MOLECULAR ANALYSIS OF SPIKE BRANCHING OBSERVED IN BLACK PEPPER (*Piper nigrum* L.) TYPE FROM IDUKKI

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Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2009

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DECLARATION

I hereby declare that this thesis entitled "**Molecular analysis of spike branching observed in black pepper (Piper nigrum. L) type from Idukki**" is a bonafide record of research work done by me in the Department of Plant Biotechnology, College of Agriculture, Vellayani during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis "**Molecular analysis of spike branching observed in black pepper (*Piper nigrum* L.) type from Idukki**" is a record of research work done independently by Mr. Vimarsha, H.S. (2006-11-135) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Vinthesh, H. S.

ACKNOWLEDGEMENT

I loved my initiative research on genes involved in flowering and inflorescence development. Good memories, discussing with my seniors, sir and madam. I was surrounded by very caring people in the laboratory about whom I would like to express my feelings.

First I would like to express my deep sense of gratitude to Dr. K. Rajmohan, Professor and Head, Department of Plant Biotechnology and Chairperson of my Advisory Committee for his two year's worth of support, critical feedback and general enthusiasm. He is a person who does not have the word "no" in his personal dictionary. He has given me almost complete independence, while always remaining available for scientific discussions-hashing out new ideas. I feel very lucky to be his student for his direct help, improving presentation, writing and styles of scientific research and constant motivations.

I have also been lucky to have a wonderful thesis committee. I am deeply indebted to Dr. K. B. Soni, Associate Professor, Department of Plant Biotechnology. She gave lot of support in my path to research. She is the one among a few people who know exactly how to help students with their research. She is the back bone of my research without her I would have gone in a wild route. Being honest, I was not knowing how to begin my research work; it was her support which helped me utmost in my research. I greatly appreciate her contribution to my research and education.

I would like to thank Dr. Swapna Alex, the best interactive person in theory aspects. I remember the days of her lecture in which every time I used to come out with one or the other question. Her guidance helped improve my presentation skills and responsibilities. I am highly indebted for her inspiring guidance.

I take this opportunity to express my profound gratitude to Dr. Roy Stephen, Associate Professor, Department of Plant Physiology, for his support, critical discussions and kind help.

No choice of words will suffice to adequately express thanks to my dear friend, brother, senior Dr. Jithesh V.G. and his lovely family for their love, care and help. I can say that "I have a life time friend now". I still remember those sad and happy moments, cooking together and fighting for no reason. I feel happy to be in Kerala as it gave me the best friend. I am indebted to his love and care.

I was fortunate to have the best course mates and seniors who shared my every happiness and sadness. Many thanks to Saritha, Ponsubrya, Lekshmi chechi and Vignsh Adiga for their friendly assistance and helpful discussions. Special thanks to Ms. Smitha Bhasi, the best senior whose everlasting smile maintained our department's happiness throughout.

Many thanks go to all of the members of our lab, past and present, for their scientific insight and support. In particular, I would like to thank Asha, Vinitha, Soni, Akhila, Sheethu chichi, Divya chichi, Priya chichi and Prajeesh Chetan.

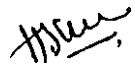
My immense gratitude to my batchmates, seniors and juniors Vidya, Bhavya, Rekha, Kumanan, Kumaran, Prabhu, Selvaraju, Divya, Renjini, Madhu Kumar. K, Tamilvel. D, Jayaramegowda, Shrikanth, Arpitha and Kavitha for their loving involvement and care during the entire period of my master's degree.

I feel happy to mention Dr. Gokulapalan, the man with a difference, who judged my precious two years in this university. He is the only person with whom I have shared me life time memories, happiness, sorrows. His friendly care, love, suggestions are encrypted in my heart forever.

How can I miss to mention my lovely sisters and brothers of Halasina Marada-Gowda family whose love, support and care has encouraged every step of my success. Special thanks to my sisters Mani, Shimshakka, Shamakka, Smithakka, Puttu, Anitakka, Shrthi, Shwetha, Theertha Varsha, Shifali, Shloka, Shabdhana and to my

cousin bro's Vaishak , Prashanth , Giridhar, Sreedhar, Rajesh and Harish who made living a pleasant experience.

Finally my love to Amma, Appa and Anna, the beats of my little heart. I am nothing without their support. There are no words in english dictionary to explain more about my family. My amma is my god. I owe my life to her happiness.



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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
AFLP	Amplified Fragment Length Polymorphism
AG	AGUMOSE gene
AP1	APETALA1 gene
AP2	APETALA2 gene
AP3	APETALA3 gene
ap1	APETALA1 mutant
bp	Base pair
CAL	CAULIFLOWER gene
cal	CAULIFLOWER mutant
CEN	CENTRODIALIS gene
CO	CONSTATNS gene
CTAB	Cetyl trimethyl ammonium bromide
CV	Coefficient of variation
DNA	Deoxy ribonucleic acid
dNTPs	deoxy Nucleotide Tri Phosphates
EDTA	Ethylene Diamino Tetra Acetic Acid
FLO	FLORICOLA gene
FT	Flowering Locus T gene
FT	Flowering Locus T protein

GA	Gibberlic Acid
ha	Hectare
HCL	Hydrochloric acid
IISR	Indian Institute of Spices Research
ISSR	Inter Simple Sequence Repeat
Kb	Kilo base
LFY	LEAFY gene
lfy	LEAFY mutant
M	Molar
Ml	Milli litre
MT	Metric Tonnes
mM	Millimolar
NaCl	Sodium Chloride
OD	Optical Density
ng	Nanogram
PCR	Polymerase Chain Reaction
pH	Per Hydrogen
PVP	Poly vinyl pyrrolidone
pM	Picomolar
PI	PISTILLATA gene
RAPD	Rndom Amplified Polymorphic DNA
RFLP	Restricted Fragment length Polymorphism
rpm	Revolution per minute
SP	SELF PRUNING gene
TFL-1	Terminal Flower-1 gene

TFL-1	Terminal Flower-1 protein
tfl1	Terminal Flower-1 mutant
TFL-2	Terminal Flower -2 gene
TAE	Tris acetate EDTA buffer
TE	Tris EDTA buffer
Tris HCl	Tris (hydroxy methyl) aminomethane Hydrochloride
UPGMA	Unweighted Pair Group Method for Arithmetic average
V	Volts

Introduction

1. INTRODUCTION

Black pepper, *Piper nigrum* L. belonging to the family *Piperaceae*, is one of the oldest and most widely used spices in the world. It is described as the 'King of Spices', since the world consumes as much black pepper as all other spices combined. India is the major producer, consumer and exporter of black pepper. India exports 32,000 tonnes of black pepper annually. Kerala accounts for 94 per cent of the total area (2,37,998 ha) and 96 per cent of the total production (87,605 tonnes) of black pepper. Even though pepper is cultivated throughout Kerala, the districts of Kozhikode, Kannur, Kottayam and Idukki account for 67 per cent of the total pepper area.

The genus *Piper* has records of the highest diversity, compared to the other genera of *Piperaceae* family. The International Plant Name Index (www.ipni.org) has recorded 6704 *Piper* species (Jaramillo and Manos, 2001). Species diversity and varietal diversity are considerable in black pepper, although, ecosystem diversity does not contribute much to its biodiversity (Hooker, 1886; Ibrahim et al., 1985c; Sasikumar et al., 1999). India is considered as the primary centre of diversity for black pepper, which is indigenous to the rain forests of south-western states. Considerable variation exists regarding its morphology, yield and quantitative traits. With respect to the spike characteristics, variability has been observed in spike length, floral composition, floral arrangement, fruit number and size.

Botanically, black pepper is a branching vine with a smooth, woody, articulate stem swollen at the joints. It is a woody climber, which may reach heights more than ten meters by means of its aerial roots. Its broad, shiny green, pointed, petiole leaves are alternately arranged. Flowers are borne in the axils of ovate, fleshy bracts in long pendant spikes, which are single in nature and appear opposite to the leaves on the plagiotropic branches. The sessile, white, small flowers on a spike may vary in number from 25 to 100, arranged in 4 to 5 rows (Parthasarathy et al., 2007). The apical buds of the plagiotropic branches transform into inflorescence. The inflorescence in its early stages of development has a convex apical meristem, subtended by a leaf and a bract (Tucker, 1982). As the inflorescence grows in length

its apical meristem diminishes. The apical meristem of the inflorescence grows in length before any organs are formed (Sasikumar et al., 2006).

Normally, black pepper species have unbranched spikes. Spike branching is very rare. The varieties 'Aimpiriyan' and 'Kathirinmelkkathir' show a tendency for spike branching. However, the branches are rudimentary and bear only very few berries. Contrary to this, recently, a black pepper type showing profuse spike branching has been reported from a farmer's field in Idukki district (Sasikumar et al., 2006). The proliferating spikes are of indeterminate growth with pronounced bracts and bear up to 30 branches with about 300 berries altogether. This is about four times the reported yield of berries from spikes of the highest yielding varieties, Panniyur-1, Panniyur-3 and Panniyur-5. Spike branching in black pepper is of great economic significance as it is a quantitative trait. Identification and characterization of genes involved in spike branching is important if they are to be transferred. Traditional crop improvement programs can be adopted for transferring the trait to superior varieties. However, this may be time consuming and may involve transfer of undesirable traits along with the branching trait. Till now there are no reports on transfer of trait in black pepper. In this context, modern biotechnological interventions have got immense potential in improving yield parameters, quality and stress resistance in black pepper varieties.

Inflorescence branching has been observed in several plant species (Brown et al., 2006). The involvement of specific genes have been reported. In Arabidopsis, genes *TFL1, LFY, CAL, FUL, AGL24, API, FT, AG* and *UFO* have been reported to be involved in determining inflorescence architecture (Espinosa-Soto et al., 2004), of which the genes *TFL1* and *FT* have prominent roles.

In this context, the present study was taken up as an initial step towards identifying and characterizing the genes involved in spike branching in black pepper, which is a prerequisite for biotechnological interventions in crop improvement. Specific objective of the study was to examine the presence of *TFL1* and *FT* genes in black pepper samples colleted from Idukki. In addition, RAPD analysis was done for seven non-spike branching cultivars and spike branching type collected from same farm for analysing marker-linked trait and genetic variability.

Review of Literature

2. REVIEW OF LITERATURE

Kerala has records of more than hundred black pepper cultivars with highly variable characters. Only a few of them have been identified and reported to be economically productive. Although India holds the prime position in production, consumption and export of black pepper (George et al., 2005), the productivity is less (368 kg/ha), compared to that of Malaysian and Indonesian black pepper (Farm guide, 2008). Distinct variations in morphological traits among Indian landraces have been reported (Mathew et al., 2006). Variations in yield traits among Indian cultivars have also been reported based on spike length, floral composition, fruit number and size (Ibrahim et al., 1985a; Ravindran and Nirmal Babu, 1994 and Prasannakumari Amma et al., 2001). However, there are only a few reports on spike branching trait in black pepper (Sasikumar et al., 2006). A farmer in Idukki district has observed profuse branching of spikes in his plantation as early as 2004.

In plants, flowering is a major developmental transition that is critical to reproductive success. Network of genetic pathways that have evolved to respond to diverse external signals such as day length, temperature, hormones and developmental controls lead to flowering. In *Arabidopsis thaliana* the phosphatidylethanolamine-binding protein homologue TERMINAL FLOWER 1 (*TFL1*) and FLOWERING LOCUS T (*FT*) are key controllers of flowering, determining when and where flowers are made. The genes LEAFY (*LFY*), APETALA1 (*AP1*), and CAULIFLOWER (*CAL*) and the floral organ identity genes APETALA3 (*AP3*) and PISTILLATA (*PI*) are interacting regulatory genes in the floral meristem (Hanzawa et al., 2005). Possibly, these genes which are determining inflorescences development in other plant species may be conserved in *Piper nigrum* (Sasikumar et al., 2006).

The present study was based on the reports on spike branching black pepper. Samples were collected from farmer's field in Idukki for molecular analysis. The objective of the study was to analyse the presence of *TFL1* and *FT* genes in the black pepper type, showing spike branching trait, using polymerase chain reaction technique. In this chapter the literature concerned with morphological variations including inflorescence branching in black pepper and several other plants have been presented.

2.1 The Family *Piperaceae*

The family *Piperaceae* belongs to the order *Piperales* and holds seven prominent genus, among which the genus *Piper* and *Piperomia* dominate in diverse speciation (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/). According to Hooker (1886), *Piper* is more primitive than *Piperomia*. De Candolle (1923) has also reported the presence of more than thousand species in *Piper* and *Piperomia* genus. Some of the indigenous *Piper* species which are commonly used for flavouring and medicinal purpose are *Piper nigrum*: the black pepper, *Piper longum, Piper colubrinum, Piper betle, Piper chaba* and *Piper brachystachyum* (Nazeem et al., 2003).

2.1.1 Biodiversity in Genus *Piper*

The International Plant Name Index (www.ipni.org) has recorded 6704 *Piper* species in their website, which is an index of huge variability in *Piperaceae* family (Jaramillo and Monos, 2001). According to Parthasarathy et al. (2006) over the thousands reported species of *Piper*, 111 are of Indian origin which are distributed throughout the tropical and subtropical regions. Central and South America has the maximum diversity (~700) for *Piper* species (Jaramillo and Manos, 2001) which accounts for 60 per cent of species diversity in *Piper* (Ravindran et al., 2005). More than 300 species were recorded from Southern

Asia, out of which *Piper nigrum* L. is believed to have originated in the Western Ghats region of India (Rahiman et al., 1984 and Joseph and Skaria, 2001).

Surveys conducted by scientists of Indian Institue of Spices Research in the Western Ghats area of Idukki district has lead to the discovery of intermediate populations, apparently composed of hybrids between *Piper nigrum*, *P. sugandhi*, *P. trichodtachyon* and *P. galeatum* and their segregating progenies, confirm the origin of this spice (Ravindran, 2000).

Kerala, the southernmost state of India, occupies a considerable portion of the Western Ghats and is a rich source of wild relatives of this spice crop (Joy et al., 2007). Local cultivar diversity is the richest in Kerala (Ravindran et al., 2005). Out of 111 species, about 16 related species are distributed in the forests of Western Ghats, and the remaining species occur in North-Eastern India (Ravindran and Nirmal Babu, 1994). Ravindran et al. (1987) has subdivided the *Piper* in to two sections; *Pippali* and *Maricha* (long pepper and black pepper) based on orientation of spike viz., erect or pendant.

Piper species occurring in South India are economically important, as they are closely related to the cultivated black pepper. *P. betle* is another economically important species which is mainly used in the paan industry. Several species of *Piper* are used as important medicinal plants. *P. longum, P. cubeba, P. retrtofractum*, etc. are some of the species used in indigenous medicine system (Nazeem et al., 2003).

2.1.2 Variation in genus *Piper*

The genus *Piper* is known to have wide distribution in tropical region. Around three thousand binomials have been reported in this genus all over the world (Ravindran et al., 1992a). Various attempts to classify the *Piper* species based on morphological, cytological (Sharma and Bhattacharya, 1959) and

chemical constituent data (Rahiman and subbiah, 1984; Sebastian and sujatha, 1996 and Sebastian et al., 2000) have been made. However, all these classifications failed to create a concrete grouping of *Piper* species (Ravindran, 2000).

According to Ravindran et al. (1992b) *Piper* is taxonomically a very difficult genus because of greater range of variability among the species and minute nature of flowers. The reasons for this variability are considered to be due to the nature of breeding systems. Efficient pollen dispersal mechanism is absent in *Piper* establishing an effective isolation barrier between population units and individuals. This barrier prevents the free gene flow and thus the population has remained discrete (Ravindran et al., 1990).

The centre of diversity for this genus is Northern, South and Central America, which together account for more than 60 per cent of the species recorded (Datta and Dasgupta, 1979). India also has rich cultivar diversity for *Piper* in Kerala, followed by Karnataka. Recent surveys by scientists of IISR in some areas of Western Ghats in Idukki district led to the discovery of intermediate populations apparently composed of hybrids and segregating progenies, indicating the presence of huge variability in this genus (Ravindran, 2000).

2.2 Black Pepper - *Piper nigrum* L.

Black pepper is a tropical vine, producing the black pepper of commerce, christened the 'King of Spices' and 'Black Gold' as judged from the volume of international trade (http://www.indianetzone.com). India is a major producer, consumer, and exporter of black pepper (George et al., 2005). This crop is planted in 2.53 lakh ha, out of which Andaman and Nicobar Islands share 697 ha. By the year 2005, India produced about 70,000 MT of black pepper, around 15,752 MT of the product was exported to developed countries and the domestic consumption was 52,000 MT (IPC, 2006).

