of genetic material (by comparison with a known standard), but numerous factors complicate this calculation. The ethidium bromide staining typically used to quantify endpoint PCR products prevents further amplification, and is only semiquantitative. PCR may not be exponential for the first several cycles, and the reaction eventually plateaus, so the amount of DNA should be measured while the reaction is still in the exponential

amplification phase, which can be difficult to determine in endpoint PCR. To address these factors, the technique of real-time quantitative PCR was developed.

In real-time PCR, the amount of DNA is measured after each cycle by the use of fluorescent markers that are incorporated into the PCR product. The increase in fluorescent signal is directly proportional to the number of PCR product molecules (amplicons) generated in the exponential phase of the reaction. Fluorescent reporters used include double-stranded DNA (dsDNA) binding dyes, or dye molecules attached to PCR primers or probes that are incorporated into the product during amplification. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

The advantages of real-time PCR include, the ability to monitor the progress of the PCR reaction as it occurs in real time, the ability to precisely measure the amount of amplicon at each cycle, an increased dynamic range of detection, the combination of amplification and detection in a single tube, which eliminates post-PCR manipulations.

Over the past several years, real-time PCR has become the leading tool for the detection and quantification of DNA or RNA. Using these techniques, one can achieve precise detection that is accurate within a two-fold range, and a dynamic range of 6 to 8 orders of magnitude.

Real-time PCR is a variation of the standard PCR technique used to quantify DNA or RNA in a sample. Using sequence-specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined.

By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles. If the sequence is scarce, amplification is observed in later cycles (Bustin, 2000; Bustin and Nolan, 2004; Anon., 2006; Song *et al.*, 2011).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2012-2014. Details regarding the experimental materials used and the methodology followed for various experiments are presented in this chapter.

3.1 PLANT SAMPLE COLLECTION

Black pepper samples of variety karimunda (spikes) were collected from the Instructional Farm, College of Agriculture, Vellayani. Samples at different developmental stages, *viz*., young leaf, mature leaf, runner bud, stage 1 spikes (immature spikes), stage 2 spikes (spikes with flowers), stage 3 spikes (spikes with immature berry) and stage 4 spikes (fully mature spikes with green berries) were collected. Each sample were labeled appropriately and then packed in a polypropylene bag. The samples for RNA isolation were snap chilled in liquid nitrogen and stored at -80^{o}C (Sanyo ultra low deep freezer, USA) for downstream analysis and the samples for microscopy studies were placed in Carnoy's fluid for fixing tissues and taken to lab for further processing.

3.2 IDENTIFICATION OF FLORAL MERISTEM IDENTITY GENES

For identification of genes associated with floral meristem identity in black pepper, the degenerate primers were designed based on the sequences of genes reported to regulate floral meristem identity in several other plant species. These primers were analyzed for amplification by PCR and RT-PCR in genomic DNA and mRNA respectively in Karimunda variety.

3.2.1 Degenerate Primer Designing

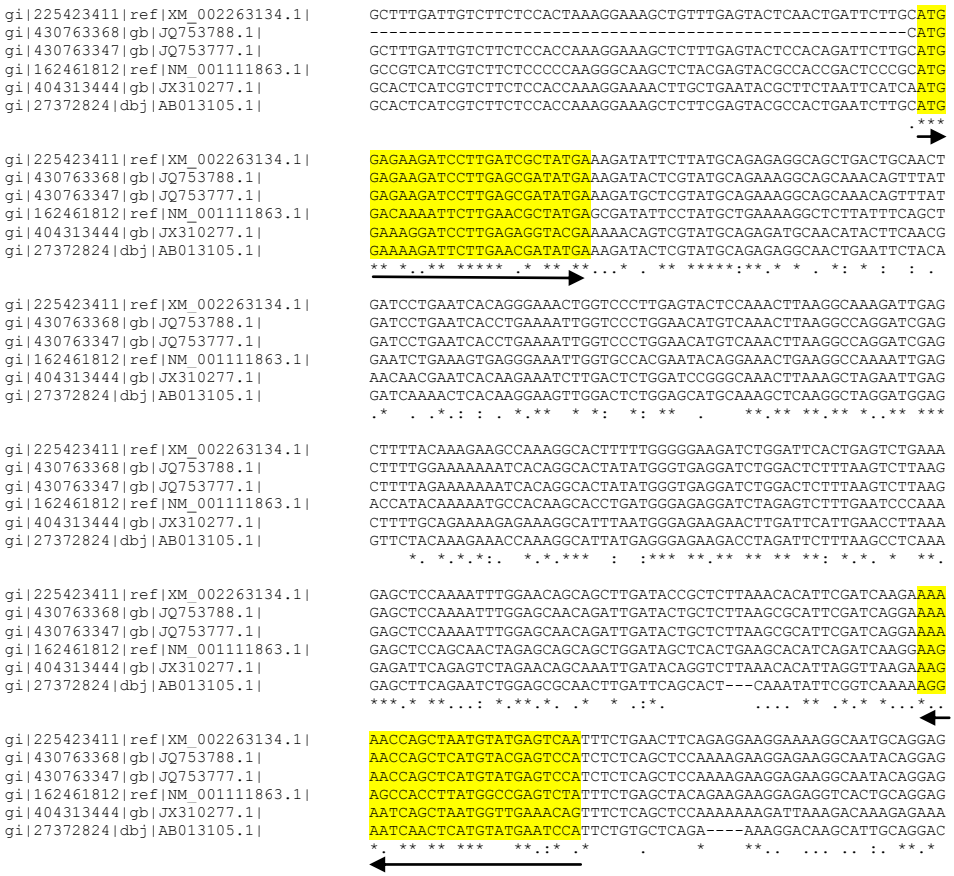
Degenerate primers were designed for two genes, namely *APETALA 1* (*AP1*) and *FRUITFUL 1* (*FUL1*) which are reported to function in floral meristem maintenance in several plant species. Nucleotide sequences of these genes (i.e. cDNA sequences) in different plant species were retrived from NCBI (National Centre for Biotechnology Information) GenBank. From the NCBI nucleotide database, nucleotide sequences of the respective genes were downloaded in FASTA format.

Nucleotide sequences of *AP1* gene from *Vitis vinifera, Cornus florida, Cornus kousa, Zea mays, Tagetes patula and Ipomoea spp.* were retrived from NCBI nucleotide database (Fig. 1). Sequences of *FUL1* gene from *Vitis vinifera (2 sequences), Malus domestica, Cornus kousa and Ipomea spp.* were also retrived from NCBI nucleotide database (Fig. 2).

The collection of FASTA sequences of each gene was then subjected to sequence alignment using clustal omega program. The best two conserved regions in the multiple alignments were identified and the primers were designed according to the sequences of those conserved regions.

3.2.1.1 Primer Analysis

The designed set of primers prior to their synthesis were checked for several parameters such as primer length, length of the PCR product, low degeneracy, maximum specificity at the 3' end. The properties such as feasible annealing temperature, an appropriate range of GC-content, potential hairpin formation and 3' complementarity were analyzed by using Oligo Calc program (http://simgene.com/OligoCalc). The sequences of the resultant primers were given to 'Merck Millipore Private Limited' for synthesis.



(*) : Conserved nucleotide

(⟶) : Region used for forward primer

(⟵) : Region used for reverse primer

Accession No.	**Plant species**
gi|225423411|ref|XM_002263134.1|	*Vitis vinifera -AP1* mRNA
gi|430763368|gb|JQ753788.1|	*Cornus florida -AP1* mRNA
gi|430763347|gb|JQ753777.1|	*Cornus kousa - AP1* mRNA
gi|162461812|ref|NM_001111863.1|	*Zea mays* - zap1 homology
gi|404313444|gb|JX310277.1|	*Tagetes patula -AP1* mRNA
gi|27372824|dbj|AB013105.1|	*Ipomoea nil -AP1* homology

Fig. 1. Multiple sequence alignment for designing degenerate *APETALA 1* (*AP1*) primer

(*) : Conserved nucleotide

(⟶) : Region used for forward primer

(⟵) : Region used for reverse primer

Accession No.	**Plant species**
gi|3947984|gb|U78948.1|	*Malus domestica* MADS box Protein
gi|205345276|dbj|AB302847.1|	*Ipomoea nil* mRNA transcription factor
gi|430763351|gb|JQ753779.1|	*Cornus kousa – FUL1* mRNA
gi|269116075|gb|GU133635.1|	*Vitis vinifera -FUL* mRNA
gi|46949181|gb|AY538747.1|	*Vitis vinifera -FUL* like protein mRNA

Fig. 2. Multiple sequence alignment for designing degenerate *FRUITFULL* (*FUL*) primer

3.2.2 Isolation of Genomic DNA

CTAB method of DNA extraction (Doyle and Doyle, 1990) with slight modifications was used for genomic DNA isolation.

β-mercaptoethanol and polyvinylpyrrolidone (PVP) were added fresh to the CTAB extraction buffer (Appendix I) to give a final concentration of 0.2 per cent (v/v) and 4 per cent (w/v) respectively. The solution was pre-heated to 65 ^{o}C in water bath (ROTEK, India). The samples were chilled and pulverized to a fine powder in liquid nitrogen using a sterile mortar and pestle. The 100 mg of powdered sample was transferred into a sterile 2 ml centrifuge tubes containing 1 ml of freshly prepared warm extraction buffer. The content was homogenized by gentle inversion. The samples were incubated at 65 ^{o}C in water bath for 1 h with intermittent shaking. To the homogenate an equal volume of 24: 1 (v/v) chloroform/isoamyl alcohol was added and mixed well by inversion for 5-10 min. The homogenate was then centrifuged (Eppendorf centrifuge 5430 R, Germany) at 7500*g* force for 10 min at 25^{o}C. The upper phase was transferred to a sterile centrifuge tube and this process with chloroform/isoamyl alcohol was repeated twice. To the aqueous phase, 0.5 ml of 5 *M* NaCl was added and mixed properly by gentle inversion. To this mixture 0.6 volume of chilled isopropanol was added and mixed by inversion. The mixture was then incubated at 4 ^{o}C overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 11,000*g* force for 10 min at 25 ^{o}C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (80%) twice, each time it was centrifuged at 5000*g* force for 7 min at 25 ^{o}C and the supernatant was discarded. The pellet was air dried for 30-40 min, 40 µl of TE buffer is added to dissolve the air dried pellet (Appendix II). The extracted DNA samples were stored at -20 ^{o}C (Lab-Line Low Temperature Cabinet, India).

3.2.2.1 Agarose Gel Electrophoresis

The most common method to assess the integrity of genomic DNA is to run an aliquot of the DNA sample on agarose gel. Horizontal gel electrophoresis unit (Genei, Bangalore) was used to run the samples on the gel. Aliquot of DNA sample (5 µl) was loaded on agarose gel (0.8 %) made in 0.5 x TBE buffer (Appendix III). The gel was run at 5 $Vcm^{-}1$ until the dyes migrated $3/4^{th}$ of the distance through the gel. The gel was visualized using the gel documentation system (BIORAD, USA) using 'Quantity One Software'.

3.2.2.2 Spectrophotometric Analysis

The absorbance of the DNA samples was recorded to determine the quantity and quality of DNA. T60 UV- Visible Spectrophotometer (Oasis Scientific, USA) was used for measuring optical density (O.D.) of the sample. Spectrophotometer was calibrated to blank (zero absorbance) at 260 nm and 280 nm wavelength with 3 ml TE buffer and absorbance of 5 µl DNA sample dissolved in 3 ml of TE buffer at respective wavelengths were recorded.

Since an absorbance value of 1.0 at 260 nm indicates the presence of 50 $ng\mu l^{-1}$ of double stranded DNA, the concentration of DNA in the extracted sample was estimated by employing the following formula:

$$\text{Amount of DNA } (ng\mu l^{-1}) = A_{260} \times 50 \times \text{dilution factor}$$

(Where A_{260} is absorbance reading at 260 nm)

DNA quality was determined by the ratio taken between A_{260}/A_{280} readings.

3.2.2.3 PCR Amplification of Genomic DNA with Degenerate Primers

The genomic DNA of Karimunda was amplified using the designed degenerate primers for the genes *AP1* and *FUL1*. The components of the mixture were optimized as listed below:

Water	:	12.9 µl
10x Taq buffer A (Tris with 15 m*M* $MgCl_2$)	:	2 µl
dNTPS (2.5 m*M* each)	:	1.6 µl
Forward primer (10 µ*M*)	:	1 µl
Reverse primer (10 µ*M*)	:	1 µl
Template DNA (50 ngμl^{-1})	:	1 µl
Taq polymerase (3 Uμl^{-1})	:	0.5 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94 ^{o}C for 4 min followed by 30 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 48 ^{o}C for 45 s and extension at 72 ^{o}C for 45 s. Final extension was done at 72 ^{o}C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with Quantum PCR Marker (low range) from 'Genei, Bangalore' were seperated on agarose gel (1.5 %). The gel was viewed under gel documentation system (BIORAD, USA).

3.2.3 RNA Isolation

Trizol reagent (Invitrogen, USA) was used for extraction of total RNA. All the materials used for RNA extraction were treated in 3 per cent hydrogen peroxide

overnight and autoclaved twice for sterilization. The double distilled water was also autoclaved twice for the same.

To extract RNA for PCR analysis, immature spikes (Stage 1 spikes) were used and for expression analysis, different tissues were used *viz*., stage 1 spikes (immature spikes), stage 2 spikes (spikes with flowers), stage 3 spikes (spikes with immature berry), stage 4 spikes (fully mature spikes with green berries), young leaf, mature leaf and runner bud.

The respected frozen samples (100 mg) were ground into a fine powder in liquid nitrogen using mortar and pestle. Powdered samples were immediately transferred into micro centrifuge tube containing 1 ml of Trizol Reagent. The content was mixed gently to homogenize and then incubated for 5 min at room temperature to permit the complete dissociation of the nucleoprotein complex. To this complex, 0.2 ml of chloroform was added and the tubes were shaken vigorously for 15 s. The tubes were incubated for 2-3 min at room temperature. The samples were centrifuged at 12,000g force for 15 min at 4 ^{o}C. The aqueous phase of the sample was transferred into a fresh tube. To the aqueous phase 0.5 ml of chilled 100 per cent isopropanol was added and incubated at room temperature for 10 min. The sample was centrifuged at 12,000g force for 10 min at 4 ^{o}C. The supernatant was removed from the tube leaving only RNA pellet. The pellet was washed with 1 ml of 75 per cent ethanol. The sample was briefly vortexed and centrifuged at 7500g force for 5 min at 4 ^{o}C. The wash was discarded and the RNA pellet was air dried for 30-40 min. The RNA pellet was resuspended in 30 µl RNase free sterile water, followed by incubation in a water bath at 55-60 ^{o}C for 10 min.

The integrity of the total RNA was determined by running 5 µl aliquot of RNA on agarose gel (1.5 %) as described in section 3.2.2.1.

The absorbance reading of extracted RNA using spectrophometer was determined as described in the section 3.2.2.2. Since an absorbance value of 1.0 at 260 nm indicates the concentration of 40 ngµl^{-1} of RNA, the concentration of RNA present in an aliquot was estimated by employing the following formula:

Concentration of RNA (ngµl^{-1}) = A_{260} x 40 x dilution factor

(Where A_{260} is absorbance reading at 260 nm)

RNA purity was determined by the ratio taken between A_{260} and A_{280}.

