Identification and characterization of *Suppressor of Overexpression of Constans1* (*SOC1*) gene in black pepper (*Piper nigrum* L.)

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Identification and characterization of *Suppressor of Overexpression of Constans1* (*SOC1*) gene 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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Certified that this thesis, entitled **"Identification and characterization of *Suppressor of Overexpression of Constans1* (*SOC1*) gene in black pepper (*Piper nigrum* L.)"** is a record of research work done independently by Mr. Manu K. Venu (2011-09-124) 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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MANU

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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
°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

Dedicated to my father, the leading light of my life

To my mother for all the struggles endured for me…..

INTRODUCTION

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), the 'King of spices' or 'Black gold' is among the important and earliest known spices produced and exported from India. The Western Ghats in South India with its dense, evergreen forests is the original home of black pepper. Black pepper belongs to the botanical family Piperaceae, which is a large pantropical family of subclass dicotyledonae and order Piperales (Cronquist, 1978).

It is predicted that global demand for pepper will increase to about 280,000 t by 2020 and that the demand will further increase to 360,000 t by 2050 (Nair, 2011). Although India is top among the producers in acreage and production, its productivity is very low compared to other countries. India exported 21,450 t of pepper valued at Rs 1,200 crore in 2014-15. Indian black pepper production had dropped to 37,000 t in 2014 before rising to 65,000 t in the current year. Brazil and Sri Lanka have increased their output in the last three years, which puts India in danger of losing its current third position behind Vietnam and Indonesia (Krishnakumar, 2015).

Flowering is one of the important processes which determines yield. 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.

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.

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. 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 the yield more accurately.

The alteration of flowering character by transfer of specific 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 provide knowledge on how to manipulate flowering behavior of the plant. This can aid in developing strategies to increase the number of flowers in the plant which in turn produces more number of berries and also to produce superior varieties of black pepper with increased productivity.

Isolating and studying flowering time genes and floral integrator genes, in turn, can help to produce spikes at any environmental conditions and control the flowering time as desired. Among the flowering time and floral integrator genes, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) has been identified as an important gene in several plants. With this in mind, the present study was carried out to isolate and characterize the flowering time gene *SOC1* from black pepper with the following specific aims:

- Design degenerate primers for the isolation of *SOC1* gene in black pepper
- Targeted isolation and sequencing of *SOC1* gene from black pepper variety Karimunda using the designed primers through PCR and RT-PCR

- Sequence analysis of the gene and characterization of expression patterns of the isolated gene through qRT-PCR/RT-PCR in different tissues and at different stages of floral development
- Track floral development in black pepper through histological studies

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Black pepper, the 'King of spices' or 'Black gold' is one among the important and earliest known spices produced and exported from India. The Western Ghats in South India, with its dense evergreen forests, is the original home of black pepper. Black pepper belongs to the botanical family Piperaceae, which is a large pantropical family of subclass dicotyledonae and order piperales (Cronquist, 1978). The family name, Piperaceae is comes from the Latin word *piper* for pepper. European names for pepper were obtained from the Sanskrit name *pippali*, and the plant was in use as long as 3,000 years ago in India (Nelson and Cannon-Eger, 2001). Piperaceae 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 1200 spp. (Callejus, 2001). American tropics have the greatest diversity of the genus *piper* (700 spp.), followed by south Asia (300 spp.) which is home to the economically important species, *Piper nigrum* L. (black pepper) and *Piper betle* L. (betel leaf) (Jaramillo and Manos, 2001). The Western Ghats and North-Eastern parts of India are hotspots of diversity of the genus *piper* in India, accommodating about 110 spp. (Purseglove *et al.*, 1981; Parthasarathy *et al.*, 2006).

More than 40 varieties of black pepper are grown worldwide of which most of the varieties were developed from India. Some of the varieties from India are Panniyur, Karimunda, Lampng, Bangka and Belontoeng. The varieties differ in berry attributes (size, color, etc.), leaf size, raceme length, resistance to pest and disease, seed quality and yield. Varieties differentially respond to climatic conditions, such as wet weather conditions or dry periods (Nelson and Cannon-Eger, 2001).

Black pepper is mostly dioecious in the wild form undergoing cross pollination (Krishnamurthy, 1969), whereas, cultivated types are gynomonoecious (bearing female and bisexual flowers in the same plant) or trimonoecious (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 have been reported in *Macropiper* and *Pothomorphe* (Jaramillo and Manos, 2011).

2.1 GENERAL MORPHOLOGY OF BLACK PEPPER PLANT

Black pepper is a perennial climber and it climbs on support trees with aerial clinging roots. The climber produces two types of branches, *viz.,* orthotropic branches (main stems) and plagiotropic branches (fruiting stems). 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, 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 broad, shiny green, pointed, petiolate leaves alternately arranged on the stem. The inflorescence is catkin (spike), 7-10 cm long, clustered with small sessile 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 FLOWER DEVELOPMENT IN BLACK PEPPER

The early available knowledge on inflorescence and flower development was based on the studies by Tucker (1982) in three *piper* spp. *viz., Piper aduncum, Piper amalgo* and *Piper marginatum,* and the process of flower development is believed to be conserved in all other species as well (Ravindran *et al.,* 2000).

According to the observations by Tucker (1982), at the early stage, Piper inflorescence is in the form of a convex apical meristem, surrounded by a vegetative leaf and a bract. The apical meristem is zonate consisting of two tunica layers, a central initial zone, a peripheral zone and a large pith rib meristem. The apical meristem of the inflorescence extensively grows in length before formation of any organs and as it grows in length the apical meristem diminishes. The bracts are initiated closer towards the apex by a periclinal division in the second tunica layer on the flanks of apical meristem. The floral development takes place in the axil of the bract. The cells in the axils are meristematic and the cells located in the outer layer divide anticlinically. The next two layers undergo very intensive cell divisions, by means of periclinal divisions before the formation of protuberance. The Cells in the outer two layers elongate anticlinically making the protuberance. This enlarges further and differentiates into a flower bud. As the primordium (protuberance) mentioned above grows, stamens are initiated from the two lateral sites. The carpels are initiated by means of periclinal divisions in the sub surface layer situated on the side of the small floral apex. Ovule initiation begins by a periclinal division in the second tunica layer situated at the centre of the floral apex (Bernier *et al., 1993*; Bernier *et al.,* 1998).

Floral bud differentiation studies in black pepper was done by Nalini (1983). It involved the microscopy study on vegetative and flowering bud and showed leaf sheath, leaf primordium, shoot primordium and spike primordium. The timing and process of transition from vegetative growth to flowering is really significant in the field of agriculture and plant breeding because flowering is the initial step of sexual reproduction. The transition from vegetative to reproductive phase is being studied by

countless physiologists and has resulted in accumulation of considerable amount of scientific information on flowering of different plant species.

Three major theories have been 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. Sachs and Hackett (1983) have postulated in the "nutrient diversion" hypothesis that the floral induction mainly depends upon the source/sink relationships which is the partitioning of assimilates such that critical areas of the shoot apical meristem (SAM) *viz.,* the relatively quiescent central zones receive high concentrations of assimilates under inductive conditions. Finally, the most promising theory, "multifactorial control" was postulated. It assumes that several chemical assimilates, phytohormones, genetic variation and also the past and present growing conditions participate in floral induction. Studies conducted in *Sinapsis 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 and also environmental factors such as light and temperature (Simpson *et al.,* 1999; Giovannini, 2006).

2.3 MOLECULAR CONTROL OF FLOWERING

The uncovering of genetic pathways leading to spike formation can be a boon in crop improvement programmes, 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 manipulate flowering.

Here, the information on flowering genes, especially 'flowering-time' gene *SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)/AGAMOUS LIKE 20 (AGL20)* 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.

The molecular mechanisms behind the transition of vegetative shoot apical meristems (SAMs) to inflorescence meristems (IMs) in Arabidopsis have been extensively studied. This transition is caused by complex pathways which regulates flowering with response to environmental and developmental signals (Blazquez *et al.*, 2003; Boss *et al.*, 2004; Mouradov *et al.*, 2002; Simpson and Dean, 2002).

The autonomous pathway controls and regulates the flowering in plants by responding to endogenous cues from various developmental stages but the flowering in short – day conditions are influenced by the Gibberellin pathway. The responses to environmental signals such as day length and cold temperature is mediated by photoperiod and vernalization pathways. Other genetic pathways which respond to changes in light quality and temperature which affect flowering process have also been proposed. The signals of these pathways converge on the transcriptional regulation of major floral pathway integrators, *FT* and *SOC1* and these in turn activate the FM identity genes such as *LFY* and *AP1* to produce FMs on the flanks of IMs (Blazquez and Weigel, 2000; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Lee *et al.*, 2000; Lee *et al.*, 2008; Liu *et al.*, 2008; Samach *et al.*, 2000). Some of the 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 may lead to the activation of the floral meristem identity genes like *LEAFY (LFY)* or *APETALA 1 (AP1)*, which specify the floral fate of the nascent lateral primordia which is produced by the SAM. An alternate possibility is

that the different promotion pathways are directly integrated at promoter levels of the floral meristem identity genes such as *LFY* and a class of 'flowering-time' genes that act in parallel with *LFY* (Schultz *et al.,* 1991; Huala *et al.,* 1992; Weigel *et al.,* 1992).

The multiple genetic pathways that promote the floral transition are integrated at the transcriptional regulation of the floral meristem identity genes and the 'flowering-time' genes such as *FT* and *SOC1*. There may be extensive crosstalk between the pathways and integrating genes (Swarup *et al.,* 1999). The flowering integration gene *SOC1* and *LFY* promote the integration of flower induced signals by the *FT* gene from the photoperiod, autonomous and vernalization pathways which in turn promotes Arabidopsis flowering (Xu Feng *et al.,* 2012).

2.3.1 Role of Floral Integrator *SOC1*

SOC1 functions by encoding a MADS-box transcription factor and also as an integrator of the day length, the gibberellic acid and the vernalization pathways. Unlike *FT, SOC1* is highly expressed in IMs. Therefore *SOC1* can contribute to the spatial specificity of FM initiation, during which a small mass of cells on the IM flank progenitor fate for a future FM (Bossinger and Smyth, 1996; Lee *et al.*, 2000; Samach *et al.*, 2000).

Four MADS-box genes in Arabidopsis such as *SOC1, SHORT VEGETATIVE PHASE (SVP), SEPALLATA 4* and *AGAMOUS-LIKE 24 (AGL24),* act directly and redundantly in order to suppress *TERMINAL FLOWER1 (TFL1)* in emerging floral meristems. This is most necessary for the important function of *AP1* in specifying floral meristems and is coupled with a conformational change of chromosome looping at the *TFL1* locus. (Liu *et al.,* 2013)

SOC1, SVP, and *AGL24* have a role in regulating the floral meristem differentiation in Arabidopsis through suppressing a class E floral organ identity gene, *SEPALLATA 3 (SEP3)* (Liu *et al.*, 2009).

A recent study has revealed that a set of MADS-box transcription factors, namely *SOC1, SVP, AGL24,* and *SEP4,* act redundantly and directly to suppress *TFL1* in emerging floral meristems. In soc1-2 agl24-3 svp-41 sep4-1 quadruple loss-of-function mutant, the main inflorescence meristem continuously generates secondary and tertiary branch shoots, which results in a massive inflorescence branching phenotype. Although *AP1* and *LFY* are normally expressed in the branch meristems in the mutant, they are not able to suppress the persistent expression of *TFL1*, which ultimately confers shoot identity rather than floral identity on branch meristems. These observations indicate that *SVP, SOC1, AGL24*, or *SEP4* are very important for the function of *AP1* and *LFY* in repressing *TFL1* (Liu *et al.*, 2009).

SVP, SOC1, and *AGL24* were identified as important flowering time genes that regulate the floral transition in Arabidopsis. Both *SOC1* and *AGL24* respond to multiple flowering genetic signals and interact with each other to promote flowering (Liu *et al.*, 2009).

The integration of the flowering signals is strictly controlled by a repressor complex that consists of two MADS-box transcription factors called *FLC* and *SVP* (Hartmann *et al.,* 2000; Li *et al.*, 2008; Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

Michaels (2009) proposed that the modulation of the chromatin structure results in repression of *FLC* by the vernalization and autonomous pathways, which promote flowering by reducing the repressive effect of *FLC* on *SOC1* and *FT* expression (Helliwell *et al.*, 2006; Searle *et al.,* 2006).

The *FT* expression in leaves is repressed by *FLC* which in turn blocks the translocation of the systemic flowering signals containing FT protein to the SAMs, a process that is mainly required for the activation of *SOC1* and *AP1* (Abe *et al.*, 2005; Corbesier *et al.*, 2007; Searle *et al.*, 2006; Wigge *et al.*, 2005). *FLC* also directly represses the expression of *SOC1* and *FT* cofactor FD in SAM (Searle *et al.*, 2006), which further inhibits the meristem response to the flowering signals.

In the vegetative seedlings, the *FLC-SVP* complex responds to flowering signals which are perceived by the autonomous, the thermosensory and GA pathways (Hartmann *et al.*, 2000; Lee *et al.*, 2007; Li *et al.*, 2008). Their mutually dependent function is directly responsible for the regulation of *SOC1* expression in whole seedlings, as well as FT expression in leaves. Therefore, most flowering pathways (with the exception of the photoperiod pathway) promote *FT* and *SOC1* mainly through de-repression mechanisms.

2.3.1.1 *Functions of SOC1*

SOC1, which encode a MADS box transcription factor, is responsible for integrating multiple flowering signals received from photoperiod, temperature, hormone, and age-related signals. *SOC1* is controlled by two flowering regulators called *CONSTANS (CO)* and *FLC*, which functions as floral activator and repressor respectively. *SOC1* is activated by *CO* mainly through *FT* but *FLC* represses *SOC1* directly by binding to the promoter. At the shoot apex, *SOC1* along with *AGL24* directly activates *LFY*, a floral meristem identity gene. *AP1* which is activated mainly by *FT*, is also an important factor responsible for establishing flower meristem identity. When *LFY* and *AP1* are established, floral development occurs at the shoot apical meristem according to the ABC model. *AP1* activates the A function during early flower development and represses flowering time genes, *SOC1*, *AGL24*, and *SVP* respectively in order to prevent floral reversion. During late flowering, such repression

is necessary to activate *SEP3* which is also a coactivator of B and C function genes with *LFY*. *SEP3* is suppressed by *SOC1*, *AGL24*, and *SVP*. Therefore, *SOC1* is necessary to prevent premature differentiation of the floral meristem (Jungeun Lee and Ilha Lee., 2010).

The *SOC1* gene is a member of the MADS box TF family and its expression is regulated by the day length and vernalization pathways (Hepworth *et al.*, 2002). The expression of *SOC1* is up-regulated under long-day (LD) conditions by *CO*, in the presence of the FT protein (Samach *et al.*, 2000; Yoo *et al.*, 2005). *SOC1* also integrates the GA-mediated flowering-time signal (Moon *et al.*, 2003).

Dorca-Fornell *et al.* (2011) said that a recently done study has shown that the Arabidopsis *SOC1* clade members like *AGL42, AGL71*, and *AGL72* contribute to the GA-mediated transition to flowering. However, *SOC1* appears to be a major player in flowering response.

FLC have a major role in the vernalization pathway, this MADS domain TF is responsible for repressing the expression of *SOC1* by binding to the *SOC1* promoter region (Hepworth *et al.*, 2002; Deng *et al.*, 2011). *FLC* also interacts with the MADS domain protein *SVP*, which acts as a floral repressor and binds *SOC1* regulatory sequences as well (Hartmann *et al.*, 2000; Li *et al.*, 2008; Tao *et al.*, 2012). These floral repressors are also influenced by *AGL24*, which acts as a direct inducer of *SOC1* and flowering (Michaels *et al.*, 2003).

Wang *et al.* (2009) proposed that *SOC1* is also controlled by an age-dependent mechanism involving SQUAMOSA-BINDING FACTOR-LIKE9 and microRNA156. In addition, the *SOC1* mRNA levels appear to be regulated via post-transcriptional changes by the RNA binding protein EARLY FLOWERING9 (ELF9), which is supposed to target *SOC1* transcripts for nonsense-mediated mRNA decay (Song *et al.*, 2009).

Wang *et al.* (2010) proposed that the *SOC1* activity is also regulated at the protein level by a PIN1-type parvulin, which is involved in cis/trans-isomerization of phosphorylated Ser/Thr-Pro motifs. Jointly, these complexes undergo regulatory mechanisms which ensure that *SOC1* reaches its threshold value for triggering the floral transition at the right time during plant development and under environmental conditions that are favorable for reproduction.

Seung *et al.* (2005) said that in Arabidopsis (*Arabidopsis thaliana*) two floral integrators, *FT* and *SOC1,* are positively regulated by *CO*. *FT* and *SOC1* act in parallel pathways downstream of *CO* based on a genetic analysis using weak *ft* alleles, since *ft soc1* double mutants showed an additive effect in suppressing the early flowering of *CO* overexpressor plants.

2.3.2 *SOC1* Expression in Annuals and Perennials

OsMADS50, which functions as a positive regulator for flowering in rice is highly homologous to Arabidopsis *SOC1* (Choong *et al.*, 2009). Million Tadege *et al.* (2003) isolated a *SOC1* homologue called *OsSOC1* from rice. The *OsSOC1* gene was isolated and used for screening the *FLC*-like genes and was found to have structural similarity to *SOC1* with seven exons and a high sequence homology. Constitutive expression of *OsSOC1* has complemented the Arabidopsis *soc1* mutation and it resulted in early flowering in the wild type, which further indicate conserved functionality (Andersen *et al.*, 2004).

Alter *et al.* (2016) discovered new key players of the flowering network in maize by analyzing the leaf transcriptomes of four lines that exhibit strong differences in flowering transition and it was found that there were strong differences among the regulated genes. Thus it was assumed that the regulation of flowering time (FTi) is a complex process in maize. It was observed that the genes encoding MADS box transcriptional regulators were up-regulated in leaves during the meristem transition.