Apart from Asian countries such as Malaysia, India, Indonesia, Thailand, Vietnam, China and Sri Lanka, black pepper is also cultivated in Brazil and Madagascar (Joy et al., 2007). Currently pepper is grown in twenty six countries. In India, Kerala holds 94 per cent of the total area and 96 per cent of the total production. Karnataka holds second place with 3.5 per cent production. Even though black pepper is cultivated throughout Kerala, the districts Kozhikode, Kannur, Kottayam and Idukki account for 67 per cent of the total area (http://www.nmce.com/commodityStudy/pepper_study.jsp).

According to Ravindran (2000), the present day *P. nigrum* cultivars are probably the descendents of segregating populations which might have originated as a hybrid and got isolated due to lack of any mechanism for active gene flow, diverged subsequently and finally lead to the present day wild as well as cultivated black pepper. According to Nirmal Babu et al. (1993) much variability might have accumulated in due course as a result of intercrossing, segregation, back crossing and accumulation of random mutation. Because of the successful vegetative propagation, and the absence of active pollen transfer, random mating and gene flow, as an isolation barrier, built up for this species.

2.2.1 General morphology of plant

By habit, black pepper is a perennial woody climber which branches dichotomously showing dimorphic growth pattern. The main stem of orthotropic shoot has indefinite growth and produces clinging roots at each node and grows up straight the support. The branches of orthotropic shoots form sympodial, fruiting branches or the plagiotrops (Prasannakari Amma et al., 2001).

Broad, shiny green, pointed, petiole leaves are alternately arranged. Flowers are borne in the axils of ovate, fleshy bracts in long pendant spikes, single in nature and appear opposite to the leaves on the plagiotropic branches

(Parthasarathy et al., 2007). In general, the genus *Piper* is characterized by very small, sessile, white, highly reduced flowers closely packed to form inflorescence (Ravindran, 2000).

2.2.2 Productivity of black pepper in India

The productivity of Indian black pepper is very less, compared to that in other countries (Ravindran, 2000). Majority of the black pepper fields are cultivated with land races or with popular high yielding hybrid varieties like Panniyur-1 (George et al., 2005). India has varieties which can yield more than 3000 kg ha^{-1}. However, the average productivity is very less (320 kg/ha) compared to Malaysia (2000 kg/ha), Brazil (1571 kg/ha) and Indonasia (800 to2000 kg/ha) (Ravindran, 2000). Irrespective of the prevailing agroclimatic conditions, indiscriminate use of cultivars may be one of the several reasons for low productivity (Ravindran and Nirmal Babu, 1988). The hybridization programmes by many research stations have yielded improved varieties with higher productivity and quality (George et al., 2005).

2.2.3 Yield variations in black pepper

Variation for yield is prominent among black pepper cultivars (Ibrahim et al., 1986). Greater amount of variation exists for yield, compared to components of yield such as spike and berry characters (Ibrahim et al., 1985b; Ravindran and Nirmal Babu, 1994). Yield variations in black pepper plants raised from different planting materials are also reported (Pillai, 1977). Fruit yield largely depends on reproductive as well as vegetative characters. The reduction in source area influence the yield attributes and photosynthate accumulation (Mathai et al., 1989 and Koshay et al., 1989). Significant positive correlations were observed between yield and reproductive characteristics such as spike yield, fresh fruit yield, number of spikes per vine and number of under developed fruits per spike (Sujatha and Namboodiri, 1995).

Prasannakumari Amma et al. (2001) observed that the local varieties viz., Karimunda, Kaniyakkadan, Neelamundi and Mundi showed significant variation for spike length, number of berries per spike and dry weight of berries. The variety, Kaniyakkadan recorded the highest number of laterals, while Karimunda recorded the highest number of spikes. The number of spikes showed no significant difference among the cultivars. However, the highest number was recorded by Jeerakamunda, followed by Karimunda.

Panniyur-1 is one of the highest yielders. However, there are reports of higher yield by Karimunda and Jeerakamunda, and according to Ravindran and Nirmal Babu (1994), the better performance of Karimunda in yield may be due to its shade tolerant behaviour.

Mathai and Nair (1990) reported that dry matter distribution in fruits is low in low yielding cultivars and high in high yielding cultivar like Panniyur-1. Mathai (1986) observed that Panniyur-1 has more laterals, spikes and berries, has higher mean berry weight, higher rate of photosynthesis and translocation and also higher yield, compared to the other cultivars of black pepper.

Significant positive correlation between nitrogen content and fresh weight of spikes has also been reported. Investigations on the effect of various micro and macro nutrients on growth and development of spike have also been carried out (Stayan and Drev, 1979). According to Kumar and Sreedharan (1984) the flag leaf of pepper has significant role on spike characters. Partial and total removal of flag leaf significantly reduced spike length, number of fruits and fruit weight in Panniyur-1.

Pillai et al. (1987) reported that Panniyur-1 showed positive heterosis for length of spike, developed fruits, bisexual flowers per spike and yield. However,

the fruit characters were found to be intermediate. The trait, fruits per spike was more prone to seasonal variations. Spike length had less impact on this trait.

George et al. (1989) reported that in many plantations in the Kodagu district of Karnataka, more than 10 kg berries per standard was observed. However, some cultivars have also yielded as low as 880 g of black pepper.

2.2.4 Morphological variations

Most of the present-day Indian cultivars of black pepper are land races representing direct introduction from the wild (Ibrahim et al., 1984). Considerable variations exist among the landraces with respect to an array of plant morphological characters giving them the status of distinct plant types (Mathew et al., 2006).

Ravindran and Nirmal Babu (1996) identified seven Principal Components (PC) that accounted for almost all the morphological variance observed among the varieties. The principal component for classification consisted of spike length, peduncle length, spike orientation, and fruit shape. One of the PC's consisted of bracts, spike length index, spike shape and spike texture. These principal components can give insight into the nature of divergence among pepper species.

Among the 51 cultivars studied for spike characters by Ravindran (2000), the smallest spike was found in the cultivar Vokkalu with a spike length of 3.4 cm. Vokkalu is a common cultivar in Sagar area of Karnataka. The cultivar Kuthiravally recorded the highest mean spike length of 17.0 cm, followed by Poonjaramundu which recorded a mean spike length of 16.4 cm. The high yielder Panniyur-1 showed a mean spike length of 14.0 cm. The peduncle length ranges from 0.5 cm in Vokkalu to 2.1 cm in Karimkotta. Although major cultivars have straight spikes, Aimpiriyan, Kalluvally, Kuriyalmundi, Narayakkodi and

Kottanadan have shown spike twisting and spike curving characters due to closeness of the flower arrangement and the high fruit setting.

The dimorphic branches of black pepper exhibit minor variations in their morphological features, though the basic structure remains the same in both. Bract type is an important species delimitation character of South Indian cultivars. Black pepper has copular bract with decurrent base. The bracts in species *P. sugandhi* and *P. sugandhi var. brevipilis* are deeply cupular and shortly stipitate with adnate base (Ravindran and Nirmal Babu, 1994).

Ravindran (1991) and Ravindran et al. (1997) reported cluster analysis for 22 morphological characters, including spike length, peduncle length, spike orientation, spike texture, spike length index and spike shape. They studied 44 major cultivars and seven wild collections of *Piper nigrum*. The analysis lead to 11 clusters. Among the 51 cultivars, 28 formed one group. The cultivars Karimunda, Kuthiravally, Vadakkan, and Panniyur-1 formed unique groups. Distribution of only 23 cultivars over 10 different clusters indicated the significant morphological variation for spike characters among existing cultivars.

Principal component analysis by Ravindran et al. (1997) could identify eight prominent components which accounted for most of the morphological variations occurring among the black pepper cultivars and thus involved in their divergence. The factors leaf length- spike length ratio, spike length, peduncle length, fruit size and fruit shape were of prominence in the analysis. Similar studies by Ravindran (1991) also indicated that Karimunda differentiated from other varieties with respect to leaf anatomical characters; but not with respect to spike characters. The cultivar Kuthiravally could be differentiated by leaf and spike characters. The natural triploid, Vadakkan varied with respect to leaf morphology, leaf anatomy, spike and stomatal characters.

Ibrahim et al. (1985c; 1987; and 1988) studied genotypic and phenotypic variability, heritability and genetic advance in black pepper using 28 lines of hybrids and open pollinated progenies. Spike yield, followed by spike number, showed the highest phenotypic coefficient of variation. The lowest variability was observed in fruit weight. Number of spikes and spike yield were the characters more amenable for improvement, as they had the highest genotypic coefficient of variation. Heritability values varied from 28 to 81 per cent, the highest for fruit weight followed by spike length. Spike yield and spike number had low heritability, indicating that these characters are highly influenced by environment. Genetic advance was the highest with respect to spike yield, indicating that selection would be advantageous.

2.2.5 Genetic variations in *Piper nigrum*

The present day black pepper populations are discrete and isolated. They lack free gene flow. According to Ravindran et al. (1990), variations in such populations occur mainly by recombination and segregation, chance crossing and segregation. Variation due to chance mutation will remain fixed as a result of vegetative reproduction, isolation of discrete populational segments and the subsequent divergence of such units. Although black pepper is vegetatively propagated by cuttings, comparative genetic variability exists within open pollinated progenies of a few varieties (Ibrahim et al. 1986).

The genus *Piper* thus warrants the application of the more relevant genotypic marker assisted classification systems for genome analysis. Nazeem et al. (2003) utilized dominant markers such as RAPD and AFLP for the evaluation of relatedness among nine *Piper* species including *Piper nigrum, P. longum, P. colubrinum, P. chaba* and *P. arboretum.* The results identified high variability among the species.

RAPD profiles for three black pepper varieties and long pepper were developed using 15 random primers (IISR, 1996). Most of them gave positive differences between these two species. Identification and examination of the genetic similarity in cultivated *P. nigrum* varieties Irumaniyan, Karimunda, Panniyur-1, Aimpiriyan and *P. attenuatum* based on RAPD markers revealed a moderate degree of diversity (Kishore, 2005). RAPD markers were found useful for analysing the genetic relationship of selected *Piper* species of South India (Raji, 2005). Molecular characterization of thirteen landraces and nine advanced cultivars of *Piper nigrum* L. using RAPD markers showed variation among the samples (Pradeepkumar et al. 2001).

Nazeem et al. (2003) were successful in evaluating the genetic diversity among the nine important *Piper* species using RAPD. Renuka (2005) also found RAPD markers useful in analyzing the diversity among seven *Piper* species. Studies on variation in yield and growth performance of cuttings derived from top, middle and bottom nodal explants of five high yielding varieties viz. Panchami, Pournami, Panniyur-1, Panniyur-3 and Panniyur-5 revealed intraclonal variability (Manoj, 2005). Molecular and morphological characterization of seven black pepper lines (Sreedevi, 2005) showed uniformity with respect to morphology and RAPD banding pattern. Assessment of genetic fidelity of black pepper plants regenerated from somatic embryos using morphological characters and RAPD profiles showed 100 per cent uniformity among regenerated plants as well as with the original parent (Das, 2005). Ajith (1997) conducted marker aided studies for verifying genetic fidelity in micropropagated black pepper plants.

2.3 Flowering in Black Pepper

Flowering in black pepper occurs in the month of May-June (Anandaraj et al., 2005). Following the monsoon rains, vegetative bud differentiation occurs. Some reports shows that the *Piper* vines can be induced to flower by copious irrigation for about 20 days (KAU, 1978). The process of spike formation and

differentiation of florets occurs during July. During flowering, the shoot apical meristem elongates and transforms into an inflorescence meristem. Spikes originate, enclosed in a prophyll, and it takes three to four weeks for the full spike emergence. The main inflorescence, which generally grows indeterminately, gives rise to hundreds of floral meristems (Coen et al., 1994).

2.3.1 Inflorescence of black pepper

The black pepper inflorescence is a catkin (spike) which is filiform and pendulous in shape. Young ones are green, whitish green or light purple. Mature ones are green or pale yellow (Menachery, 1993). The colour of the inflorescence is purple in certain collections of *P. nigrum*, in other species the spikes are white or light green or green (Ravindran et al., 1992b).

Not much detailed information is available on inflorescence and flower development in black pepper. Tucker (1982) studied the inflorescence and flower development in *P. aduncum, P. amlgo* and *P. marginatum.* The spikes of black pepper and closely related species are either pendulous or erect. Based on shape, the spikes can be filiform, cylindrical or globose. According to Ravindran (1991) spikes are glabrous in most of the species, except in some collections of *P. nigrum* (*P. nigrum* var. *hirsutellosum*) and in *P. sugandhi* var. *brevipilis.* Spike length varies much. Peduncle is glabrous, bracts are oblong, decurrent, sessile with free upper margin, develop into a shallow cup in female spikes. Rachis and bracts are glabrous.

The reproductive transition in black pepper starts at shoot apex by forming the dome shaped apical meristem, leaf initials and axillary bud initials (Ravindran, 2000). The terminal buds of the plagiotropic branches produce the spike and growth is continued by axillary buds. The growing axillary bud pushes the spike aside and inflorescence appears to be leaf opposed (Ravindran et al., 1990). Based on shape the spikes of *Piper* can be filiform, cylindrical or globous. Spike length

varies from 1 to 18 cm. Studies by Tucker (1982) showed that the initial stage of inflorescence development has a convex apical meristem, subtended by vegetative leaf and bract. The inflorescence meristem appears to be terminal; so that the growth is sympodial. The apical meristem of the pepper inflorescence grows extensively in length, before any organs are formed.

The number of apical buds which eventually transforms into functional inflorescence varies among cultivars. In one particular year the figures were 20, 43 and 73 per cent in three cultivars viz., Panniyur-1, Karimunda and Kalluvally, respectively (CPCRI, 1979).

2.3.2 Flowers on spike

Flowers in black pepper arise in the axil of bracts (Tucker, 1982). Cells in the axils remain meristematic and the cells in the outer layer divide anticlinally. The continued anticlinal divisions lead to protuberance which further differentiates into flower bud.

In the genus *Piper*, male, female, and hermaphrodite forms exist. Most of the Indian *Piper* species has dioecious flowers, while Central and South American species are mostly monoecious (Tebbs, 1989). The wild forms of *P. nigrum* bear dioecious flowers, but most cultivated ones are bisexual (Ravindran et al., 2005). Male spikes, female spikes and bracts are strong factors in clustering and grouping, which separates *P. argyrophyllum, P. attenuatum* and *P. sugandhi* from the remaining species. *Piper* flowers are much reduced; however, they exhibit definite patterns of variation that have been important for the classification of the genus (Parthasarathy et al., 2006).

Flower composition of the spike varies among cultivars (Ravindran, 2000). Some are completely bisexual as in Karimunda, while in others varying

number of unisexual flowers occurs. In Panniyur-1, 97 per cent are bisexual flowers and the rest are pistillate.

2.3.3 Spike branching

Kellog (2000) proposed a generalised model for inflorescence development which explained the wide variety of inflorescence forms found in plants. The model states that meristem can produce either more meristems or a set of determinate floral organs on their flanks.

Spike branching in black pepper is very rare, though, considerable variation in spike length, floral composition, fruit number and size are reported (Ravindran, 2000). In cultivar Aimpiriyan, formation of rudimentary spikes on main spikes are also reported. The concept of spikelet matches to the proliferating spikes, where spikelet literally means "little spike" referring to its similarity to an indeterminate branching inflorescence, developing within the larger inflorescence (Ikedia et al., 2004).

Mutation in the floral meristem of black pepper could also result in branching of inflorescence. Scientists from Indian Institute of Spices Research have reported a new spike variant with hundred percent of its spikes, proliferating. The vegetative progenies observed to be true-to-type (Sasikumer et al., 2006).

The variety Aimpiriyan occasionally show rudimentary spike branching trait. Recently, cultivar "Kathirinmelkkathir" has been reported to have profuse spike branching character (Bavappa et al., 2007). However, the details regarding growth pattern and quality are not available.

2.4 Genes and flowering

Over the past decade several classes of genes involved in GA pathway, autonomous pathways, vernalization pathways, light dependent pathways are decoded which are the developmental switches from a vegetative meristem to a floral meristem (Fig.1) in both model plant systems and agronomic species (Ahearn et al., 2001). From the studies on floral development and floral meristem identity genes in *Arabidopsis thaliana*, *Antirrhinum majus*, cauliflower, maize, rice, etc. it is now known that most floral genes and meristem identity genes are highly conserved in the plant kingdom across species (Goto et al., 2001 and Jack, 2004).