3.2.3.1 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RT-PCR is the most sensitive technique for mRNA detection and quantitation among currently available techniques such as Northern blot analysis and RNase protection assay. RT-PCR can be used to quantify mRNA levels from much smaller samples.

RT-PCR was carried out using AMV RT-PCR Kit (GeNei^{TM}). RT-PCR is essentially a two step process. The first step involves the use of reverse transcriptase to synthesize cDNA from single stranded mRNA. The second step is the amplification of cDNA by PCR. To avoid any RNase contamination, the sterile working environment was maintained. All the materials used were pre-treated with 3 per cent hydrogen peroxide overnight and autoclaved twice.

3.2.3.1.1 Synthesis of cDNA Using AMV RT-PCR Kit

To a sterile RNase free micro-centrifuge tube, 5 µl RNA and 1 µl oligo $(dT)_{18}$ primer was added, and made up the volume to 10 µl with nuclease free water. The tubes were warmed at 65 ^{o}C for 10 min and then kept at room temperature for 2 min

to remove secondary structures in RNA. The tubes were spun briefly and the components were added in the order given below:

RNase inhibitor	:	1 µl
10 X Assay Buffer	:	2 µl
30 m*M* dNTP mix	:	1 µl
AMV – Reverse Transcriptase	:	1 µl
Nuclease Free Water	:	5 µl
Total Volume	:	10 µl
Overall Total Volume	:	20 µl

The contents were mixed well and incubated at 42 ^{o}C for 1 h. The tubes were then incubated at 92 ^{o}C for 2 min and quickly placed the tubes in ice and spun briefly (this step denatures RNA-cDNA hybrids). The cDNA samples were stored at -20 ^{o}C (Lab-Line Low Temperature Cabinet, India) until PCR amplification.

3.2.3.1.2 PCR Amplification of cDNA with Degenerate Primers

The cDNA samples were subjected to PCR with two degenerate primers afore mentioned in section 3.2.1. The PCR mixture of the total volume of 20 µl was prepared similarly as described in the section 3.2.2.3 except that 3 µl of cDNA was used as template instead of 1 µl of genomic DNA.

The PCR programme was set with initial denaturation temperature of 94 ^{o}C for 1 min, followed by 30 cycles of denaturation at 94 ^{o}C for 45 s, annealing (different temperatures screened) for 45 s and extension at 72 ^{o}C for 45 s. The final extension was set at 72 ^{o}C for 5 min. The PCR products were separated on agarose gel (1.5 %) and the gel was observed using gel documentation system.

3.2.3.2 Sequencing of the Amplicons Produced by Degenerate Primers

The amplicons produced by the degenerate primer pair was eluted from the agarose gel and purified using GeNei^{TM} Gel Extraction Kit. The eluted product was given to Regional facility for DNA finger printing at RGCB (Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram) for sequencing.

3.2.3.2.1 Gel Elution Using Gel Extraction Kit (GeNei^{TM})

Gel piece containing the amplicon was weighed and placed in a centrifuge tube. The gel was then crushed and 2.5 volume of sodium iodide solution was added. This mixture was kept at 55 ^{o}C for 5 min to solubilize the gel. To this 15 µl of silica solution was added and left in room temperature for 10-15 min with intermittent shaking. The mixture was centrifuged at 8000 rpm for 1-2 min at room temperature and supernatant was discarded. To the pellet 200 µl of wash buffer was added, vortexed and the solution was again centrifuged at 8000 rpm for 1-2 min (this step is repeated). After the second wash the vial was incubated at 37 ^{o}C (dry bath) to ensure complete drying of pellet. To the pellet add 30 µl of TE buffer and place at 55 ^{o}C for 10 min to ensure dissolution of pellet. The solution was centrifuged at 8000 rpm for 1-2 min at room temperature and stored at -20 ^{o}C until sequencing.

3.2.3.2.2 Sequence Analysis of the Amplicons

The resultant sequence of the amplicon from sequencing was used for analysis using bioinformatic tools inorder to identify the sequence *viz.,* BLAST (tblastx), blastn suite-GEO (Gene expression omnibus), NCBI conserved Domain Search and Phylogeny.fr. The BLAST programme used was tblastx i.e., translated nucleotide query is used to search in translated nucleotide sequence database. blastn suite-GEO programme takes a nucleotide query and compares it with the gene expression databases, NCBI conserved domain search also uses nucleotide query and finds the

conserved domain for which it belongs and finally the phylogeny.fr was used to create phylogenetic tree and to show which gene is related to the nucleotide query, this application is known as "one click" phylogeny analysis this requires a group of nucleotide sequence in FASTA format as an input file to generate a tree (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi).

3.3 EXPRESSION STUDIES

3.3.1 Semi Quantitative Analysis

Semi quantitative analysis was based on the studies done by Preston and Kellog (2006). It was done by doing a PCR using **c**DNA from young leaf, mature leaf, runner bud, stage 1 spikes and stage 2 spikes as template with *FUL1*, Ubiquitin and RAPD primers (RAPD and Ubiquitin primers were used as endogenous control). The PCR mixture of 20 µl total volume was prepared similarly as described in the section 3.2.2.3 except that 3 µl of cDNA was used as template instead of 1 µl of genomic DNA.

The PCR programme was set with initial denaturation temperature of 94 ^{o}C for 1 min, followed by 30 cycles of denaturation at 94 ^{o}C for 45 s, annealing (different temperatures, according to the primers) for 45 s and extension at 72 ^{o}C for 45 s. The final extension was set at 72 ^{o}C for 5 min. The PCR products were separated on agarose gel (1.5 %) and the gel was observed using gel documentation system.

3.3.2 Quantitative Reverse Transcriptase - PCR (qRT-PCR)

Quantitative reverse transcriptase PCR was done using the specific primers designed for the sequenced fragment. Primers were designed by using primer3 tool (http://primer3.ut.ee/).

qRT-PCR study was designed based on the study by Song *et al*. (2011)A sterile qRT-PCR plate was taken, to a well of a sterile qRT-PCR plate 10 µl of SYBR® green master mix, 1 µl of each primer i.e., forward and reverse specific primers, 3 µl of cDNA from different tissues (young leaf, mature leaf, runner bud, stage 1 spikes, stage 2 spikes, stage 3 spikes and stage 4 spikes) and 5 µl of distilled water were added. Similarly each components were added to each wells respectively. After adding all the components, it was vortexed and centrifuged briefly to spin down the contents and to avoid air bubbles. This plate was used to run the reaction.

Absolute quantification (i.e. creation of amplification plot and dissociation curve) was done based on the study by Bustin (2000). Reaction conditions were, enzyme activation at 95 ^{o}C for 10 min, denaturation 95 ^{o}C for a 15s and annealing and extension 60 ^{o}C for 1 min this cycle was repeated for 40 cycles to get an amplification plot. Similarly to get a dissociation curve, enzyme activation at 95 ^{o}C for 15 s, denaturation at 95 ^{o}C for 15 s and annealing and extension at 60 ^{o}C for 15 s were followed. This was carried out in RGCB.

3.4 MICROSCOPY STUDIES

The spike samples were collected from different tissues (stage 1 spikes, stage 2 spikes, stage 3 spikes and stage 4 spikes) and placed in test tubes containing chilled Carnoy's fluid (Appendix IV) that had been pre-stored at -20 ^{o}C for temperature stabilization overnight. The samples were kept at -20 ^{o}C overnight. Next day, the spikes were either viewed directly under the stereomicroscope (Leica ez4hd,

Germany) or subjected to dehydration in a series of alcohol *viz*., 70 per cent ethanol for 1 h (twice), 80 per cent ethanol for 1 h (twice), 95 per cent ethanol for 1 h (twice), 100 per cent ethanol for 1 h (thrice) and finally with 3 changes of xylene, each change for a duration of 1 h.

The samples were then washed in 100 per cent ethanol and then hand sectioned using a new razor blade. The sections were placed in watch glass containing water, then it was transferred to 100 per cent ethanol (2 changes of 3 min each) followed by 2 changes of 95 per cent ethanol for 3 min and these sections were placed on glass slide. The sections were either directly observed under the microscope or subjected to staining. For staining the sections were placed in 100 per cent ethanol for 2 min (twice) then in 70 per cent ethanol for 2 min followed by 30 min of safranin stain. The safranin stained sections were visualized under microscope. Few safranin stained sections were subjected to secondary stain i.e., fast green. This was done by placing the safranin stained sections in 70 per cent ethanol for 2 min then the sections were placed in watchglass containing fast green stain for 30 s, excess stain was removed by placing the sections in 100 per cent ethanol for 2 min (twice). These sections were then observed under microscope.

RESULTS

4. RESULTS

The study entitled “Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.)”, was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2012-2014. The results related to the study are presented in this chapter.

4.1 IDENTIFICATION OF FLORAL MERISTEM IDENTITY GENES

4.1.1 Degenerate Primer Designing

Degenerate primers for the genes governing floral meristem identity, *viz*., *AP1* and *FUL1* were designed based on the conserved regions in the multiple aligned nucleotide sequences and named as (*AP1*-F, R) and (*FUL1*-F, R) respectively. The details of the primers designed are given in Table 1.

4.1.1.1 Primer Analysis

The analysis of primers using Oligo Calc programme revealed desirable GC content and annealing temperature, and also none of the designed primers exhibited any hairpin formation and 3’ complementarity.

4.1.2 DNA Isolation

Young leaves and immature spikes (stage 1 spikes) of Karimunda variety were used for genomic DNA extraction. The agarose gel electrophoresis (0.8%) of the extracted genomic DNA showed the presence of good quality unsheared DNA bands (Plate 1). Lane 1, 2 and 3 shows the genomic DNA from young leaves from different Karimunda plants named A, B and C. Lane 4, 5 and 6 shows the genomic DNA from immature spikes from different Karimunda plants (D, E and F). Further absorbance reading of the extracted genomic DNA by using spectrophotometric method revealed good quality and quantity of DNA (Table 2).

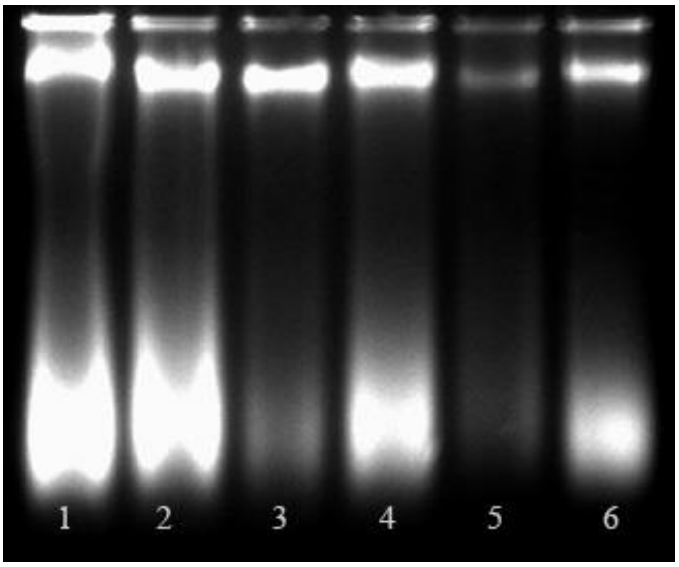
Table 1. Sequences of designed degenerate primers

Sl. No.	Target gene	Primer name	Primer sequence (5' to 3')	No. of bases	GC content (%)	T_m (^oC)
1	*AP1*	*AP1*- F	ATGGAVARRATYCTTGADMGVTAYGA	26	38.5	54.8
		AP1- R	AYDGWYTCRDMCATDAGBTGRYTYYT	26	40.5	56.3
2	*FUL1*	*FUL1*- F	AGRGGWAGGGTKSARYTGAAG	21	52	59.8
		FUL1- R	AATCAKTGGMRTACTCRAASAGC	23	43	58.9

Degenerate code		Bases
M	:	AC
R	:	AG
W	:	AT
S	:	CG
Y	:	CT
K	:	GT
V	:	ACG
H	:	ACT
D	:	AGT
N	:	ACGT

Table 2. Quality and quantity of isolated genomic DNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield ($ng\mu l^{-1}$)
1	(Leaf) Karimunda A	0.2257	0.1121	2.0133	3385.5
2	(Leaf) Karimunda B	0.1739	0.0894	1.9451	2608.5
3	(Leaf) Karimunda C	0.2108	0.1219	1.7161	3162
4	(Spike) Karimunda D	0.1689	0.1071	1.5770	2533.5
5	(Spike) Karimunda E	0.1467	0.0802	1.8291	2200.5
6	(Spike) Karimunda F	0.0917	0.0494	1.85	1375.5



DNA from leaves

Lane 1 - Karimunda A

Lane 2 - Karimunda B

Lane 3 - Karimunda C

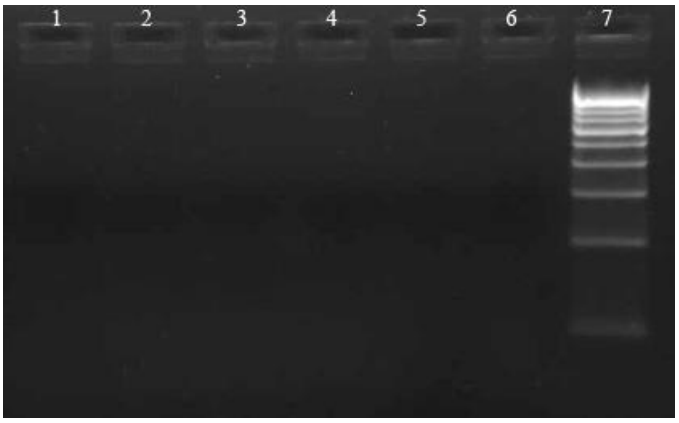
DNA from immature spikes

Lane 4 - Karimunda D

Lane 5 - Karimunda E

Lane 6 - Karimunda F

Plate 1. Agarose gel electrophoresis of black pepper genomic DNA from different karimunda samples named A, B, C, D, E and F



Lane 1 to 6 - PCR product of genomic DNA

Lane 1 to 3 – *AP1*

Lane 4 to 6 – *FUL1*

Lane 7 – 100bp Ladder

Plate 2. Agarose gel electrophoresis of PCR product of black pepper genomic DNA with designed primers

4.1.2.1 PCR Analysis of Genomic DNA with Degenerate Primers

Genomic DNA from immature spikes were used as template. Out of the degenerate primers designed for *AP1* and *FUL1*, None of the primer pairs, i.e., (*AP1*-F, R) and (*FUL1*-F, R) produced any amplification in the genomic DNA of Karimunda variety of black pepper. Lane 1, 2 and 3 shows the PCR product with *AP1*-F, R primers, Lane 4, 5 and 6 shows the PCR product with *FUL1*-F, R primers and Lane 7 shows 100bp ladder (Plate 2).

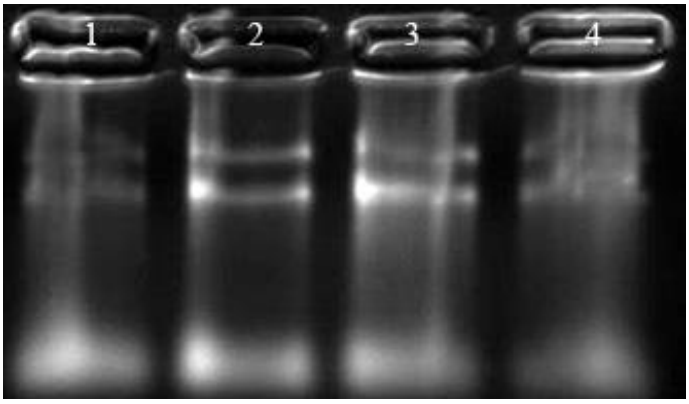
4.1.3 RNA Isolation

Four stage 1 spike samples were used for extraction of total RNA. Distinct intact rRNA (ribosomal RNA) bands with no apparent RNA degradation was observed on the gel in case of samples 2 and 3 and no genomic DNA contamination was observed on agarose gel (0.8 %) showing good quality RNA extraction for these samples (Plate 3). RNA samples 2 and 3 were used to prepare cDNA and for PCR.