Alter *et al.* (2016) selected *ZmMADS1* for functional studies, as it represents a functional ortholog of the central *FTi* integrator *SOC1* of Arabidopsis. It was observed that the RNA interference mediated down regulation of *ZmMADS1* gene resulted in a delay of *FTi* in maize. Also, the strong overexpression of *ZmMADS1* caused an early-flowering phenotype, pointing out its role as a flowering activator. *ZmMADS1* functions as a positive *FTi* regulator that shares an evolutionarily conserved function with *SOC1* which may serve as an ideal starting point to study the integration and variation of *FTi* pathways also in maize (Alter *et al., 2016*).

Voogd *et al.* (2015) studied the mechanism behind the regulation of dormancy and flowering in kiwifruit, *SOC1*-like genes from kiwifruit were identified and characterized. It was observed that the ectopic expression of *AcSOC1e, AcSOC1i*, and *AcSOC1f* in *Actinidia chinensis* did not have any role in the establishment of winter dormancy and as a result failed to induce precocious flowering, but it was observed that *AcSOC1i* decreased the duration of dormancy in the absence of winter chilling. These findings helped to understand in detail about the *SOC1-like* gene family and the potential diversification of *SOC1* function in woody perennials.

Wang *et al.* (2014) isolated six flowering genes *PrSOC1, PdSOC1, PsSOC1, PsSOC1-1, PsSOC1-2,* and *PsSOC1-3* from peony tree species namely *Paeonia rockii, Paeonia delavayi*, and *Paeonia suffruticosa* respectively. Sequence comparison analyses showed 99.41 % nucleotide identity which indicated that these six genes were highly conserved. *PsSOC1*'s expression patterns in flower buds and vegetative organs were studied in detail and the results indicated that *PsSOC1* could be expressed both in vegetative and reproductive organs. While the expression of *PsSOC1* in different developmental stages of buds was different, it was observed that high expression levels of *PsSOC1* occurred in the bud at the bud sprouting stage.

Zhang *et al.* (2015) cloned two MADS-box genes *GhSOC1* and *GhMADS42* from upland cotton CCRI36 (*Gossypium hirsutum* L.) and transformed it into Arabidopsis. In addition upland cotton plants were transformed with *GhSOC1* gene. Sequence conservation between *GhSOC1* and *GhMADS42* was observed during the comparative analysis. Tissue-specific expression analysis of *GhSOC1* and *GhMADS42* was done and the results revealed spatiotemporal expression patterns involving high transcript levels in leaves, shoot and apical buds. It was also revealed that spatiotemporal overexpression of *GhSOC1* and *GhMADS42* in Arabidopsis accelerated flowering, with *GhMADS42* transgenic plants showing abnormal floral organ phenotypes.

Cseke *et al.* (2003) identified a vascular tissue-specific MADS-box gene, *Populus tremuloides MADS-box 5* (*PTM5*) which is expressed in differentiating primary and secondary xylem and phloem. Phylogenetic analysis revealed that *PTM5* is a member of the *SOC1/TM3* class of MADS-box genes. These results led to the conclusion that the expression of *PTM5* with other vascular developmental genes may play a major role in the complex events that lead to the formation of the woody plant body.

In order to understand the molecular basis of flowering regulation in soybean, Xiaofan *et al.* (2013) isolated *GmSOC1* and *GmSOC1-like*, two putative soybean orthologs for the Arabidopsis *SOC1/AGL20*. Expression studies of the isolated *GmSOC1-like* was done by means of qRT-PCR in *Zigongdong-dou*, a photoperiod-sensitive soybean cultivar. It was revealed that *GmSOC1-like* was expressed at different levels in most organs of the soybean. However, the highest expression was seen in the shoot apex during the early stage of floral transition.

The *Vitis vinifera* L. flowering genes *VvFT* and *VvMADS8* from the grapevine cultivar 'Cabernet Sauvignon' have been isolated by Sreekantan and Thomas (2006).

The isolated Sequences of *VvFT* and *VvMADS8* were highly homologous to the floral integrators, *FT* and *SOC1*, respectively, from Arabidopsis. The expression studies done by means of real-time PCR revealed that expression of *VvFT* was low in axillary buds and high in developing inflorescence and berries. It was also observed that the *VvMADS8* was highly expressed in the axillary buds at the time when inflorescence primordia were being initiated in these buds suggesting that *VvMADS8* is a gene involved in the early stages of inflorescence development.

2.4 IDENTIFICATION OF CANDIDATE GENE

An unknown sequence related to known sequence can be isolated in order to investigate the biological function. Genes that are unknown in one organism but are homologous to the sequences of known genes from different organisms can be isolated by PCR methods (Rose *et al.*, 1998).

Degenerate primers which are designed from the conserved motifs of resistant genes have been found to be successful for isolating resistance genes in 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.

Candidate gene approach using degenerate primers could be advantageous in exploring the presence of flowering genes as many of these genes 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).

2.4.1 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 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 interfere in the isolation process as they bind to the nucleic acids during DNA isolation (Puchooa and Khoyratty, 2004; Mishra *et al.*, 2008). Browning of the DNA is caused by polyphenol oxidation and co-precipitation (Varma *et al.*, 2007; Mishra *et al.*, 2008). Loading of the DNA becomes difficult due to the presence of polysaccharides which make it viscous (Sharma *et al.*, 2000; Sablok *et al.*, 2009). Several biological enzymes like ligases, polymerases and restriction endonucleases have been reported to be interfered by these metabolites (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).

During DNA extraction increasing the concentrations of NaCl and CTAB can effectively remove the polysaccharides (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).

2.4.2 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 enzymes are heat-stable and refold following heat denaturation. They are difficult to get inactivated as they do not require cofactors. Strong denaturant agents have always been used in intact RNA isolation to inhibit endogenous RNases. The most common isolation methods are of two classes, *viz.,* utilizing guanidinium thiocyanate and utilizing phenol and SDS (Doyle, 1996).

A guanidinium based salt is a strong protein denaturant and RNase inhibitor. Therefore, it is often 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).

Natalia Kolosovo *et al.* (2004) reported that cDNA library construction and RNA microarray analysis are difficult procedures due to RNA degradation and RNA contamination with secondary metabolites or polysaccharides (e.g., polyphenolics and oleoresin terpenoids) as they inhibit reverse transcription. All solutions except Tris buffer were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. 0.1% DEPC-treated water was used for preparation of Tris buffers.

Several existing methods deal with a number of problems while isolating RNA from conifers. A widely used method described by Chang *et al.* (2009) as well as a number of recent protocols for RNA isolation use preheated CTAB and extraction buffers with high concentrations of NaCl to remove high levels of proteins and polysaccharides (Natalia Kolosovo *et al.*, 2004).

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

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, which is followed by its exponential amplification in a PCR reaction. The complete RT-PCR process takes place by using RNA and DNA-dependent DNA polymerases, either in separate or in single reactions. Separation of the RT and PCR steps has the advantage of generating a stable cDNA pool which can be stored 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 minimize 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 where all the chemical and physical 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 affect the ability of the reverse transcriptase enzyme to generate cDNA (Buell *et al.*, 1978).

The two commonly used RT enzymes are *Moloney murine* leukemia virus reverse transcriptase (MMLV-RT) and avian myeloblastosis virus reverse transcriptase (AMV-RT). AMV-RT is more robust than MMLV-RT and retains significant polymerization activity up to 55°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 makes it a better choice for the amplification of full-length cDNA molecules (Bustin, 2000).

Specific primers, random hexamers or oligo-dT primers can be used for the RT step. Background priming is decreased by the use of mRNA-specific primers, whereas the use of random and oligo-dT primers maximizes the number of mRNA molecules which 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 because 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).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Identification and characterization of *Suppressor of Overexpression of Constans1* (*SOC1*) gene in black pepper (*Piper nigrum* L.)", was conducted at the Integrated Biotechnology Block, College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2016. This chapter discusses about the experimental materials used and the methodology followed for various experiments.

3.1 PLANT SAMPLE COLLECTION

Black pepper samples of variety Karimunda (spikes, leaves and roots) 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 berries), stage 4 spikes (fully mature spikes with green berries) and tender roots were collected. Each sample was labeled appropriately and then packed in a polypropylene bag. The samples for RNA isolation were snap chilled in liquid nitrogen and stored at -80°C (Panasonic MDF U55V-PE) for downstream analysis and the samples for microscopy studies were fixed in freshly prepared FAA(Formaldehyde-acetic acid-ethanol) and preserved in 70% ethanol for further processing.

3.2 ISOLATION OF *SOC1* GENE

For isolation of the *SOC1* gene from black pepper, degenerate primers were designed based on the sequences of *SOC1* genes reported in several other plant species obtained from NCBI database. 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 the flowering time gene *SOC1*. Nucleotide sequences of these genes (i.e. cDNA sequences) in different plant species were retrieved from NCBI (National Centre for Biotechnology Information) GenBank and downloaded in the FASTA format.

Nucleotide sequences of *SOC1* gene equivalents from *Magnolia virginiana, Oryza sativa, Brassica napus, Arabidopsis thaliana, Mangifera indica* and *Cajanus cajan* were selected for primer designing. The collection of FASTA sequences of each gene was then subjected to sequence alignment using clustal Omega program. The best conserved regions in the multiple alignments were identified and the primers were designed according to the sequences of those conserved regions. A set of nested primers were designed taking conserved regions within the forward and reverse primers. In addition to the nested primers, an inner nested forward primer was designed taking a conserved region within the nested primers.

3.2.1.1 *Primer Analysis*

The designed set of primers were checked for several parameters such as primer length, length of the PCR product, low degeneracy and maximum specificity at the 3' end, before their synthesis. 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 'Sigma Aldrich' for synthesis.

3.2.3 RNA Isolation

Five ml CTAB buffer was taken in a 15 ml oakridge tube and pre-warmed at 60-65°C in a water bath (ROTEK, India). Half gram of tissue was immersed in liquid nitrogen in a prechilled mortar and pestle. While the liquid nitrogen was gradually evaporating the tissues were ground into fine powder using the pestle.

In a fume hood, 2% β- mercaptoethanol was added to the pre-warmed CTAB buffer and mixed well. Using a pre-chilled spatula the powdered tissue was quickly transferred to the oakridge tube containing CTAB buffer. The contents in the tube were mixed thoroughly, incubated for 15 min and shaken vigorously by hand every 5 min.

To the contents in the oakridge tube, equal amount of chloroform: isoamyl alcohol (24:1) was added. The contents were centrifuged (Hermle centrifuge) for 20 min at 12000 rpm in 4°C. After centrifugation a top aqueous layer was formed with clear yellow color. Using a 1000 µl pipette this aqueous layer (supernatant) was transferred to a fresh sterile 15 ml Oakridge tube. To this 1/3 volume of 8M LiCl solution was added and kept for overnight incubation at 4°C.

The next day, the Oakridge tube was centrifuged at 12000 rpm, 4°C for 20 min. The supernatant was carefully removed by slowly pouring it off into a clean beaker, or by using a transfer pipette. The pellet at this stage was often difficult to see at the bottom of the translucent Oakridge tubes. The pellet was suspended in 50µl RNAse free distilled water.

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

3.2.3.2 *Spectrophotometric Analysis*

The absorbance of the RNA sample was recorded to determine the quantity and quality of RNA. AV-2701 UV-Visible Double Beam Spectrophotometer (SYSTRONICS, INDIA) was used to measure the absorbance of the sample. Spectrophotometer was first calibrated to blank (absorbance reading is zero) at 260 nm and 280 nm wavelength with 2 ml TE buffer and absorbance of 2 µl RNA sample dissolved in 2 ml of distilled water at respective wavelengths were recorded. Since an absorbance value of 1.0 at 260 nm indicates a 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 ($ng\mu l^{-1}$) = A_{260} x 40 x dilution factor

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

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

3.2.3.3 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 (Roche). RT-PCR was essentially a two-step process. The first step involved the use of reverse transcriptase to synthesize cDNA from single stranded mRNA. The second step was the amplification of cDNA by PCR. To avoid any RNase contamination, a sterile working environment was maintained. All the materials used were pre-treated with 3% hydrogen peroxide overnight and autoclaved twice.

3.2.3.3.1 Synthesis of cDNA Using AMV RT-PCR Kit

To a sterile RNase free micro-centrifuge tube, 1µl 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 70° C for 5 min and then kept on ice immediately 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
dNTP mix	:	2 µl
AMV – Reverse Transcriptase:		1 µl
$MgCl_2$	:	4 µl
Total Volume	:	10 µl
Overall Total Volume	:	20 µl

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

3.2.3.3.2 PCR Amplification of cDNA with Degenerate Primers

The cDNA samples were subjected to PCR with two degenerate primers among the designed primers. The PCR mixture of the total volume of 20 µl was prepared using 2µl of cDNA as template. The components of the reaction mixture is given below.

Distilled Water	:	12 µl
10x Taq buffer A	:	2 µl

(Tris with 15m*M* $MgCl_2$)

dNTPS (2.5m*M* each)	:	1 µl
Primer (10µ*M* each)		
Forward	:	1 µl
Reverse	:	1 µl
Template cDNA (50ngµl^{-1}):		2 µl
Taq polymerase (1Uµl^{-1})	:	1 µl
Total volume	:	20 µl

The PCR programme was set with initial denaturation temperature of 94 °C for 1 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 53 °C for 45 s and extension at 72 °C for 45 s. Final extension was set at 72 °C for 5 min. The PCR products were separated on agarose gel (1.5 %) and the gel was observed using gel documentation system.

3.2.3.3.3. Sequencing of the Amplicons Produced by Degenerate Primers

The amplicons produced by the degenerate primer pair were eluted from the agarose gel and purified using HipurA™ PCR product and Gel purification combo kit. The eluted product was given to Scigenom, Kochi for sequencing.

3.2.3.3.3.1 Gel Elution Using Gel Extraction Kit (Himedia™)

Agarose gel electrophoresis was performed to fractionate DNA fragments. After adequate separation of bands had occurred, the DNA bands were excised from the ethidium bromide stained gel with a clean razor blade or scalpel using 312 nm UV light. The weight of the gel slice was determined and accordingly three volumes of combo binding buffer (CB) (DS 0115) was added per gel slice volume.

The mixture was incubated at 55-60^0 C for 7 min until the gel had completely melted. The contents of the tube were mixed after every 2-3 minutes so that the agarose was completely dissolved.

The lysate was loaded into HiElute miniprep spin column and the combo binding buffer mixture was applied to a HiElute miniprep spin column and centrifuged at 10,000g (12000rpm) for 1 min at room temperature. The flow-through was discarded and the column was placed back into the same collection tube. Three hundred µl of combo binding buffer was added into the column and centrifuged for 1 min at 10,000 g at room temperature to wash the membrane. The flow through was discarded and the collection tube reused.

The column was placed into the same collection tube and 700 µl of diluted wash solution was added and centrifuged for 1 min at 12,000 rpm, room temperature. The flow- through was discarded and the collection tube reused. The empty column was centrifuged for two min at maximum speed to dry the column membrane.

The column was placed into a new uncapped 2.0 ml collection tube and 30-50 µl of elution buffer (ET) (10mm Tris-Cl, PH-8.5) (DS0040) was added directly onto the column membrane and incubated at room temperature for 1 min. The assembly was centrifuged for 1 min at maximum speed ≥ 14,000 rpm to elute the DNA and the eluate was transferred into capped 2 ml collection tube. Aliquots were sent for sequencing and the rest was stored at -20^{0}C.

3.2.3.3.3.2 Cloning of PCR products for Sequencing

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

3.3 COMPETENT CELL PREPARATION

The ability of a cell for up taking a plasmid DNA is called as competence and the cells having the competency that used for transformation are the competent cells. *E. coli* cells treated with cold Calcium chloride ($CaCl_2$) takes up plasmid DNA by transformation. $CaCl_2$ is thought to enhance binding of plasmid DNA to cell surface by affecting plasma membrane permeability.

The *E. coli* strain used for the transformation was *E. coli* DH5 α. From a freshly streaked LB agar (2%) plate, *E. coli* was inoculated into 50 ml LB broth directly. The culture was then incubated at 37° C overnight with shaking. After incubation, 500 µl of the culture was inoculated into 50 ml LB broth in 250 mL flask and allowed to grow for 2-3 h till the OD – 600 values reached 0.3 to 0.4 (silky appearance). The cells were then harvested by spinning the bacterial culture taken in 2ml Eppendorf tubes at 4000 rpm for 5 min. The supernatant was discarded and pelleting was also repeated using fresh culture until sufficient amount of bacterial pellet was obtained. The pellet was then suspended in 1 ml chilled 0.1M $CaCl_2$ and centrifuged at 4000 rpm for 5 min 4° C. The supernatant was discarded and the pellet was again resuspended in 1 ml chilled 0.1M $CaCl_2$ and kept on ice for 30 min. After centrifuging at 4000 rpm for 5 mins at 4°C, the supernatant was discarded and the pellet was once again resuspended in 200 µl chilled 0.1 M $CaCl_2$ and stored at 4 °C.

3.3.1 Ligation of PCR Products

The ligation of the PCR products was done using TA Cloning® Kit (Invitrogen). One vial containing pCR®2.1 vector was centrifuged to collect all the liquid in the bottom of the vial. The components of the ligation mixture are listed below:

Fresh PCR product	:	2 µl
5X T4 DNA Ligase Reaction Buffer	:	2 µl
pCR®2.1 vector (25 ng μl^{-1})	:	2 µl
Water	:	3 µl
ExpressLink™ T4 DNA Ligase (5U)	:	1 µl
Final volume	:	10 µl

The ligation reaction was then incubated at room temperature for 20 min and stored at -20 °C.