The floral developmental pathway in *Arabidopsis thaliana* is composed of several interacting regulatory genes (Olsen et al., 2002), including the inflorescence architecture gene *TERMINAL FLOWER1* (*TFL1*), the floral meristem identity genes *LEAFY* (*LFY*), *APETALA1* (*AP1*), and *CAULIFLOWER* (*CAL*), and the floral organ identity genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) all of which have regulatory roles in maintaining inflorescence architecture (Fig. 2).

According to Yanofsky (1995) during floral transition, the shoot apical meristem adopts the fate of an inflorescence meristem, which is accompanied by the expression of the floral meristem identity genes within the inflorescence apex and at the flanking floral meristems that eventually generate flowers. More than 80 flowering time genes, floral-meristem identity genes and floral-organ identity genes have been identified (Levy and Dean, 1998). It appears that many of the genes, particularly flowering time genes, in all three pathways are mainly expressed in leaves. However, meristem-identity genes, such as *LFY, AP1, TFL1*, show higher expression in inflorescence and floral meristem (Colsanti et al., 1998).

2.4.1 Genes responsible for the formation of floral meristems

APETALA (*AP*) Gene family

According to Ferrandiz et al. (2000) *Arabidopsis thaliana* has *AP1*, *AP 2* and *AP3* genes, and three APETALA like genes, *AP1*, CAULIFLOWER (*CAL*) and FRUITFULL (*FUL*). All three of these genes act redundantly in the transition from vegetative to inflorescence development by specifying the identity of the floral meristem that gives rise to flowers. The genes *LFY, AP1, CAL* and *FUL* are closely related MADS-box transcription factors which play major role in specifying cell fates in the inflorescence and the floral meristems. *LFY* encodes a novel transcription factor that is very specific to plants (Weigel et al., 1992).

LEAFY (*LFY*)

LEAFY (*LFY*) in *Arabidopsis thaliana* (Weigel et al., 1992) is a floral meristem identity gene that signals the transition from an indeterminate inflorescence meristem to a determinate floral meristem (Mandel and Yanofsky, 1995). *LFY* is expressed in both vegetative and reproductive tissues, and low expression during the vegetative phase prevents premature flowering. Upregulation of *LFY* serves as a reliable indicator of the transition to a floral meristem from an inflorescence meristem with the associated cessation of further shoot elongation.

According to William et al. (2004) the spatial and temporal expression of *LFY* plays a central role in the degree of inflorescence branching. The role of *LFY* homologues in determining meristem fate is well established in several plant species, and its interactions with both upstream and downstream genes involved in regulation and signal transduction in meristems and floral organs has been demonstrated.

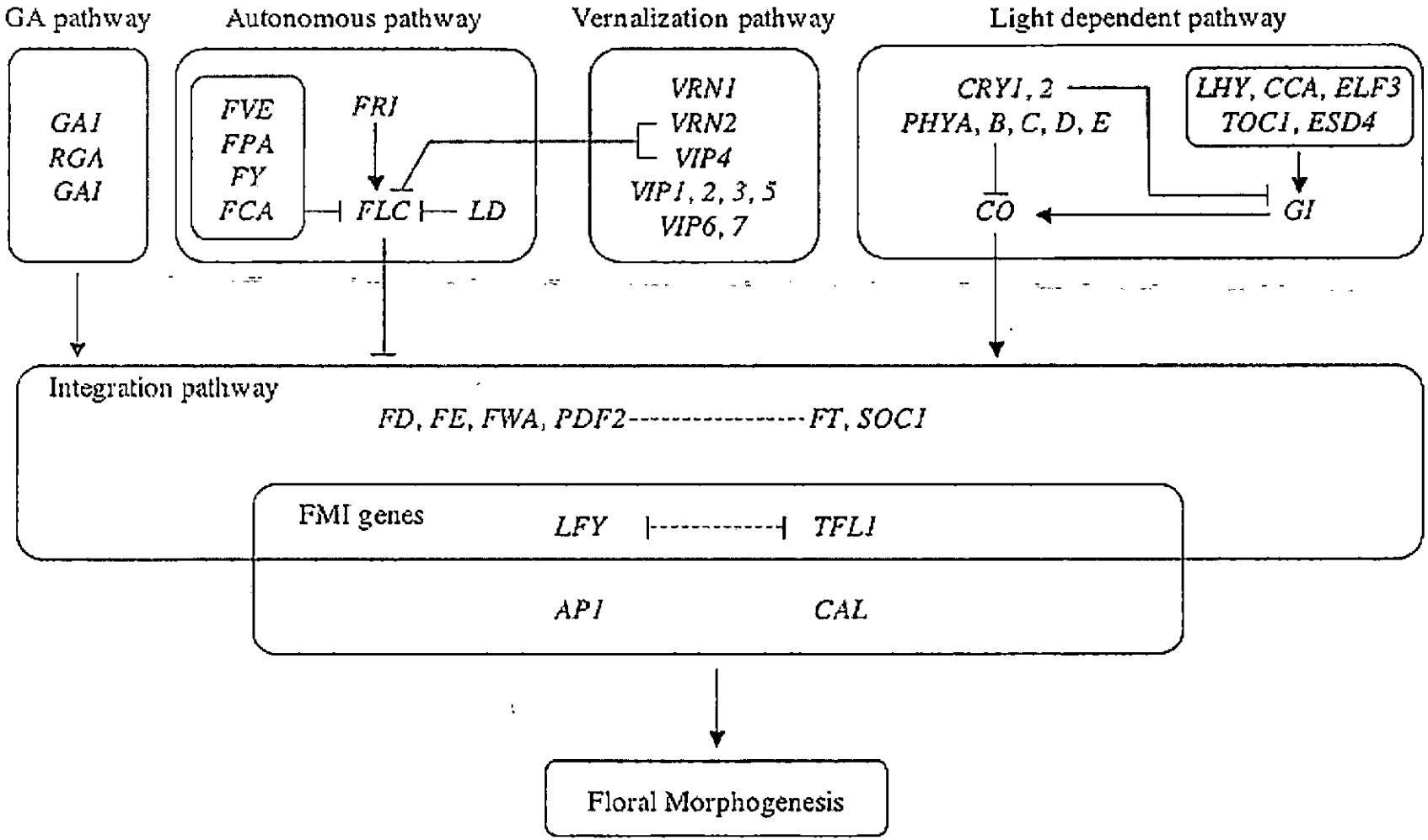


Fig. 1. The genetic pathways of flowering in *Arabidopsis thaliana* showing integration patways of regulation. Positive (arrows) and negative (T-lines) interactions are described. Dotted lines show undescribed interaction. (Komeda, 2004)

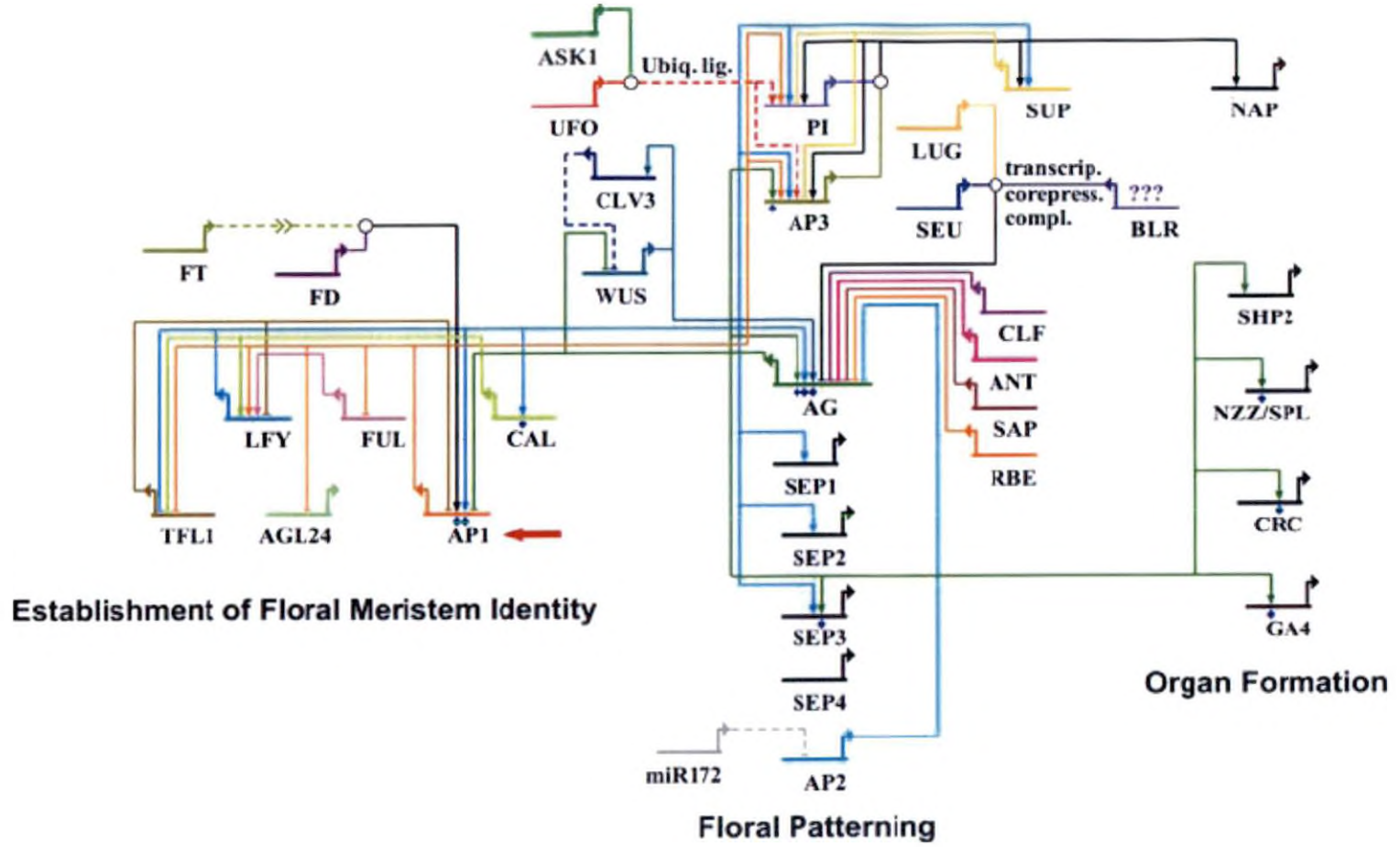


Fig. 2. Gene Regulatory Network Controlling Early Flower Development in *Arabidopsis thaliana* (Wellmer et al., 2006)

Bradley et al. (1997) stated that *TFL1* negatively regulates *LFY* expression during the vegetative phase. The indeterminate architecture of the arabidopsis inflorescence is the result of the interplay between genes that confer floral identity, including, *LFY*, and genes that promote shoot identity, which includes *TFL1*. *TFL1* represses the *LFY* expression in shoots and *LFY* represses *TFL1* expression in flowers.

The genes controlling the most important taxonomic features of the inflorescence structure have been identified not only in *A. thaliana* but also in other flowering plants. In a plant from the family Scrophulariaceae, snapdragon (*Antirrhinum majus*), the formation of floral meristems is controlled by a gene FLORICAULA *(FLO)* orthologus to *LFY* gene, whose protein product has a high homology (70 per cent) to protein LFY and which, like gene *LFY*, is present in the genome in single copy (Coen and Nugget, 1994).

CAULIFLOWER (*CAL*)

The gene *CAULIFLOWER (CAL)* partially duplicates the function of gene *AP1*. Mutants *cal* have normal phenotype, but in double mutants *ap1 cal*, floral meristems are transformed into the inflorescence meristems and the inflorescence resembled that of cauliflower (Bowman et al., 1993). Gene *CAL* encodes a MADS-containing transcription factor and has a high homology with gene *AP1* (Kempin et al., 1995).

2.4.2 Genes suppressing the terminal flower development

TFL: The Inflorescence Architecture gene

TFL1 gene is a part of small gene family that regulates the onset of flowering and meristem identity in *Arabidopsis thaliana*. *TFL1* is expressed at high levels in the inflorescence apex. In wild plants, transformation of the apical meristems of the main and laterals shoots into floral meristems is prevented by

genes *TERMINAL FLOWER -1 (TFL1)* and *TERMINAL FLOWER-2 (TFL-2)* (Ohshima et al., 1997 and Larsson et al., 1998). *TFL1* acts to maintain the inflorescence meristem identity causing continued inflorescence (meristem indeterminacy) elongation by preventing *LFY* expression in the meristem cells (Liljegren et al., 1999).

Overexpression of *TFL1* in *Arabidopsis thaliana* greatly extends both vegetative and reproductive phases, resulting in larger plants with heavily branched inflorescences (Ratcliffe et al., 1998).

The *TFL1* gene encodes a putative phosphatidylethanolamine-binding and nucleotide-binding protein (Ohshima, et al., 1997). The shoot apical meristem or inflorescence meristem remains undetermined, and to maintain this state the *TFL1* and *TFL-2* genes are essentially required. The *TFL1* gene is strongly expressed in a group of cells just below the apical dome of the inflorescence in accordance with a role in this meristem (Bradley et al., 1997).

2.4.2.1 *TFL* Homologs and orthologs

Members of *TFL/SP/CEN* family control inflorescence determinacy and architecture (Shannon and Meeks-Wagner, 1991). *LpTFL1*, a terminal flower like-1 gene from the perennial ryegrass, *Lolium perenne* has been implicated as functioning in floral transition and axillary meristem identity (Jensen et al., 2001).

SELF-PRUNING (*SP*) protein in Tomato is a functional homologue of *TFL* of *Arabidopsis thaliana* (Pnueli et al., 1998) and *TFL1* ortholog CENTRORADIALIS (*CEN*) from snapdragon (Banfield and Brady, 2000). *CET* in tobacco is a *CEN* homolog of snapdragon (Amaya et al., 1999) have been identified as a group of genes that control inflorescence architecture and length of the vegetative phase.

Over-expression of *RCN1* and *RCN2*, *TFL1/CEN* orthologs in rice orthologs resulted in a delayed transition to the reproductive phase and more branched panicles due to a delayed switch from the branch shoot to floral meristem (Zhu et al., 2003).

In pea, an *AP1/CAL* ortholog has been shown to correspond to the inflorescence identity gene PROLIFERATING INFLORESCENCE MERISTEM (*PIM*) (Taylor et al., 2002).

2.5 Role of Regulatory Proteins and Transcription Factors

Morphological novelty occurs through the creation or modification of developmental pathways (Petersen et al., 2006). Theses pathways involve multiple interacting proteins, many of which are transcription factors. In plants, some of the best studied transcription factors are members of the MIKC-type MADS-box family.

Most of the floral homeotic genes are members of the MADS-box gene family (Schwarz-Sommer et al., 1990). MADS-box genes encode proteins containing a highly conserved 56 amino acid MADS domain which is involved in DNA binding. This protein occurs in a diverse group of eukaryotic organisms including yeasts, mammals, insects, amphibians and plants (Shore and Sharrocks, 1995).

MADS-box genes function at many developmental stages. However, they are not limited to floral development, and are involved in diverse roles within various organs, including fruit (Boss et al., 2001), tubers, leaves (Brunner et al., 2000) and embryonic tissues and roots (Perry et al.,1999); although, they are best characterized for their role in inflorescence and floral development (De Folter et al., 2005). Several members of the MADS-box family proteins are important regulators of inflorescence meristem and floral organ identity genes (Theissen et

al., 2000). Many MADS-box genes belonging to the APETALA/ SEPALLATA group have defined roles in the early developmental decisions affecting the fate of inflorescence and floral meristem.

These MADS-box proteins share a highly-conserved DNA binding domain (the MADS domain) at or near the N-terminus, a less conserved Intervening (I) domain, a keratin like (K) domain, and finally a highly variable C terminus (Riechmann et al., 1996).

The floral meristem identity genes include LEAFY (*LFY*) (Schultz and Haughn, 1991), APETALA1 (*AP1*) (Irish and Sussex, 1990), CAULIFLOWER (*CAL*) (Bowman et al., 1993) and APETALA2 (*AP2*) (Irish and Sussex, 1990) all of which appear to encode transcription factors. These genes promote determinate floral development in lateral primordial, while the antagonist TERMINAL FLOWER (*TFL*) promotes maintenance of inflorescence meristem identity in the indeterminate shoot apex (Shannon and Meeks-Wagner, 1991).

Organ identity in the flower is instructed by the overlapping expression patterns of floral homeotic genes. These genes encode members of a family of transcription factors called MADS box genes, which share homology with transcription factors in yeast and animals (Jarvis et al., 1989).