For expression studies, young leaf, mature leaf, runner bud, stage 1 spikes, stage 2 spikes, stage 3 spikes and stage 4 spikes were used for extraction of total RNA. Distinct intact rRNA bands with no RNA degradation was observed on the gel. No DNA contamination was observed on agarose gel (0.8 %) showing good quality RNA extraction (Plate 4). Further absorbance reading of the extracted RNA by using spectrophotometric method revealed good quality and quantity of RNA (Table 3).

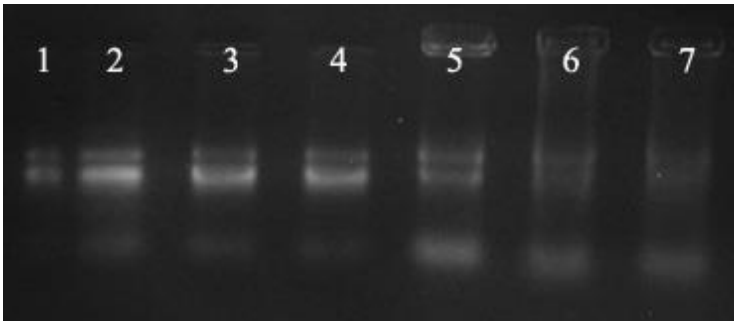
Table 3. Quality and quantity of isolated total RNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	RNA Yield ($ng\mu l^{-1}$)
1	Stage 1 spikes	0.054	0.025	2.16	648
2	Stage 2 spikes	0.055	0.028	1.96	660
3	Stage 3 spikes	0.049	0.026	1.88	588
4	Stage 4 spikes	0.053	0.023	1.15	636
5	Runner bud	0.057	0.026	2.2	684
6	Young leaf	0.052	0.028	1.85	624
7	Mature leaf	0.054	0.024	2.25	648



Lane 1 to 4 – Total RNA of black pepper from immature spike samples 1, 2, 3 and 4.

Plate 3. Agarose gel electrophoresis of black pepper total RNA from four immature spike samples



Lane 1 - Stage 1 spike
Lane 2 - Stage 2 spike
Lane 3 - Stage 3 spike
Lane 4 - Runner bud
Lane 5 - Mature leaf
Lane 6 - Young leaf
Lane 7- Stage 4 spike

Plate 4. Agarose gel electrophoresis of black pepper total RNA from different tissues for expression studies

4.1.3.1 RT-PCR with Degenerate Primers

The reverse transcription of the extracted RNA from immature spikes (stage 1 spikes) was carried out shortly to synthesize the first strand cDNA followed by PCR with the designed degenerate primers. The reaction did produce two amplicons with *FUL*-F, R degenerate primer, bigger band is ~400bp and the smaller band is ~150bp. Lane 1 shows the PCR product of cDNA with *FUL*-F, R primers and Lane 2 shows 100bp ladder (Plate 5).

4.1.3.2 Sequencing of the Amplicons Produced by Degenerate Primers

The two amplicons produced by the degenerate primer pair is eluted from the agarose gel and purified using GeNei^{TM} Gel Extraction Kit, the eluted products was given to Regional facility for DNA finger printing of RGCB for sequencing. Four sequences were generated (Two sequences from an amplicon - one from forward primer and another from reverse primer).

Sequences of the sequenced amplicons are given below.,

(409bp) *FUL1* - F→

CCCGCAAACCTGGAGTCTGCACTAGGCAGGCCTCAAGTCTCGGATGAGAATG
AGGACGAGAAGAATAAGACGGAATCCTTGAACGAGAAGTTTGAGGGCTTAT
GCATGGTGATCAAGGATGTCTTGGGAGATAAGGTGGAGAAAGTTGTAGTTTC
TGACCGTGTGGTGGACTCCCCCTGTTGTTTGGTAACTGGTGAGTATGGTTGGA
CTGCCAACATGGAGAGGATCATGAAGGCCCAGGCACTGAGGGACTCTAGCAT
GGCGGGATATATGTCTTCCAAAAAGACTATGGAGATCAACCCCGAGAATGCG
ATCATGGAGGAGCTCAGAAAGAGGGCAGATGCTGACAAGAACGACAAGTCC
GTGAAAGATTTGGTGCTGCTGCTCTTCGAGTACCCCAATGATTACA

(402bp) *FUL1* - R→

GGGGGTACGAAGAGTGTCTTACTAGACAGCATCTGCCATCTTTATGAGCTCCT
CCATGATCGAATTCTCGGGGTTGATCTCCATATTCTTTTTGGAACACATATAT
CCCGCCATGCTAGAGTCCCTCAGTGCCTGGGCCTTCATGATCCTCTCCATGTT
GGCAGTCCAACCATACTCACCAGTTACCAAACAACAGGGGGAGTCCACCACA
CGGTCAGAAACTACAACTTTCTCCACCTTATCTCCCAAGACATCCTTGATCAC

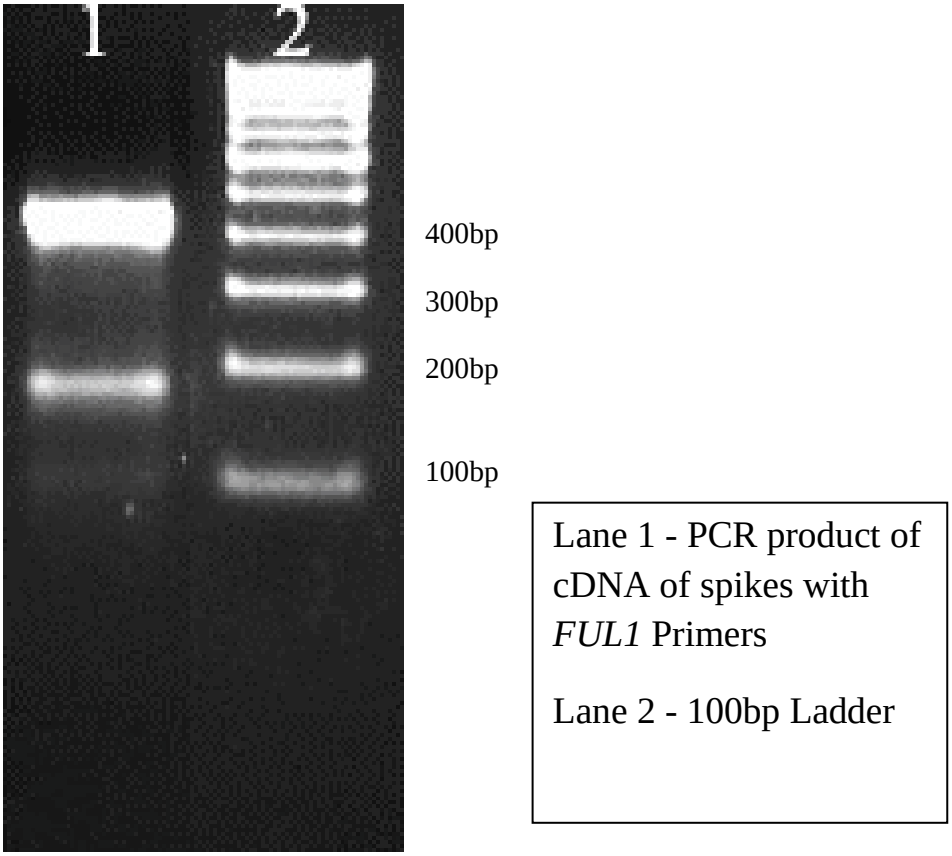


Plate 5. Agarose gel electrophoresis of PCR product from cDNA of spike using *FUL1* primers

CTTGCATAAGCCCTCAAACTTCTCTTTCAAGGATTCCTTCTTTTTCTTCTCGTC
CTCATTCTCATCGAGGTTGAGGCCTTCCTTGGTTGCAGACACCAACTTCTTCC
CCTCAAACTCCTTCAGCTCCACCCTACCACT

(*143bp*) *FUL1* - F→
TAGAGGATAAGCATGTGAGTTCTCGAGAGGAGGTTGGCCGGCTGCGACGCGT
TGACATCTCCGTGCTTTGCGACGCCAAGGTAGCGCTGTTGTCTTCTCCGCACG
GGCGAGTAGTTCGAGTACGCCACTGATTAAATACCGTC

(*152bp*) *FUL1* - R→
AAAGGGAAACAGGGGTGGGTCAACTTGGCTCTTTACAGACACCGCTGTCTGA
GACGACTCCACGTACAGGTCTGACCTCCTCTTGGAGAACGTCACCTGCCTGTT
GATCTTGTTCTCTATCCTAATAAACTGCACCCTTCCCCTAACTATGT

4.1.3.3 Sequence Analysis of the Amplicons

The resultant sequence of the amplicon from sequencing was used for analysis using bioinformatic tools *viz.,* BLAST, blastn suite-GEO (Gene expression omnibus), NCBI conserved Domain Search and Phylogeny.fr

The BLAST programme used was tblastx i.e., translated nucleotide query is used to search in translated amino acid database, this result showed that the bigger fragment belongs to mRNA of HSP90 (Plate 6a,b) whereas smaller fragment belongs to mRNA of MADS box transcription factor and APETALA 1 protein (Plate 7a,b). Table 4 gives the detailed results of the tBLASTx.

Table 4. tBLASTx results of the sequenced fragments

Sequence identifier	tBlastx hits	Gene
(409bp) FUL1 - F	773 Blast Hits on the Query Sequence	mRNA of HSP90
(402bp) FUL1 - R	784 Blast Hits on the Query Sequence	mRNA of HSP90
(143bp) FUL1 - F	116 Blast Hits on the Query Sequence	Nonspecific BLAST hits
(152bp) FUL1 - R	274 Blast Hits on the Query Sequence	mRNA of MADS box Transcription factor and *APETALA 1* protein

Basic Local Alignment Search Tool

NCBI/ BLAST/ tblastx/ **Formatting Results - HBF930MV015**
Formatting options
Download
Blast report description

Nucleotide Sequence (402 letters)

RID HBF930MV015 (Expires on 03-05 18:46 pm)

Query ID lcl|22515
Description None
Molecule type nucleic acid
Query Length 402

Database Name nr
Description Nucleotide collection (nt)
Program TBLASTX 2.2.29+

Graphic Summary

Distribution of 784 Blast Hits on the Query Sequence

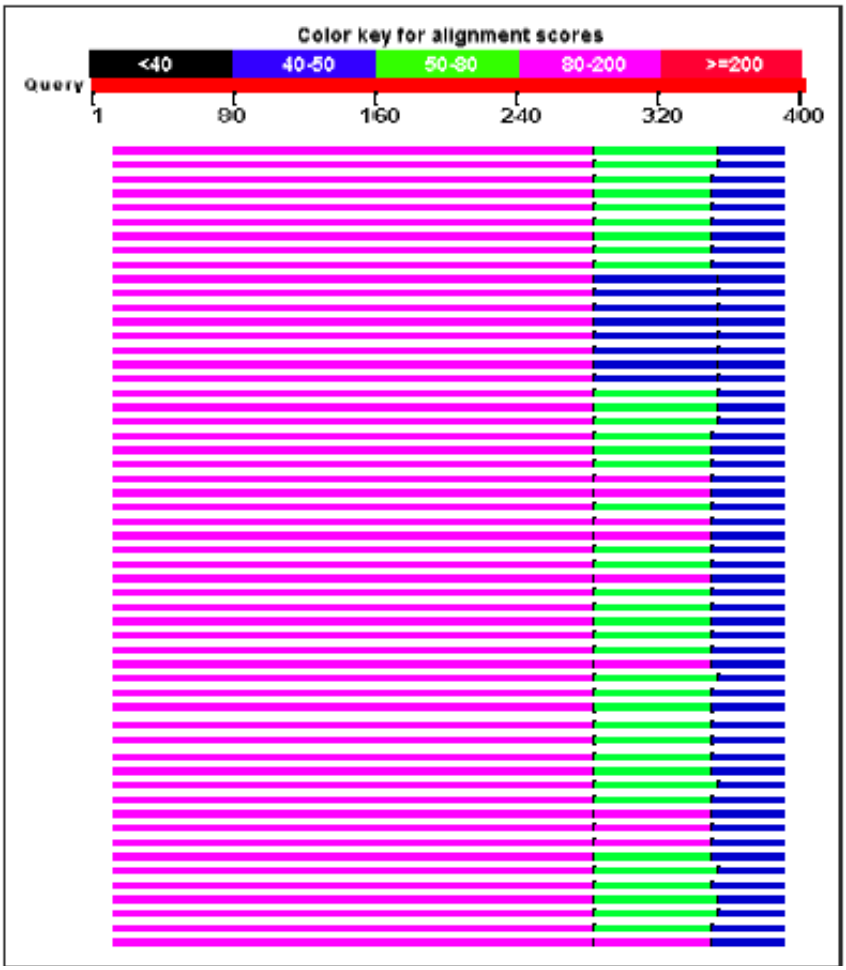


Plate 6a. Screenshot showing the result of tBlastx (402bp) *FUL1* – F

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	N	Accession
Arabidopsis thaliana chromosome 5, complete sequence	145	2166	93%	5e-40	Sumn2	CP002688.1
Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MDA7	145	1704	93%	5e-40	Sumn2	AB011476.1
Nicotiana tabacum heat shock protein 90 (ONtHsp90) mRNA, complete cds	145	649	93%	5e-40	Sumn2	AY519499.1
Nicotiana tabacum hsp90 mRNA for heat shock protein 90, complete cds	145	649	93%	5e-40	Sumn2	AB264546.1
Nicotiana attenuata heat shock protein 90-2 mRNA, complete cds	145	627	93%	5e-40	Sumn2	GU265723.1
Nicotiana tabacum NtHsp90 mRNA for Heat shock protein 90, complete cds	145	649	93%	5e-40	Sumn2	AB689674.1
Nicotiana tabacum cultivar Bright Yellow 2 molecular chaperone Hsp90 mRNA, complete cds	145	649	93%	5e-40	Sumn2	HQ834904.1
Nicotiana benthamiana molecular chaperone Hsp90-3 mRNA, partial cds	145	646	93%	5e-40	Sumn2	GQ845021.1
Nicotiana benthamiana molecular chaperone Hsp90-1 mRNA, complete cds	145	650	93%	5e-40	Sumn2	AY368904.1
Arabidopsis thaliana heat shock protein 81-2 mRNA, complete cds	145	515	93%	5e-40	Sumn2	NM_124985.4
Arabidopsis thaliana clone RAFL09-76-I24 (R19621) putative heat shock protein 81-2 (HSP81-2) (At5g56030) mRNA, complete cds	145	515	93%	5e-40	Sumn2	BT000717.1
Arabidopsis thaliana HEAT SHOCK PROTEIN 81-2 (HSP81-2) (MDA7.7) mRNA, complete cds	145	515	93%	5e-40	Sumn2	AY062750.1
Arabidopsis thaliana heat shock protein 81-2 mRNA, complete cds	145	515	93%	5e-40	Sumn2	NM_001203624.1
Arabidopsis thaliana Unknown protein mRNA, complete cds	145	515	93%	5e-40	Sumn2	BT002535.1
Arabidopsis thaliana heat shock protein 81-2 (At5g56030) mRNA, complete cds	145	515	93%	5e-40	Sumn2	AY128805.1
Arabidopsis thaliana clone C105057 putative heat-shock protein HSP81-2 (At5g56030) mRNA, complete cds	145	515	93%	5e-40	Sumn2	BT001944.1
Arabidopsis thaliana mRNA for HEAT SHOCK PROTEIN 81-2, partial cds, clone: RAFL22-66-H11	145	515	93%	5e-40	Sumn2	AK230157.1
Lactuca sativa heat shock protein 90 (Hsp90) mRNA, complete cds	145	665	93%	7e-40	Sumn2	JX312559.1
Vitis vinifera contig VV78X135759.6, whole genome shotgun sequence	144	666	93%	9e-40	Sumn2	AM426751.1
PREDICTED: Vitis vinifera heat shock cognate protein 80-like (LOC100246643), mRNA	144	657	93%	9e-40	Sumn2	XM_003634988.1
Solanum lycopersicum chromosome 7 clone C07SLm0119A22, complete sequence	144	662	93%	1e-39	Sumn2	AC210371.2
Tomato heat shock cognate protein 80 gene, 3' end	144	662	93%	1e-39	Sumn2	M96549.1