3.3.2 Transformation of Ligated PCR Products into Competent Cells

The tubes containing ligation reactions was first centrifuged briefly and kept on ice. Ten µl of the ligated products was added to the tubes containing 200 µl of competent *E. coli* DH5α cells and the tubes were inverted gently to mix and placed them on ice for 30 min. The cells were then allowed to heat shock at 42 °C for 2 min in water bath without shaking and immediately kept on ice for 2 min. To each of the tubes containing the cells, 800 µl of LB broth was added. The culture was then incubated for 1 h at 30° C with shaking at 50 rpm in shaker cum incubator. It was done for allowing the bacteria to recover and to express the antibiotic resistance marker encoded by the vector. After the incubation, 200 µl of the transformed culture was poured on LB/Amp/IPTG/X-Gal plates and spread plating was done using a sterile L-rod. The culture plates were then allowed to dry and incubated at 30 °C overnight without shaking in shaker cum incubator (ROTEK-LES).

3.3.2.1 Plasmid isolation from transformed colonies

Transformed colonies from freshly inoculated plates were used to inoculate 5 ml LB broth (containing appropriate antibiotics) taken in test tubes. The culture was incubated at 37° C overnight with shaking at 180 rpm in shaker cum incubator. After incubation, the culture was then poured into 1.5 ml tube and centrifuged at 4000 rpm for 4 min to pellet out the cells. The supernatant was removed and resuspended the bacterial pellets in 200 µL GET buffer. Three hundred µl freshly prepared Lysis buffer (0.2 N NaOH/ 1% SDS) was added and mixed the contents of the tubes by gentle inversion. After incubation on ice for 5 min, the solution was neutralized by adding 300 µl Neutralizing solution (3M potassium acetate) and mixed by gentle inversion, followed by incubation on ice for 5 min. The solution was then centrifuged at 15,000 rpm for 10 min at room temperature, for the removal of cellular debris and transferred the supernatant to a clean tube. 400 µl of Chloroform was added and mixed by inversion for 30 s. The solution was then centrifuged at 13,000 rpm for 1 min to separate phases and transferred the upper aqueous phase to a fresh tube. This step was done 2 times. An equal volume of 100% isopropanol was added and mixed the contents by inversion. Centrifuged at maximum speed (14,000 rpm) for 10 min at room temperature and removed the isopropanol. The pellet was then washed with 500 µl 70% ethanol and centrifuged at 10,000 rpm for 5 min. After the centrifugation, the pellet was allowed to air dry for 30 min in laminar hood. The pellet was resuspended in 60 µl double distilled water and stored at -80 °C for further use.

3.3.2.2 Sequence Analysis of the Amplicons

The *SOC1* sequences that were cloned into DH5 alpha were stored at -80 °C for future studies. The resultant sequence of the amplicon from sequencing was used for analysis using bioinformatics tools in order to identify the sequence *viz.,* BLAST (tblastx). The BLAST programme used was tblastx, i.e., translated nucleotide query used to search in translated nucleotide sequence database. Sequences were also

analyzed for Conserved Domains using NCBI conserved domain search and phylogenetic tree construction.

3.4 MICROSCOPY STUDIES

3.4.1 Fixation of Pepper Tissue Samples Using FAA

The spike samples were collected from different tissues and placed in separate Oakridge tubes containing chilled FAA (Formaldehyde-acetic acid-ethanol) solution that had been pre-stored at -20 °C (Samsung RS21HUTPN1 freezer) for temperature stabilization overnight. The samples were kept at 4° C overnight.

3.4.2 Dehydration, Paraffinization and Microtoming of Pepper Tissue Samples

The next day after fixation, the tissues fixed in FAA were washed and stored in 70% ethanol at 4° C. After draining off the 70% ethanol, the tissue samples were kept subsequently in 85% ethanol and 95% ethanol 60 min each. The samples were kept in 100% ethanol two times 30 min each and was followed by rinsing the tissue sections again in 100% ethanol two times 60 min each. After draining off the 100% ethanol, the tissue samples were kept subsequently in different proportions of Xylol (Xylene plus ethanol), *viz*., 25% Xylene plus 75% ethanol, 50% Xylene plus 50% ethanol and 75% Xylene plus 25% ethanol 30 min each. Then, the samples were kept in 100% Xylene two times 60 min each. The tissue samples were then dipped in 100% Xylene and one-fourth of the volume was filled with paraffin chips and kept at 42 °C overnight for complete melting. Then, the paraffin chips were again added to that in one-fourth of the volume and kept at 60°C for several hours until completely melted. After that, the melted wax was replaced with freshly melted wax overnight at 60°C. Then, the tissue samples were allowed for 6 subsequent wax changes separated by 4-6 h. After all of the wax changes, freshly melted wax was poured in several molds and placed the tissue

samples in each of the wax molds for embedding and then stored at 4 °C for setting of wax, which is important for proper microtoming process.

Thin sections of the spike tissues which were embedded inside the wax mold were taken using rotary microtome (SIPCON) facility available at Department of Botany, Kerala University, Karyavattom Campus. The ribbon like sections obtained were dipped in pre-warmed (42 °C) water and placed on polylysine coated clean microscopic slides. The slides were kept at 42 °C in hot air oven (LABLINE).

3.4.3 Deparaffinization and staining of tissue samples

The slides with tissue sections were dipped in 100% xylene for deparaffinization by leaving the tissue sections to stick on to the slides because of the polylysine coating. After deparaffinization, the tissue sections were rinsed three times with 70% ethanol, leaving tissue in each rinsing solution for at least 10 mins. After draining off the third rinse of 70% ethanol, it was replaced with fresh ice cold 70% ethanol and kept in refrigerator before sectioning. The thin sections were placed in watch glass containing 1 per cent (w/v) Safranin O stain solution, 2 -24 h for staining. The stained sections were then rinsed in fresh 70% ethanol and followed by rinsing in distilled water two times for 5 min each, with gentle agitation. The tissue sections were then subjected to a series of dehydration process. The sections were first washed in 95% ethanol plus 0.5% picric acid for 15 sec, followed by washing in 95% ethanol plus four drops of Ammonium hydroxide for 10 sec to 1 min (don't go longer because it will destain the tissue completely.). Final dehydration was done by dipping in 100% ethanol for 10 sec. The sections were then counter stained in 0.05 % (w/v) Fast Green stain solution for just 5 to 15 sec taking care of not over-staining by the fast green. The sections were washed in clearing solution by dipping for 10 sec, followed by the removal of clearing solution residues by dipping in the xylene plus 2-3 drops of 100% ethanol. The sections were then allowed to clear in 100% xylene with 2 washes of 5-

10 min each. After the clearing process, the sections were placed in clean microscopic slide and a drop of glycerol was added over each section. A clean coverslip was mounted over the sections and they were viewed directly under the stereomicroscope (Leica ez4hd, Germany).

RESULTS

4. RESULTS

The study entitled Identification and characterization of *Suppressor of Overexpression of Constans* (*SOC1*) gene in black pepper (*Piper nigrum* L.)", was carried out at the Integrated Biotechnology Block, College of Agriculture, Vellayani during 2015-2016. The results related to the study are presented in this chapter.

4.1 IDENTIFICATION OF FLOWERING TIME GENES

4.1.1 Degenerate Primer Designing

Degenerate primers for the flowering time gene *SOC1* was designed based on the conserved regions in the multiple aligned nucleotide sequences (fig. 1, 2) and named as SOC1Fw-1, SOC1Fw-2, SOC1Fw-3, SOC1rev-1, SOC1rev-2, SOC1rev-3 and SOC1rev-4 respectively. The primer sequences are presented in Table 1.

4.1.2 RNA Isolation

Three different tissue samples, namely bud, mature leaf and roots were used for extraction of total RNA. Distinct, intact rRNA (ribosomal RNA) bands with no apparent RNA degradation were observed on the gel in all three samples and no genomic DNA contamination was observed on agarose gel (1.5%) showing good quality RNA extraction for these samples (Plate 1). The RNA samples were used to prepare cDNA and for PCR.

For expression studies, stage 1 spike, mature leaf, roots, orthotropic shoots and plagiotropic shoots were used for extraction of total RNA. Distinct, intact rRNA bands with no RNA degradation were observed on the gel. No DNA contamination was observed on agarose gel (1.5 %) showing good quality RNA extraction. Further, absorbance reading of the extracted RNA by using spectrophotometric method revealed good quality and quantity of RNA (Table 2).

```
CLUSTAL O(1.2.3) multiple sequence alignment

gi|259014666|gb|GQ489241.1|     ------------------------------------------------------------
HC084627.1                      ------------------------------------------------------------
ENA|AFH41826|AFH41826.1         ------------------------------------------------------------
AY007726.1                      CCTATACATACACAAACCCTTTATCCTCGAAAGCTTCCTCCTGGTTAGGTTTTTATCAAA
ENA|ADX97324|ADX97324.1         ------------------------------------------------------------
ENA|KYP64350|KYP64350.1         ------------------------------------------------------------


gi|259014666|gb|GQ489241.1|     ------------------------------------ATGGTGAGGGGGAAAACGCAG
HC084627.1                      -----------GTTGGTTCATCGGCGAT-----CGAAGATGGTGCGGGGGAAGACGCAG
ENA|AFH41826|AFH41826.1         ------------------------------------ATGGTGAGGGGCAAAACTCAG
AY007726.1                      CCCTTTTAGCCAATCGGGAAATTAACTAAAGAAGAAGATATGGTGAGGGGCAAAACTCAG
ENA|ADX97324|ADX97324.1         ------------------------------------ATGGTGAGAGGCAAAACCCAA
ENA|KYP64350|KYP64350.1         ------------------------------------ATGGTGAGAGGAAAGACTCAG
                                                                     ******.*.** **.** **.


gi|259014666|gb|GQ489241.1|     ATGAGGAGGATAGAGAACGCGACGAGCAGGCAGGTGACGTTCTCGAAAAGGAGGAATGGG
HC084627.1                      ATGAAGCGGATAGAGAACCCCACGAGCCGCCAGGTCACCTTCTCCAAGCGCCGCAACGGC
ENA|AFH41826|AFH41826.1         ATGAAGCGAATAGAGAATGCAACAAGCAGACAAGTCACTTTCTCTAAACGAAGGAATGGT
AY007726.1                      ATGAAGAGAATAGAGAATGCAACAAGCAGACAAGTGACTTTCTCCAAAAGAAGGAATGGT
ENA|ADX97324|ADX97324.1         ATGAGACGCATAGAGAACAATACAAGCAGGCAAATCACCTTCTCCAAGCGGCGAAATGGG
ENA|KYP64350|KYP64350.1         ATGAGGCGCATAGAGAACGCCACAAGCAGGCAAGTCACTTTTTCAAAACGCCGTAATGGT
                                ****...* ********  . **.***.* **..* ** ** ** **..* .* ** ****


gi|259014666|gb|GQ489241.1|     TTGTTTAAGAAGGCATTTGAGCTGTCTGTACTGTGCGATGCTGAGGTGGCATTGATCGTC
HC084627.1                      CTGCTCAAGAAGGCCTTCGAGCTCTCCGTCCTCTGCGACGCCGAGGTCGCGCTCATCGTC
ENA|AFH41826|AFH41826.1         TTGTTGAAGAAAGCTTTTGAGCTCTCAGTGCTTTGTGATGCTGAAGTTTCTCTGATCATC
AY007726.1                      TTGTTGAAGAAAGCCTTTGAGCTCTCAGTGCTTTGTGATGCTGAAGTTTCTCTTATCATC
ENA|ADX97324|ADX97324.1         CTGCTTAAGAAAGCCTTTGAGCTCTCAGTTCTTTGCGATGCCGAGGTTGCCCTTATCATC
ENA|KYP64350|KYP64350.1         TTGTTGAAGAAGGCCTTTGAACTTTCAGTCCTTTGTGATGCTGAAGTTGCTCTCATCATT
                                ** * *****.** ** **.** ** ** ** ** ** ** **.**  *  * ***.*
```

(*) : Conserved nucleotide

Accession no:	Plant species
gi|259014666|gb|GQ489241.1|	*Magnolia virginiana*
HC084627.1	*Oryza sativa*
ENA|AFH41826|AFH41826.1	*Brassica napus*
AY007726.1	*Arabidopsis thaliana*
ENA|ADX97324|ADX97324.1	*Mangifera indica*
ENA|KYP64350|KYP64350.1	*Cajanus cajan*

Fig. 1. Multiple sequence alignment for designing degenerate primers (forward) for *SOC1* gene.

(*) : Conserved nucleotide

Accession no:	Plant species
gi|259014666|gb|GQ489241.1|	*Magnolia virginiana*
HC084627.1	*Oryza sativa*
ENA|AFH41826|AFH41826.1	*Brassica napus*
AY007726.1	*Arabidopsis thaliana*
ENA|ADX97324|ADX97324.1	*Mangifera indica*
ENA|KYP64350|KYP64350.1	*Cajanus cajan*

Fig. 2. Multiple sequence alignment for designing degenerate primers (reverse) for *SOC1* gene.

Table 1. Sequences of designed degenerate primers for *SOC1*

Sl. No.	Primer name	Primer sequence (5' to 3')	No. Of bases	GC content (%)	T_m (°c)
1	SOC1fw-1	TGGTGMGRGGVAARACBCAGATGA	24	41.6	61.0
2	SOC1fw-2	CAGATGARGMGVATAGAGAA	20	35	49.5
3	SOC1fw-3	GBAAYGGBYTGYTBAAGAARGC	22	31.8	54.9
4	SOC1rev-1	CWATGAAYAAYTSRGTYTCBACTTC	25	24	56.3
5	SOC1rev-2	TCCTTYTSYTTDAGYTGCWSAAT	23	21.7	55.4
6	SOC1rev-3	CCTTCTCCCAAVAGYTTDCGYTT	23	39.1	69.2
7	SOC1rev-4	TTCAAGDTKYTCRATYTTCTT	21	19.0	55.4

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 total RNA

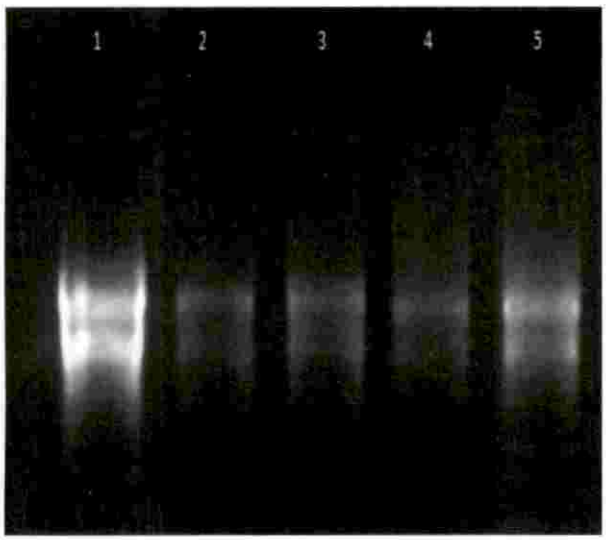
Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	RNA Yield ($ng\mu l^{-1}$)
1	Stage 1 spikes	0.017	0.010	1.7	680
2	Mature leaf	0.0081	0.039	2.07	3240
3	Root	0.023	0.013	1.76	920
4	Plagiotropic shoot	0.014	0.008	1.75	560
5	Orthotropic shoot	0.041	0.023	1.78	1640

4.1.3 RT-PCR with Degenerate Primers

The reverse transcription of the extracted RNA from the stage 1 spikes was carried out to synthesize the first strand cDNA followed by PCR with the designed degenerate primers. Different combinations of the primers were used to do PCR reactions. The first reaction was done using the combination of SOC1fw-1 and SOC1rev-1 which yielded no bands. Subsequently the PCR product from the first reaction was taken as template for nested reactions. Two of the reactions using primers SOC1-fw2 with SOC1-rev1 and SOC1fw-2 with SOC1-rev3 yielded desired amplification at the expected size of 640 bp and 330 bp respectively (Plate 2).

4.1.4 Sequencing of the Amplicons Produced by Degenerate Primers

The two amplicons produced by the two degenerate primer pairs were eluted from the agarose gel and purified using Genei™ Gel Extraction Kit, the eluted products



Lane 1 – Mature leaf

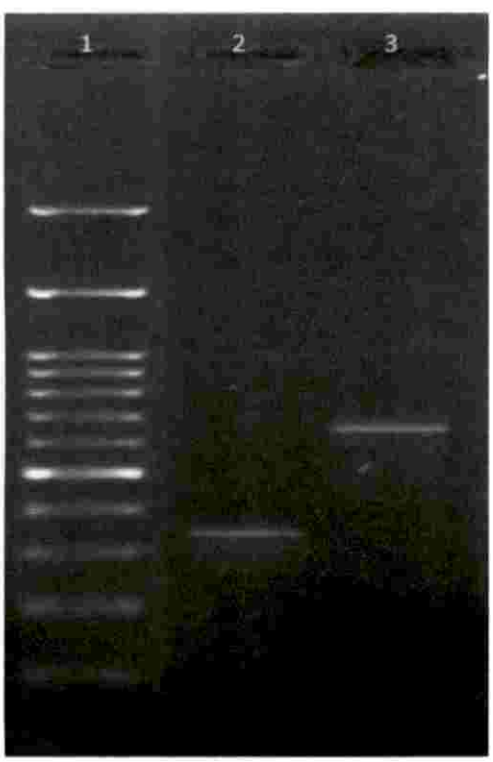
Lane 2 – Root

Lane 3 – Orthotropic shoot

Lane 4 – Plagiotropic shoot

Lane 5 – Spike

Plate 1. Agarose gel electrophoresis of black pepper total RNA from different plant parts



Lane 1 – 100 bp DNA ladder

Lane 2 –PCR amplification of *SOC1* primers (330bp)

Lane 3 - PCR amplification of *SOC1* primers (640bp)

Plate 2. Agarose gel electrophoresis of PCR product from cDNA of stage 1 spike RNA using *SOC1* primers

were sequenced at Scigenom, Kochi. Four sequences were generated (Two sequences each from an amplicon - one from forward primer and the other from reverse primer).

Sequences of the amplicons are given below.