In *Arabidopsis thaliana* subclass of MADS-box genes, the APETALA1/ FRUITFUL (*AP1/FUL*) genes are involved in meristem identity, and floral organ identity, establishing these genes as potential candidate for the evolution of inflorescence form in other angiosperms. Studies by Kellogg (2000) showed that grasses have more MADS box genes than *Arabidopsis* and other eudicots, allowing many more possibilities for protein interactions.

2.6 The *TFL1* and *FT* family proteins

The TFL/FT-like protein is also a PEBP, sharing 50 per cent identity and 70 per cent sequence similarity with TFL, and 48 per cent identity and 64 per cent similarity with FT. Interestingly, the antagonistic proteins TFL and FT share 56 per cent identity and 74 per cent sequence similarity (Alvarez et al., 1992). Given that TFL inhibits the vegetative to reproductive transition in the shoot apical meristem and FT promotes this transition, it is difficult to predict exactly how the TFL/FT-like protein is influencing the decision to flower (Pnueli et al., 1998).

The predicted polypeptides encoded by the Arabidopsis *FT* and *TFL1* genes are 175 and 177 amino acids long, respectively, with only 39 residues involving neoconservative changes, including substitutions and insertions/deletions. Each gene has four exons, with conserved exon/intron boundaries (Ezhova and Sklyarova, 2001).

2.6.1 The Flowering Locus T (FT) gene

FT is a floral promoter that takes part in the photoperiodic pathway (Kardailsky et al., 1999 and Kobayashi et al., 1999). According to Bamnolker and Samach (2005) the transition to flowering involves major changes in the shoot apical meristem and in the fate of existing leaf primordia. Transcripts of the *Arabidopsis thaliana* flowering-promoting gene FLOWERING LOCUS T (*FT*) are present in leaf tissue but can also promote flowering when artificially introduced into the meristem. *FT* may normally act in the leaf and/or the meristem, initiating or constituting a mobile flower-promoting signal. Studies by Pnueli et al. (2001) showed that the gene *FT* encodes a 20 kD protein which belonging to the CETS (*CEN, TFL1, SP*) family proteins which are key players in floral meristem fate and overexpression of *FT* causes early flowering.

The FT gene plays a central role in integrating flowering signals in *Arabidopsis thaliana* because its expression is regulated antagonistically by the photoperiod and vernalization pathways. *FT* belongs to a family of six genes characterized by a phosphatidylethanolamine-binding protein (PEBP) domain. In rice, 19 PEBP genes were previously described, 13 of which are *FT*-like genes (Faure et al., 2007). FT transcripts are expressed in leaves, but can travel through the vascular system to the shoot apex and interact with a bZIP transcription factor, FD, to activate floral identity genes such as APETALA1 (*AP1*) and promote flowering under long days (Abe et al., 2005)

The function of several genes involved in photoperiod responsiveness is conserved between *Arabidopsis thaliana* and *Oryza sativa*, and suggesting that the difference between long-day and short-day responsive plants result from a different regulatory interaction between two genes, CONSTANS (*CO*) and FLOWERING LOCUS T (Hayama et al., 2003).

FT is a key target and integrator of many flowering pathways, and induction of *FT* expression leads to activation of flowering. In contrast, induction of TFL1 results in a suppression of flowering (Hanzawa et al., 2005). In *Arabidopsis thaliana FT* is a member of a six-gene family that includes another important flowering-related gene, *TFL1*. Unlike FT, TFL1 acts to delay the transition to flowering and also promotes the indeterminacy of the primary inflorescence (Ratcliffe et al., 1998).

2.7 Mutational studies

Recently a number of mutants affecting the shoot apical meristem have been described. This review focuses on how such mutants have contributed conversion of floral meristem to inflorescence spikelet meristem. Several mutations in *A. thaliana* either directly or pleotropically effect this normal process of axillary meristem formation (Hecht et al., 2005).

Many mutations that affect inflorescence development have been characterized and several of the genes responsible for these mutations have been identified (McSteen et al., 2000). Mutations in the floral meristem identity genes result in varying degrees of transformation of flowers into inflorescence shoots.

Additionally, it has been demonstrated that constitutive expression of either LFY or *AP1* or *CAL* is sufficient to convert shoots into flowers, similar to the phenotype observed in *tfl* mutants (Weigel and Nilsson, 1995).

2.7.1 Mutational studies in *AP1*, *CAL* and *FUL* relatives

Carpenter et al. (1995) showed that mutations in the related Arabidopsis genes *AP1*, *CAL* and *FUL* cause loss of floral meristem identity and proliferation of inflorescence meristem in positions normally occupied by developing flowers. Strengthening the results, the mutational experiments by Ferrandiz et al. (2000) in both *CAL* and *AP1* of *Arabidopsis thaliana* resulted in the proliferation of inflorescence meristem instead of flower.

Kempin et al. (1995) noticed that more inflorescence meristems proliferate when AP1 mutations are combined with mutations in paralogous genes CAULIFLOWER (*CAL*) and *FUL*. He suggested that all the three genes thus act together during inflorescence development to specify the identity of inflorescence meristem that gives rise to flowers.

2.7.2 Mutation in *TFL1*

Mutant *tfl* plants show a phenotype similar to plants over expressing *LFY*. Mutations in *TFL1* resulted in early flowering, replacement of coflorescences by flowers, and determinate growth of the apical meristem, which develops into a flower (Meeks-Wagner, 1991). The *tfl* mutation showed ectopic expression of

LFY and *API* in the apical meristem (Blazquez et al., 1998). Therefore, it appears that the *tfl1* mutant fails in negatively regulating *LFY* and *API*, thereby promoting early flowering with the formation of a terminal flower. It is also found that terminal flower 1 (*tfl1*) mutants is characterized by reduced branching due to secondary shoot terminating in a flower (Ohshima et al., 1997).

2.7.3 Mutations in *LFY*

The key role in the formation of floral meristems in *A. thaliana* is played by gene *LFY* which is present in the genome in one copy and encoding the transcription activator that has no homologies with any known protein of animals and microorganism (Olsen et al., 2002). Mutations in LFY results in an increase in the number of secondary inflorescence and transformation of flowers into shoot-like structures (Weigel et al., 1992).

2.8 Role of Plant Hormones and Flowering

One of the phytohormones, Gibberellins, was shown to act in flowering time control. Application of GA3 or constitutive activation of the GA signalling pathway promotes flowering (Jacobson and Olszewski, 1993). In contrast, blocking GA signalling or biosynthesis results in delay in flowering (Wilson and Somerville, 1995). *LFY*, a major floral meristem identity gene is also an effecter of the gibberellins- dependent pathway. Exogenous application of gibberellins can up-regulate the expression of the gibberellins biosynthesis mutants (Blazquez et al., 1998).

Elucidation of genetic control mechanisms in angiosperm flower development has provided new prospects for plant improvement. The identification and characterization of floral organ identity genes, first from *Arabidopsis thalianus* and *Antirrhinum majus*, led to the ABC model of angiosperm flower development (Bradley et al., 1996)

Biological and mechanical systems for incorporating novel genes into diverse organisms have already greatly impacted agricultural crops through transgenic expression of genes conferring beneficial traits. The biotechnological tools, DNA fingerprinting technologies such as restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLP) have increased our ability to assess genetic diversity, that allowed breeders to take full advantage of intra or interspecies genetic potential. Furthermore, the ability, through genetic engineering, to reach beyond species or even family levels to different kingdoms for genetic variation offers seemingly endless potential for developing novel traits in plants. In this aspect, isolation and characterization of gene pool which are associated with spike branching in black pepper has utmost importance, as spike branching is a quantitative trait in *Piper nigrum*. In that order, PCR based methods are applied to isolate *TFL1* and *FT* homologs in black pepper.

2.9 Primer designing

Primer design is the largest variable in PCR applications and is an important factor, determining the result of PCR reactions. Innis et al. (1990) suggested a set of rules for primer sequence design which include, length of primer (17-28 bases), G+C content (50-60 per cent), the presence of G or C, or CG or GC at 3' end , and a preferred melting temperature between 55-80°C. Primer 3 software designs primers for PCR reactions, according to the conditions specified by the user (http//www.fokker.wi.mit.edu/primer3/). Primer 3.0 considers conditions like melting temperature, concentration of various solutions in PCR reactions, primer bending and folding, and many other conditions when attempting to choose the optimal pair of primers for a reaction. These factors are essential to ensure that a primer is able to bind to a template and initiate extension by the ability of a primer to bind to a single location within the initial pool of DNA (Boutros and Okey, 2004).

2.10 Polymerase Chain Reaction

Since its origin in 1986 (Mullis et al., 1986) polymerase chain reaction has become an essential part of molecular biology. Don et al. (1991) did touch down PCR to overcome the problems with melting temperature differences between primers pair, in which annealing temperature was lowered by 0.5° per cycle during first few cycles. SPCR is one of the program that can help to choose a PCR primer pair giving the least possible non target products (Coa et al., 2005). Its algorithm is based on the hypothesis that the annealing temperature of a primer to a template is an information transfer process. *In situ* PCR (isPCR) is designed for predicting possible PCR products that two primers could produce (www.Soe.ucsc.edu/kent.scr/unzipped/isPcr).

2.11 RAPD in detection of genetic variability

RAPD technique, to investigate genetic variability was found to be efficient and reliable (Brown et al., 1993 and Munthali et al., 1996). According to Nazeem et al. (2003) RAPD technique can be successfully used to evaluate the genetic diversity among the *Piper* species. Renuka (2005) also found RAPD markers useful in analyzing the diversity among *Piper* species. Studies on variation in yield and growth performance of cuttings derived from top, middle and bottom nodal explants of five high yielding varieties viz. Panchami, Pournami, Panniyur-1, Panniyur-3 and Panniyur-5 revealed intraclonal variability (Manoj, 2005).

RAPD can be used to detect genetic variation at the intra as well as interspecific level (Abo-elwafa et al., 1995). RAPD markers were found to be very useful in assessing the genetic variability in vegetatively propagated crops. Morphological variations observed on these crops may or may not result in variations on molecular analysis.

According to Palacios and Gonzales (1997) no genetic variability was observed in the rare and endangered *Limonium cavanillesii* using RAPD markers and this was the lowest level of genetic variation detected in plants using RAPD markers. RAPD analysis of *Allium ampeloprasum* var. *babingtonii* revealed no polymorphism suggesting that all sampled individuals are part of a single clone (Treu et al., 2001). Vega et al. (2001) reported one of the lowest levels of polymorphism 0.8 per cent detected for a plant species by RAPD analysis was for *Agave tequilana* var *azul* plants. Seven population of *Alternanthera philoxeroides*, a clonally propagated aquatic plant, on molecular analysis using RAPD and ISSR markers showed that its genetic diversity is extremely low (Wang et al., 2005). According to Li et al. (2006) RAPD and ISSR markers used to analyze genetic structure of six populations of invasive plant *Eichhornia crassipes* indicate that the genetic diversity is extremely low.

2.12 Crop improvement in black pepper

As India is the primary centre of diversity of *P. nigrum*, the indigenous genetic resources are reservoirs of useful genes for plant improvement programmes (Pradeepkumar et al., 2001). Majority of the present-day Indian black pepper cultivars, numbering about 100, are land races representing direct introduction from the wild (Ibrahim et al., 1984) and most of the varieties released for cultivation are clonal selections from the existing land races (George et al., 2005).

In black pepper combining yield and quality parameters has been a perennial goal for improvement programmes (Pradeepkumar et al., 2003). One of the major research aims of different institutions is to develop high yielding, good quality varieties of black pepper with tolerance to diseases and pests (Saji and Sasikumar, 2006). Although the development of improved cultivars through hybridization has made a major contribution to increased productivity and quality of plants in different crop plants (George et al., 2005), the productivity of Indian black pepper (368 kg ha^{-1}) is less (Farm guide, 2008). More emphasis has to be

given in improvement programmes for quality parameters like piperine, oleoresin and oil content rather than bulk pepper as the export of value added products is now gaining importance.

3. MATERIALS AND METHODS

The study entitled "Molecular analysis of spike branching observed in black pepper (*Piper nigrum* L.) type from Idukki" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2006-2008. Details regarding the experimental materials used and methodology adopted for various experiments are presented below.

3.1 Plant Sample Collection

Plant samples of black pepper showing spike branching (Pepper Thekkan – as named by the farmer) were collected from an innovative farmer, Thomas.T.T. of Kanjiar village, near Kakkatikada, Kattappana taluk, Idukki district. Plant samples of seven varieties and cultivars from the same farm viz., Karimunda, Kumbakal Kodi, Arayan Mundi, Vella Mundi, Naraya Kodi, Panniyur 2 and Panniyur 4 were also collected. Semi mature leaves were collected and transported to laboratory in ice boxes. Samples were then thoroughly washed and rinsed with distilled water and stored at -80 °C (Sanyo Ultra Low deep freezer).

3.2 Molecular analysis

Homology of the primers, designed based on the genes Terminal Flower 1 (*TFL1*) and Flowering Locus T (*FT*), with the DNA samples of the eight black pepper varieties was analysed, using PCR technique. Also, the samples were subjected to RAPD analysis to determine the genetic variability and closest relative of spike branching black pepper in the farm of the farmer.

3.2.1 Identification and characterisation of genes *TFL1 and FT*

Many molecular genetic laboratory methods are used for the identification and characterisation of genes. Polymerase chain reaction, cloning, restriction analysis, gene specific oligonucleotide hybridization and DNA sequencing are frequently used for this purpose. Amplification and sequencing of the genes using gene specific primers; which are made using genomic sequences from online databases is one of the important approaches. However, there are no reported sequences available for black pepper in GenBank database. In the present study, based on the assumption that, the meristem identity gene *TFL1* and flowering gene *FT* are conserved across species, the primers were designed using gene sequences of *Arabidopsis thaliana* and multiple sequence alignment for homologus genes of other species.

3.2.2 DNA isolation

Modified C-TAB method (Doyle & Doyle, 1987) developed by Kalisz Lab (http://www.pitt.edu/Kaliszlab/Protocols/DNAExtraction.doc) was used for genomic DNA isolation of all eight black pepper samples. For five milliliter of extraction buffer (2% w/v C-TAB; 3 M NaCl; 100 mM Tris-HCl of pH 8, 0.5M EDTA) 200 mg (4% w/v) of Poly Vinyl Pyrrolidone (PVP) was added prior to DNA extraction. The PVP was dissolved completely by warming the buffer, using a water bath. Thereafter, 25 µl (0.5% v/v) of β-mercaptoethanol was added to the extraction buffer, mixed well and kept warm in water bath. Next, the deep frozen leaves were washed and dried using sterile blotting papers. One gram of leaf sample was chopped and transferred to a dry, sterile mortar. Chopped material was ground well to a fine powder using liquid nitrogen. Warm extraction buffer was added immediately and the mixture was homogenized. The extract was then subjected to incubation at 55 to 60 ^{0}C for sixty minutes with intermittent shaking. The tubes were then brought back to room temperature. The mixture was centrifuged at 10,000 rpm for 8 minutes at 4°C and the supernatant was collected. For each 500µl of supernatant, 250 µl of phenol:

chloroform: isoamyl alcohol (25:24:1) was added and slowly mixed by gentle inversions for 2-3 minutes and centrifuged at 7000 rpm for 7 minutes. Then, the supernatant was extracted twice with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 7000 rpm. The DNA from the supernatant was then precipitated with chilled 100 per cent ethanol and stored overnight at -20^{0}C. The precipitate was then centrifuged at 14,000 rpm and washed twice with 70 per cent ethanol and the pellet was air dried for 15 minutes. Thereafter, the pellet was slowly dissolved in 60µl of TE buffer (10 mM Tris-HCl (pH 8.0), 1mM EDTA) and stored at -20^{0}C (Rotek deep freezer).

3.2.3 Spectrophotometric analysis

Spectrophotometric analysis of the extracted DNA samples was made for determining quality and quantity of DNA. Spectronic Genesis 5 spectrophotometer with deuterium lamp as UV source was used for measuring absorbance measurements at 260nm and 280nm. Spectrophotometer was calibrated for sterile distilled water which was used as blank further. Optical density of 5 µl of DNA dissolved in 3 ml of sterile distilled water was measured.

3.2.3.1 Quantification of DNA

An Optical Density value of 1.0 at 260 nm indicates the presence of 50µg of double stranded DNA in an ml of solution. So the quantity of the DNA present in the extracted sample was estimated by employing the following formula:

$$\text{Amount of DNA } (\mu g/g) = A_{260} \times 50 \times \text{dilution factor}$$

(where, A_{260} is absorbance value at 260 nm)

3.2.3.2 Purity of DNA

Although it is possible to estimate concentration of solution of nucleic acid and oligonucleotides by measuring their absorption at single wavelength (260 nm), the absorbance of sample should be measured at several wavelengths since ratio of absorbance at 260 nm to the absorbance at other wavelength is a good indicatior of purity of preparation. Absorption at 280 nm indicates presence of protein, because aromatic amino acids absorb strongly at 280 nm. So, the quality of the DNA was easily ascertained by the ratio of O.D. for the sample recorded at both 260 nm and 280 nm. A value ranging from 1.79 to 1.90 is good indicator of reduced contamination from proteins. Samples showing variation from the above values were subjected to DNA purification by chloroform-isoamyl treatment.