Plate 6b. Screenshot showing the result of tBlastx (402bp) *FUL1* – F

Basic Local Alignment Search Tool

NCBI/ BLAST/ tblastx/ **Formatting Results - HBFATCSM015**
Formatting options
Download
Blast report description

Nucleotide Sequence (152 letters)

RID HBFATCSM015 (Expires on 03-05 18:47 pm)

Query ID lcl|6005
Description None
Molecule type nucleic acid
Query Length 152

Database Name nr
Description Nucleotide collection (nt)
Program TBLASTX 2.2.29+

Graphic Summary

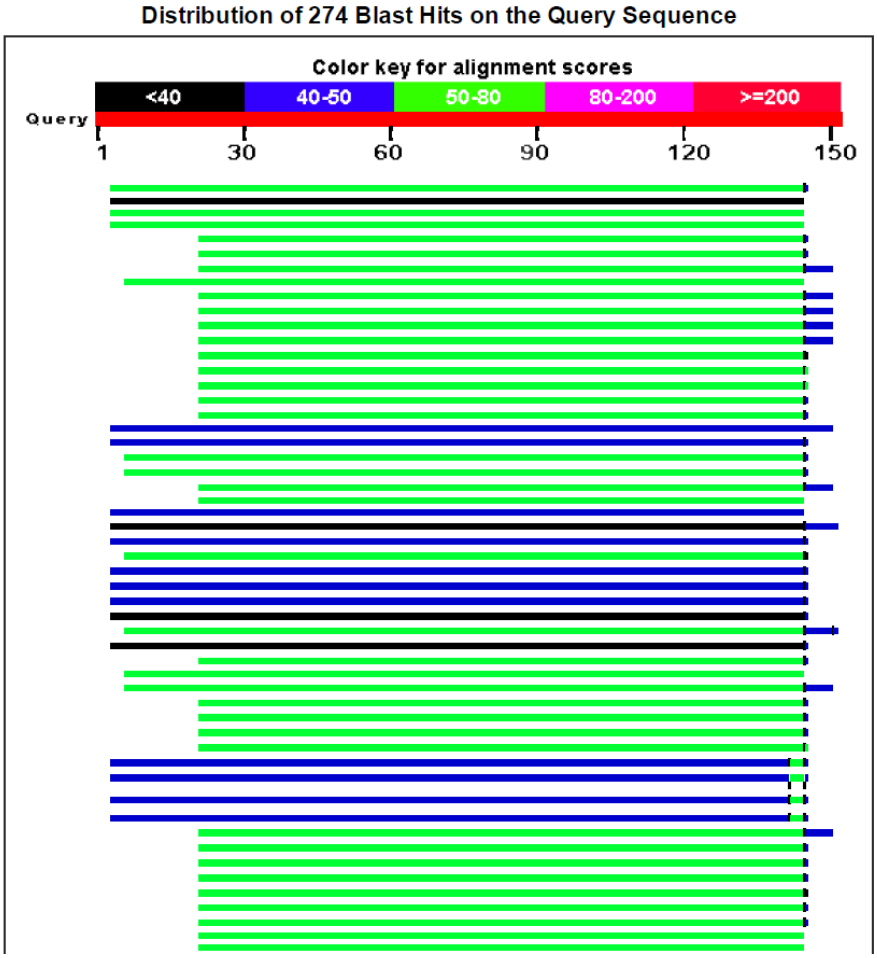


Plate 7a. Screenshot showing the result of tBlastx (152bp) *FUL1* - R

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	N	Accession
Houttuynia cordata HcAP1 mRNA for MADS-box transcription factor, complete cds	61.6	239	93%	8e-08	Sumn1	AB089153.1
Triticum urartu clone 5 VRN1 (Vrn1) gene, Vrn1-A1g allele, exon 1 and partial cds	59.3	134	92%	4e-07	Sumn1	GQ482973.1
Triticum turgidum subsp. turanicum isolate 34b5_17_02_09 vernalization protein (Vrn1) gene, Vrn1-B1a allele, promoter region, exon1 and partial cds	57.9	132	92%	1e-06	Sumn1	GQ451772.1
Triticum turgidum subsp. turanicum isolate 34b4_17_02_09 vernalization protein (Vrn1) gene, Vrn1-B1a allele, promoter region, exon1 and partial cds	57.9	132	92%	1e-06	Sumn1	GQ451771.1
Gossypium hirsutum MADS box protein MADS43 mRNA, complete cds	57.0	140	81%	2e-06	Sumn1	KC155638.1
Romneya coulteri FRUITFULL-like protein (FL1) mRNA, partial cds	57.0	99.7	81%	2e-06	Sumn1	KF500128.1
Gossypium hirsutum fiber-specific MADS protein (MADS11) mRNA, complete cds	57.0	142	84%	2e-06	Sumn1	HM989877.1
Mangifera indica MAP1 (MAP1) mRNA, MAP1-1 allele, complete cds	57.0	57.0	90%	2e-06	Sumn1	FJ529206.1
Fragaria x ananassa AP1-like transcription factor (AP1) gene, complete cds	56.5	142	84%	3e-06	Sumn1	JN788263.1
Litchi chinensis APETALA1-like protein (AP1) mRNA, partial cds	56.5	142	84%	3e-06	Sumn1	JQ062978.1
Litchi chinensis APETALA1 (AP1) mRNA, complete cds	56.5	142	84%	3e-06	Sumn1	JN214349.1
Rosa hybrid cultivar APETALA1-like protein (AP1-1) mRNA, complete cds	56.5	135	84%	3e-06	Sumn1	FJ970026.2
Lotus japonicus genomic DNA, clone: LjT13A22, TM1930, complete sequence	56.5	232	81%	3e-06	Sumn1	AP009769.1
PREDICTED: Glycine max agamous-like MADS-box protein AGL8 homolog (LOC100784742), transcript variant X2, mRNA	56.1	188	81%	4e-06	Sumn1	XM_006578457.1
PREDICTED: Glycine max agamous-like MADS-box protein AGL8 homolog (LOC100784742), transcript variant X1, mRNA	56.1	188	81%	4e-06	Sumn1	XM_003522936.2
Gossypium hirsutum MADS box protein MADS65 mRNA, complete cds	56.1	102	81%	4e-06	Sumn1	KC155660.1
Gossypium hirsutum MADS box protein MADS44 mRNA, complete cds	56.1	102	81%	4e-06	Sumn1	KC155639.1
Berberis thunbergii FRUITFULL-like protein (FL1) mRNA, partial cds	56.1	136	96%	4e-06	Sumn1	KF500149.1
Pyrus pyrifolia var. culta PpMADS3-1 mRNA for MASDS-box protein, complete cds	56.1	189	93%	4e-06	Sumn1	AB623165.2
Sedirea japonica AP1-like protein (MADS1) mRNA, complete cds	56.1	97.4	91%	4e-06	Sumn1	JQ776636.1

Plate 7b. Screenshot showing the result of tBlastx (152bp) *FUL1* – R

The (152bp) *FUL1* – R sequence was used for further sequence analysis,

- Blastn suite-GEO programme is another powerful bioinformatics tool which takes a nucleotide query and compares it with the gene expression databases. The programme gave seven blast hits on the query sequence showing that the query sequence is similar to the *Oryza sativa* MADS box protein (Plate 8a, b).

- The NCBI conserved Domain Search programme. This uses nucleotide query and finds the conserved domain for which the query sequence belongs to. It showed that the query sequence belonged to MADS/Type II subfamily of MADS (Agamous) domain. (Plate 9)

- Phylogeny.fr was used to create phylogenetic tree and to show which gene is related to the nucleotide query, "one click" phylogeny analysis tool was used. For these different sequences of *APETALA1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS, FRUITFULL1, AGAMOUS* and *PISITALLA* from *Arabidopsis, Vitis vinifere and, Malus* were chosen to create the tree, it was found that the pepper query sequence i.e., (152bp) FUL1 – R sequence was closer to *APETALA 1* of *Arabidopsis* (Fig. 3).

4.2 EXPRESSION STUDIES OF THE IDENTIFIED GENE

4.2.1 Semi Quantitative Analysis

Semi quantitative analysis was based on the studies done by Preston and Kellog (2006). It was done by doing a PCR using **c**DNA from different tissues *viz*., leaf, mature leaf, runner bud, stage 1 spikes and stage 2 spikes as template with *FUL1*, Ubiquitin and RAPD primers. The PCR products were separated on agarose gel (1.5%) and the gel was observed using gel documentation system. The gel pictures showed that there was amplification only in stage 1 spike when *FUL1* primers were used (Plate 10). Gel pictures of PCR product with Ubiquitin primers showed amplification in all the wells except in blank (Plate 11). Similarly gel picture containing PCR product with RAPD primers showed amplification in all

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite-GEO/ **Formatting Results - S8AMZ4K1015**

Formatting options

Download

Blast report description

filtered DNA sequence consisting of 152 bases.

RID	S8AMZ4K1015 (Expires on 05-28 14:03 pm)
Query ID	lcl|42515
Description	filtered DNA sequence consisting of 152 bases.
Molecule type	nucleic acid
Query Length	152
Database Name	GEO/GEO
Description	Gene Expression Omnibus (GEO)
Program	BLASTN 2.2.29+

Graphic Summary

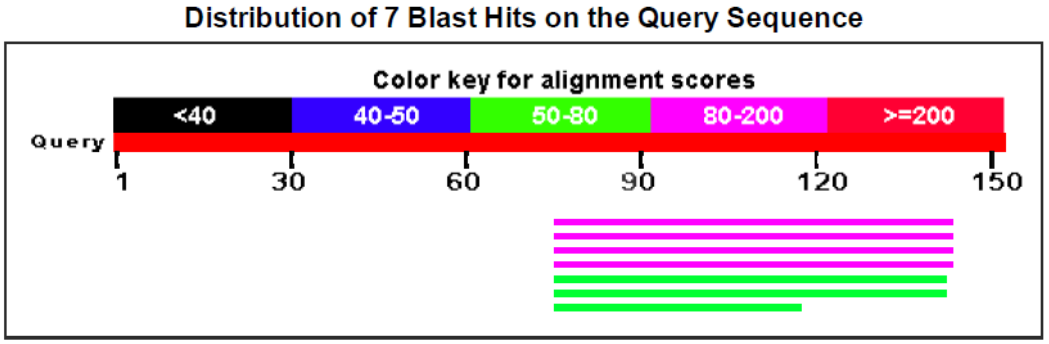


Plate 8a. Screenshot showing the result of Blastn suite-GEO programme

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Oryza sativa (japonica cultivar-group) MADS-box protein RMADS215 mRNA, complete cds	91.6	91.6	44%	5e-17	91%	AY551920.1
Oryza sativa Japonica Group cDNA clone:J023140K24, full insert sequence	91.6	91.6	44%	5e-17	91%	AK072683.1
Oryza sativa MADS15 protein mRNA, complete cds	91.6	91.6	44%	5e-17	91%	AF058698.1
Oryza sativa Japonica Group mRNA for MADS box-like protein, complete cds, clone:E31864	91.6	91.6	44%	5e-17	91%	AB003325.1
Oryza sativa Japonica Group cDNA clone:J023143P16, full insert sequence	67.6	67.6	44%	8e-10	85%	AK072867.1
Oryza sativa MADS box protein (OsMADS8) mRNA, complete cds	67.6	67.6	44%	8e-10	85%	U78892.1
Oryza sativa putative MADS box protein (OsMADS24) mRNA, partial cds	62.1	62.1	27%	4e-08	93%	U32109.1