>SOC1fw-2 (534 bp)

CTCAAGAAGTCGATGTCTGTCATGCTCATACTGTGATCATTCAAGAAGCC
GTTCGAGCTCTCCGTCCTTTGCGACGAGGAGTCGCTCTCATCGTCTTCTCC
CCCCGCGGCAGCTCTATGAGTTCGCCGCTCCAGCATGCAACAGACTATTG
AACGCTACCAGAAGTACACTGCAGGTGTTTTTTGAAAGATAAACCTCTGA
ACAGCACATTCAGCAATGGAGATTGAAGCTGGAAAATGGCGAAAAAATT
GAATTTTGGAAGTTTCAAAAGGAACTATGGGTGAAGACTTGGATCTTGTT
CCCTTGAAGAACTTCAGAAATGGAAGCAGCTGAGAAGGCCTAGGCAAGT
AGAGGAAGGAAGATCTCCGTTTAGAGAACAGATGGATGACTAGAGGAAA
AGAGTGAGGTTAATTAAAGAGAATTCGCTACTGCGGCAATGTTTGTTGTC
ATGAACAAGACGTAGATAAAAATGGTAGAGGAAATTGCCCTGATGATGT
AGTGGCAAGGTTTGAAGTCAAACCATTTTTTTTGATT

>SOC1-rev1 (570 bp)

TGACCACAAAATAAGACTGTAGAGGACATATGACTATCACCATTTTTATT
CTCACGTTCTTGTTGAGAACTTCATCTTTTGCCGCAGTAGCGAGTTCTCCT
TAATTAAGCTCCTCTCATTTTCCTCTAGTCGTTCAATCTGTTCTCTAAACA
GGAGGTTCTTCCTTCCTCTAATTTTGCCTAGGCTTTGCTCTAGGTGGCCTT
CCAATTGCTGAAGTTCTTCAAGGGAACAAGATCCAAGGCCTTCACCCAAT
AGTTTCCTTTTGGAAACTTCCAAAAATTCAAGTTTTTTCGCCATATTTCCA
GCTTCAAATCTACATTGCTGAATGTGCTGTTCAGAGATTTTGTTGTTCAAG
CCAATGCTTGCGGTGTACTTCTGGTAGCGTTCAATAGTCTTTTGCATGCTG
GAGCTGGCGAATTCATAGAGCTTGCCCGCGGGGGGAGAAGACGATGAGA

GCGAGCTCGGCGTCGCAAAAGGACGGAGAGCTCGAAGGCCTTCTTGAGG
AGGCGACTACGGCGCTTGGAGAAGGTAACCCTGCCGGCTGTGGCGTTCTC
TATCCTCCTCATCTGA

>SOC1-fw2 (242bp)

AAGGCCTTCGAGCTCTCCGTCCTTTGCGACGCCGAGCTCGCTCTCATCGTC
TTCTCCCCCCCGCGGCAAGCTCTATGAATTCGCCAGCTCCAGCATGCAAAA
GACTATTGAACGCTACCAGAAGTACACCGCAGGCATTGGCTTGAACAACA
AAACCTCTGAACAGCACATTCAGCAATGTAGATTTGAAGCTGGAAATATG
GCGAAAAAACTTGAATTTTTGGAAGTTTTCAAACGCAAACT

>SOC1-rev (178bp)

TTGCCGCGGGGGGAGAAGACGATTAGAGCGAGCCCGGCGTTTTAAGGAC
GGATAGCTCGAAGGCCTTCTTGAGGAGGCCTCTACGGCGCTTGGAGAAGG
TAACCTGCCGGCTGGTGGCGTTCTCTATTCGCCTCATCTGAATTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTT

4.1.5 Analysis of the sequences

The resultant sequence of the amplicon from sequencing was used for analysis using bioinformatics tools *viz.,* BLAST, NCBI conserved Domain Search and Phylogeny tree.

The BLAST programme used was tblastx i.e., translated nucleotide query is used to search in translated amino acid database. The results showed that the sequences belonged to mRNA of *SOC1* gene. Table 3 gives the summary results of the tblastx. The SOC1fw2 sequence was used for further sequence analysis of Conserved Domain Study. This was carried out using the NCBI Conserved Domain Search program.

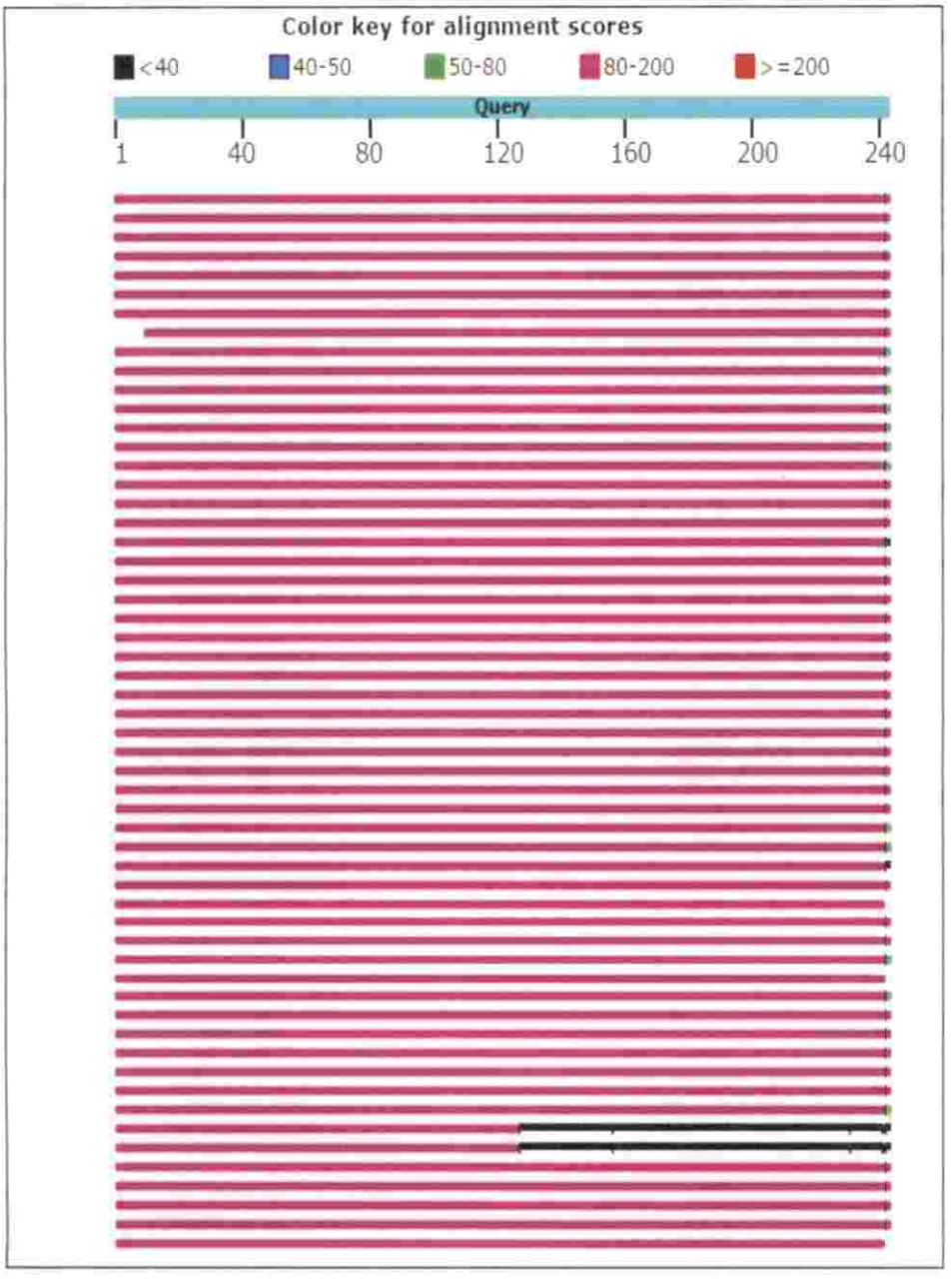


Fig. 3a. Screenshot showing the result of tblastx (242bp) alignment score for SOC1fw-2

Description	Max score	Total score	Query cover	E value	N	Accession
PREDICTED: Amborella trichopoda MADS-box protein SOC1 (LOC18424466), transcript variant X2, mRNA	132	412	100%	2e-28	1	XM_006829053.2
Magnolia virginiana SUPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) mRNA, complete cds	132	268	100%	2e-28	1	GQ489241.1
Pachysandra terminalis suppressor of overexpression of constans 1 mRNA, partial cds	131	269	100%	5e-28	1	JX266557.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X4, mRNA	130	269	100%	1e-27	1	XM_018964843.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X3, mRNA	130	269	100%	1e-27	1	XM_018964842.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X2, mRNA	130	269	100%	1e-27	1	XM_018964840.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X1, mRNA	130	269	100%	1e-27	1	XM_018964839.1
Magnolia praecocissima mRNA for putative MADS-domain transcription factor MpMADS9, partial cds	129	263	96%	2e-27	1	AB050651.1
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X7, mRNA	128	262	100%	3e-27	1	XM_006477293.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X6, mRNA	128	262	100%	3e-27	1	XM_006477292.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X5, mRNA	128	262	100%	3e-27	1	XM_006477289.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X4, mRNA	128	262	100%	3e-27	1	XM_006477290.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X3, mRNA	128	262	100%	3e-27	1	XM_006477288.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X2, mRNA	128	262	100%	3e-27	1	XM_015529665.1

Fig. 3b. Screenshot showing the result of tblastx (242bp) prediction for SOC1fw-2

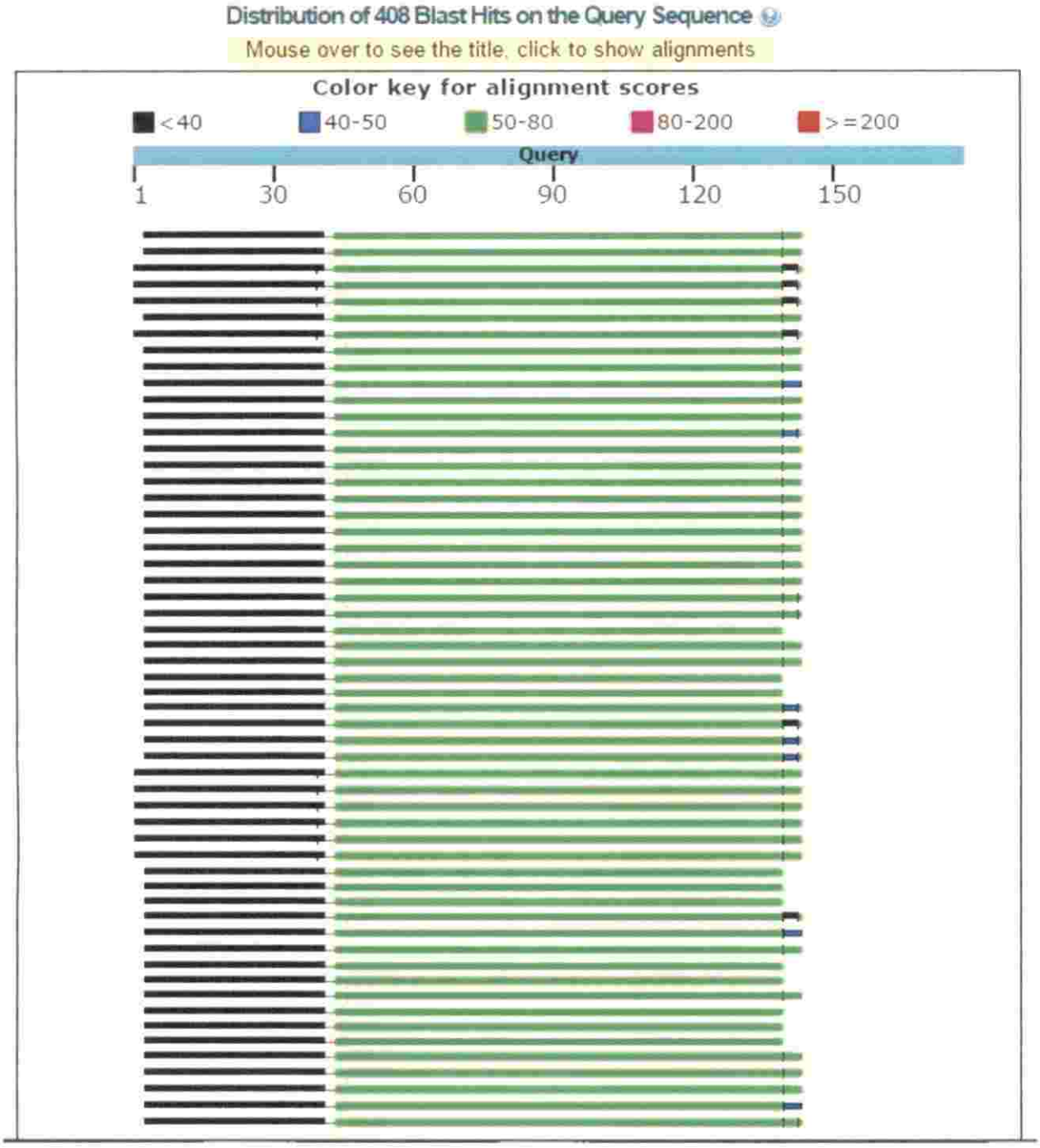


Fig. 4a. Screenshot showing the result of tblastx (178bp) alignment scores for SOC1rev-3

Description	Max score	Total score	Query cover	E value	N	Accession
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X4, mRNA	70.3	185	78%	2e-12	2	XM_018964843.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X3, mRNA	70.3	185	78%	2e-12	2	XM_018964842.1
PREDICTED: Theobroma cacao MADS-box protein SOC1 (LOC18614250), transcript variant X3, mRNA	70.3	215	79%	2e-12	2	XM_018114171.1
PREDICTED: Theobroma cacao MADS-box protein SOC1 (LOC18614250), transcript variant X2, mRNA	70.3	215	79%	2e-12	2	XM_018114170.1
PREDICTED: Theobroma cacao MADS-box protein SOC1 (LOC18614250), transcript variant X4, mRNA	70.3	215	79%	2e-12	2	XM_018114172.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X2, mRNA	70.3	185	78%	2e-12	2	XM_018964840.1
PREDICTED: Theobroma cacao MADS-box protein SOC1 (LOC18614250), transcript variant X1, mRNA	70.3	215	79%	2e-12	2	XM_007051917.2
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X1, mRNA	70.3	185	78%	2e-12	2	XM_018964839.1
PREDICTED: Vigna angularis MADS-box protein SOC1-like (LOC108328026), mRNA	70.3	275	78%	2e-12	2	XM_017561799.1
PREDICTED: Ziziphus jujuba MADS-box protein SOC1 (LOC107432650), mRNA	70.3	288	78%	2e-12	2	XM_016043834.1
PREDICTED: Arachis duranensis MADS-box protein SOC1-like (LOC107485538), transcript variant X2, mRNA	70.3	150	78%	2e-12	2	XM_016106073.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1-like (LOC107639802), transcript variant X4, mRNA	70.3	151	78%	2e-12	2	XM_016343402.1
PREDICTED: Ricinus communis MADS-box protein SOC1 (LOC8258485), transcript variant X2, mRNA	70.3	218	78%	2e-12	2	XM_015728902.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1-like (LOC107639802), transcript variant X3, mRNA	70.3	151	78%	2e-12	2	XM_016343401.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1-like (LOC107639802), transcript variant X1, mRNA	70.3	151	78%	2e-12	2	XM_016343399.1
PREDICTED: Arachis duranensis MADS-box protein SOC1-like (LOC107485538), transcript variant X1, mRNA	70.3	150	78%	2e-12	2	XM_016106072.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1 (LOC107626026), transcript variant X4, mRNA	70.3	150	78%	2e-12	2	XM_016328795.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1 (LOC107626026), transcript variant X3, mRNA	70.3	150	78%	2e-12	2	XM_016328794.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1 (LOC107626026), transcript variant X2, mRNA	70.3	150	78%	2e-12	2	XM_016328793.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1 (LOC107626026), transcript variant X1, mRNA	70.3	150	78%	2e-12	2	XM_016328792.1
PREDICTED: Arachis ipaensis MADS-box protein SOC1-like (LOC107639802), transcript variant X2, mRNA	70.3	151	78%	2e-12	2	XM_016343400.1
Camellia sinensis SOC2 (SOC2) mRNA, complete cds	70.3	181	78%	2e-12	2	KT327078.1
PREDICTED: Vigna radiata var. radiata MADS-box protein SOC1-like (LOC106752989), mRNA	70.3	331	78%	2e-12	2	XM_014634771.1
Vigna radiata genotype Vaibhav suppressor of overexpression of constans 1 mRNA, partial cds	70.3	331	78%	8e-13	2	KT025629.1
PREDICTED: Daucus carota subsp. sativus MADS-box protein SOC1-like (LOC108206428), mRNA	70.3	188	75%	1e-12	2	XM_017376732.1

Fig. 4b. Screenshot showing the result of tblastx (178bp) prediction of SOC1rev-3