3.2.3.3 Agarose Gel Electrophoresis

Both Genomic DNA and PCR amplicons were confirmed by horizontal gel electrophoresis using the unit supplied by the Bangalore Genei. Gel was run at 5 V/cm. Electrophoresis of genomic DNA was done on 0.8 % Low EEO agarose made of 1X TAE buffer, whereas for PCR amplicons 1.4 per cent agarose gel was used. The DNA samples were mixed with required volume of gel loading buffer comprising 0.25 per cent (w/v) of bromophenol blue: 30 per cent glycerol: 70 per cent sterile water. Each well was loaded with 13µl of sample and run for about one hour. After electrophoresis, gel was visualized using BIORAD gel documentation unit and the data was recorded using 'Quantity One software'.

3.3 Primer Designing

PCR technique is an essential and ubiquitous tool in genetics and molecular biology. The primers, not more than 25 nucleotides length are used to determine the DNA fragment to be amplified by the PCR process. Many primer designing tools are

available online; one among them is Primer 3.0 which is widely used. Various parameters can be adjusted on the primer designer form to specify the primer properties. Specifications of primers and different primers designed using Primer 3.0 tool for the genes *TFL1* and *FT* for sequences from *Arabidopsis thaliana* and homologus sequences of other species using multiple sequence alignment program - Clustal W (http://align.genome.jp) are described below.

Primers TFL1-F1 & TFL1-R1

Forward and reverse primers were designed using Primer 3.0 software for the fourth exonic sequence which is the longest and conserved. 1172 bp DNA sequence (c1024640-1025811) of the gene *TFL1* of locus (NC_003076.4) from chromosome 5 of the model plant *Arabidopsis thaliana* was used for the purpose. Factors were set to achieve the priming position in fourth exonic sequence (833 bp -1172 bp).

Primers FT-F1 & FT-R1

Studies have indicated that gene *FT* has conserved fourth exon same as *TFL1*. Primers were designed for the 2506 bp DNA sequence of the gene FT (GI: 42592260) belonging to the locus NC_003070.5 of chromosome 1 in *Arabidopsis thaliana*. Factors were set to achieve the primer sequence in fourth exonic sequence (2039 bp - 2507 bp) which is the biggest.

Primers TFL1-F2 & TFL1-R2

First primer pair was specifically made on exonic sequence without consideration of intronic gaps. However, Primers (TFL1-F2 & TFL1-R2) were designed using the conserved sequences obtained using the multiple sequence alignment program (Clustal W) for the homologus sequences for the gene *TFL1*. GI:

30680238(*Arabidopsis thaliana*), GI: 187761640 (*Malus domestica*), GI: 149250805 (*Picea abies*), GI: 82791228 (*Aquilegia formosa*), GI: 83628279 (*Populus trichocarpa*) sequences were used to find conserved sequence. Position of left primer was fixed and reverse primer was designed using Primer 3.0 tool.

Primers FT-F2 & FT-R2

Multiple sequence alignment was done for the homologus sequences of the gene *FT* using Clustal W program. Conserved sequence of GI: 30697201 (*Arabidopsis thaliana*), GI: 157429034 (*Chenopodium rubrum*), GI: 164457874 (*Citrus unshiu*) were used to fix the left primer sequence. Primers were generated using Primer 3.0 software.

3.3.1 Primer Analysis

Primer sequences need to be chosen to uniquely select for a region of DNA, avoiding the possibility of mishybridization to a similar sequence nearby. A commonly used method is BLAST search whereby all the possible regions to which a primer may bind can be seen. Primers were analyzed using BLAST N (Atschul et al., 1997) similarity matrix to find binding region and primer specificity. Oligonucleotide Properties Calculation program (www.Basic.northwester.edu/bitools/oligocalc.html) was used to find the 3' complementarity, hairpin formation and self annealing properties of designed primer.

3.4 PCR using the designed primers

The Genomic DNA of black pepper was amplified using specific primers designed for the genes *TFL1* and *FT*. A 20µl of reaction mixture was prepared in 0.2 ml flat capped eppendorf PCR tubes with the reaction mixture constituents as

mentioned below. PCR was done using Eppendorf Mastercycler and PTC-100 thermocyclers.

Component	Volume (µl)
Genomic DNA sample (20 ng/µl)	2.0
10x assay buffer A	3.0
Taq DNA Polymerase (3U/ µl)	0.5
dNTP mix(10 mM)	2.0
10 pM Forward Primer	1.0
10 pM Reverse Primer	1.0
Sterile distilled water	10.5
Total Volume	20.0

Thermocycler program was set for 44 PCR cycles with initial denaturation at 94°C for 4 minutes followed by repeated cycles of denaturation at 94°C for 1minute, annealing at 51°C for 1 minute and extension at 72°C for 2 minutes. Final extension was done for 4 minutes at 72°C. Varied annealing temperatures were screened. Control was set without DNA to distinguish the target product from non target products and primer dimers.

3.5 RAPD Analysis

Many RAPD studies have been reported at the molecular level with the genus *Piper*. RAPD analysis is one of the necessary techniques in order to begin an extensive molecular taxonomic study on spike branching black pepper. Furthermore, no reports on molecular comparisons of spike branching pepper with landraces, local cultivars and improved varieties of black pepper are available.

The DNA samples of 7 non-spike branching and single spike branching black pepper type were screened with many arbitrarily designed decamer primers supplied by Operon Inc., CA, USA. Out of all, 8 primers which produced good banding pattern were selected for DNA amplification of collected cultivars/varieties.

The components of the reaction mixture were optimized and a typical 20µl PCR mixture comprised of 30 ng genomic DNA; 2.5 µl 10X assay buffer; 3 µl dNTP mix (4mM each); 1 pM primer. PCR reaction was carried out in a Programmable Thermal Cycler (PTC 100, M J Research, Inc). Pre-denaturation was done at 94°C for about 4 minutes, and then 40 cycles of amplification were set at 94°C for 1 minute, 35°C for 45 seconds, 72°C for 1minute and 30 seconds. Final extension was set at 72°C for 5 minutes. The amplified products were separated on 1.4 per cent Agarose gel in 1x TAE buffer.

3.5.1 RAPD Data Analysis

The PCR products were scored for the presence (+) or absence (-) of bands on electrophorated gels. The numbers of monomorphic and polymorphic bands were recorded. Thus, banding pattern of all eight primers for eight samples were scored as 1 and 0 in Microsoft XL sheet and subjected for statistical analysis.

The genetic similarity matrix was constructed using Jaccard's similarity coefficient values and this matrix was subjected to an unweighted pair-group method for arithmetic average analysis (UPGMA) to generate dendrogram using average linkage procedure. All these computations were carried out using NTSYS-pc version 2.02 (Rohlf, 1998) software and the dendrogram constructed was used to asses the association and distance between the varieties under study.

Results

4. RESULTS

The results of the study entitled "Molecular analysis of spike branching observed in black pepper (*Piper nigrum* L.) type from Idukki", carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2006-2008 are presented below.

4.1 Morphological studies

Observations on different morphological traits were made on the black pepper type showing spike branching. Height of plant (m), lateral branch pattern, leaf length (cm), leaf lamina shape, petiole length (cm), nodes per lateral branch, peduncle length (cm) and spike characters were recorded.

4.1.1 General morphology of plant

The height of plant varied from 3 to 5 meters. Plagiotropic branches were protruding out prominently from main shoot axis (Plate 1). Shoot tip colour was light green. Lateral branches were semi erect and the average branch length was more than 30 cm, consisting of 7 to 12 nodes per lateral branch.

4.1.2 Leaf characters

Leaf length was medium ranging from 11 to 15 cm and average leaf width was 7 cm. Leaf petiole length varied from 1 to 2.5 cm. Leaf lamina was ovate elliptic in shape with the widest axis at the midpoint of the leaf. Leaf base shape was acute, with margin straight to convex, forming a terminal angle of 40 to 50 degrees. Leaf margin was smooth without any indentations or incisions on margins.

4.1.3 Spike characters

Young spikes were greenish yellow in colour and did not show any branching, but almost all matured spikes were profusely branching (Plate 2).

Branching spikes had peculiar protruding bracts, compared to normal ones (Plate 3). Peduncle length was 1.3 to 2.1 cm. Wide variation in spike length was also observed, ranging from 9.4 cm to 18.6 cm. Although main spikes were branching irregularly, they showed complete indeterminate growth status. Seed setting was also irregular and loose on branched spikes and number of berries per spike varied from 60 to 240. More than 4 spikes per lateral branches were observed. Number of spike per plant varied from 87 to 162. Berry shape was round and bold.

4.2 Anatomical studies of the spike

The spikes with pronounced bracts were subjected to anatomical studies. The cross section of the branching spike showed the formation of inflorescence primordia at the base of floral meristem (Plate 4a). Longitudinal sections of young spikes observed under 4x magnification (Nikon SMZ 10A) showed the variation at the floral junction (Plate 4b).

4.3 Molecular studies

The DNA extracted from the 8 samples were analysed for the presence of homologus sequences of *TFL1* and *FT* genes. RAPD analysis was done for seven non spike branching cultivars and spike branching type collected from same farm for analyzing genetic variability and trait linked marker. Results of analysis are listed further.

4.3.1 Plant DNA isolation

Deep frozen, semi mature leaves were used for DNA isolation. Modified Doyle and Doyle CTAB DNA extraction protocol was used which yielded good quality genomic DNA. Spectrophotometric analysis provided crucial information about quality and quantity of genomic DNA. All eight samples showed O. D. ratios ranging from 1.50 to 1.88 (Table 1). The genomic DNA yield ranged from 180 to 630 µg/g.

Plate 1. The spike branching pepper type from the farmer's plot at Idukki district

Plate 2. The farmer with branched pepper spike

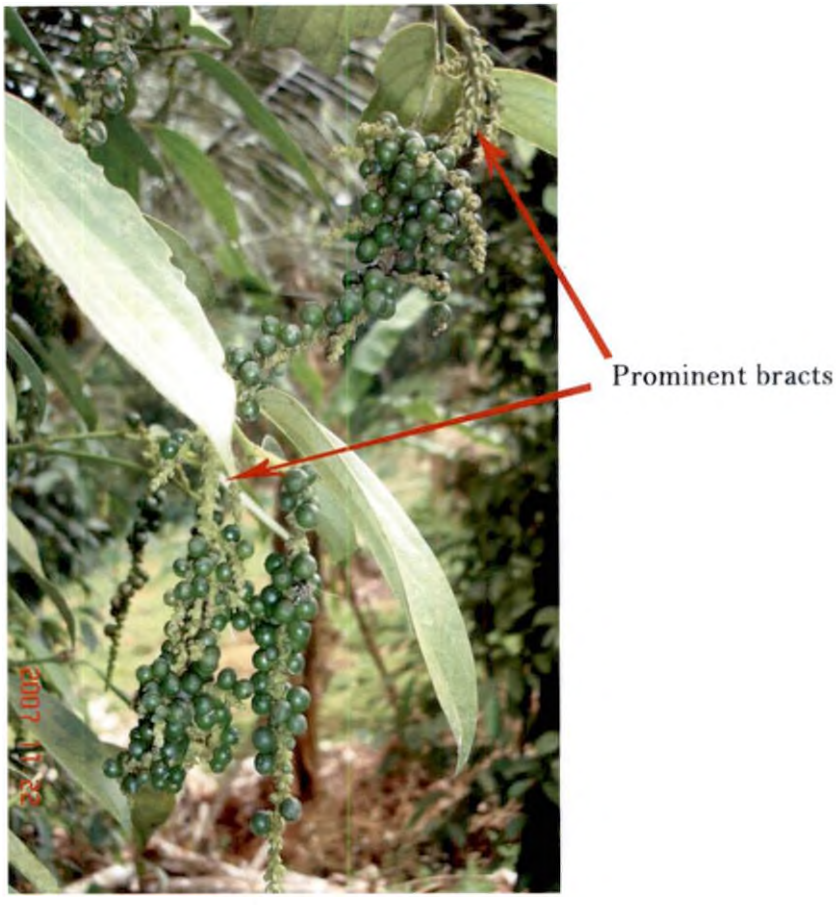


Plate 3. Spikes showing pronounced bracts at the base.

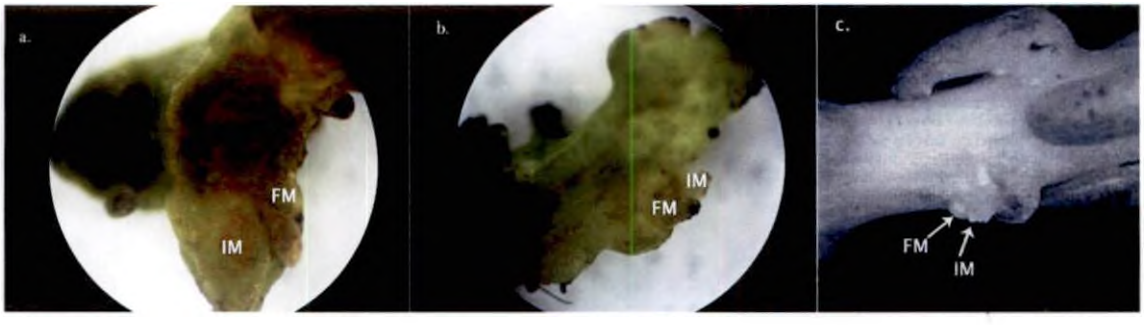


Plate 4. Formation of IM at the FM junction at the axils of pronounced bracts.
a. C.S of young spike. **b.** L.S. of young spike **c.** Initiation of IM at the floral junction (IM- Inflorescence meristem, FM- Floral meristem)(x4)

Table 1: Quality and quantity of DNA isolated from the eight cultivars/varieties of black pepper.

Sl. No.	Plant	A_{260}	A_{280}	O. D. Ratio (A_{260}/ A_{280})	DNA Yield (µg/g)
1	Karimunda	0.012	0.007	1.71	360
2	Kumbukal Kodi	0.007	0.004	1.75	210
3	Arayan Mundi	0.015	0.008	1.87	450
4	Spike branching type	0.014	0.008	1.75	420
5	Vellamundi	0.006	0.004	1.50	180
6	Naraya Kodi	0.021	0.012	1.75	630
7	Panniyur 2	0.017	0.009	1.88	510
8	Panniyur 4	0.020	0.011	1.81	600

4.3.2 Agarose gel electrophoresis

The electrophoresis of genomic DNA of black pepper on 0.8 per cent agarose gel, indicated the presence of good quality unsheared DNA, forming a single band without any smear (Plate 5).

4.4 Primer designing

For identifying sequence homology to *TFL*1 and *FT* genes in spike branching black pepper type, primers were designed for gene sequences of other plants using Primer 3.0 software (Table 2), as there are no reported sequences are available for black pepper in GenBank database. Based on the assumption that the meristem identity gene *TFL1* and flowering gene *FT* are conserved across species, the primers were designed using gene sequences of *Arabidopsis thaliana* and multiple sequence alignment for homologus genes of other species.

Forward and reverse primers (TFL1-F1 and TFL1-R1) were designed for the gene *TFL*1 using 1172 bp DNA sequence (c1024640-1025811) of the gene *TFL1* which belongs to locus NC_003076.4 on chromosome 5 of the model plant *Arabidopsis thaliana*.

Primers (FT-F1 and FT-R1) were designed for the 2506 bp DNA sequence of the gene FT (GI: 42592260) which belongs to locus NC_003070.5 of chromosome 1 of *Arabidopsis thaliana*. Both primers were 20 nt in length with an average of 52.5 per cent GC content. The priming sequence was confined to the longest fourth exonic region of the gene (2039 bp -2507 bp).

Primers (TFL1-F2 and TFL1-R2) were designed for the conserved sequence of *TFL* homologue sequences GI: 30680238 (*Arabidopsis thaliana*), GI: 187761640 (*Malus domestica*), GI: 149250805 (*Picea abies*), GI: 82791228 (*Aquilegia formosa*) and GI: 83628279 (*Populus trichocarpa*). Conserved sequence was manually selected as forward primer and appropriate reverse primer was designed using Primer 3.0 software. The forward and reverse primers were of 20 nucleotide length, with 45 and 40 per cent GC content, respectively.