Plate 8b. Screenshot showing the result of Blastn suite-GEO programme

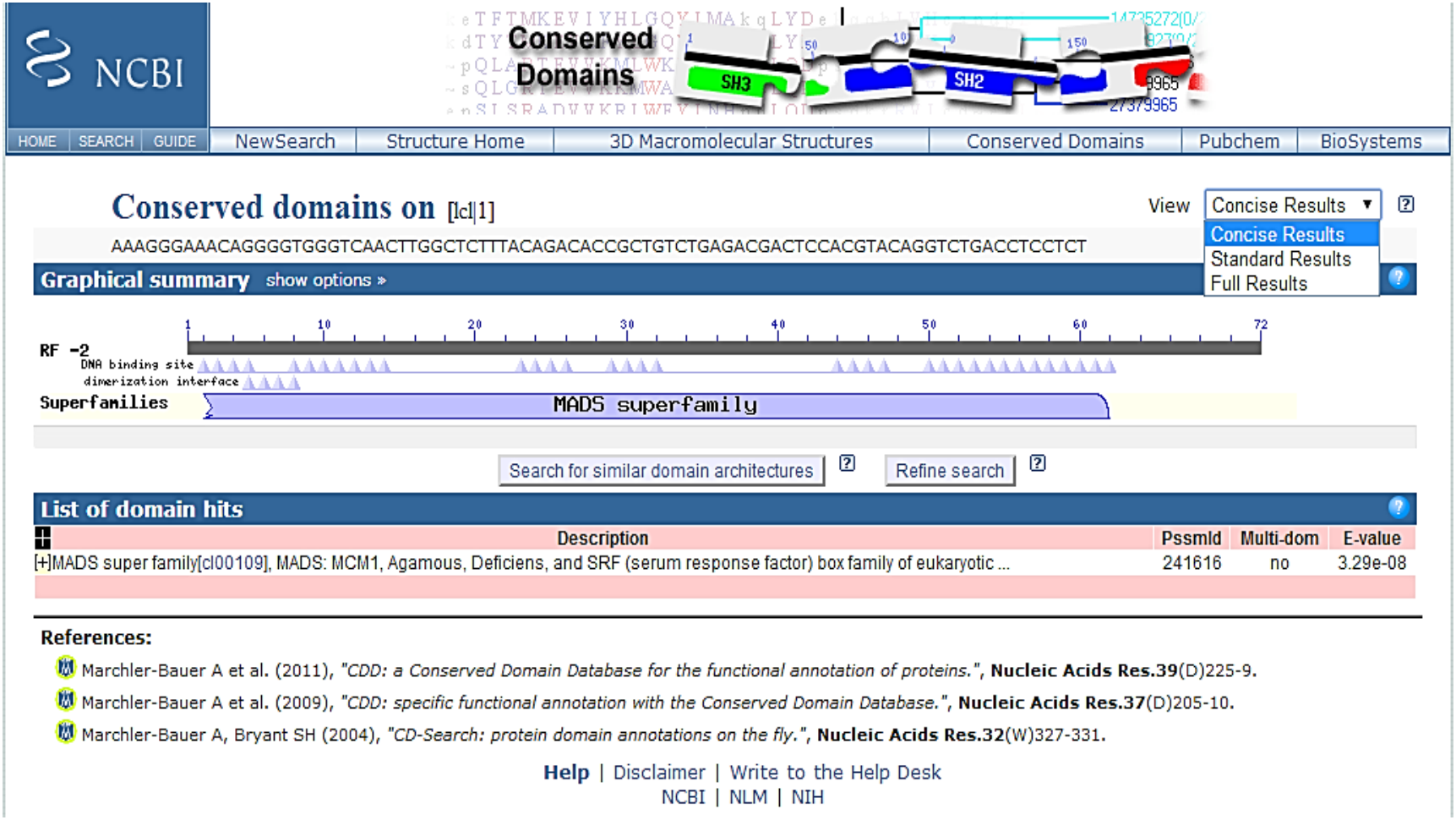


Plate 9. Screenshot showing the result of NCBI conserved Domain Search programme

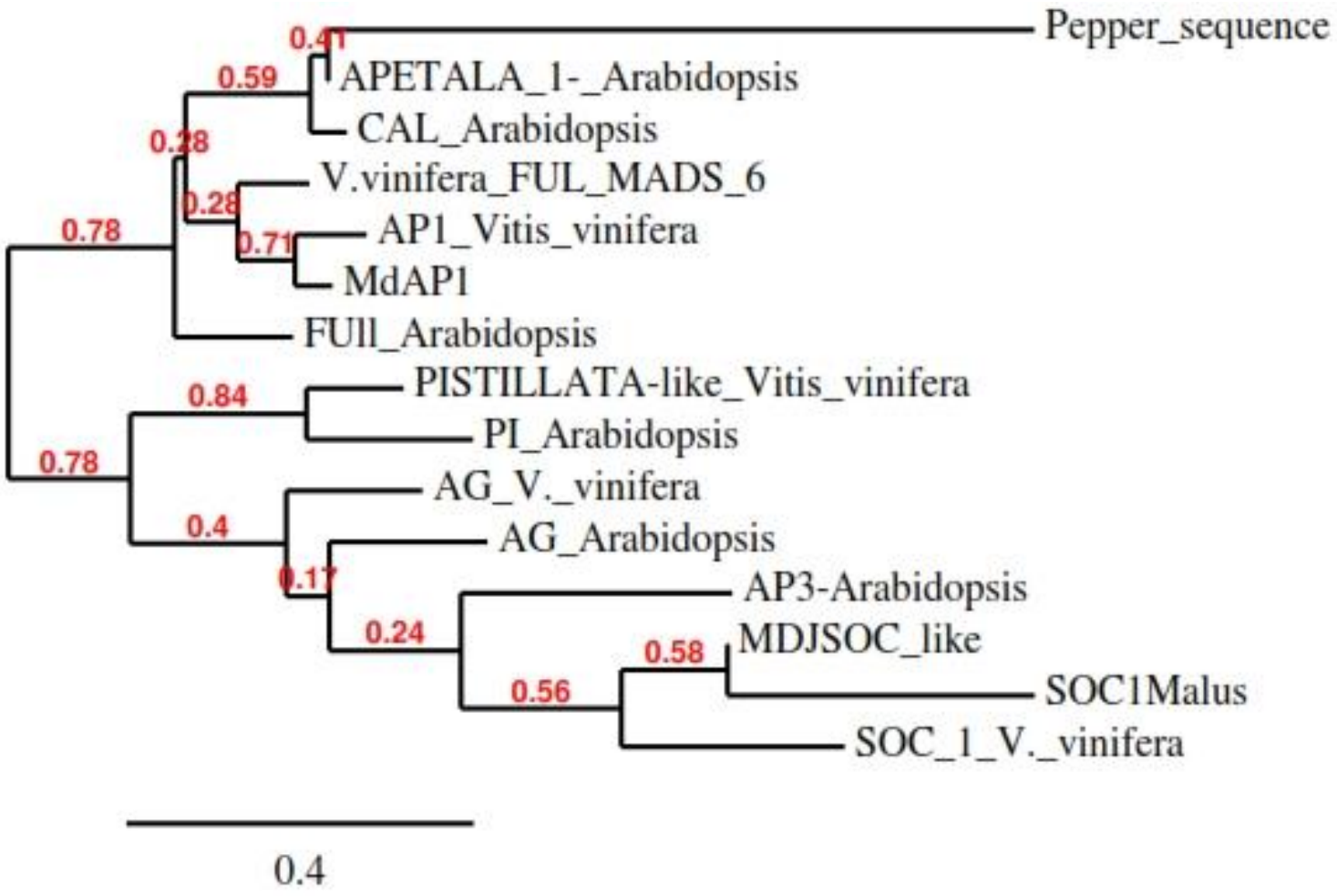
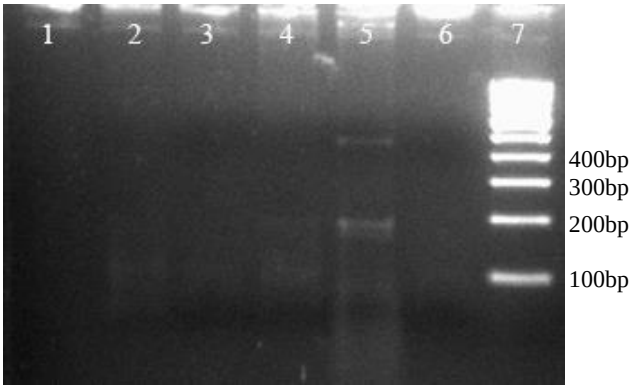
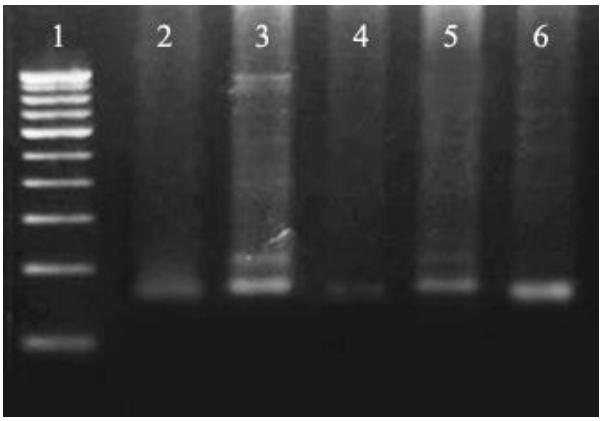


Fig. 3. Phylogenetic tree created using Phylogeney.fr



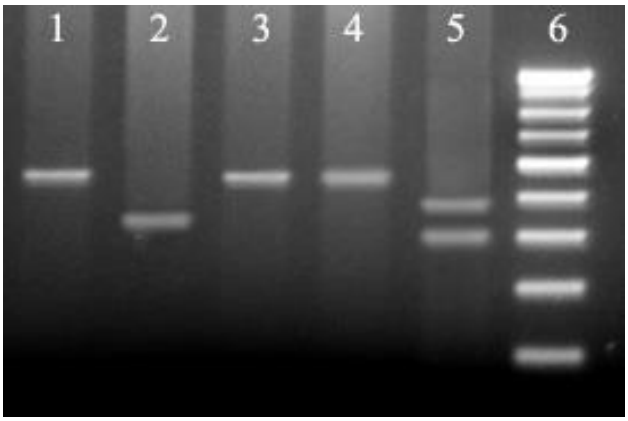
Lane 1 - Mature leaf
Lane 2 - Runner bud
Lane 3 - Young leaf
Lane 4 - Stage 2 spike
Lane 5 - Stage 1 spike
Lane 6 - Blank
Lane 7 - 100bp Ladder

Plate 10. Agarose gel electrophoresis of PCR product of cDNA from different tissues with *FUL1* primers



Lane 1 – 100bp Ladder
Lane 2 – Mature leaf
Lane 3 - Runner bud
Lane 4 – Young leaf
Lane 5 - Stage 2 spike
Lane 6 - Stage 1 spike

Plate 11. Agarose gel electrophoresis of PCR product of cDNA from different tissues with Ubiquitin primers



Lane 1 – Mature leaf
Lane 2 – Runner bud
Lane 3 – Young leaf
Lane 4 - Stage 2 spike
Lane 5 - Stage 1 spike
Lane 6 - 100bp Ladder

Plate 12. Agarose gel electrophoresis of PCR product of cDNA from different tissues with RAPD

the wells except in blank (Plate 12). Table 5 shows the sequence of the primers used in semi quantitative analysis.

Table 5. Primers used for semi quantitative analysis and qRT- PCR

Sl. No.	Target gene	Primer name	Primer sequence (5' to 3')	No. of bases	GC content (%)	T_m (^oC)
1	*BPFul-sp*	*BPFul* - F	AAACAGGGGTGGGTCAACTT	20	50	60.6
		BPFul - R	AGGGGAAGGGTGCAGTTTAT	20	50	59.8
2	ACTIN	*ACT*- F	CTGGTGATGGTGTGAGCCAC	20	60	62.5
		ACT - R	CATGAAATAGCTGCGAAACG	20	45	56.4
3	Ubiquitin	*UBI* - F	ATGCAGATCTTCGTTAAGACT	21	38	48.5
		UBI - R	ACCACCACGGAGCCTGAGAAC	21	62	58.3
4	RAPD	RAPD	AGCCTGAGCC	10	70	34
5	*FUL1*	*FUL1*- F	AGRGGWAGGGTKSARYTGAAG	21	52	59.8
		FUL1- R	AATCAKTGGMRTACTCRAASAGC	23	43	58.9

4.2.2 Quantitative Reverse Transcriptase - PCR (qRT-PCR)

qRT-PCR study was designed based on the study by Song *et al*. (2011). It was carried out in RGCB. cDNA from different samples were used as template *viz*., stage 1 spikes, stage 2 spikes, stage 3 spikes, stage 4 spikes, young leaf, mature leaf and runner bud with gene specific primer (specially designed for qRT-PCR based on the (152bp) *FUL1* – R sequence, *BPFul*-sp Primer sequence is in Table 5) and Actin primer as endogenous control. Thermal cycling for absolute quantification i.e., amplification plot and dissociation curve was done. As an output, amplification plot and dissociation curve was generated by the software after carrying out the reactions. Fig. 4, Fig. 5 and Fig. 6 shows the graph showing amplification curve of gene specific primer, amplification curve of Actin primer and dissociation curve of both gene specific and Actin primers respectively. Absolute quantification was done based on the study by Bustin (2000). Table 5 shows the sequence of the primers used in qRT-PCR.

Table 6 and Table 7 gives the data derived from the curves, which was then analyzed. As shown in Table 6, amplification was found only in stage one spikes when gene specific primer was used for analysis, whereas table 7 shows amplification in all samples except in stage 4 spikes when Actin primer was used as endogenous control (Actin primer sequence is in Table 5).

Table 6. qRT-PCR results with different samples with gene specific primer

Sample name	Detector Name	Reporter	Ct value	Amplification
Young leaf	*BPFul*-Sp	SYBR	Undetermined	Negative
Mature leaves	*BPFul*-Sp	SYBR	Undetermined	Negative
Runner bud	*BPFul*-Sp	SYBR	Undetermined	Negative
Stage 1 spikes	*BPFul*-Sp	SYBR	30.37803	Positive
Stage 1 spikes	*BPFul*-Sp	SYBR	31.25589	Positive
Stage 1 spikes	*BPFul*-Sp	SYBR	32.70245	Positive
Stage 2 spikes	*BPFul*-Sp	SYBR	Undetermined	Negative
Stage 3 spikes	*BPFul*-Sp	SYBR	Undetermined	Negative
Stage 4 spikes	*BPFul*-Sp	SYBR	Undetermined	Negative

Table 7. qRT-PCR results with different samples with Actin primer

Sample name	Detector Name	Reporter	Ct value	Amplification
Young leaf	Actin	SYBR	19.49402	Positive
Mature leaves	Actin	SYBR	4.133297	Positive
Runner bud	Actin	SYBR	21.84232	Positive
Stage 1 spikes	Actin	SYBR	24.02548	Positive
Stage 1 spikes	Actin	SYBR	19.03152	Positive
Stage 1 spikes	Actin	SYBR	27.50447	Positive
Stage 2 spikes	Actin	SYBR	25.34346	Positive
Stage 3 spikes	Actin	SYBR	20.851372	Positive
Stage 4 spikes	Actin	SYBR	undetermind	Negative