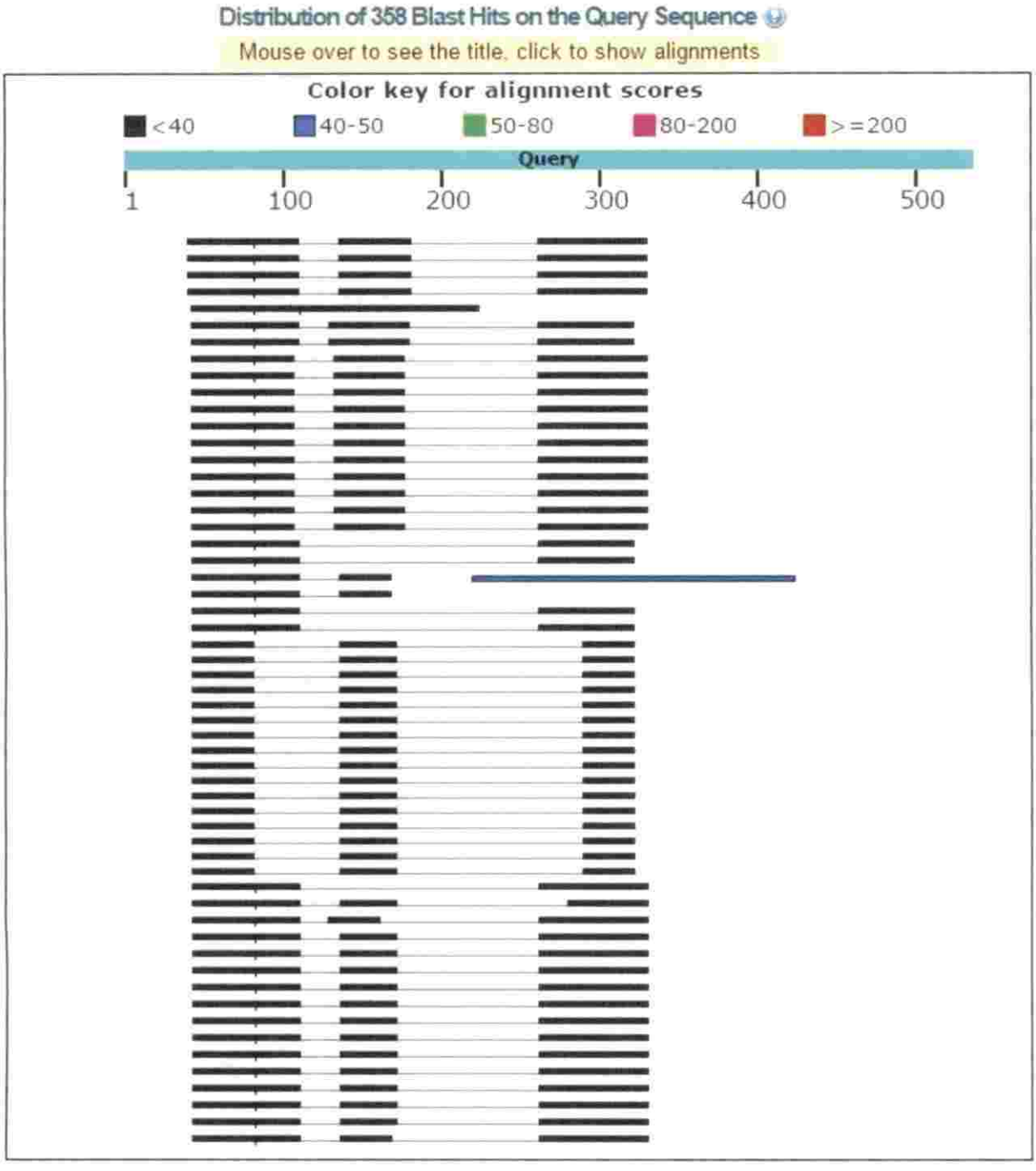


Fig. 5a. Screenshot showing the result of tblastx (534bp) alignment scores for SOC1fw-2

Description	Max score	Total score	Query cover	E value	N	Accession
PREDICTED: Amborella trichopoda MADS-box protein SOC1 (LOC18424496), transcript variant X2, mRNA	98.7	716	86%	3e-27	4	XM_006829053.2
Pachysandra terminalis suppressor of overexpression of constans 1 mRNA, partial cds	89.5	564	83%	1e-35	4	JX266557.1
PREDICTED: Elaeis guineensis MADS-box transcription factor 50-like (LOC105043288), transcript variant X2, mRNA	89.0	591	89%	8e-38	4	XM_010920779.1
PREDICTED: Elaeis guineensis MADS-box transcription factor 50-like (LOC105043288), transcript variant X1, mRNA	89.0	591	89%	8e-38	4	XM_010920778.1
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X7, mRNA	88.1	427	88%	4e-39	4	XM_006477293.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X6, mRNA	88.1	427	88%	4e-39	4	XM_006477292.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X2, mRNA	88.1	427	88%	4e-39	4	XM_015529665.1
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X5, mRNA	88.1	349	83%	2e-36	4	XM_006477289.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X4, mRNA	88.1	349	83%	2e-36	4	XM_006477290.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X3, mRNA	88.1	349	83%	2e-36	4	XM_006477288.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X1, mRNA	88.1	349	83%	2e-36	4	XM_006477291.2
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X1, mRNA	87.7	620	84%	3e-37	4	XM_018964839.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X2, mRNA	87.7	620	84%	3e-37	4	XM_018964840.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X3, mRNA	87.7	620	84%	3e-37	4	XM_018964842.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X4, mRNA	87.7	620	84%	3e-37	4	XM_018964843.1
PREDICTED: Phoenix dactylifera MADS-box transcription factor 50-like (LOC103702697), mRNA	85.8	537	86%	9e-37	4	XM_008785233.1
Vigna unguiculata VuSOC1 mRNA for suppressor of overexpression of constans 1, complete cds	78.5	500	80%	3e-33	4	AB588746.1
Arabis alpina SOC1 (SOC1) mRNA, complete cds	79.9	300	79%	2e-32	4	JF436957.1
PREDICTED: Daucus carota subsp. sativus MADS-box protein SOC1-like (LOC108209100), mRNA	81.7	496	90%	1e-16	4	XM_017379845.1
Cardamine flexuosa MADS-box protein (AGL20) mRNA, complete cds	78.0	322	79%	6e-32	4	AY257542.1
Pimpinella brachycarpa transcription activator (MADS1) mRNA, complete cds	78.0	539	89%	5e-33	4	AF082531.1
Glycine max SOC1 (LOC100037477), mRNA	83.1	483	85%	7e-20	4	NM_001249448.1
Populus tremuloides MADS-box protein PTM5 mRNA, complete cds	77.6	479	83%	2e-32	4	AF377868.1
PREDICTED: Brassica oleracea var. oleracea MADS-box protein SOC1-like (LOC106336135), transcript variant X1, mRNA	77.1	321	79%	2e-31	4	XM_013734871.1

Fig. 5b. Screenshot showing the result of tblastx (534bp) prediction of SOC1fw-2

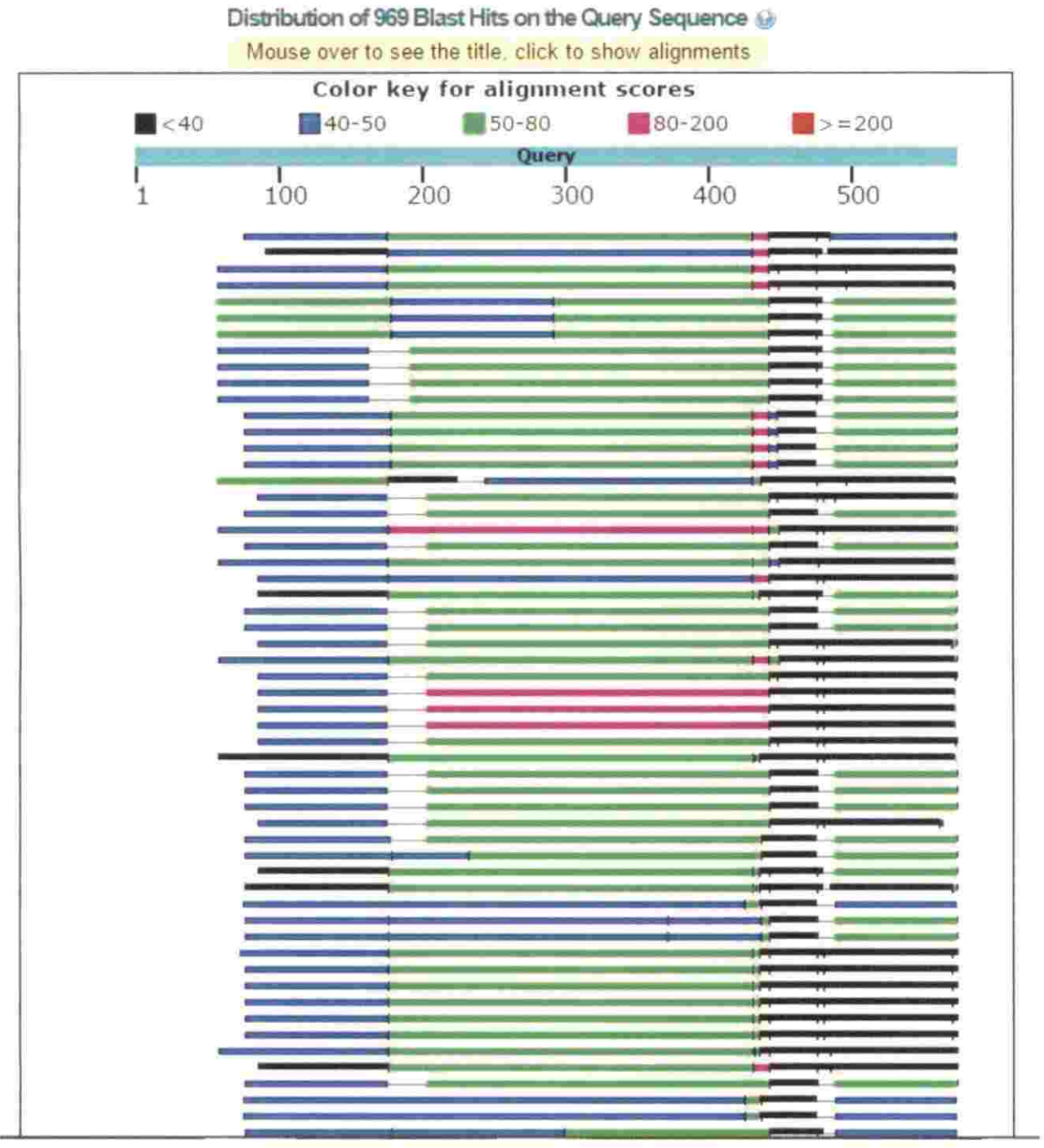


Fig. 6a. Screenshot showing the result of tblastx (570bp) alignment scores for SOC1rev-1

Description	Max score	Total score	Query cover	E value	N	Accession
PREDICTED: Amborella trichopoda MADS-box protein SOC1 (LOC18424466), transcript variant X2, mRNA	98.7	716	86%	3e-27	4	XM_006829053.2
Pachysandra terminalis suppressor of overexpression of constans 1 mRNA, partial cds	89.5	564	83%	1e-35	4	JX266557.1
PREDICTED: Elaeis guineensis MADS-box transcription factor 50-like (LOC105043288), transcript variant X2, mRNA	89.0	591	89%	8e-38	4	XM_010920779.1
PREDICTED: Elaeis guineensis MADS-box transcription factor 50-like (LOC105043288), transcript variant X1, mRNA	89.0	591	89%	8e-38	4	XM_010920778.1
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X7, mRNA	88.1	427	88%	4e-39	4	XM_006477293.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X6, mRNA	88.1	427	88%	4e-39	4	XM_006477292.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X2, mRNA	88.1	427	88%	4e-39	4	XM_015529665.1
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X5, mRNA	88.1	349	83%	2e-36	4	XM_006477289.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X4, mRNA	88.1	349	83%	2e-36	4	XM_006477290.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X3, mRNA	88.1	349	83%	2e-36	4	XM_006477288.2
PREDICTED: Citrus sinensis agamous-like MADS-box protein AGL19 (LOC102626103), transcript variant X1, mRNA	88.1	349	83%	2e-36	4	XM_006477291.2
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X1, mRNA	87.7	620	84%	3e-37	4	XM_018964839.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X2, mRNA	87.7	620	84%	3e-37	4	XM_018964840.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X3, mRNA	87.7	620	84%	3e-37	4	XM_018964842.1
PREDICTED: Juglans regia MADS-box protein SOC1-like (LOC108990767), transcript variant X4, mRNA	87.7	620	84%	3e-37	4	XM_018964843.1
PREDICTED: Phoenix dactylifera MADS-box transcription factor 50-like (LOC103702697), mRNA	85.8	537	86%	9e-37	4	XM_008785233.1
Vigna unguiculata VuSOC1 mRNA for suppressor of overexpression of constans 1, complete cds	78.5	500	80%	3e-33	4	AB588746.1
Arabis alpina SOC1 (SOC1) mRNA, complete cds	79.9	300	79%	2e-32	4	JF436957.1
PREDICTED: Daucus carota subsp. sativus MADS-box protein SOC1-like (LOC108209100), mRNA	81.7	496	90%	1e-16	4	XM_017379845.1
Cardamine flexuosa MADS-box protein (AGL20) mRNA, complete cds	78.0	322	79%	6e-32	4	AY257542.1
Pimpinella brachycarpa transcription activator (MADS1) mRNA, complete cds	78.0	539	89%	5e-33	4	AF082531.1
Glycine max SOC1 (LOC100037477), mRNA	83.1	483	85%	7e-20	4	NM_001249448.1
Populus tremuloides MADS-box protein PTM5 mRNA, complete cds	77.6	479	83%	2e-32	4	AF377868.1
PREDICTED: Brassica oleracea var. oleracea MADS-box protein SOC1-like (LOC106336135), transcript variant X1, mRNA	77.1	321	79%	2e-31	4	XM_013774871.1

Fig. 6b Screenshot showing the result of tblastx (570bp) prediction of SOC1rev-1

Conserved domains on [lcl|S2_F2_32973-1_9291_Raw]

View Concise Results

S2_F2_32973-1_9291,Raw Sequence(304 bp)

Protein Classification

Graphical summary Zoom to residue level show extra options »

RF +2

putative phosphorylation site

dimerization interface

protein interaction site

Superfamilies MADS superfamily

Search for similar domain architectures | Refine search

List of domain hits

	Name	Accession	Description	Interval	E-value
[+]	MADS super family	cl00109	MADS: MCM1, Agamous, Deficiens, and SRF (serum response factor) box family of eukaryotic ...	29-181	1.10e-25

References:

Marchler-Bauer A et al. (2015), *"CDD: NCBI's conserved domain database."*, **Nucleic Acids Res.43**(D)222-6.

Marchler-Bauer A et al. (2011), *"CDD: a Conserved Domain Database for the functional annotation of proteins."*, **Nucleic Acids Res.39**(D)225-9.

Marchler-Bauer A et al. (2009), *"CDD: specific functional annotation with the Conserved Domain Database."*, **Nucleic Acids Res.37**(D)205-10.

Marchler-Bauer A, Bryant SH (2004), *"CD-Search: protein domain annotations on the fly."*, **Nucleic Acids Res.32**(W)327-331.