Based on multiple sequence alignment of the homologous sequences of genes FT; GI: 30697201 (*Arabidopsis thaliana*), GI: 157429034 (*Chenopodium rubrum*), GI: 164457874(*Citrus unshiu*) the conserved region was selected manually from Clustal W program. The consensus sequence was used as a forward primer (FT-F2) and appropriate reverse primer (FT-R2) was designed using Primer 3.0 software. The forward and reverse primers were of 20 nucleotide length, with 45 and 40 per cent GC content, respectively.

4.4.1 Primer analysis

Primers designed were analyzed using Oligonucleotide Properties Calculator program (Table 3). BLASTN analysis was carried out to find the binding sites of the designed primers in nucleotide sequences of various plant species (Fig 3a and 3b and Table 4) to find binding region and primer specificity None of the designed primers had hairpin formation, 3' complimentarity and self annealing properties.

4.5 PCR analysis with gene specific primers

Polymerase Chain Reaction was carried out using the primers designed for the genes *TFL1* and *FT*. Genomic DNA of spike branching pepper type and seven non-spike branching cultivars were used as template DNA. PCR carried out using the primers TFL1-F1 and TFL1-R1 yielded a specific amplicon equivalent to 700 bp DNA ladder. The amplified DNA fragment was separated on 1.4 per cent agarose gel. The amplification was prominent at 51.5°C and the product was reproducible in Karimunda, Spike branching pepper type, and Vellamundi cultivar/varieties (Plate 6).

Plate 5. Agarose gel electrophoresis of black pepper genomic DNA

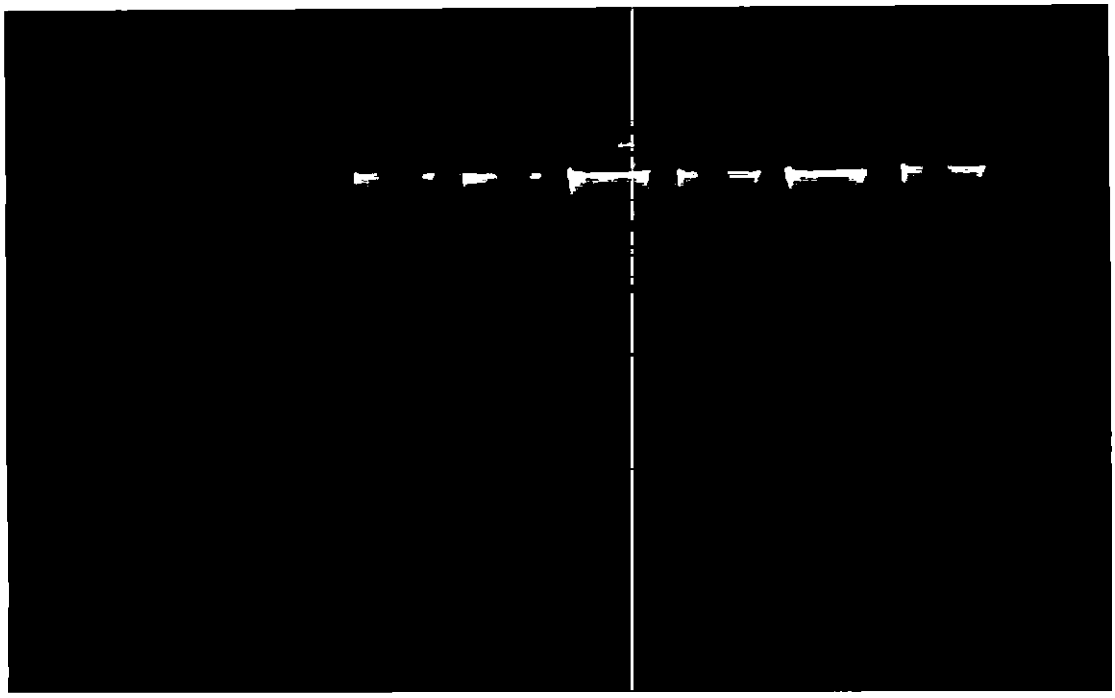


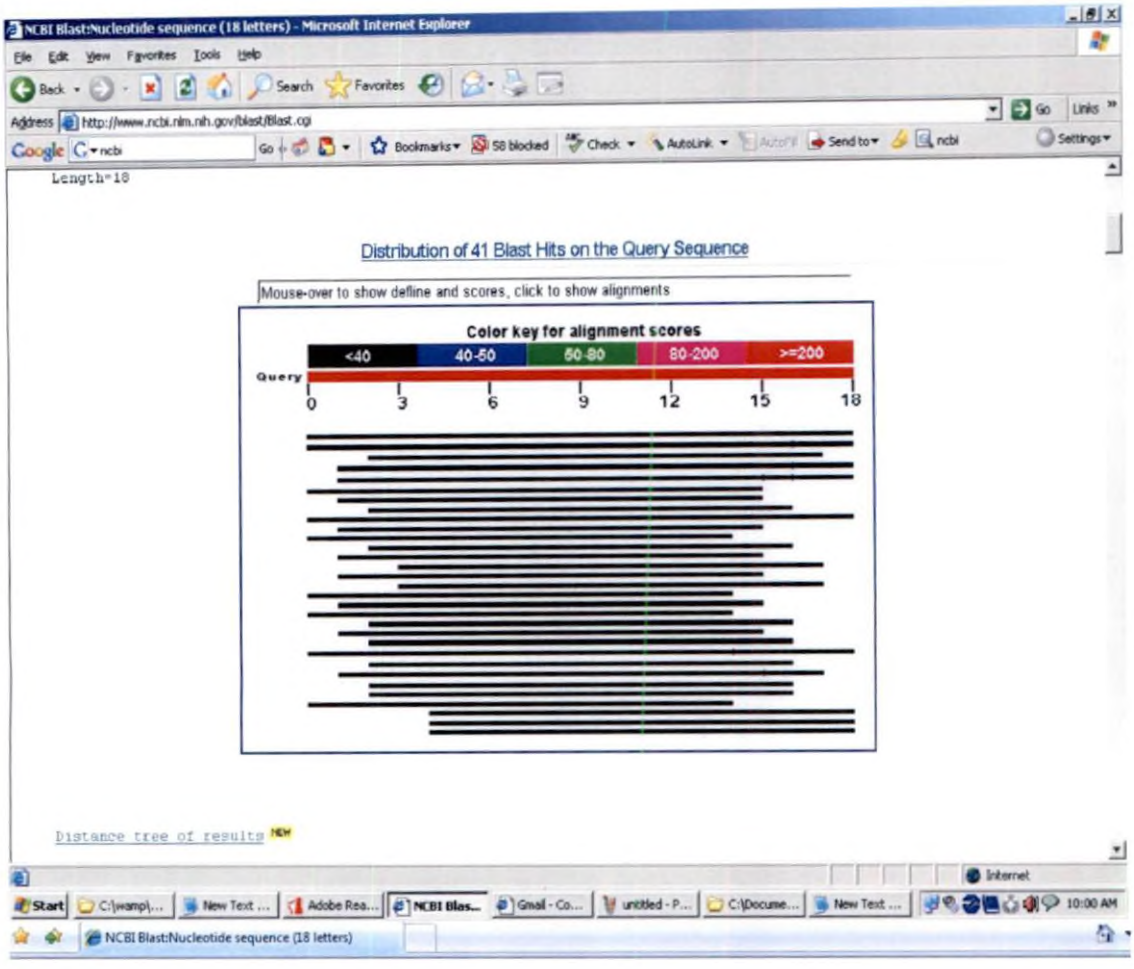
Table 2. Primers designed

Sl.No	Gene identity	Primer notation	Tm (°C)	GC %	Primer length (nt)	Sequence of Primer
1	TFL1 Arabidopsis. Exonic region 4	TFL1-F1	54.0	55.5	18	5'CAGGCAGAAGCAAAGACG3'
		TFL1-R1	56.2	55.5	18	5'ACTAGCGTTTGCGTGCAG3'
2	FT Arabidopsis Exonic region 4	FT-F1	56.7	55.0	20	5'GCGCCAGAACTTCAACACTC3'
		FT-R1	54.5	50.0	20	5'TCATCACCGTTCGTTACTCG3'
3	Multiple Sequence Alignment (gi: 30680238,187761640, 149250805,82791228, 83628279)	TFL1-F2	50.5	45.0	20	5'CACTTTGGTGATGATAGACC3'
		TFL1-R2	47.8	40.0	20	5'GATCTCTCGAAGGGATATTA3'
4	Multiple Sequence Alignment (gi : 30697201, 157429034, 164457874)	FT-F2	48.4	36.8	19	5'ATTGGTTGGTGACTGATAT3'
		FT-R2	49.5	40.0	20	5'TTGTAGATCTCAGCAAACTC3'

Table 3. Primers analyzed using oligonucleotide property calculation program

Sl. No.	Primer	Hairpin formation	3'Complimentarity	Self annealing
1	TFL1-F1	None	None	None
2	TFL1-R1	None	None	None
3	FT-F1	None	None	None
4	FT-R1	None	None	None
5	TFL-F2	None	None	None
6	TFL-R2	None	None	None
7	FT-F2	None	None	None
8	FT-R2	None	None	None

Fig 3a. BLASTN analysis of primer sequence TFL1-F1 (Forward primer)



Distance tree of results

Sequences producing significant alignments:		Score (Bits)	E Value
ref|NC_003076.4|	Arabidopsis thaliana chromosome 5, complete ...	36.2	0.020
ref|NC_003071.3|	Arabidopsis thaliana chromosome 2, complete ...	32.2	0.32
ref|NT_107206.1|	Oryza sativa (japonica cultivar-group)	30.2	1.2
ref|NC_003075.3|	Arabidopsis thaliana chromosome 4, complete ...	30.2	1.2
ref|NC_003070.5|	Arabidopsis thaliana chromosome 1, complete ...	30.2	1.2
gb|DV442201.1|	CV01006B1C02.f1 CV01-normalized library Maniho...	30.2	1.2
ref|NT_107239.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107216.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107181.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107179.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107178.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107175.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107174.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107151.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107132.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107114.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107082.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107079.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_107077.1|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_080067.1|	Oryza sativa (japonica cultivar-group) chrom...	28.2	4.9
ref|NT_080060.1|	Oryza sativa (japonica cultivar-group) chrom...	28.2	4.9
ref|NT_079947.2|	Oryza sativa (japonica cultivar-group)	28.2	4.9
ref|NT_079927.2|	Oryza sativa (japonica cultivar-group)	28.2	4.9

Fig 3b. BLASTN analysis of primer sequence TFL1-R1 (Reverse primer)

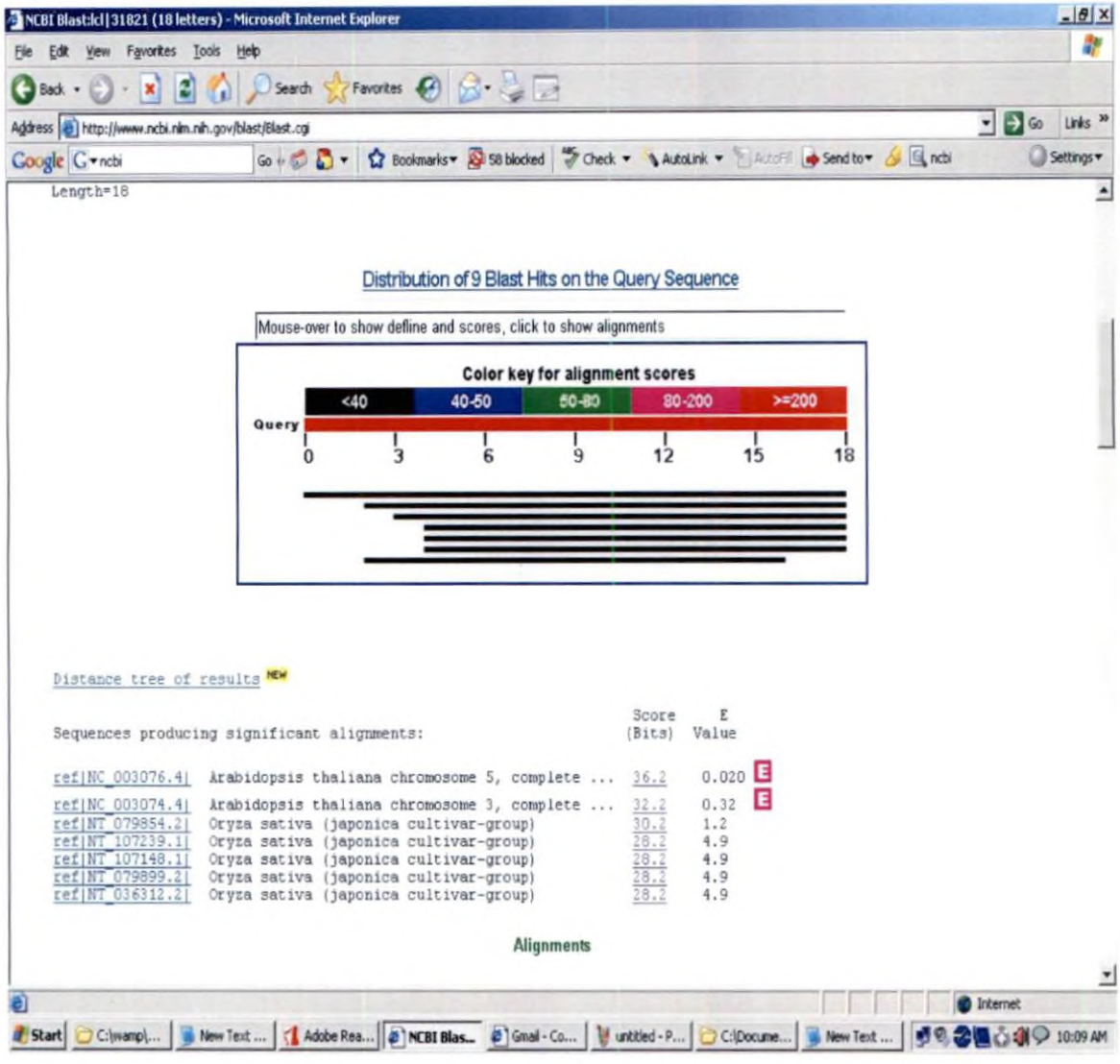


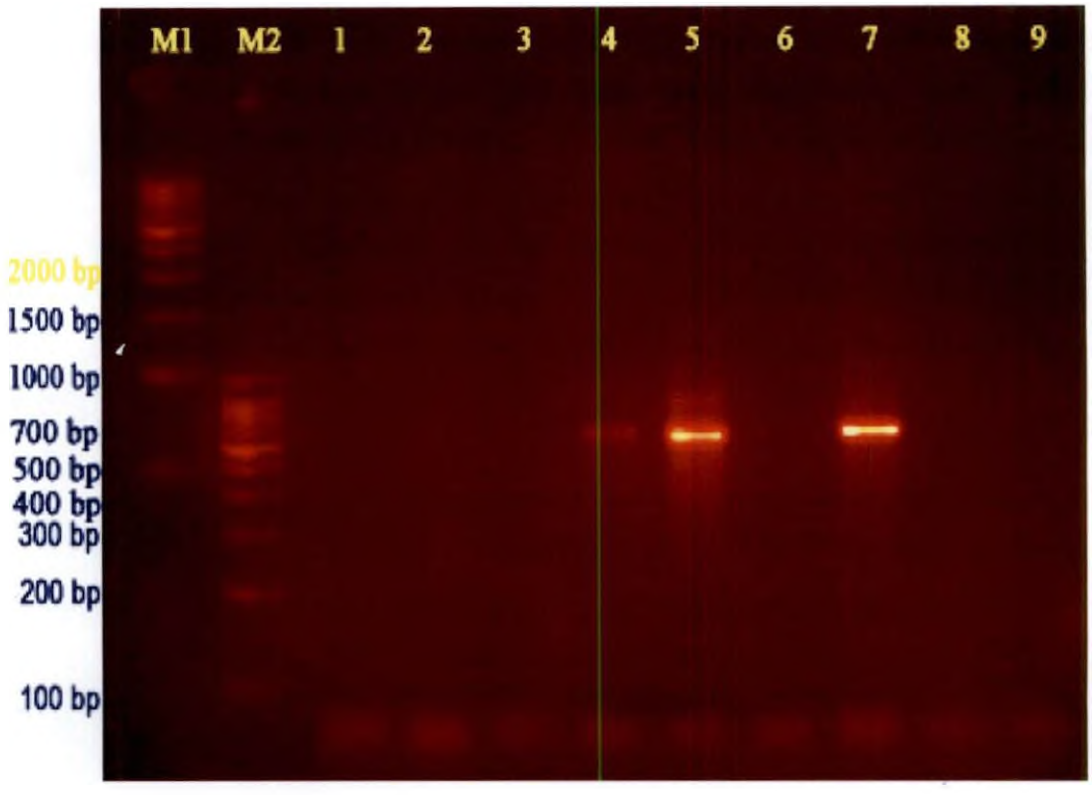
Table 4. BLASTN analysis of primer sequences

Sl. No.	Primer	Plant	Gene Identity No.	Query seq. Length(nt)	Binding position
1	FT-F1	*B. rapa* *A. thaliana*	27804462 30697201	1-20	101-120 496-515
2	FT-R1	*A. thaliana* *A. thaliana*	6117977 30697201	1-20	12851-12870 702-721
3	TFL1-F2	*A. thaliana* *A. thaliana*	18415488 30697201	1-20	308-326 383-401
4	TFL1-R2	*A. thaliana* *A. thaliana*	30680238 89111877	1-20	220-239 204-223
5	FT-F2	*P. trifoliate* *C. unshiu*	166850555 164457876	1-19	308-326 383-401
6	FT-R2	*A. thaliana* *A. thaliana*	18415488 30697201	1-20	467-486 518-537

Plate 6. Amplification of black pepper genomic DNA by TFL-F1 and TFL-R1 primers

M1	500 bp DNA marker
M2	100 bp DNA marker
1	Panniyur-4
2	Naraya Kodi
3	Panniyur-2
4	Karimunda
5	spike branching black pepper type
6	Arayan mundi
7	Vellamundi
8	Kumbakal kodi
9	Contol reactiion

Plate 6. Amplification of black pepper genomic DNA by TFL-F1 and TFL-R1 primers



The primers TFL-F2, TFL-R2, FT-F1, FT-R1, FT-F2 and FT-R2 did not yield any amplification for the genomic DNA of spike branching pepper type. Varied annealing temperature and PCR conditions were tried to find possible amplicons. In spite of varied PCR replications for these primers, they did not yield any amplification.