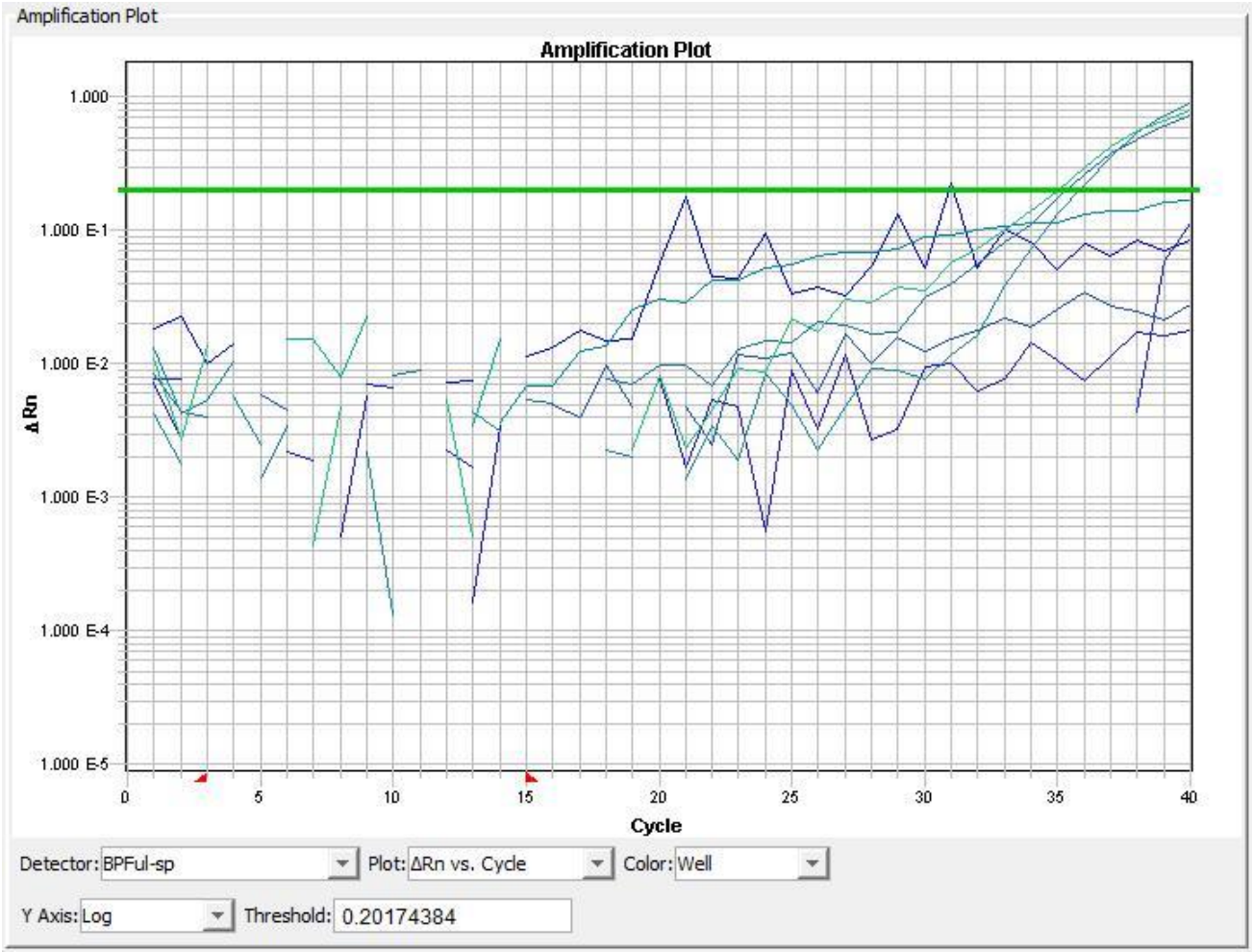


Fig. 4. Amplification curve of gene specific primer

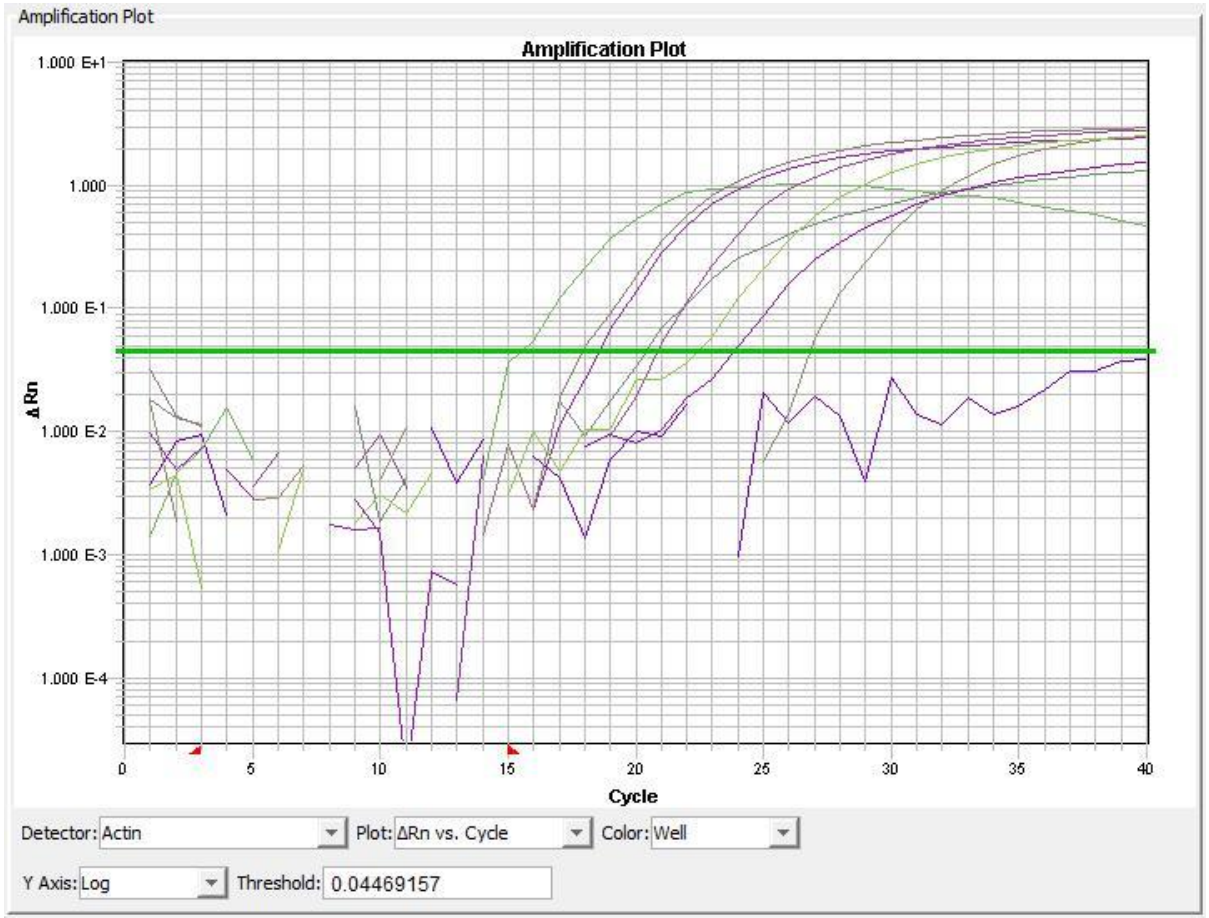


Fig. 5. Amplification curve of Actin primer

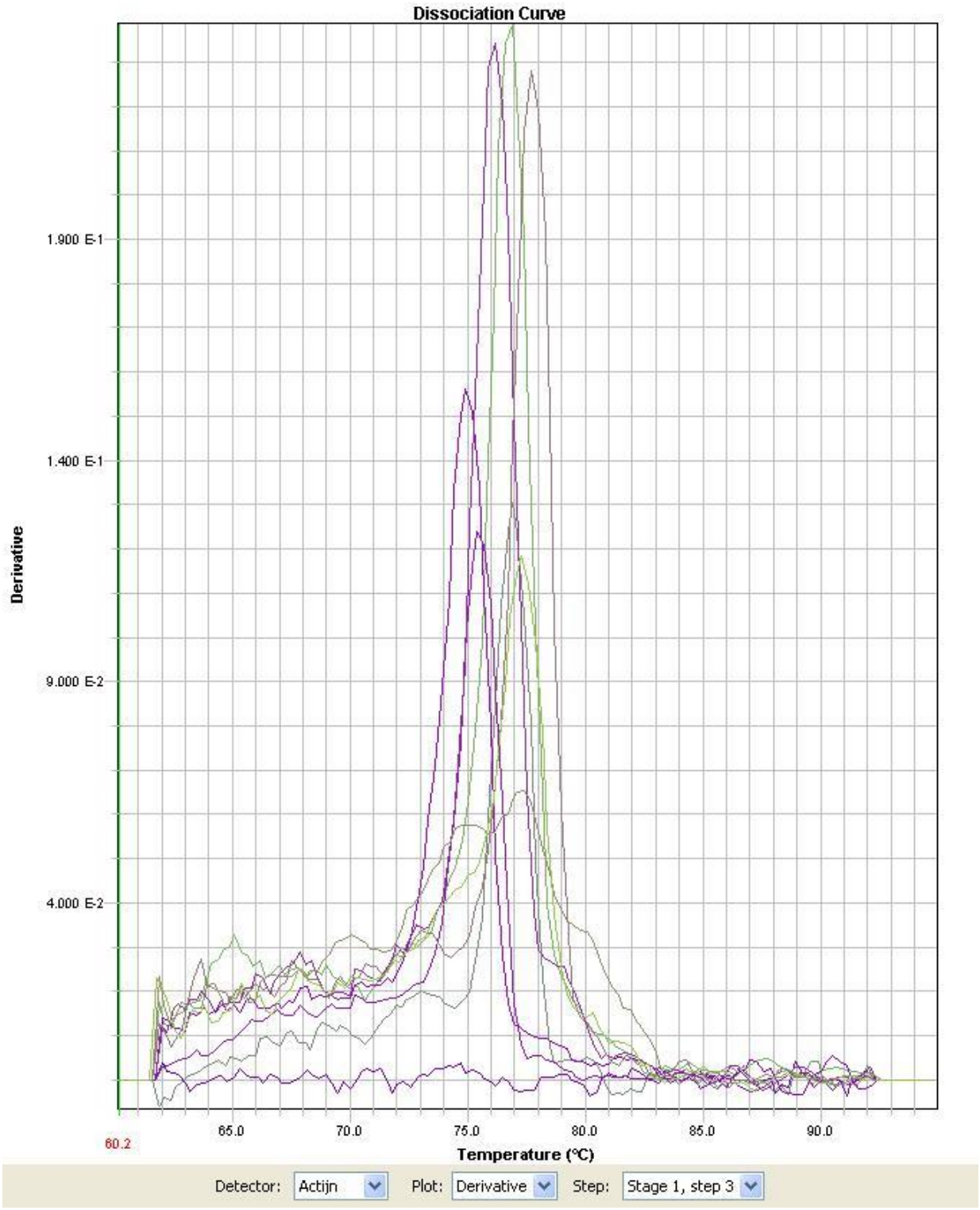


Fig. 6. Dissociation curve of both gene specific and Actin primers

4.3 MICROSCOPIC ANALYSIS

Plate 13 shows the general view of the black pepper spikes of different stages showing the development, starting from its emerging stage. It also shows the classified spikes *viz*., stage 1 spikes, stage 2 spikes, stage 3 spikes and stage 4 spikes. A 15 cm ruler is kept for the clarification of the actual sizes of the pepper spikes.

Plate 14 shows the magnified sections of the stage 1 pepper spikes under 8X magnification, this plate contains sections of both stained and non-stained sections.

Plate 15 shows the magnified sections of the stage 1 and stage 2 pepper spikes under 4X and 20X magnification which are not stained.

Plate 16 shows the magnified sections of the stage 2, stage 3 and stage 4 pepper spikes under 35X magnification. This plate contains sections of both stained and non-stained sections.

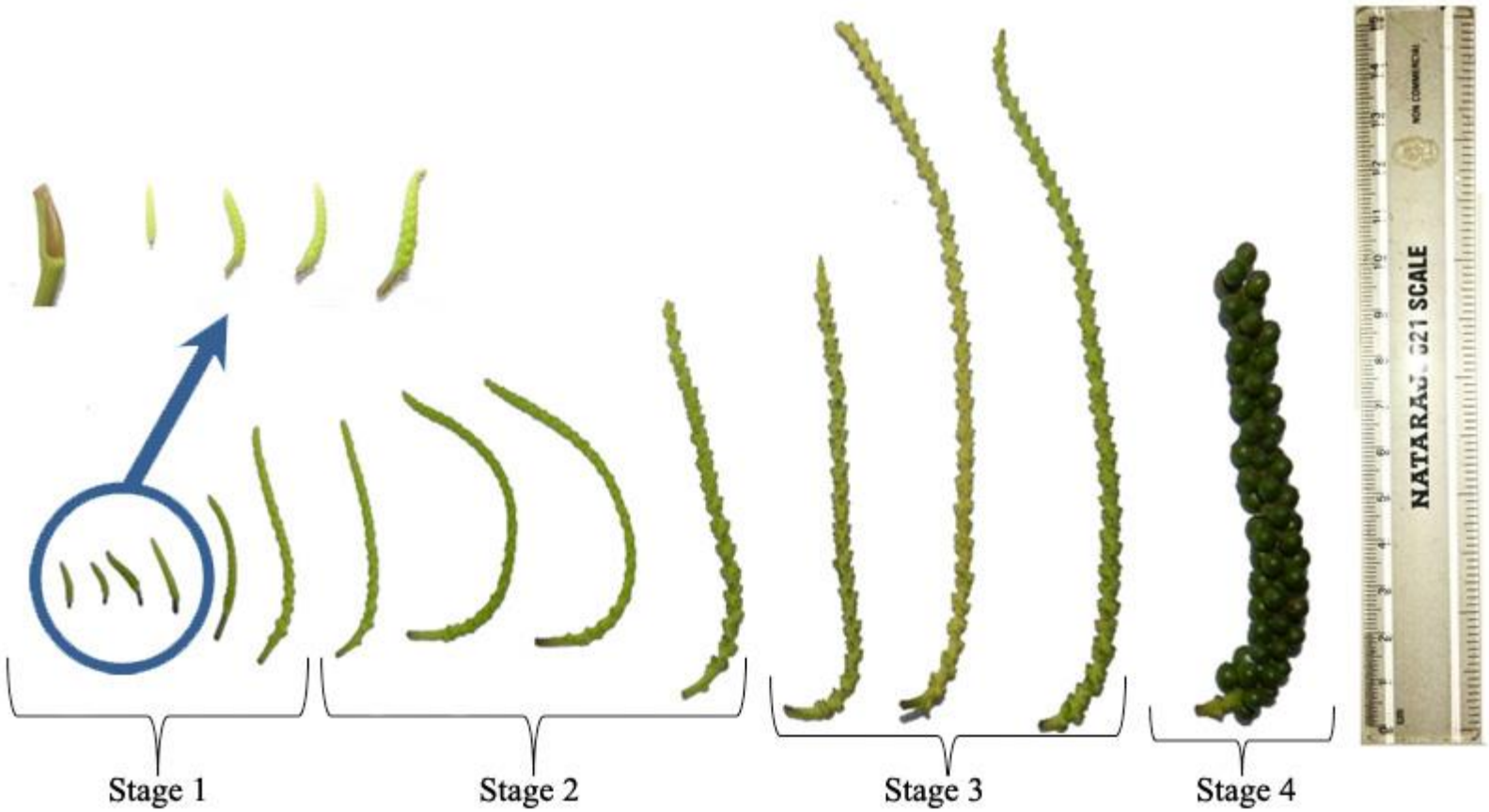


Plate 13. General view of different stages of black pepper spikes used for microscopy studies