Fig. 7. Screenshot showing the result of NCBI conserved Domain Search programme

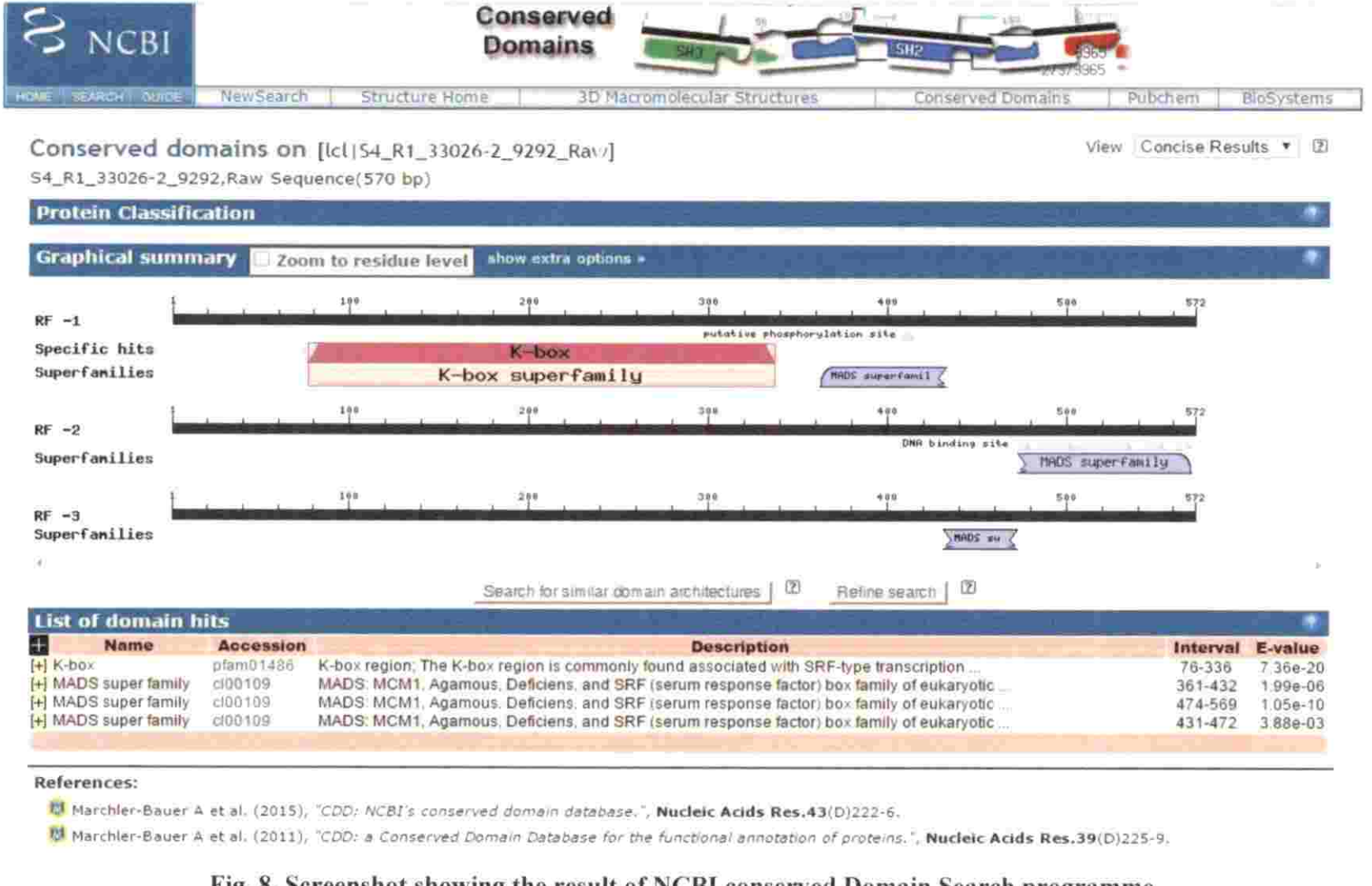


Fig. 8. Screenshot showing the result of NCBI conserved Domain Search programme

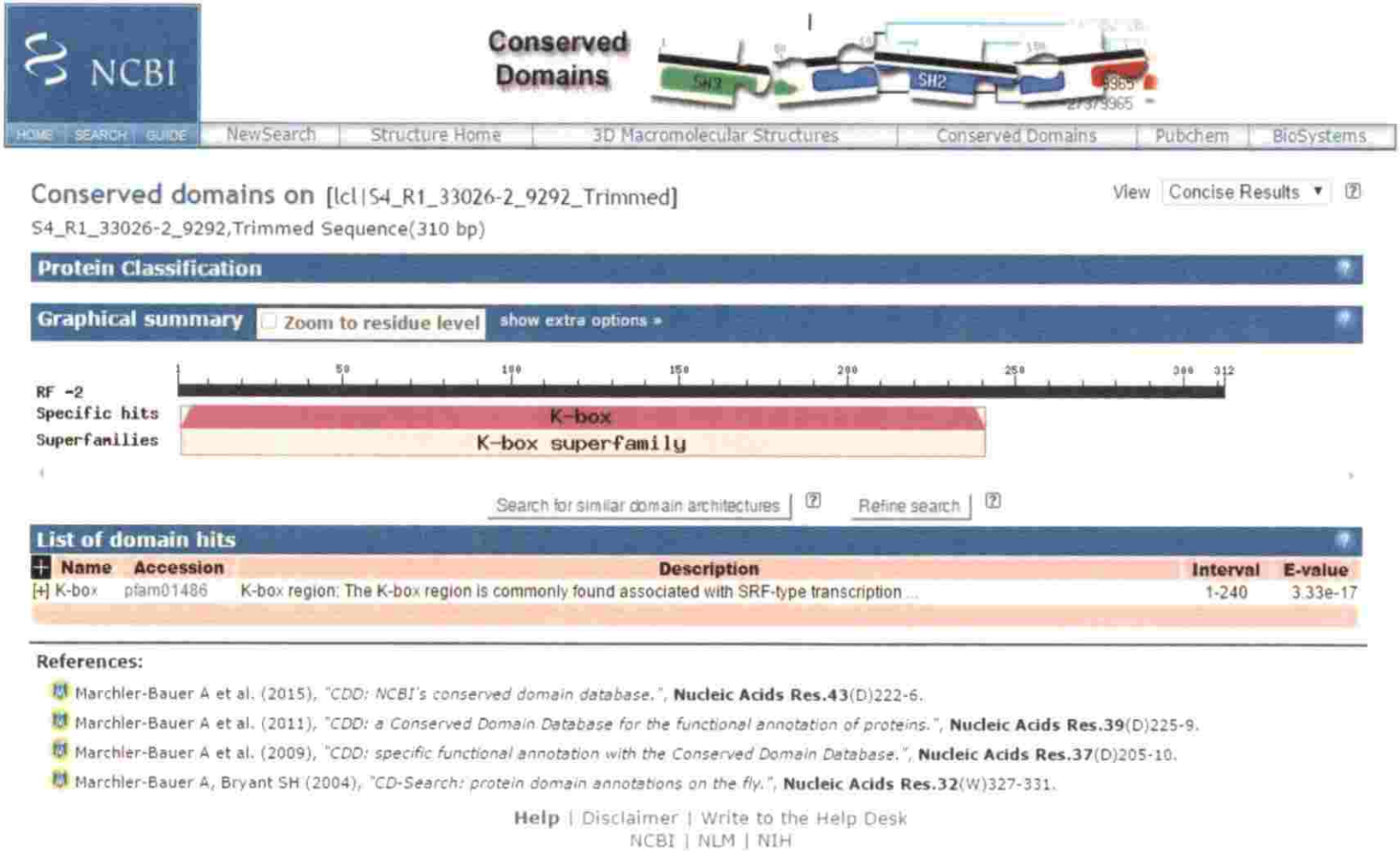


Fig. 9. Screenshot showing the result of NCBI conserved Domain Search programme

Fig. 10. Screenshot showing Phylogenetic tree created using Clustal Omega

Table 3. The tblastx search result of the sequenced fragments

Sequence identifier	tblastx hits	Gene
SOC1fw2	358	*SOC1*
SOC1rev1	969	*SOC1*
SOC1fw2	365	*SOC1*
SOC1rev3	408	*SOC1*

This uses nucleotide query and finds the known conserved domains within the sequences. The analysis showed that the query sequences carried MADS/K-Box domains (Fig. 7, 8 and 9). Phylogenic tree was constructed using Clustal Omega program and the sequence similarity of *SOC1* among black pepper and different plants was studied (Fig. 10). The tree revealed that the isolated SOC1 sequences from black pepper were closely related to SOC1 genes from other plants and belonged to a clade that contained orthologues of the gene from *Nelumbo nucifera, Magnolia virginiana and Amborella tricopoda.*

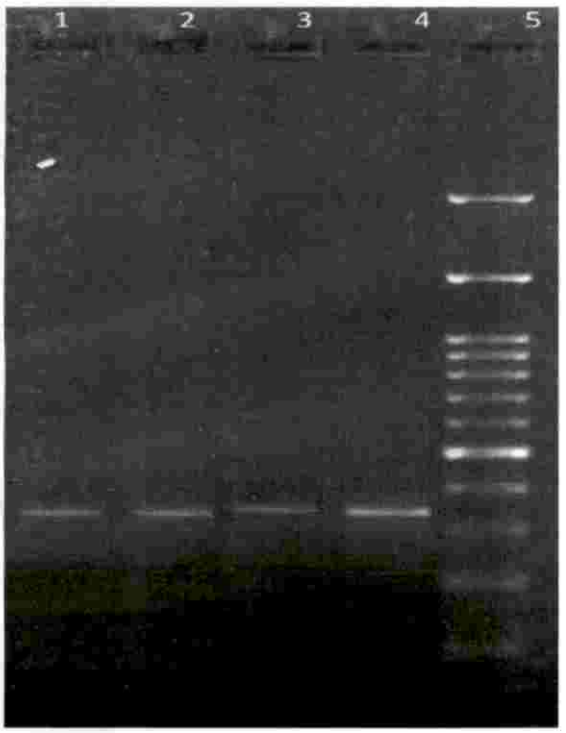
4.2 EXPRESSION STUDY OF THE GENE IN DIFFERENT TISSUES

4.2.1 Semi quantitative analysis

Expression of the *SOC1* gene in different tissues was studied by RT-PCR. The tissues selected for the study were mature leaf, root, orthotropic shoots and plagiotropic shoots. Expression was seen in all the above mentioned tissues (Plates 3 & 4).

4.3 MICROSCOPY STUDIES

The spikes of pepper were viewed under a stereo microscope to view their floral characteristics. Longitudinal sections were observed under 10X magnification.



Lane 1 – Mature leaf

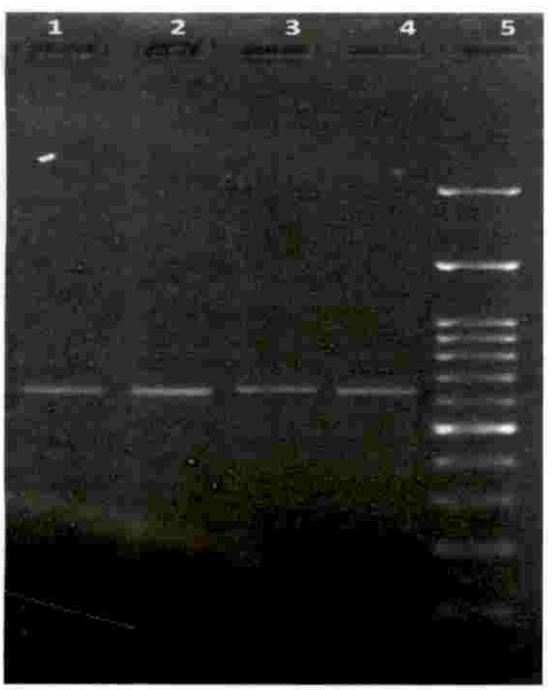
Lane 2 – Root

Lane 3 – Orthotropic shoot

Lane 4 – Plagiotropic shoot

Lane 5 – 100 bp DNA ladder

Plate 3. Expression of *SOC1* gene with SOC1fw-2 and SOC1rev-3 primer



Lane 1 – Mature leaf

Lane 2 – Root

Lane 3 – Orthotropic shoot

Lane 4 – Plagiotropic shoot

Lane 5 – 100 bp DNA ladder

Plate 4. Expression of *SOC1* gene with SOC1fw-2 and SOC1rev-1 primer

It was observed that the ovary was superior in pepper. The anthers were seen below the gynoecium as dark stained region and numbered 1 to 10. Bract primordia seemed to develop as small bumps on very immature spikes. After bracts had formed, undifferentiated floral meristems then formed in the axils of the bract (Plate 5). The development of the carpels were more advanced than the development of stamens which were just emerging as anther primordia indicating that the flowers were protogynous and the flowers were covered by bracts (Plate 6). Plate 7 shows the anthers in the LS of pepper spike. A longitudinal section of the stage 3 spike was observed under stereo microscope 4X to study the different floral primordia in the same spike (Plate 8). The various tissues such as plagiotropic shoots, orthotropic shoots and roots were also viewed under the stereo microscope (Plates 9 & 10) and development of floral parts such as the carpel and stamens could be seen.

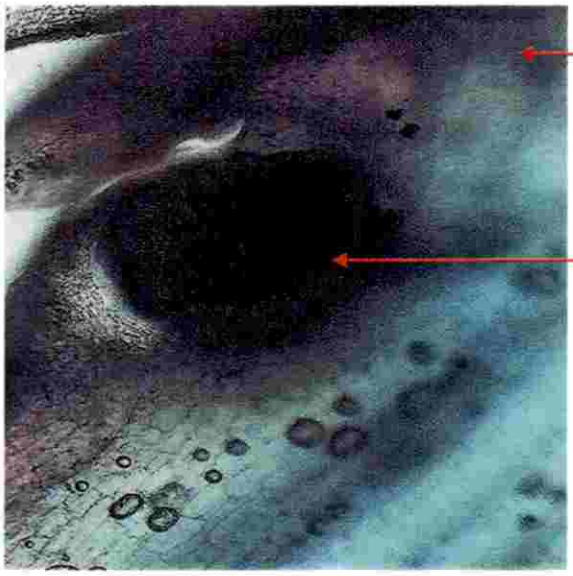


Plate 5. Undifferentiated floral meristem in axil of bract in immature spike

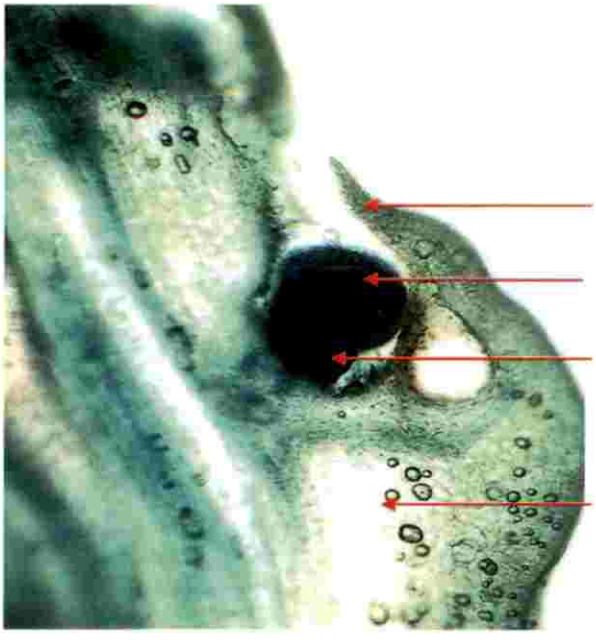


Plate 6. General view of LS of black pepper spikes under microscope (10X)

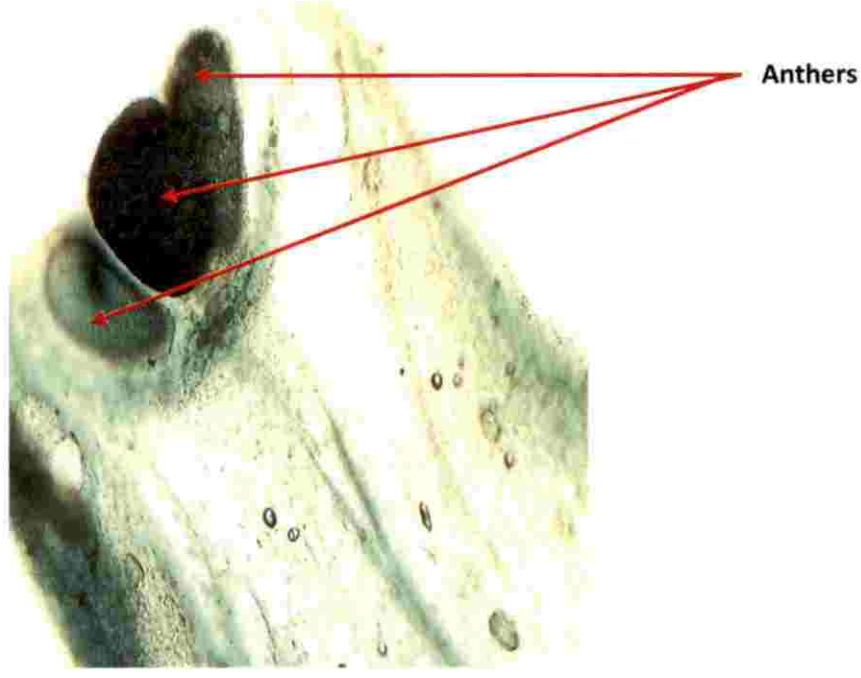


Plate 7. LS showing the anthers (10X)

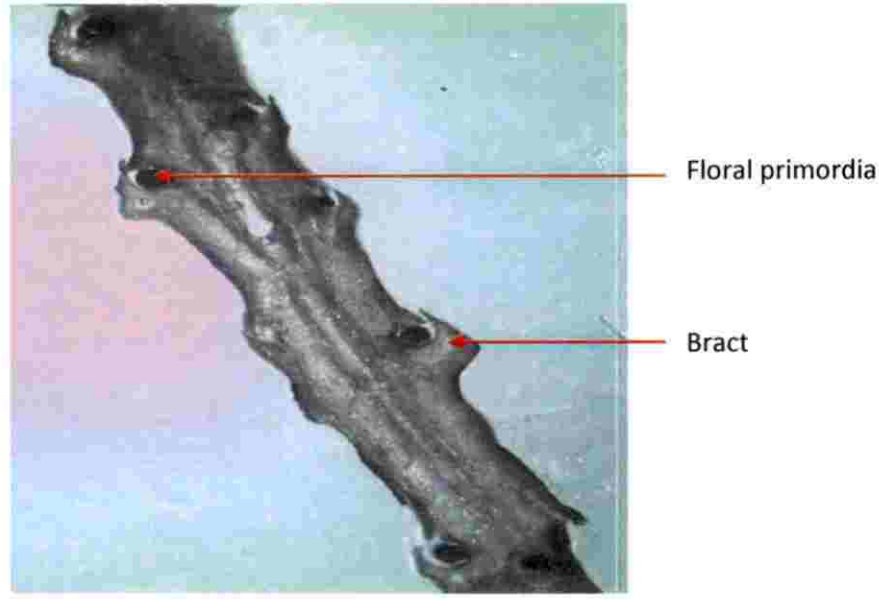


Plate 8. LS of black pepper spike (4X)

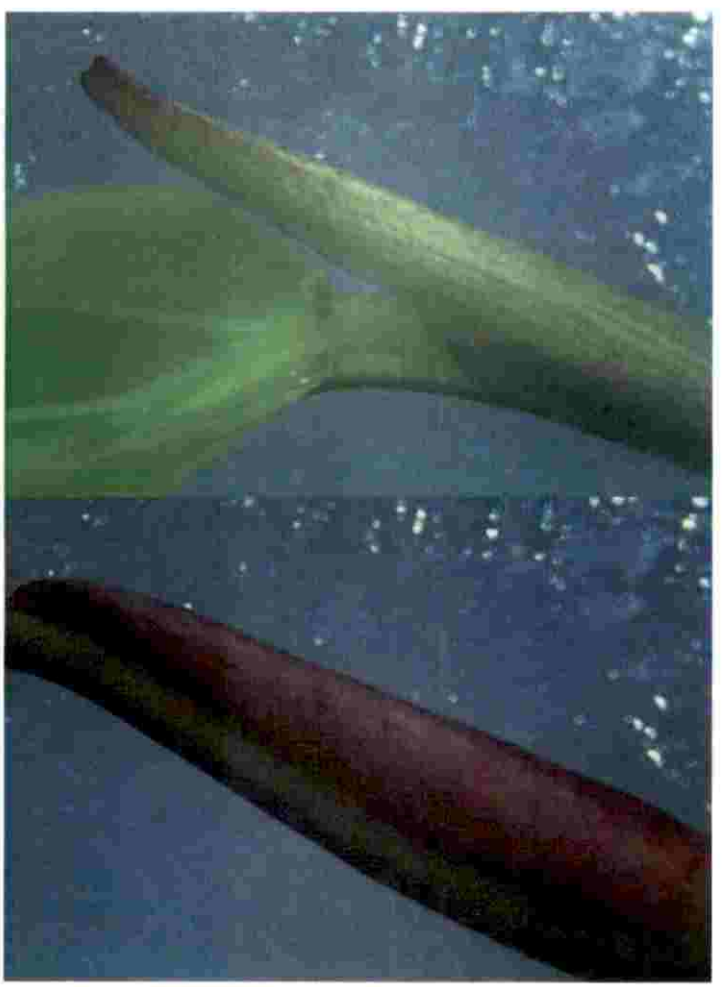


Plate 9. General view of plagiotropic shoottip (top) and orthotropic shoottip (bottom)

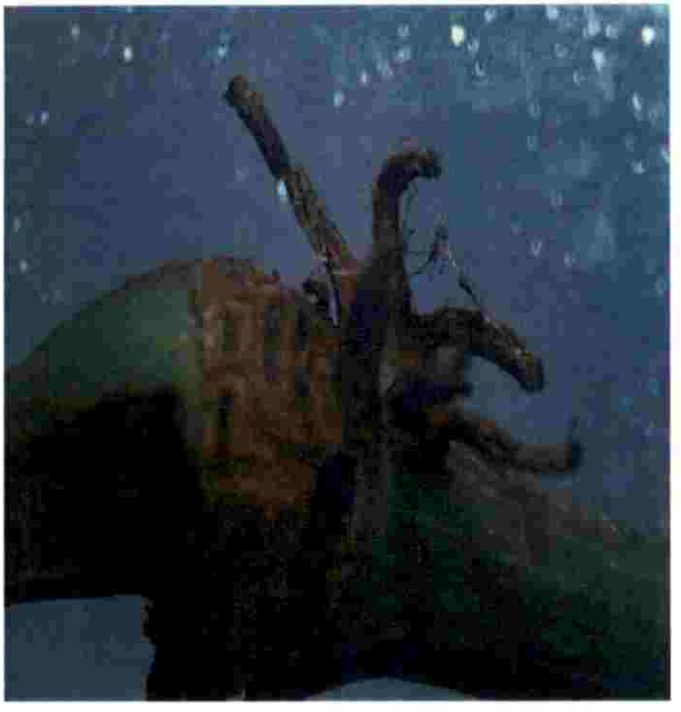


Plate 10. General view of aerial roots of black pepper

DISCUSSION

5. DISCUSSION

The Western Ghats region of India serves as the center of origin of 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, including about 110 species (Purseglove *et al.*, 1981; Parthasarathy *et al.*, 2006) but still the productivity in India is very low. Flowering is a critical development process that determines yield. However, there is little information on genes involved in flowering in black pepper. A partial orthologue of only one gene viz., *FRUITFULL* has been isolated so far (Hemanth, 2014). It is in this background that this study entitled "Identification and characterization of *Suppressor of Overexpression of Constans1* (*SOC1*) gene in black pepper (*Piper nigrum* L.)" was undertaken at the Integrated Biotechnology Block, College of Agriculture, Vellayani during 2015-2016. Discussions pertaining to this study are discussed in this chapter.