4.6 RAPD analysis

Among the 16 primers tested for amplification, eight primers were selected for the RAPD analysis (Table 5). The number of bands resolved per amplification was primer dependent and varied from 7 to maximum of 14. The primers altogether generated 83 scorable bands with an average of 10.37 bands per primer. Out of these 83 bands produced altogether, 13 bands were monomorphic and 70 bands were polymorphic showing 83.57 per cent polymorphism (Table 6).

Primer OPA 10 gave maximum of 3 monomorphic bands and OPB 8 amplified maximum of 14 polymorphic bands. All primers showed polymorphism greater than 70 per cent for all eight varieties.

The primer OPA 8 produced 7 scorable bands. Out of 7, six of the bands were polymorphic and one was monomorphic. This primer produced 85.71 per cent of polymorphism (Plate 7).

The primer OPA 10 produced 10 scorable bands. Out of 10, seven bands were polymorphic and three were monomorphic. This primer produced about 70.00 per cent polymorphism (Plate 8).

Maximum of 15 bands were produced by the primer OPB 8. Bands showed maximum of 93.33 per cent polymorphism with a single monomorphic band at 600 bp (Plate 9). Eight scorable bands were produced by the primer OPB 10. Out of these 8 bands, two were monomorphic at 200 bp and 500 bp (Plate 10).

Table 5. Sequence of the selected primers used for DNA amplification of eight black pepper cultivars/varieties

Sl.No.	Primer	Sequence	GC content (%)
1	OPA 8	5' GTGACGTAGG 3'	60
2	OPA 10	5' GTGATCGCAG 3'	60
3	OPB 8	5' GTCCACACGG 3'	70
4	OPB10	5' CTGCTGGGAC 3'	70
5	OPF 3	5' CCTGATCACC 3'	60
6	OPP 1	5' GTAGCACTCC 3'	60
7	OPS12	5' CTGGGTGAGT 3'	60
8	OPU 13	5' GGCTGGTTCC 3'	70

Plate 7 & 8: RAPD profile using primer OPA-8 and OPA-10, respectively

M1: 100 bp DNA marker
M2: 500 bp DNA marker
1: Karimunda
2: Kumbakal Kodi
3: Arayan mundi
4: spike branching type
5: Vellamundi
6: Naraya Kodi
7: Paniyur-2
8: Panniyur-4

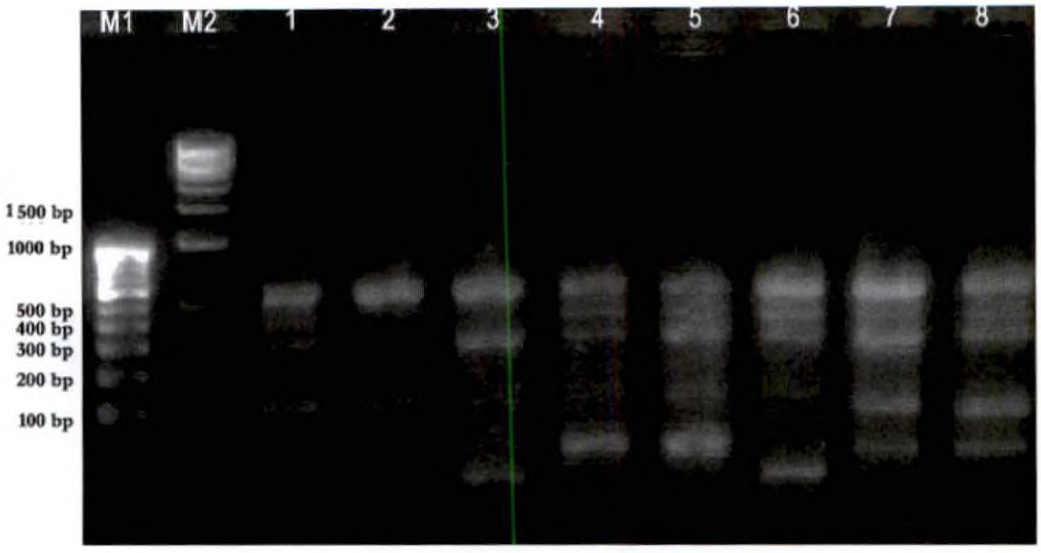


Plate 7: RAPD profile using primer OPA 8

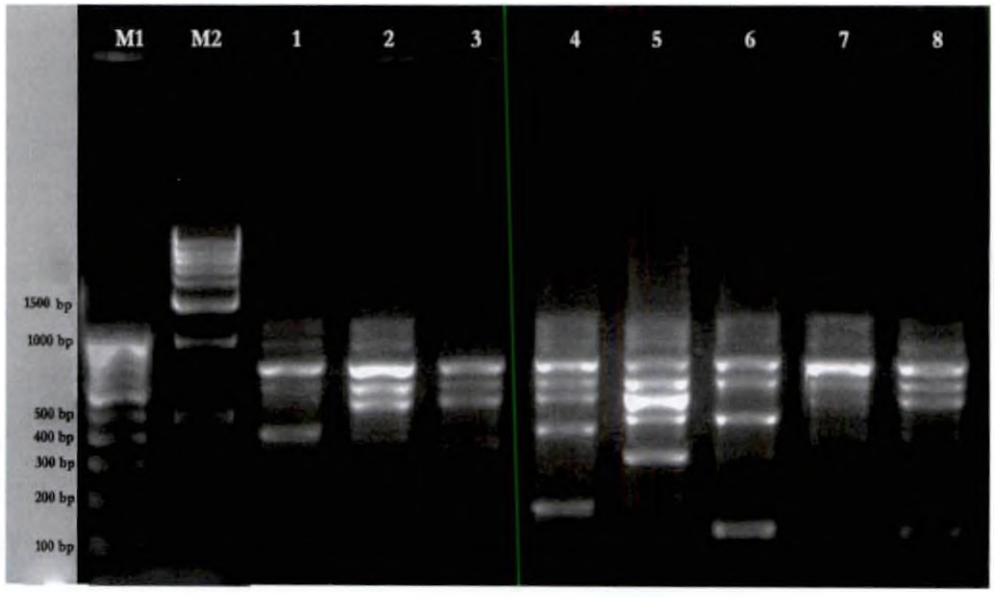


Plate 8: RAPD profile using primer OPA 10

Plate 9 & 10: RAPD profile using primer OPB 8 and OPB 10, respectively

M1: 100 bp DNA marker
M2: 500 bp DNA marker
1: Karimunda
2: Kumbakal Kodi
3: Arayan mundi
4: spike branching type
5: Vellamundi
6: Naraya Kodi
7: Paniyur-2
8: Panniyur-4

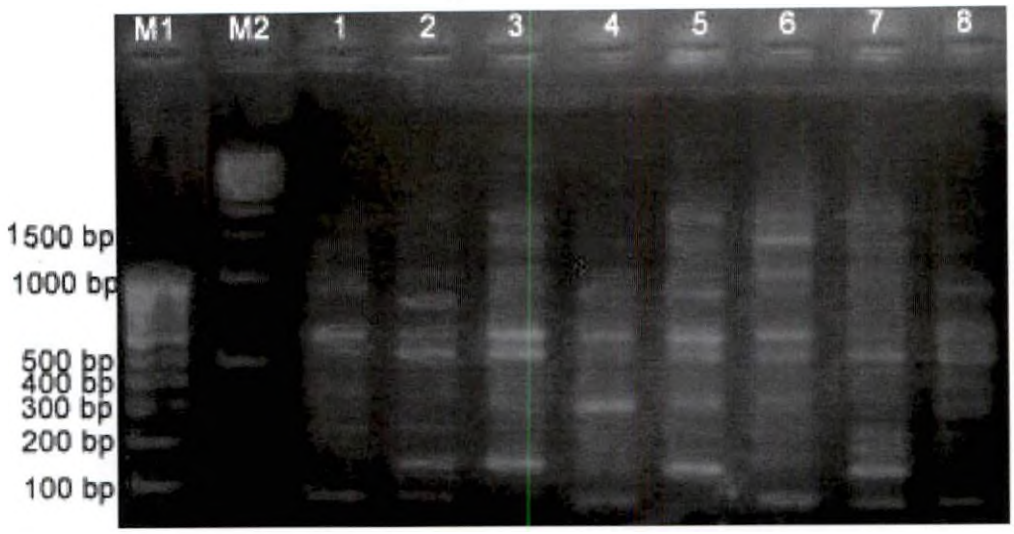


Plate 9: RAPD profile using primer OPB 8

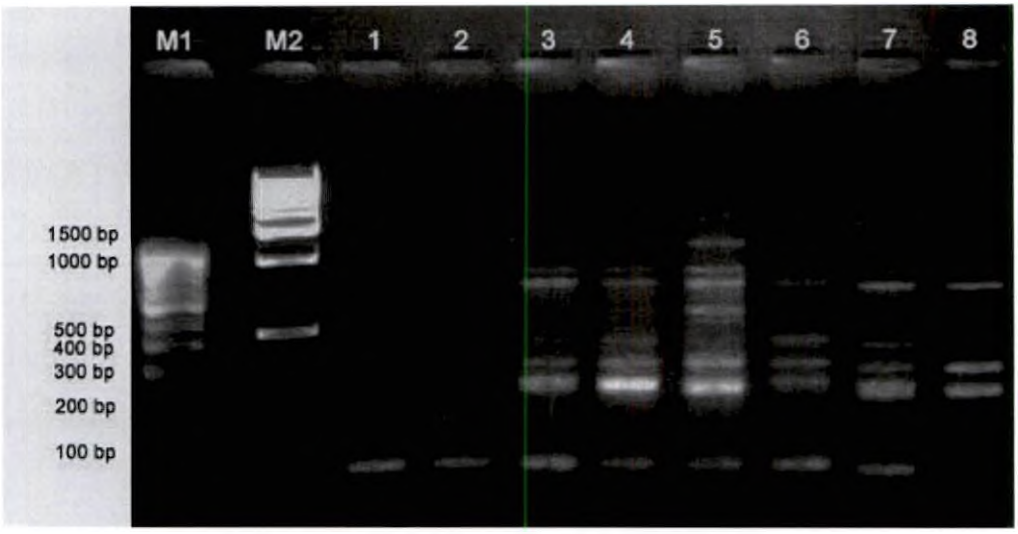


Plate 10: RAPD profile using primer OPB 10

Plate 11 & 12: RAPD profile using primer OPF 3 and OPP 1, respectively

M1: 100 bp DNA marker
M2: 500 bp DNA marker
1: Karimunda
2: Kumbakal Kodi
3: Arayan mundi
4: spike branching type
5: Vellamundi
6: Naraya Kodi
7: Paniyur-2
8: Panniyur-4

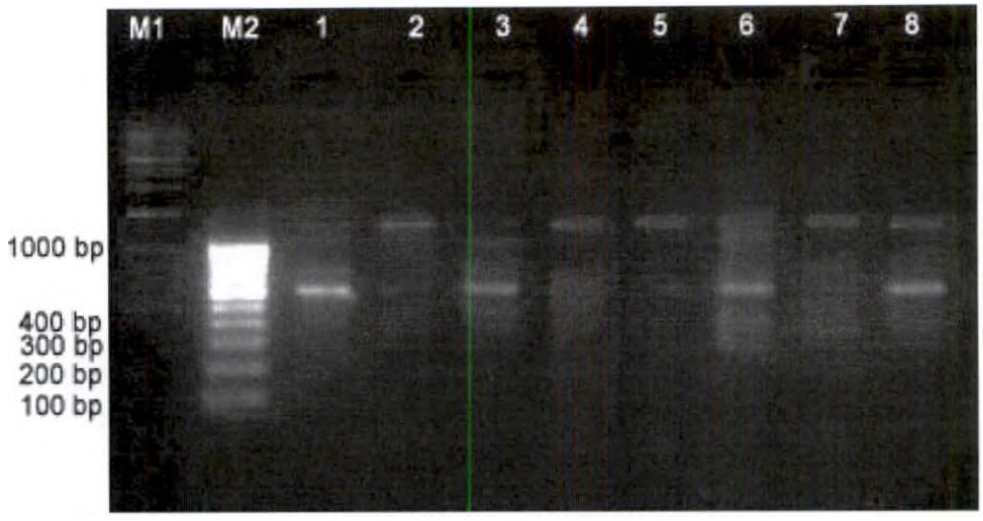


Plate 11. RAPD profile using primer OPF 3

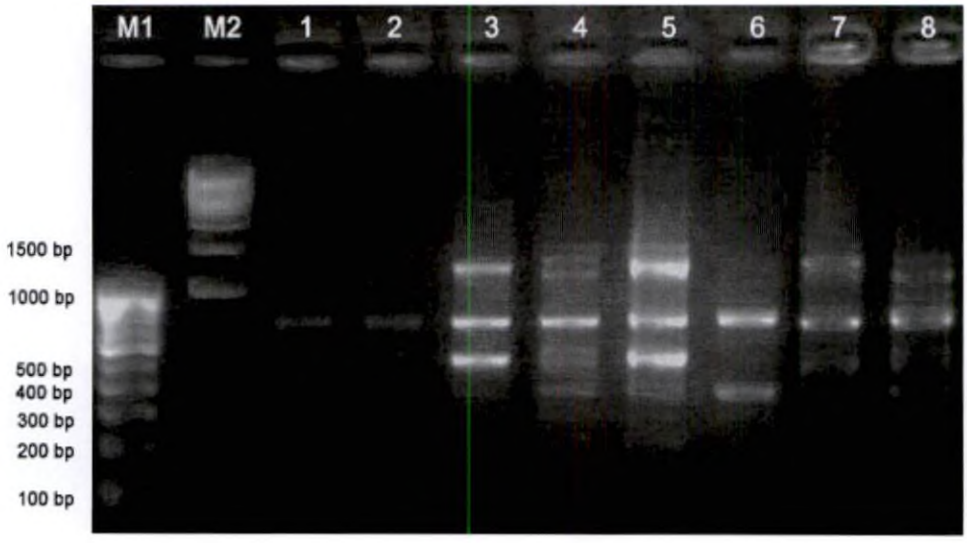


Plate 12. RAPD profile using primer OPP 1

Plate 13 & 14: RAPD profile using primer OPS 12 and OPU 13, respectively

1: Karimunda
2: Kumbakal Kodi
3: Arayan mundi
4: Spike branching type
5: Vellamundi
6: Naraya Kodi
7: Paniyur-2
8: Panniyur-4
M1: 100 bp DNA marker
M2: 500 bp DNA marker