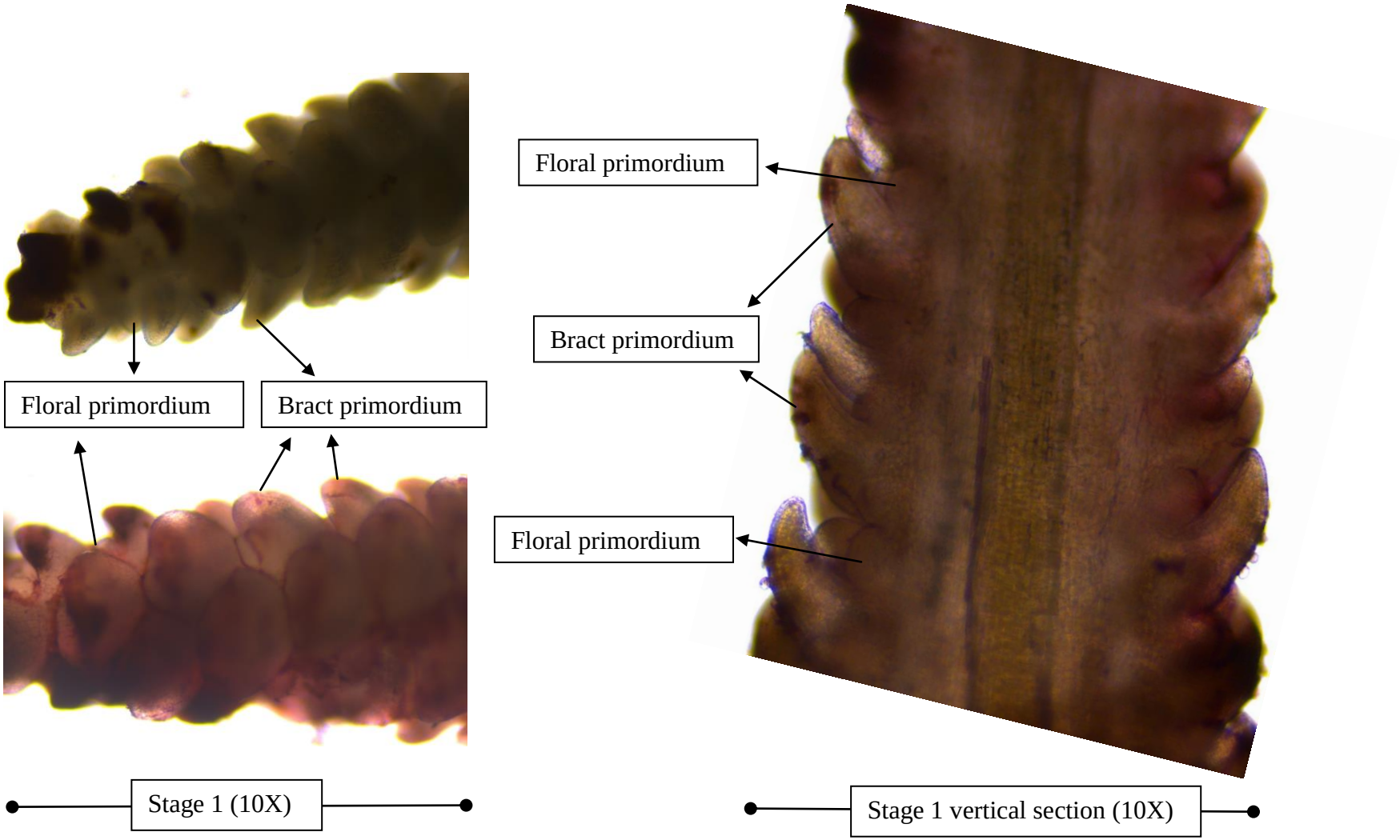


Plate 14. General view of Stage 1 black pepper spikes under microscope

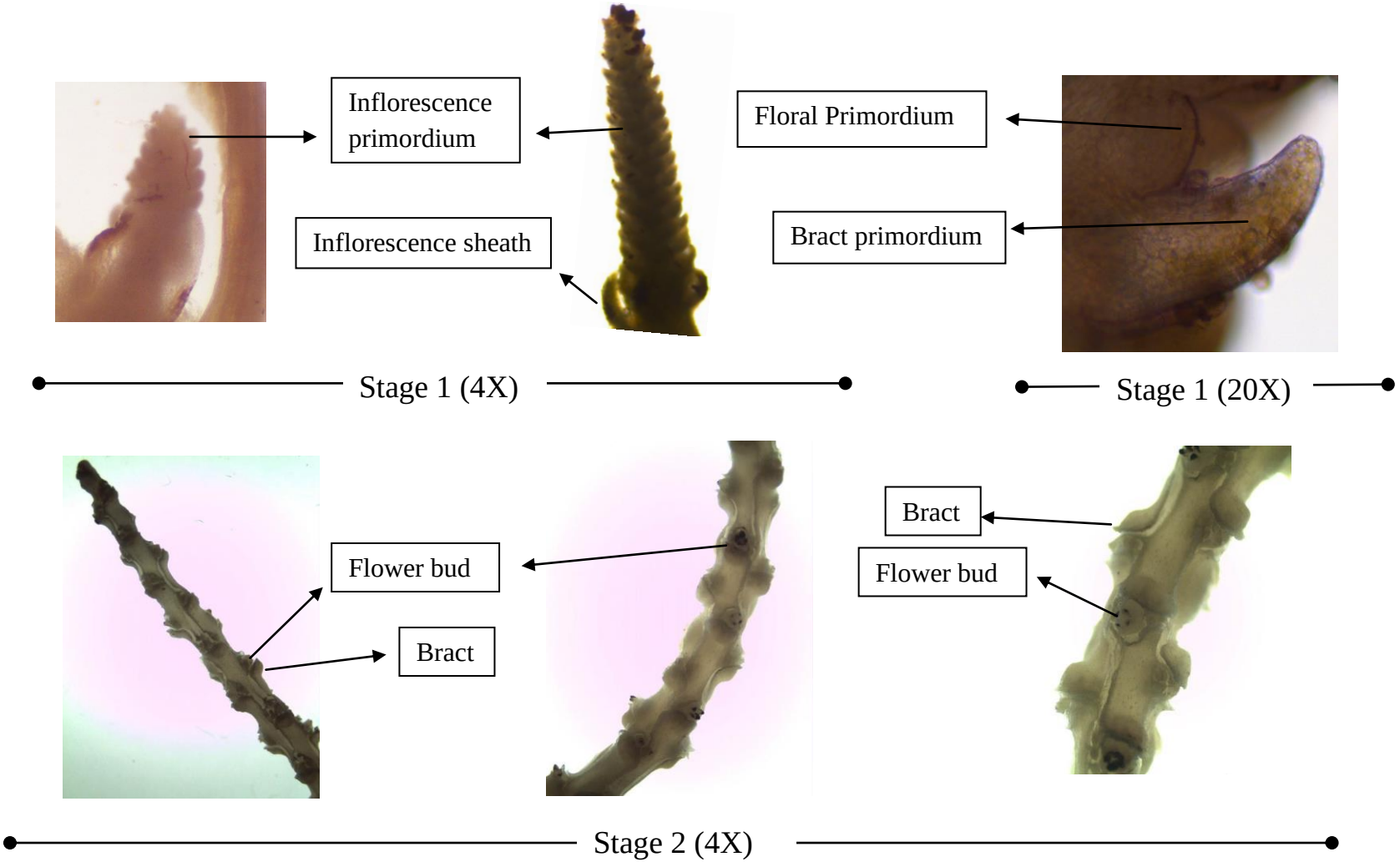


Plate 15. General view of Stage 1 & 2 of black pepper spikes under microscope

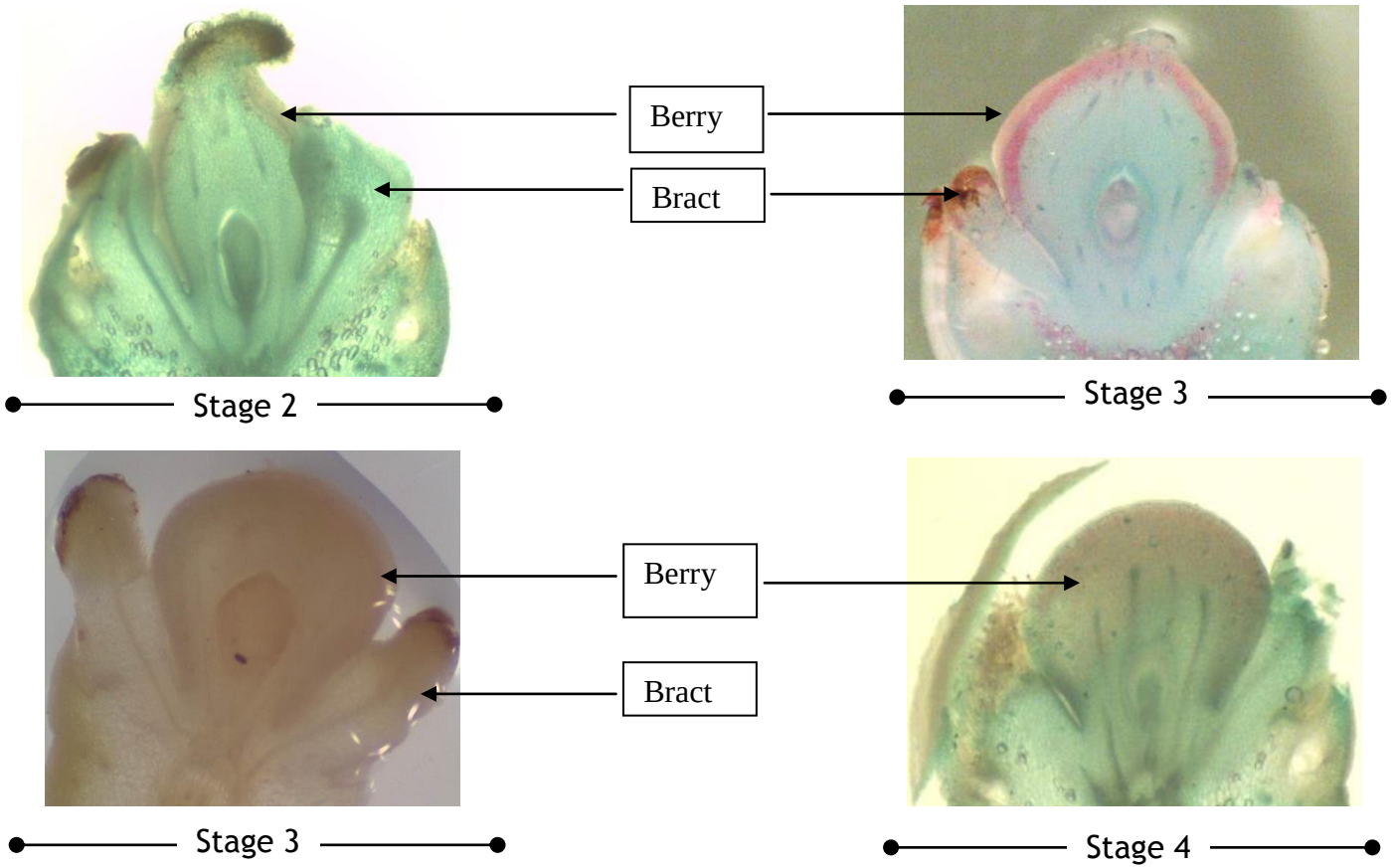


Plate 16. Microscopic images of Stage 2, 3 and 4 of black pepper spikes sections under 35X magnification

DISCUSSION

5. DISCUSSION

The Western Ghats region of India serves as the center of origin of the black pepper (*Piper nigrum*), the most important commercial spice of the world. Western Ghats of South India and the North-Eastern India are the two hotspots of diversity of the genus Piper in India, inhabiting about 110 species (Purseglove *et al*., 1981; Parthasarathy *et al*., 2006) but still the productivity in India is very low. So in order to increase the yield at the gene level initial steps have been taken in this study entitled "Molecular analysis of floral meristem identity genes in black pepper (Piper nigrum L.)". Which was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2012-2014. Discussions pertaining to this study are discussed in this chapter.

5.1 IDENTIFICATION OF MERSISTEM IDENTITY GENE

5.1.1 DNA Isolation and PCR Analysis

Recently, many genes have been identified to have a role in the floral meristematic development in different plants, though no such study in black pepper is reported till date. Most of these genes are found to be conserved among diverse plant species (Benlloch *et al*., 2007).

Based on the nucleotide sequences of two different genes, *viz*., *AP1* and *FUL1* involved in regulating meristem identity in many other plant species such as *Arabidopsis*, the degenerate primers were designed. The degenerate primers are preferred over the specific primers since the former can amplify the weakly conserved sequences of the homologous genes in different organisms (Shen *et al*., 1998). These genes were analyzed by PCR and RT-PCR techniques in genomic DNA and mRNA samples respectively.

Obtaining a good quality DNA is a prerequisite for a reliable PCR reaction. In black pepper, the isolation of DNA was affected by high level of polysaccharides and polyphenols. CTAB method of DNA isolation (Doyle and Doyle, 1990) has been mostly accepted in plant species and was used in this

study. Nonetheless, the method needed few modifications in order to procure a good quality and quantity of DNA from black pepper samples. CTAB is reported to reduce the visible amount of polysaccharides in DNA samples. An effective removal of polysaccharides was obtained by increasing the concentration of CTAB along with NaCl in the extraction buffer (Syamkumar *et al*., 2005**;** Sahu *et al*., 2012). An increase in the volume of extraction buffer per sample tissue to 1 ml per 100 mg of tissue resulted to be effective as reported by Dhanya *et al.* (2007).

Since black pepper is rich in secondary metabolites, a very careful and quick processing of the sample mainly at the time of grinding was required. The inclusion of PVP and β-mercaptoethanol in extraction buffer which are known to prevent the oxidation of secondary metabolites in the disrupted plant material (Prittila *et al.*, 2001) avoided the brown pigmentation in the sample, increasing the yield and quality of DNA.

Phenol: chloroform: isoamyl alcohol (25:24:1) has been reported to give high purity DNA in many plant species (Sablok *et al*., 2009). DNA degradation and contaminations were avoided by carrying out all the steps at 25 ^{o}C (room temperature), except the isopropanol precipitation step which gave good result at 4 ^{o}C in the present work.

The absorbance ratio (A_{260}/A_{280}) in the range of 1.8 to 2.0 indicates a high level of purity of DNA (Weising *et al*., 2005). The isolated genomic DNA from black pepper spikes confirmed high purity with (A_{260}/A_{280}) value ranging from 1.6-2.

The PCR amplification of the genomic DNA was tried using the set of designed degenerate primers. The primers (*FUL1*-F, R) and (*AP1*-F, R) did not produce any amplicons. Absence of amplicons may be related to the absence of primer binding site in the DNA because primers were designed based on the cDNA sequences this could be the probable reason for the absence of the amplicons.

5.1.2 RNA Isolation and RT-PCR Analysis

The genes regulating floral meristem identity (*AP1* and *FUL1*) have been reported to express during the initial stages of flower development. Therefore, the isolation of RNA was carried out from the immature spikes (stage 1 spikes) of Karimunda.

The success of RNA extraction is governed by the quality, quantity and integrity of the RNA recovered. The Trizol reagent was used for the isolation of RNA from spikes of black pepper in this study. The maintenance of strictly aseptic condition and the use of sterile materials to inhibit the RNA degradation by RNase are the most important factors determining the quality of the isolated RNA (MacRae, 2007). The bands in agarose gel showed clear and discrete ribosomal RNAs confirming good quality. The spectrophotometric absorbance ratio (A_{260}/A_{280}) ranging from 1.7 to 2.0 is regarded to have little or no protein contamination (Accerbi *et al*., 2010). The ratios obtained in this study (1.85-2.16) unveiled that the extracted RNA was of good quality except for the stage 4 spikes whose ratio was 1.15 because the quality of the RNA from this tissue is poor, this is due to the hardy nature of the stage 4 spike which happens to be a fully mature spike.

The extracted RNA was used for RT-PCR with the designed degenerate primers at specific annealing temperatures. No amplification was detected with *AP1*-F, R primers. The possible reason may be the degradation of cellular functional mRNA facing stress while handling the samples or the absence of the gene activity at that stage, other probable reason could be the absence of primer binding site in the cDNA which could indirectly mean that the conserved region from which the *AP1* primer were designed is not present in black pepper. However amplification was detected with *FUL1*-F, R primers which produced two bands of size ~150 bp and ~400 bp. Primers produced two bands because of degenerate primer i.e., one or more positions of the degenerate nucleotide can be

occupied by one of several possible nucleotides (Kwok *et al*., 1994). The use of low annealing temperatures could also be the probable cause.

5.1.3 Sequencing and Sequence Analysis

The two bands were eluted, purified and sequenced. Even though the expected size of desired amplicon was not around 400bp (i.e. the bigger amplicon) still it was sent for sequencing. This was done due to fact that the DNase was not used for RNA extraction, which included the possibility of targeted gene getting amplified because of the presence of the DNA with some introns causing it to be bigger than the expected size.

The sequences of the amplicons were analyzed using bioinformatic tools in order to identify the gene *viz*., tBLASTx, blastn suite-GEO (Gene expression omnibus), NCBI conserved Domain Search and Phylogeny.fr.