5.1 ISOLATION OF *SOC1* GENE

5.1.1 RNA Isolation and RT-PCR Analysis

The *SOC1* gene has 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. Cethyl trimethylammonium bromide (CTAB) 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 of the isolated RNA. 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 also confirmed that the extracted RNA was of good quality.

The extracted RNA was used for RT-PCR with the designed degenerate primers at specific annealing temperatures. No amplification was detected in the first reactions using SOC1-fw1 and SOC1-rev1 primers. It was assumed that this may be due to the low copy numbers in the samples and hence lower amplification products. Therefore nested reactions using the PCR product as template were carried out which resulted in two bands being visible on the agarose gel in which the products of the nested PCR products were run. One among the two bands was obtained using the primers SOC1-fw2 and SOC1-rev1, amplified at 640 bp whereas the other band was obtained using the primers SOC1-fw2 and SOC1-rev3, amplified at 330 bp. Both the bands were of the expected size when the position of the primers was considered in the sequence alignment carried out for primer designing.

5.1.2 Sequencing and Sequence Analysis

The two bands were gel-eluted, purified and sequenced. The sequences of the amplicons were analyzed using Bioinformatic tools in order to identify the gene *viz.*, tBLASTx, NCBI conserved Domain Search and Phylogeny tree. The tBLASTx programme showed that the sequences belonged to MADS box transcription factors. The MADS box is a conserved domain of 150 bps and is involved in DNA binding and further activation of downstream genes. Many of the flowering genes isolated from other plants are MADS box genes. *SOC1* has been reported to be a MADS box gene (Bossinger and Smyth, 1996) and is a floral integrator that integrates signals from environmental, hormonal and developmental cues and triggers downstream flowering genes such as *FT*. The BLAST hits presented in Fig. 3 to 6 clearly showed that the isolated sequences were partial orthologues of *SOC1* from black pepper. The identities

and positives in the BLAST search were as high as 69 and 83 confirming once again that the sequences isolated in this study were *SOC1*. Since no earlier gene publications on floral integrator genes in black pepper could be noted in NCBI database, the gene identified in the present study could be the first MADS box floral integrator gene to be identified in black pepper. It could also be said that this was the first ever floral integrator to be isolated from black pepper apart from *FT* which has also been isolated in a contemporary study in the same department (Sooraj *et al.*, 2016). It has been reported that *SOC1* gene belongs to the MIKC type of MADS transcription factors that contain a MADS domain followed by an intervening region and then a K box region and finally the C terminal which gives specificity to the gene. The NCBI Conserved Domain Search program, showed that the isolated sequence contained a MADS box and K-box region. There was a short intervening region in between. The fragment isolated was towards the 5' end of the gene and hence the MADS domain and the K box region could be seen in the isolated sequence confirming that it exhibited features of the *SOC1* gene from other plants.

The phylogenetic analysis showed close relationship between the isolated *SOC1* sequences of pepper and *Amborella tricopoda.* Pepper also showed similarity toward *Magnolia virginiana* and *Nelumbo nucifera.*

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 expressed similar to the studies done by Preston and Kellog (2006). It was done by performing a PCR using **c**DNA from different tissues *viz.*, mature leaf, root, orthotropic shoot and plagiotropic shoot as template with *SOC1* primers. 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 all the tissues (Plates 3 & 4). This is because the *SOC1* gene is present in the phloem in addition to the flowering stages. So we can conclude that the *SOC1* gene is present in the shoots, leaves and roots of the pepper plant in addition to the spikes. One of three *SOC1/ tm3*-like MADS- box gene from *Eucalyptus ssp. Bicostata ETL* (*Eucalyptus TM3 Like*), is expressed in both vegetative and reproductive organs, including shoot meristems, roots, and floral organ primordia (Decroocq *at al.,* 1999). Although *SOC1* in *Arabidopsis* is expressed predominantly in the meristem tissues, it is ubiquitously expressed in various tissues, including roots, leaves, shoots, inflorescences, and stems. Probably, *SOC1/TM3*-like genes in dicots are widely expressed in various tissues and the regulatory functions of these genes may be more diversified (Lee and Lee, 2010).

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 conducted by Nalini (1983) which included the microscopy study of vegetative bud and flowering bud showing leaf sheath, leaf primordium, shoot primordium, and spike primordium.

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% ethanol for light microscopy study.

The various tissues such as plagiotropic shoots, orthotropic shoots and roots were also viewed under the stereo microscope (Plates 9 & 10). Roots were found on runner/ orthotropic shoots. The orthotropic shoots seemed to be more pigmented than the plagiotropic/fruitful shoots.

On observing the fixed and sectioned young spikes, the flowers seemed to form in the axils of bracts (Plate 5). Tucker (1982) had also observed that in several *Piper* species, bract initiation precedes the initiation of flower bud. It was noted that the perianthless flower had a superior ovary. The anthers were relatively more stained with safranin and were seen around the gynoecium. A longitudinal section of the stage 3 spike was observed under stereo microscope 4X to study the different floral primordia in the same spike (Plate 8). The development of the flowers seemed to progress from the base to the tip of the spike. More immature flowers and floral primordia were found towards the tip while more developed flowers were found at the base of the spike. Plate 7 shows the anthers in the LS of pepper spike. Anthers were more visible in the basal flowers rather than in the ones towards the tip of the spike. Pepper spikes are pendulous and this helps in pollination of flowers at the tip of the spike by pollen washed down from the basal flowers.

In the present study, thus a partial orthologue of *SOC1*, a floral integrator and flowering time gene, was successfully isolated and sequenced. The sequence analysis conclusively confirmed the fragment isolated was *SOC1*. Expression studies showed *SOC1* expression in other tissues such as aerial roots. The expression pattern also conforms to other reports of the activity and function of the gene (Lee and Lee, 2010).

The microscopy studies looked at development of pepper inflorescence and flowers in the present project. However, tracking the progress of floral development in relation with gene expression patterns can be attempted as future line of work. Similarly, obtaining the complete sequence of the full length gene and transformation

studies can also be undertaken as future work to characterize the role of the black pepper *SOC1* gene.

SUMMARY

6. SUMMARY

The study entitled "Identification and characterization of *Suppressor of Overexpression of Constans1* (*SOC1*) gene in black pepper (*Piper nigrum* L.)" was conducted at the Integrated Biotechnology Block, College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2016. The objective of the study was to isolate and sequence genes homologous to the floral integrator gene *SOC1* in black pepper (variety - Karimunda) and functionally characterize these genes by studying their expression patterns.

Degenerate primers were designed for *SOC1* gene sequences from NCBI database (forward and reverse primers). RNA was isolated using CTAB method followed by synthesis of cDNA using AMV RT (Avian myeloblastosis virus reverse transcriptase).

PCR (Polymerase chain reaction) was done using the cDNA. Amplification was obtained in the nested reactions. Two bands were obtained in two reactions using different primer combinations. The bands were of the size of 330 bp and 640 bp as expected.

These two bands were eluted, purified and sequenced using the relevant reverse and forward primers. Four sequences were obtained of 242 bp, 178 bp, 534 bp and 570 bp each. The sequences were analyzed using bioinformatics tools in order to identify the gene *viz*., tBLASTx, NCBI conserved Domain Search and phylogeny tree.

The tBLASTx programme showed that both the isolated fragments were MADS box transcription factors. The bigger fragment contained MADS-box and K-box domains whereas the smaller fragment encoded for just the MADS box region.

The sequence data of the two fragments were of SOC1 protein of black pepper which also happens to be the floral integrator gene making them the partial fragments of the first ever MADS box floral integrator gene to be identified in black pepper.

Phylogenetic tree created from Clustal Omega also showed that the 242 bp sequence was closer to *Amborella tricopoda, Magnolia virginiana* and *Nelumbo nucifera*.

Semi quantitative analysis with *SOC1* primers showed that the *SOC1* gene in black pepper was expressed in the leaves, shoots and roots also.

Microscopy studies were done using FAA 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.

Tracking the progress of floral development in relation with gene expression patterns can be attempted as future line of work. Similarly, obtaining the complete sequence of the full length gene and transformation studies can also be undertaken as future work to characterize the role of the black pepper *SOC1* gene.

REFERENCES

7. REFERENCES

Aarts, M. G., Hekkert, B., Holub, E. B., Beynon, J. L., Stiekema, W. J., and Pereira, A. 1998. Identification of R-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. *Mol. Plant Microbe. Interact.* 11: 251-258.

Abd-Elsalam, K. A. 2003. Bioinformatic tools and guideline for PCR primer design. *Afr. J. Biotechnol.* 2: 91-95.

Ambrose, B. A., Lerner, D. R., Ciceri, P., Padilla, C. M., Yanofsky, M. F., and Schmidt, R. J. 2000. Molecular and genetic analyses of the *SILKY1* gene reveal conservation in floral organ specification between eudicots and monocots. *Mol. Cell* 5: 569-579.

Andersen, C. H., Jensen, C. S., and Petersen, K. 2004. Similar genetic switch systems might integrate the floral inductive pathways in dicots and monocots. *Trends in plant sci.* 9: 105-107.

Alter, P., Bircheneder, S., Zhou, L., Schlüter, S., Gahrtz, M., Sonnewald, U., and Dresselhaus, T. 2016. Flowering Time-Regulated Genes in Maize Include the Transcription Factor ZmMADS1. *Plant Physiol.* 172: 389-404.

Azevedo, H., Lino-Neto, T., and Tavares, R.M. 2003. An improved method for high-quality RNA isolation from needles of adult maritime pine trees. *Plant Mol. Biol. Reporter* 21: 333-338.

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. 2005. FD, a bZIP protein mediating

signals from the floral pathway integrator FT at the shoot apex. *Sci.* 303: 1052-1056.

Benlloch, R., Berbel, A., Serrano-Mislata, A., and Maduen, F. 2007. Floral initiation and inflorescence architecture: a comparative view. *Ann. Bot.* 100: 659-676.

Bernier, G. 1988. The control of floral evocation and morphogenesis. *Annu. Rev. Plant Physiol. and Plant Mol. Biol.* 39: 175-219.

Bernier, G., Havelange, A., Houssa. C., Petitjean, A. and Lejeune, P. 1993. Physiological signals that induce flowering. *The Plant cell* 5: 1147-1155.

Blázquez, M. A., Soowal, L. N., Lee, I., and Weigel, D. 1997.*LEAFY* expression and flower initiation in *Arabidopsis*. *Dev.*124: 3835-3844.

Brooks, E. M., Sheflin, L. G., and Spaulding, S. W. 1995. Secondary structure in the 3' UTR of EGF and the choice of reverse transcriptases affect the detection of message diversity by RT-PCR. *BioTechniques* 19: 806-815.

Buell, G. N., Wickens, M. P., Payvar, F., and Schimke, R. T. 1978. Synthesis of full length cDNAs from four partially purified oviduct mRNAs. *J.Biol. Chem.* 253: 2471-2482.

Bugos, R.C., Chiang, V. L., Zhang, X. H., Campbell, E. R., Podila, G. K., and Campbell, W.H. 1995. RNA isolation from plant tissues recalcitrant to extraction in guanidine. *BioTechniques* 19: 734-737.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J.Mol. Endocrinol.* 25:169-193.

Bustin, S. A. and Nolan, T. 2004. Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction. *J. Biomol. Tech.* 15: 155-166.

Callejas, R. 2001. Piperaceae. In: Stevens, W. D., Ulloa, C., Pool, A., and Montiel, O. M. (eds), *Flora de Nicaragua, Tomo III*. Missouri Botanical Garden Press, St. Louis, pp. 1928-1984.

Chang, L., Zhonghui, T., and Hao, Y. 2009. Coming into bloom: the specification of floral meristems. *Dev.* 136: 3379-3391.

Chaveerach, A., Mokkamu, P., Sudmoon, R., and Tanee, T. 2006. Ethnobotany of the genus *Piper* (Piperaceae) in Thailand. *Ethnobot. Res. Appl.* 4: 223-231.

Chen, L., Cheng, J. C., Castle, L., and Sung, Z. R. 1997. EMF genes regulate *Arabidopsis* inflorescence development. *Plant Cell* 9: 2011-2024.

Choong., Lee, S., Cho, L., Kim, S. L., Lee, Y., Choi, S. C., Jeong, H. J., Yi, J., Park, J. and Gynheung, A. 2009. OsMADS50 and OsMADS56 function antagonistically in regulating long day (LD)-dependent flowering in rice. *Plant Cell and Environ.* 32: 1412–1427.

Chiocchia, G. and Smith, K. A. 1997. Highly sensitive method to detect mRNAs in individual cells by direct RT-PCR using Tth DNA polymerase. *BioTechniques* 22: 312-318.

Cseke, L. J., Zheng, J. and Podila, G. K. 2003. Characterization of PTM5 in aspen trees: a MADS-box gene expressed during woody vascular development. *Gene* 318: 55-67.

Cronquist, A. 1981. *An integrated system of classification of flowering plants*. Columbia university press, New York, 1262p.

Cronquist, A. 1978. Once again, what is a species? In: Rhomberger, J. A. (ed.), *Biosystematics in Agriculture*. John Wiley and Sons, New York, pp. 1-20.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Sci.* 316: 1030-1033.

Donoghue, M. J. and Doyle, J. A. 1989. Phylogenetic analysis of angiosperms and the relationships of Hammameliidae. In: Crane, P.R. and Blackmore, S. (eds.), *Evolution, systematics, and fossil history of the Hammamelidae. Vol. 1.* Clarendon, Oxford, UK, pp. 17-45.

Doyle, K. 1996. *The Source of Discovery: Protocols and Applications Guide*, Promega, Madison, Wisconsin, USA.

Deng, C. and Davis, T. M. 2001. Molecular identification of the yellow fruit color (c) locus in diploid strawberry: a candidate gene approach. *Theor. Appl. Genet.* 103: 316-322.

Decroocq, V., Zhu, X., Kauffmann, M., Kyozuka, J., Peacock, W. J., Dennis, E. S., and Llewellyn, D. J. 1999. A TM3- like MADS- box gene from Eucalyptus expressed in both vegetative and reproductive tissues. *Gene* 228: 155-160.

Dhanya, K. and Sasikumar, B. 2010. Molecular marker based adulteration detection in traded food and agricultural commodities of plant origin with special reference to spices. *Curr. Trends Biotechnol. Pharm.* 4: 454-489.

Ding, L. W., Sun, Q. Y., Wang, Z. Y., Sun, Y. B., and Xu, Z. F. 2008. Using silica particles to isolate total RNA from plant tissues recalcitrant to extraction in guanidine thiocyanate. *Anal. Biochem.* 374: 426-428.

George, K. J., Ganga, G., Varma, R. S., Sasikumar, B., and Saji, K.V. 2005. Identification of hybrids in black pepper (*Piper nigrum* L.) using male parent-specific RAPD markers. *Curr. Sci.* 88: 206-218.

Gerard, G. F., Fox, D. K., Nathan, M., and Alessio, J. M. 1997. Reverse transcriptase. The use of cloned Moloney murine leukemia virus reverse transcriptase to synthesize DNA from RNA. *Mol. Biotechnol.* 8: 61-77.

Ghawana, S., Paul, A., Kumar, H., Kumar, A., Singh, H., Bhardwaj, P. K., Rani, A., Singh, R.S., Raizada, J., Singh, K., and Kumar, S. 2011. An RNA isolation system for plant tissues rich in secondary metabolites. *BMC Res. Notes* 4: 85-89.

Giovannini, A. 2006. Flowering. *J. Crop Improv.* 17: 227-244.

Goto, K., Kyozuka, J., and Bowman, L. 2001. Turning floral organs into leaves, leaves into floral organs. *Curr. Opin. Genet. Dev.* 11: 449-456.