- The tBLASTx programme showed that the bigger amplicon (~400bp) belongs to mRNA of HSP90 whereas smaller amplicon (~150bp) belongs to mRNA of MADS box transcription factor and *APETALA 1* protein.

The sequence of the bigger amplicon which is around ~400bp was published in the NCBI database as the HSP90 partial mRNA sequence of black pepper [Accession number is KJ534563]. This is the first ever heat shock protein gene in black pepper to be identified, sequenced and published in the NCBI database and this accidental discovery is due to the use of degenerate primers and its permutation and combination of the degenerate nucleotides which made it possible for the primer to anneal to the mRNA sequence of the HSP90 (Aarts *et al*., 1998; Shen *et al*., 1998; Deng and Davis, 2001; Lang and Orgogozo, 2011).

The sequence of the smaller fragment which is around 152bp [(152bp) *FUL1* – R sequence] is the partial sequence of the *APETALA 1* like protein of black pepper which also happens to be the floral meristem identity gene. Since no earlier gene publications on flowering genes in black pepper could be noted in NCBI database, the gene identified in the present study could be the first ever

floral gene to be identified in black pepper. Further sequence analysis were done using this sequence which includes,

- The NCBI conserved Domain Search programme, showed that this sequence contains a MADS box region which is similar to the MADS/Type II subfamily of MADS (Agamous) domain.

- Blastn suite-GEO programme, gave Seven Blast Hits on the (152bp) *FUL1* – R sequence showing that the query sequence is similar to the Oryza sativa MADS box protein.

- Phylogenetic tree created from Phylogeny.fr, shows two separate clades, one which belongs to floral meristem identity genes and the other that belongs to organ identity genes. Phylogenetic analyses of the identified pepper sequence (i.e., (152bp) *FUL1* – R) belong to the clade of floral meristem identity genes. The tree also shows that this sequence is closer and related to the MADS box domain containing *APETALA 1* gene of *Arabidopsis*. Bootstrapping was done to verify the solidity of each node and the tree was scaled, the scale bar represents the evolutionary distance of 0.4 K_{nuc} (K_{nuc} = average number of nucleotide changes per sequence position). Bootstrap values are given at the respective nodes.

These sequence analysis reaffirms that the (152bp) *FUL1* – R sequence is a partial sequence of a gene belonging to a MADS box family and is probably *APETALA 1* making it a floral meristem identity gene. The identified gene could thus be important in the specification and development of the flowers based on the studies conducted by Coen and Meyerowitz (1991).

5.2 EXPRESSION STUDIES OF THE IDENTIFIED GENE

5.2.1 Semi Quantitative Analysis

Semi quantitative analysis was done because it provides good information on relative transcript abundance and to identify the exact tissue in which the

identified gene is expressing similar to the studies done by Preston and Kellog (2006). It was done by performing a PCR using **c**DNA from different tissues *viz*., leaf, mature leaf, runner bud, stage 1 spikes and stage 2 spikes as template with *FUL1*, Ubiquitin and RAPD primers. Ubiquitin and RAPD primers were used as a positive control to show that the cDNA is stable and is of good quality. The PCR products were separated on agarose gel (1.5 %) and the gel was observed under gel documentation system.

The gel picture showed that there was amplification in only (Stage 1 spikes) immature spikes when *FUL1* primers were used (Plate 10). This is because the meristem identity gene is active only in the immature stage of the spikes and in other tissues like runner bud, stage 3 spikes, mature leaves and stage 2 spike this gene activity is absent.

Gel picture containing PCR product with Ubiquitin primers showed amplification in all the wells except in blank (Plate 11). This is because ubiquitin is a housekeeping gene which is expressed in all the stages of the plant and it also proves that the cDNA from different tissues is of good quality.

Similarly gel picture containing PCR product with RAPD primers showed amplification in all the wells except in blank (Plate 12). It also proves that the cDNA from different tissues is of good quality.

This analysis proves that the identified gene is expressed only in (stage 1 spike) immature spikes making it a floral meristem identity gene.

5.2.2 Quantitative Reverse Transcriptase - PCR (qRT-PCR)

qRT-PCR was done based on the study conducted by Song et al. (2011). It was carried out in RGCB. cDNA from different samples were used as template *viz*., stage 1 spikes (immature spikes), stage 2 spikes (spikes with flowers), stage 3 spikes (spikes with immature berry), stage 4 spikes (fully mature spikes with green berries), young leaf, mature leaf and runner bud with gene specific primer and Actin primer as endogenous control. The gene specific primer is specifically

designed for qRT-PCR based on the (152bp) *FUL1* – R sequence, it was named as *BPFul*-Sp (The sequence of the *BPFul*-Sp primers is given in Table 5). Thermal cycling for absolute quantification (i.e., amplification plot and dissociation curve was done) was done based on the study by Bustin (2000). As a result of absolute quantification amplification plot and dissociation curve was generated by the software after carrying out the reactions.

Fig. 4 shows the amplification curve of *BPFul* specific primer. Graph shows that the three lines are crossing the green threshold line, these three lines represents the fluorescence produced by the PCR product of the (immature spikes) stage 1 spikes and rest of the fluorescence is below the threshold meaning that the others are not amplified. This result is in sync with the results from the semi quantitative analysis.

Fig. 5 shows the amplification curve of Actin primer, actin primer is used as an endogenous control. Graph shows that the all of the samples are producing fluorescence except from the stage 4 spikes. PCR product is produced in all the tissue samples because actin is an endogenous control which means that the cDNA used for analysis is of good quality. The reason for no amplification in stage 4 spikes is because of the problem in RNA isolation from the mature spikes which is hard especially to crush the mature berries.

And finally the Fig. 6 shows the dissociation curve of primers. Dissociation curve gives us the idea of the specificity of the primers, its ability to form primer dimers and it also gives us the information about the nonspecific fluorescence due to primer dimer formation, based on this the ct value is given. Ct value gives the relative concentration/ copy number of the PCR product formed.

Results obtained by the study conducted by Song *et al.* (2011) are similar to the results obtained in qRT-PCR based on this we can conclude that the identified gene sequence ((152bp) *FUL1* – R) is expressed only in the stage 1 spikes, which happens to be the immature spikes and the reason for not getting

any amplification in other tissues is solely because of the absence of the gene expressions in them.

5.3 MICROSCOPY STUDIES

The microscopy study of floral meristem structure linked to the expression of the flowering genes has not been reported till date. But the floral bud differentiation studies in black pepper has been done by Nalini (1983) which includes the microscopy study of vegetative bud and flowering bud showing leaf sheath, leaf primodium, shoot primodium, and spike primodium.

Tucker (1982) did the study on inflorescence and flower development in three *Piper* spp., namely *Piper aduncum*, *Piper amalgo* and *Piper marginatum* which are believed to be conserved in other species as well (Ravindran, 2000).

Sokoloff *et al*. (2006) reported the fixing of samples in either formalin-aceto-alcohol (FAA) or 70 per cent ethanol for light microscopic study. However, Carnoy's fluid which has greater penetrating power than FAA is remarked as better fixative for plants with high phenolics (Sreekantan, 2002). In this study, the spikes stored at -80 ^{o}C when directly used or fixed in Carnoy's fluid stored at room temperature soon turned brown and were unfit for the study. On the other hand, fixing the samples overnight in Carnoy's fluid pre-stored at -20 ^{o}C inhibited the browning of the sample as reported by Sreekantan (2002).

The spikes were then viewed directly under the stereomicroscope (Leica ez4hd, Germany) or subjected to dehydration and then hand sectioned using a new razor blade. These sections were either directly observed under the microscope or subjected to staining. For staining, the safranin stain was used as primary stain. The safranin stained sections were visualized under microscope. Few safranin stained sections were subjected to secondary stain i.e., fast green. These sections were then observed under microscope.

Plate 13 shows the general view of the black pepper spikes of different stages showing the different stages of development starting from its emergence till

full maturity. It also shows the classification of spikes *viz*., stage 1 spikes, stage 2 spikes, stage 3 spikes and stage 4 spikes

Plate 14 shows the magnified image of the stage 1 pepper spikes under 10X magnification. Stage 1 spikes shown in this plate are newly emerged pepper spikes and this is the actual stage of spike where the meristem identity gene is active and expressing. This plate shows that the floral primordium is completely enclosed with bract primordium, it is more prominent in the vertical section of the stage 1 spike.

Plate 15 shows the magnified image of the stage 1 and stage 2 pepper spikes under 4X and 20X magnification. Stage 1 spikes shown in this plate are newly emerged pepper spikes. This plate also shows the microscopy image of vertical section of stage 1 spike under 20X magnification which shows floral meristems in the region of floral primordium. Stage 2 spikes are under 4X magnification, it shows the gradually developing bract and flower bud.

Plate 16 shows the magnified image of the stage 2, stage 3 and stage 4 pepper spike sections under 35X magnification. The sections in this plate are taken by horizontal hand sections of the pepper spikes. This plate gives an idea about the pepper berry formation, stage after stage. In stage 2 spike, the flower is fertilized by rain drop and it is preparing for the formation of berry, the stage 3 spike sections shows the immature berry formation finally stage 4 section shows the mature berry. Expression of floral meristem identity gene is absent in all the stages shown in this plate (Davies, 2006).

Based on the semi quantitative analysis and qRT-PCR results one can confidently say that the identified floral meristem identity gene is expressed only in stage 1 spikes and it is completely absent in stage 2, stage 3 and stage 4 spikes. This is because the floral meristem identity gene is only active at the earlier stages of flowering, where the floral meristems are still in the process of differentiation. But the floral meristem identity gene activity is absent in tissues where the floral meristems are completely differentiated (Davies, 2006).

SUMMARY

6. SUMMARY

The study entitled "Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2012-2014. The objective of the study was to isolate and sequence genes homologous to key floral meristem identity genes in black pepper (variety - Karimunda) and functionally characterize these genes by studying their expression patterns.

Two floral meristem identity genes *viz*., *FRUITFULL 1* and *APETALA 1 (AP1)* were chosen to identify in black pepper. Degenerate primers were designed for the above said genes based on the gene sequences from NCBI database (*FUL1* and *AP1* forward and reverse primers) which were used to isolate and identify the genes. Genomic DNA of black pepper was isolated using modified CTAB method and RNA was isolated using Trizol reagent method followed by synthesis of cDNA using AMV RT (Avian myeloblastosis virus reverse transcriptase).

PCR (Polymerase chain reaction) with degenerate primers using DNA as template showed no amplification. When cDNA was used as a template for PCR no amplification was found with degenerate *AP1* primers. However with *FUL1* primers, two bands of size ~400bp and ~150bp were produced when cDNA of immature spikes were used as a template for PCR.

These two bands were eluted, purified and sequenced. The sequences of the amplicon were analysed using bioinformatics tools inorder to identify the gene *viz*., tBLASTx, blastn suite-GEO (Gene expression omnibus), NCBI conserved Domain Search and Phylogeny.fr.

The tBLASTx programme showed that the bigger fragment belongs to mRNA of HSP90 whereas smaller fragment belongs to mRNA of MADS box transcription factor and *APETALA 1* protein.

The sequence of the bigger fragment was published in the NCBI database as the HSP90 partial mRNA sequence of black pepper [Accession number is KJ534563]. This is the first ever Heat shock protein 90 gene to be identified and sequenced in black pepper.

The sequence of the smaller fragment which is around 152bp is the sequence of the *APETALA1* like protein of black pepper which also happens to be the floral meristem identity gene making it the partial fragment of the first ever floral gene to be identified in black pepper. Further sequence analysis were done using this sequence.

The NCBI conserved Domain Search programme, showed that this sequence contains a MADS box region which is similar to the MADS/Type II subfamily.

Blastn suite-GEO programme, showed that (152bp) *FUL1* – R sequence was similar to the Oryza sativa MADS box protein. Phylogenetic tree created from Phylogeny.fr also showed that the 152bp sequence is closer to *APETALA 1* of *Arabidopsis* containing MADS box domain and grouped with the clade of floral meristem identity genes.

Specific primers were designed to 152bp smaller fragment for qRT-PCR to study the expression of the floral gene. It was confirmed that this gene was expressed only in immature spikes of black pepper. Semi quantitative analysis with *FUL1* primers also gave the same results.

Microscopy studies were done using carnoy's fluid as fixative, sectioning the tissues and staining with safranin and fast green were carried out to see the changes occurring in different development stages of spikes from immature spike to complete spike with berries. This helped to clearly identity that the expression of the identified floral meristem identity gene was in the immature stage (stage 1 spike) of the spike i.e. when floral meristems are developing on the spike. These

spatial expression of the identified gene at this stage also strengthened the possibility of it being a floral meristem identity gene.

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7. REFERENCES

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ABSTRACT

Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.)

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ABSTRACT
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ABSTRACT

The present study entitled "Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, during 2012-2014. The study envisaged isolation and sequencing of genes homologous to key floral meristem identity genes in black pepper (variety - Karimunda) and functional characterization of these genes by studying their expression patterns.

Two floral meristem identity genes *viz*., *FRUITFULL 1 (FUL1)* and *APETALA 1 (AP1)* were chosen to identify in black pepper. Degenerate primers were designed for the above said genes based on the gene sequences from NCBI database (*FUL1* and *AP1* forward and reverse primers) which were used to isolate and identify the genes. Genomic DNA of black pepper was isolated using modified CTAB method and RNA was isolated using Trizol reagent method followed by synthesis of cDNA using AMV RT (Avian myeloblastosis virus reverse transcriptase).

PCR (Polymerase chain reaction) with degenerate primers using DNA as template showed no amplification. When cDNA was used as a template for PCR no amplification was found with degenerate *AP1* primers. However with *FUL1* primers, two bands of size ~400bp and ~150bp were produced when cDNA of immature spikes were used as a template for PCR. These fragments were sequenced and analyzed, analysis of sequences showed that the bigger fragment i.e. ~400bp showed similarity to HSP90 (Heat shock protein 90) and the sequence of the smaller fragment which is around 152bp showed similarity to the floral meristem identity gene *APETALA1* making it the first ever floral gene to be identified in the black pepper.

The sequence of the bigger fragment was published in the NCBI database as the HSP90, partial mRNA sequence of black pepper [Accession number is

KJ534563]. This is the first ever Heat shock protein gene to be identified, sequenced and published in black pepper.

Specific primers were designed for qRT-PCR (Quantitative RT-PCR) to study the expression of the sequenced floral meristem identity gene, it was confirmed that this gene was expressed only in immature spikes of black pepper.

Microscopy studies were carried out to see the changes occurring in different development stages of spikes from immature spike to complete spike with berries and also to correlate the expression of the identified floral meristem identity gene in the different tissues. This study helped to clearly identify the expression of the identified floral meristem identity gene, which is expressed in the immature stage of the spike.

APPENDIX I

CTAB Extraction Buffer

C-TAB	2.5 %	
Tris- HCl (pH 8.0)	100 mM	
EDTA	25 mM	
NaCl	1.5 M	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA
PVP	4 % (w/v)	extraction

APPENDIX II

TE buffer

Tris- HCl (pH 8.0)	10 mM
EDTA	1 mM

APPENDIX III

TBE Buffer (5X) for 1 liter solution

Tris base	54 g	(0.445M)
Boric acid	27.5 g	(0.445M)
0.5 M EDTA (pH 8.0)	20 ml	(0.01M)

APPENDIX IV

Carnoy's fluid

Ethyl alcohol (100 %)	60 ml
Glacial acetic acid	10 ml
Chloroform	30 ml