Gu, Q., Ferra´ndiz, C., Yanofsky, M. F., and Martienssen, R. 1998. The *FRUITFULL*MADS-box gene mediates cell differentiation during *Arabidopsis* fruit development. *Dev.* 125: 1509-1517.

Freeman, W. M., Vrana, S. L., and Vrana, K. E. 1996. Use of elevated reverse transcription reaction temperatures in RT-PCR. *BioTechniques* 20:782-783.

Hartmann, U., Hohmann, S., Nettesheim, K., Wisman, E., Saedler, H., and Huijser, P. 2000. Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. *Plant J.* 21(4): 351-360.

Hemanth. 2014. Molecular Analysis of Floral Meristem Identity Genes in Black Pepper (*Piper nigrum* L.). MSc (Ag) thesis, Kerala Agricultural University, Trissur.

Hepworth, S. R. 2002. Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. *EMBO J.* 21: 4327–4337

Huala, E. and Sussex, I. M. 1992. *LEAFY* interacts with floral homeotic genes to regulate *Arabidopsis* floral development. *Plant Cell* 4: 901-913.

Jaramillo, M. A. and Manos, P. S. 2001. Phylogeny and patterns of diversity in the genus Piper (Piperaceae). *Amer. J. Bot.* 88: 706-716.

Joshi, M. and Deshpande, J. D. 2010. Polymerase chain reaction: methods, principles and application. *Int. J. Biomed. Res.* 1: 81-97.

Kampke, T., Kieninger, M., and Mecklenburg, M. 2001. Efficient primer design algorithms. *Bioinforma.*17: 214-225.

Kanno, A., Saeki, H., Kameya, T., Saedler, H., and Theissen, G. 2003. Heterotropic expression of class B floral homeotic genes supports a modified ABC model for tulip (*Tulipa gesneriana*). *Plant Mol. Biol.* 52: 831-841.

Kojima, K. and Ozawa, S. 2002. Method for isolating and purifying nucleic acids. United State patent US 2002/0192667 A1.

Kolosov, N., Miller, B., Ralph, S., Ellis, B. E., Douglas, C., Ritland, K., and Bohlmann, J. 2004. Isolation of high-quality RNA from gymnosperm and angiosperm trees. *BioTechniques* 35: 821-824.

Koonjul, P. K., Brandt, W. F., Farrant, J. M., and Lindsey, G. G. 1999. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nucleic Acids Res.* 27: 915-916.

Koornneef, M., Alonso-Blanco, C., Peeters A. J. M., and Soppe, W. 1998. Genetic control of flowering time in *Arabidopsis*. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49: 345-370.

Krishnamurthi, A. 1969. *The Wealth of India*: *Raw Materials*. Publication Information Directorate, CSIR, New Dehli, 8: 350p.

Krishnakumar, P. K. 2015. Indian black pepper production to decline in 2016 due to inclement weather. http://economictimes.indiatimes.com.

Kwok, S., Chang, S.Y., Sninsky, J. J., and Wang, A. 1994. A guide to the design and use of mismatched and degenerate primers. *PCR Methods Appl.* 3: S39-47.

Lang, A. 1984. Die photoperiodische regulation von fbrderung undhemmung der blütenbildung. *Ber. Deutsch. Bot. Ges.* 97: 293-314.

Lang, M. and Orgogozo, V. 2011. Identification of homologous gene sequences by PCR with degenerate primers. *Methods Mol. Biol.* 772: 245-256.

Lee, J. and Lee, I. 2010. Regulation and function of SOC1, a flowering pathway integrator. *J. Exp. Bot.* 61: 2247-2254.

Lin, P. K. T. and Brown, D. M. 1992. Synthesis of oligodeoxyribonucleotides containing degenerate bases and their use as primers in the polymerase chain reaction. *Nucleic Acids Res.* 19: 5149-5152.

Linhart, C. and Shamir, R. 2005. The degenerate primer design problem: theory and applications. *J. Comput. Biol.* 12: 431-456.

Liu, C., Thong, T., and Yu. 2009. Coming into bloom: the specification of floral meristems. *Dev.* 136: 3379-3391.

Ma, H. 1998. To be, or not to be, a flower – control of floral meristem identity. *Trends Genet.* 14: 26-32.

Ma, H. and dePamphilis, C. 2000. The ABCs of flower evolution. *Cell* 101: 5-8.

MacRae, E. 2007. Extraction of Plant RNA. *Methods Mol. Biol.* 353: 15-24.

Mandel, M. A. and Yanofsky, M. F. 1995a. A gene triggering flower formation in *Arabidopsis*. *Nat.* 377: 522-524.

Mandel, M. A. and Yanofsky, M. F. 1995b. The *Arabidopsis* AGL8MADS box gene is expressed in inflorescence meristems and is negatively regulated by *APETALA1*. *Plant Cell* 7: 1763–1771.

Mattheus, N., Ekramoddoullah, A. K. M., and Lee, S. P. 2003. Isolation of high quality RNA from white spruce tissue using a three-stage purification method and subsequent cloning of a transcript from the PR-10 gene family. *Phytochem. Anal.* 14: 209-215.

Mishra, K. M., Rani, S. N., Ram, A. S. and Sreenath, H. L. 2008. A simple method of DNA extraction from coffee seeds suitable for PCR analysis. *Afr. J. Biotechnol.* 7: 409-413.

Moyo, M., Amoo, S. O., Bairu, M. W., Finnie, J. F., and Van Staden, J. 2008. Optimising DNA isolation for medicinal plants. *South Afr. J. Bot.* 74: 771-775.

Myers, T. W. and Gelfand, D. H. 1991. Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochem.* 30:7661-7666.

Nair, P. K. P. 2011. *Agronomy and economy of Black pepper and Cardamom*. Elsevier publications, London, pp. 62-70.

Nair, R. R., Sasikumar, B., and Ravindran, P. N. 1993. Polyploidy in a cultivar of black pepper (*Piper nigrum* L.) and its open pollinated progenies. *Cytologia*. 58: 27-31.

Nalini, P. V. 1983. Flower bud differentiation in pepper (*Piper nigrum* L.). M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 83p.

Neto, E. D., Harrop, R., Correa-Oliveira, R., Wilson, R. A., Pena, S. D., and Simpson, A. J. 1997. Minilibraries constructed from cDNA generated by arbitrarily primed RT-PCR: an alternative to normalized libraries for the generation of ESTs from nanogram quantities of mRNA. *Gene* 186:135-142.

Ng, M. and Yanofsky, M. F. 2001. Function and evolution of the plant MADS-box gene family. *Nat. Rev. Genet.* 2: 186-195.

Parcy, F., Nilsson, O., Busch, M. A., Lee, I., and Weigel, D. 1998. A genetic framework for floral patterning. *Nat.* 395: 561-566.

Parenicova, L., de Folter, S., Kieffer, M., Horner, D.S., Favalli, C., Busscher, J., Cook, H. E., Ingram, R. M., Kater, M. M., Davies, B., Angenent, G. C., and Colombo, L. 2003. Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in *Arabidopsis*: new openings to the MADS world. *Plant Cell* 15: 1538-1551.

Parthasarathy, U., Saji, K. V., Jayaraj, K., and Parthasarathy, V. A. 2006. Biodiversity of *Piper* in South India and application of GIS and cluster analysis in search of its distribution. *Curr. Sci.* 91: 652-658.

Prittila, A. M., Hirsicorpi, M., Kamarainen, T., Jakkola, L., and Hohtala, A. 2001. DNA isolation method for medicinal and aromatic plants. *Plant Mol. Biol. Reporter* 19: 273-273.

Puchooa, D. and Khoyratty, S. S. S. 2004. Genomic DNA extraction from *Victoria amazonica*. *Plant Mol. Biol. Reporter* 22: 195-196.

Purseglove, J. W., Brown, E. G., Green, C. L., and Robbins, S. R. J. 1981. *Spices*. Vol. 1. Longman: New York, pp. 10-99.

Putterill, J., Robson, F., Lee, K., Simon, R., and Coupland, G. 1995. The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* 80: 847-857.

Ravindran, P. N., Nirmal Babu K., Sasikumar, B., and Krishnamoorthy, K. S. 2000. Botany and crop improvement of black pepper. In. Ravindran, P. N. (ed.). *Black pepper* (*Piper nigrum* L.). Harwood Academic Publishers, Amsterdam, The Netherlands, pp. 23-142.

Rose, T. M., Schultz, E. R., Henikoff, J. G., Pietrokovsk, S., McCallum, C. M., and Henikoff, S. 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Res*. 26: 1628-1635.

Roux, K. H. 1995. Optimization and trouble shooting in PCR. *Genome Res*. 4: 185-194.

Ryu, C., Lee, S., Cho, L., Kim. S. L., Lee, Y., Choi, S. S., Jeong, H. J., Yi, J., Park, S. J., Han C. D., and Gynheung. 2009. OsMADS50 and OsMADS56 function

antagonistically in regulating long day (LD)-dependent flowering in rice. *Plant Cell and Environ.* 32: 1412-1427.

Sablok, G., Gahlot, P., Gupta, A. K., Pareek, K., and Shekhawat, N. S. 2009. Extraction of PCR usable DNA from trees of arid environment. *Plant Omics J.* 2: 103-109.

Sachs, R. M. and Hackett, W. P. 1983. Source-sink relationships and flowering in Beltsville Symposia in Agricultura1 Research. W. J. Meudt. (ed.). *6 Strategies of Plant Reproduction.* Osmun Publishing, Allanheld, Totowa, NJ, pp. 263-272.

Sahu, S. K., Thangaraj, M., and Kathiresan, K. 2012. DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen. *ISRN Mol. Biol.* 1: 1-6.

Salzman, R. A., Fujita, T., Zhu-Salzman, K., Hasegawa, P. M., and Bressan, R. A. 1999. An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. *Plant Mol. Biol. Reporter* 17: 11-17.

Sangwan, N. S., Sangwan, R. S., and Kumar, S. 1998. Isolation of genomic DNA from the antimalerial plant *Artemisia annua. Plant Mol. Biol. Reporter* 16: 1-9.

Schultz, E. A. and Haughn, G. W. 1991. *LEAFY*, a homeotic gene that regulates inflorescence development in *Arabidopsis. Plant Cell* 3: 771-781.

Schultz, E. A. and Haughn, G. W. 1993. Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis. Dev.* 119: 745-765.

Seung, K. Y., Chung, K. S., Kim, J., Lee, J. H., Hong, S. M., Yoo, S. J., Yoo, S. Y., J. S., and Ahn, J. H. 2005. CONSTANS Activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to Promote Flowering in Arabidopsis. *Plant Physiology.* 139: 770-778.

Sharma, A. D., Gill, P. K., and Singh, P. 2003. RNA isolation from plant tissues rich in polysaccharides. *Anal. Biochem.* 314: 319-321.

Sharma, K. K., Lavanya, M., and Anjaiah, V. 2000. A method for isolation and purification of peanut genomic DNA suitable for analytical applications. *Plant Mol. Biol. Reporter* 18: 393-393.

Simpson, G. G. and Dean, C. 2002. *Arabidopsis*, the Rosetta stone of flowering time. *Sci.* 296: 285-289.

Simpson, G. G., Gendall, A. R., and Dean, C. 1999. When to switch to flowering. *Annu. Rev. Cell. Dev. Biol.* 15: 519-550.

Singh, G., Kumar, S. and Singh, P. 2003. A quick method to isolate RNA from wheat and other carbohydrate-rich seeds. *Plant Mol. Biol. Reporter* 21: 93a-93f.

Singh, V. and Kumar, V. 2010. An optimised method of DNA isolation from highly mucilage rich okra (*Abelmoschus esculentus* L.) for PCR analysis. *Pelagia Res. Libr.* 3: 1809-1813.

Song, J., Clemens, J., and Jameson, P. E. 2011. Expression of floral identity genes in *Clianthus maximus* during mass inflorescence abortion and floral development. *An. of Bot.* 107: 1501-1509.

Sooraj, J. R. and Sreekantan, L. Identification and characterization of *Flowering Locus T* (*FT*) gene in black pepper (*Piper nigrum* L.). M. Sc. Thesis, Kerala Agricultural University, Thrissur.

Sreekantan, L. 2002. Molecular studies of flowering in *Metrosideros excelsa*. Ph. D. thesis, Massey University, Palmerston North, New Zealand, 175p.

Sreekantan, L. and Thomas, M. R. 2006. VvFT and VvMADS8, the grapevine homologues of the floral integrators FT and SOC1, have unique expression patterns in grapevine and hasten flowering in Arabidopsis. *Functional Plant Biol.* 33: 1129-1139.

Sung, Z. R., Belachew, A., Shunong, B., and Bertrand-Garcia, R. 1992. EMF, an *Arabidopsis* gene required for vegetative shoot development. *Sci.* 258: 1645-1647.

Swarup, K., Alonso-Blanco, C., Lynn, J. R., Michaels, S. D., Amasino, R. M., Koornneef, M., and Millar, A. J. 1999. Natural allelic variation identifies new genes in the *Arabidopsis* circadian system. *Plant J.* 20: 67-77.

Syamkumar, S., Jose, M., and Sasikumar, B. 2005. Isolation and PCR amplification of genomic DNA from dried capsules of cardamom (*Elettaria cardamomum* M.). *Plant Mol. Biol. Reporter* 23: 417-417.

Tadege, M., Sheldon, C. C., Helliwell, C. A., Upadhyaya, N. M., Dennis, E. S., and Peacock, W. J. 2003. Reciprocal control of flowering time by *osSOC1* in transgenic Arabidopsis and by *FLC* in transgenic rice. Plant Biotech. J. 1: 361-369.

Thangaselvabal, T., Justin, C. G. L., and Leelamathi, M. 2008. Black pepper (*Piper nigrum* L.) 'The king of spices' - a review. *Agric. Rev.* 29: 89 -98.

Tucker, S. C. 1982. Inflorescence and flower development in the Piperaceae. II. Floral ontogeny of *Piper*. *Amer. J. Bot.* 69(9): 1389-1401.

Varma, A., Padh, H., and Shrivastava, N. 2007. Plant genomic DNA isolation: an art or a science. *Biotechnol. J.* 2: 386-392.

Voogd, C., Wang, T. and Gasic, E. V. 2015 Functional and expression analyses of kiwifruit SOC1-like genes suggest that they may not have a role in the transition to flowering but may affect the duration of dormancy. *J. Exp. Bot.* 66 (15): 4699-4710.

Wang, S., Beruto, M., Xue, J., Zhu, F., Liu, C., Yan, Y., and Zhang, X. 2015. Molecular cloning and potential function prediction of homologous SOC1 genes in tree peony. *Plant Cell Rep* 34: 1459-1471.

Wang, T. and Brown, M. J. 1999. mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal. Biochem.* 269: 198-201.

Wang, X., Tian, W., and Li, Y. 2008. Development of an efficient protocol of RNA isolation from recalcitrant tree tissues. *Mol. Biotechnol.* 38: 57-64.

Warude, D., Chavan, P., Joshi, K., and Patwardhan, B. 2003. DNA isolation from fresh, dry plant samples with highly acidic tissue extracts. *Plant Mol. Biol. Reporter* 21: 467-467.

Weigel, D. and Nilsson, O. 1995. A developmental switch sufficient for flower initiation in diverse plants. *Nat.* 377: 495-500.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F., and Meyerowitz, E. M. 1992. *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* 69: 843-859.

Xiaofan, N., Jian, B., Yao, W., Wu, C., Hou, W., Jiang, B., Bi, Y., and Han, T. 2013. Cloning and functional analysis of the flowering gene GmSOC1-like, a putative SUPPRESSOR OF OVEREXPRESSION CO1/AGAMOUS-LIKE 20 (SOC1/AGL20) ortholog in soybean. *Plant Cell Rep.* 32: 1219-1229.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, Y., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. 2000. Hd1, a major photoperiod sensitivity QTL in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell* 12: 158-167.

Zhang, J. and Byrne, C.D. 1999. Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse-transcriptase PCR. *Biochemical J.* 337: 231-241.

Zhang, X., Wei, J., Fan, S., Song, M., Pang, C., Weib, H., Wanga, C., and Yu, S. 2015. Functional characterization of GhSOC1 and GhMADS42 homologs from upland cotton (*Gossypium hirsutum* L.). *Plant Sci.* 242: 178-186.

APPENDICES

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 RNA 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

FAA Solution (For) 200 ml

Ethyl alcohol (95%)	100 ml
Glacial acetic acid	10 ml
Formaldehyde	20 ml
Distilled water	70 ml

ABSTRACT

Identification and characterization of *Suppressor of Overexpression of Constans1 (SOC1)* gene in black pepper (*Piper nigrum* L.)

By

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(2011-09-124)

Abstract of Thesis

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ABSTRACT

The present study entitled "Identification and characterization of *Suppressor of Overexpression of Constans1* gene in Black Pepper (*Piper nigrum* L.)" was conducted at the Integrated Biotechnology Block, College of Agriculture, Vellayani, during 2015-2016. The study envisaged isolation and sequencing of *SOC1,* a flowering integrator gene in black pepper (variety - Karimunda) and functional characterization of the gene by studying the expression patterns.

Degenerate primers were designed for the above said gene based on the gene sequences from NCBI database (*SOC1* forward and reverse primers) which were used to isolate and identify the gene. Total RNA of black pepper was isolated using modified CTAB method followed by synthesis of cDNA using AMV RT (Avian myeloblastosis virus reverse transcriptase).

PCR (Polymerase chain reaction) with degenerate primers was done using cDNA as the template. However no amplifications were observed after the first reactions. Therefore nested PCR reactions were done using the PCR products of the first reaction as the template. Two bands of size 640 bp and 330 bp were produced in the nested reactions. Sequencing of the product yielded four sequences with each of the sequence showing similarity to the *SOC1* gene, when done sequence analysis, thus making it the first flowering integrator gene to be identified in black pepper.

Microscopy studies were carried out to see the floral characters of black pepper in detail. Microscopy studies were done using FAA 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.