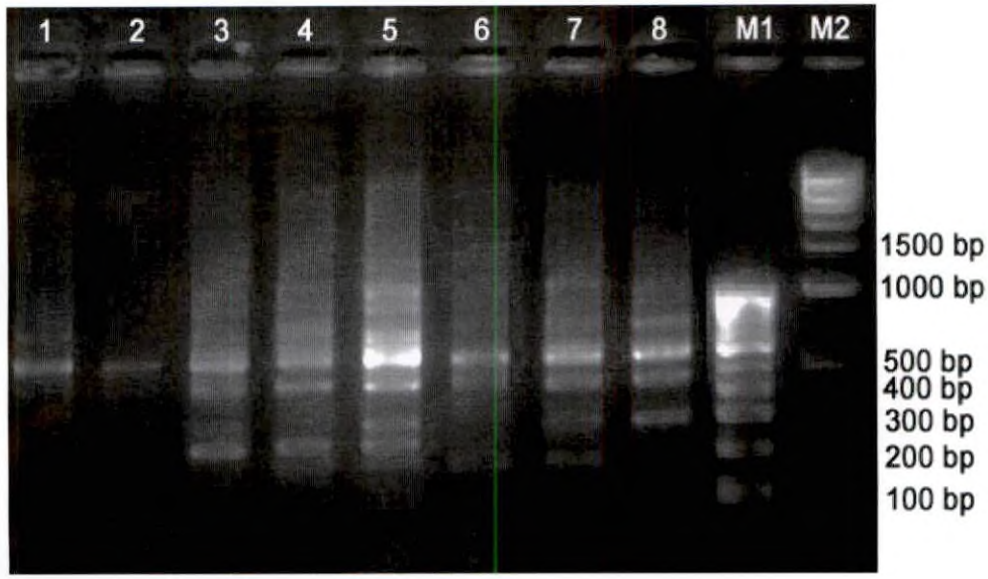


Plate 13.RAPD profile using primer OPS 12

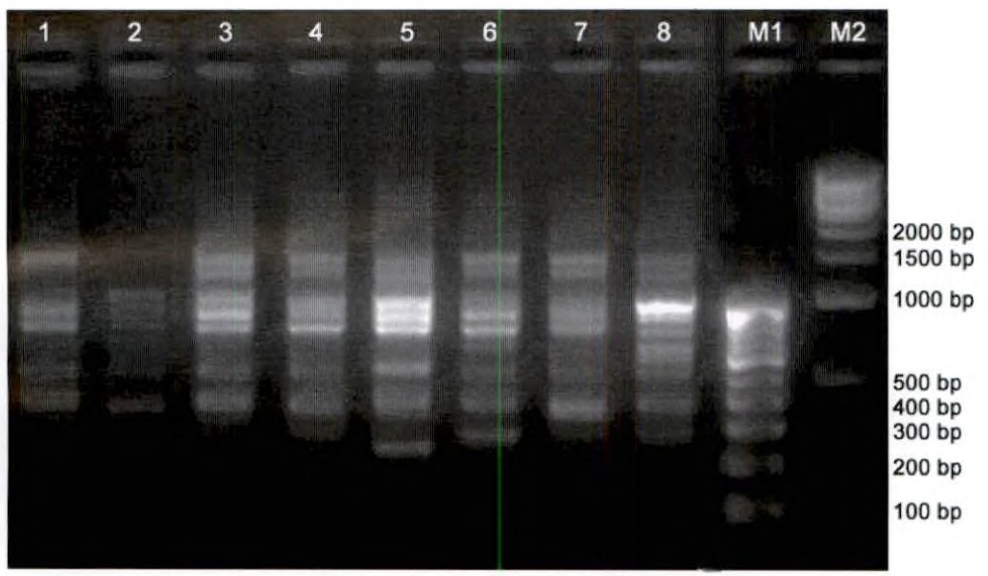


Plate 14. RAPD profile using primer OPU 13

Among seven scorable bands amplified by the primer OPF 3 one was monomorphic. The primer produced distinct bands although very less bands were

formed. Six bands from this primer accounted for 85.71 per cent polymorphism among varieties (Plate 11).

Primer OPP 1 produced 11 scorable bands. Out of these 11 bands, 1 band (~ 900 bp) was monomorphic and remaining 10 bands showed 90.9 per cent polymorphism (Plate 12).

The primer OPS 12 produced 11 bands. 81.8 per cent polymorphism was exhibited by this primer with 9 polymorphic bands and 2 monomorphic bands (Plate 13).

OPU 13 primer produced 12 scorable bands. Out of those 12 bands, 10 were polymorphic and 2 were monomorphic exhibiting 83.33 per cent polymorphism (Plate 14).

4.6.1 Statistical Analysis

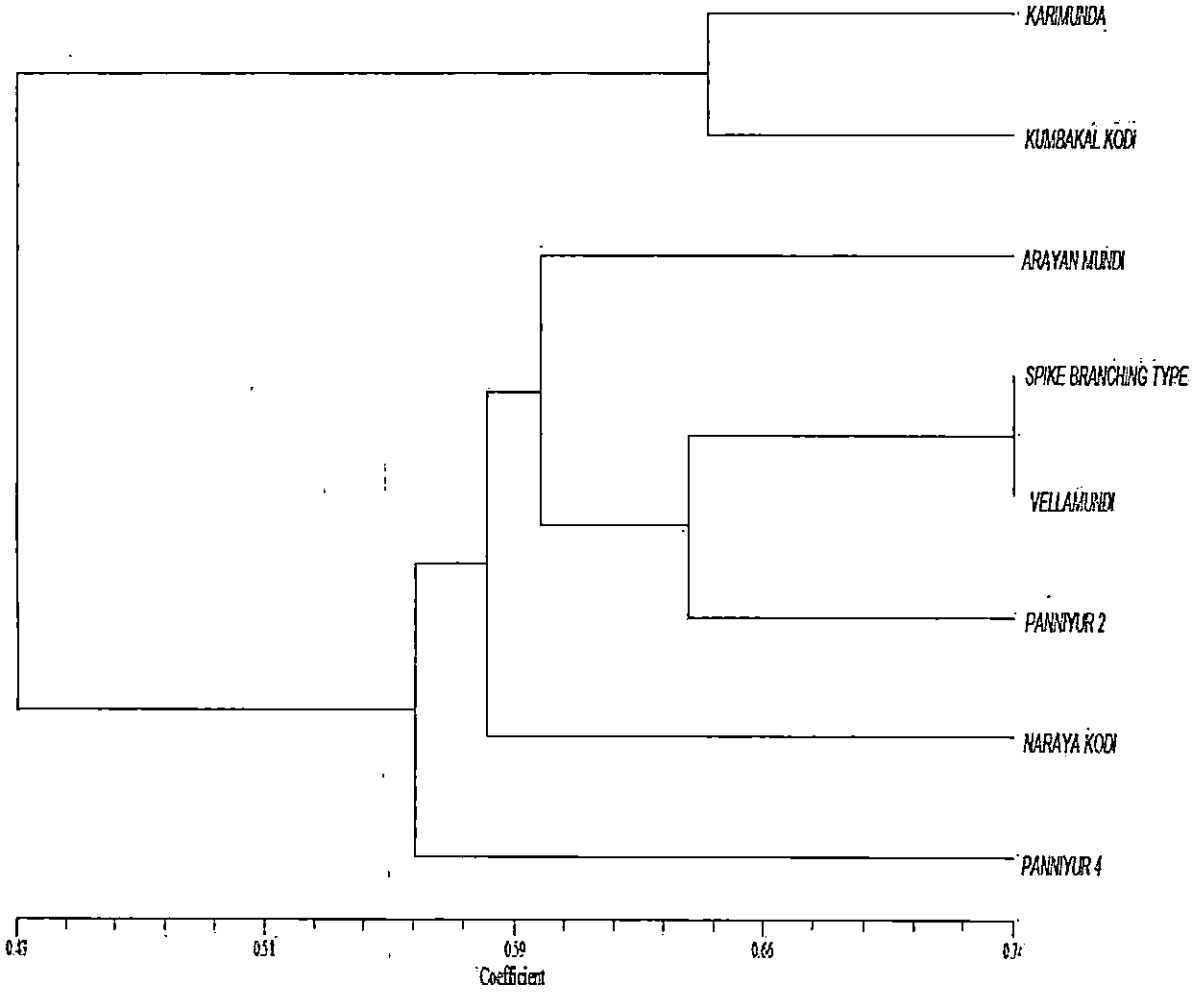
Jaccard's similarity coefficient values for each pair wise comparison between the plants were calculated and a similarity coefficient matrix was constructed. The matrix was subjected to un-weighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram (Fig. 4) using average linkage procedure. All these computations were carried out using NTSYS-pc software version 2.02.

In the dendrogram, divergence for all the varieties under study started at 0.43 coefficient, indicating only 43 per cent similarity among cultivars/varieties. Two big clusters formed at the same similarity index. Two of the varieties; Karimunda and Kumbakal kodi formed a separate cluster, indicating the wide divergence over other six varieties. However, both the cultivars showed only 64.6 per cent similarity. Divergence for the biggest cluster started at coefficient of 0.556 indicating the initiation of divergence for six of the varieties of same cluster. Maximum of 74 per cent similarity was obtained for the cultivar Vellamundi and Spike branching black pepper type.

Table 6. Polymorphism exhibited by different primers for eight black pepper cultivar/varieties

Primer	No. of bands	No. of Monomorphic bands	No. of Polymorphic bands	Percent polymorphism
OPA 8	7	1	6	85.71
OPA 10	10	3	7	70.0
OPB 8	15	1	14	93.33
OPB 10	8	2	6	75.0
OPF 3	7	1	6	85.71
OPP 1	11	1	10	90.9
OPS 12	12	2	10	83.33
OPU 13	13	2	11	84.61

Fig. 4. Dendrogram generated from molecular analysis of eight black pepper cultivars and varieties from Idukki.



Discussion

5. DISCUSSION

The Trans-Gangetic and South Deccan regions are considered to be two independent centres of origin of the genus *Piper* (Hooker, 1886). *Piper nigrum* is believed to have originated in the Western Ghats. Kerala which forms part of the Western Ghats holds the richest species diversity and cultivar diversity in black pepper. It harbours a major share of the gene pool of this crop. About 111 *Piper* species have been identified so far, in Kerala (Parthasarathy et al., 2006) and there are 70 distinct black pepper cultivars. Ravindran (2000) reported the presence of several uncharacterised hybrids of *Piper* species in the Western Ghats of Kerala, indicating rich biodiversity. Wide variability has been observed among the cultivars of black pepper with respect to economically important traits like spike length, number of berries per spike and size of berries. Spike characteristics have been studied in detail (Ravindran, 2000). Spike branching is a rare phenomenon. The variety 'Aimpiriyan' has been observed to have a tendency for spike branching. However, only rudimentary branches are often produced, bearing only a few berries. Sasikumar et al. (2006) reported an uncharacterised black pepper type from Idukki district, showing spike branching. Bavappa et al. (2007) mentioned about the spike branching trait of the cultivar 'Kathirinmelkkathir'.

The present study was undertaken as an initial step for understanding spike branching trait in black pepper, using the samples reported by a farmer from Idukki district. Black pepper type in his farm has profuse spike branching and more berry yield per spike, compared to cultivar 'Kathirinmelkkathir'. He has a few plants, vegetatively propagated, showing typical spike branching with about 30 branches and 250 to 350 berries per spike. Three to four times more berry yield per spike has been observed in this case, compared to the leading commercial varieties like Panniyur series, indicating the significance of this trait in crop improvement. These plants have not been characterised so far or subjected to detailed study.

A number of factors like expression/regulation of inflorescence specific genes, hormones, genetic mutations, environmental factors like altitude, temperature, etc. can be responsible for the branching trait. Molecular analysis with respect to inflorescence branching in many other plant species has been done. In *Arabidopsis thaliana*, *TFL1*, *LFY*, *CAL*, *FUL*, *AGL24*, *AP1*, *FT*, *AG* and *UFO* have been reported to be involved in determining inflorescence architecture and inflorescence branching (Espinosa-Soto et al., 2004), of which the genes *TFL1* has been shown to have major role in determining the shape of inflorescence. The main objective of present study was to find out the presence of two important genes (*TFL1* and *FT*) among the network of genes involved in inflorescence branching in modal plant *Arabidopsis thaliana*, using the designed gene specific primers.

The results of the study are discussed below:

On-farm observations were made on the morphological traits of the spike branching type of black pepper from Idukki. Although young spikes appeared to be normal, bracts were long and prominent than the other cultivars/varieties. Individual flower buds appeared to be transformed into branches. Length of branches varied widely. However, this transformation was not uniform and some of the individual flower buds remained untransformed and developed into flowers and berries ultimately. The spike branching black pepper type reported by Sasikumar et al. (2006) had morphological similarity to the cultivar 'Karimunda'. In the present study also, such a similarity was evident for leaf characters. However, the spike characters did not resemble very much to that of Karimunda. Young spikes appeared to be normal, except for the long bracts in some of the spikes and formation of inflorescence meristem in the axils of such bracts. Anatomical studies have revealed presence of such a transformation. Leaf samples from these plants were collected for further molecular studies.

The modified C-TAB method (Doyle and Doyle, 1987) used in this study yielded good quality DNA from leaf samples. Grinding with liquid nitrogen and use of β-mercaptoethanol were found to be effective. DNA yield ranged from 180 to 630 µg/g of leaf sample. The O.D. ratios varied from 1.71 to 1.88. Quality of DNA was confirmed by spectrophotometric analysis and agrose gel electrophoresis. Some of the

fresh leaf samples used for DNA extraction using the same procedure yielded sheared genomic DNA. However, deep frozen leaves which were stored at -80 °C yielded higher molecular weight DNA with least shearing. Mondal et al. (2000) reported that tender leaves yielded more quantity of DNA. However, the extraction procedure on young, fresh leaves yielded fragmented, sheared genomic DNA.

Two sets of primers were designed for the genes *TFL1* and *FT* using the cDNA sequences of *Arabidopsis thaliana* and other plant species, as there are no reported sequences for any genes of black pepper. The first primer pair, TFL1-F1 and TFL1-R1 was designed using Primer 3.0 software based on the fourth exonic sequence which is the longest and conserved. Factors were set to achieve the priming position in fourth exonic sequence (833 bp -1172 bp). PCR was done using TFL1-F1 and TFL1-R1 primer pair. Annealing temperature and PCR conditions were optimized. PCR using the primer pair produced a band of ~700 bp in 3 out of the 8 black pepper samples analysed. The amplification was prominent in the varieties Vellamundi and spike branching black pepper type. However, Karimunda produced a faint band. Amplification might have resulted due to conserved 4th exonic sequence of the *TFL1* gene in black pepper, as primers were designed specifically within fourth exonic sequence.

Presence of sequence homology for the gene *TFL1* indicated the possible involvement of *TFL1* gene, which had been reported to be associated with inflorescence branching in *Arabidopsis thaliana* (Bradley et al., 1997) in the spike branching trait of the black pepper type. This result is significant, as five out of the eight cultivars tested did not give any positive response for the primer pair designed based on *TFL1* gene. However, two non-spike branching varieties including Karimunda and Vellamundi have also shown amplification for the *TFL1* primer pair.

Amplifications for *TFL1* specific primers in the three cultivars need to be analysed in detail for verification of the presence of full length gene. Presence of the sequence similarity for *TFL1* specific primers in non-spike branching cultivars is interesting. This necessitates detailed analysis for the presence of full length gene, as well as investigations on gene expression. TFL1 is a well known regulator of gene expression. It belongs to the MADS box transcription regulator. The expression level

of the gene rather than its presence, may be critical for the spike branching trait. It would be worthwhile to analyse for the presence and expression of *TFL1* gene in the cultivars Aimpiriyan and Kathirinmelkathir, showing rudimentary spike branching. Such an analysis as well as sequencing of the amplicons might help to confirm that the amplification was related to spike branching trait.

The *FT* gene also has been reported to have a conserved fourth exon, similar to that in *TFL1* gene. The *FT* gene shows strong homology to the *TFL1* gene (Weigel, 1992). In the present study, primers FT-F1 and FT-R1 were designed for the 2506 bp DNA sequence of the gene *FT* (GI: 42592260) belonging to chromosome 1 in *Arabidopsis thaliana*. Factors were set to achieve the primer sequence in fourth exonic sequence (2039 bp -2507 bp) which is the biggest. The PCR reactions were carried out using FT-F1 and FT-R1 primers, at varied annealing temperatures. However, no DNA amplification was observed. Lack of homologus sequences for the primers of fourth exonic sequence of *FT* gene in black pepper is a possible reasons for non-amplification. Primers designed on other exonic sequences of *FT* gene have to be tried.

Similarly, primers TFL1-F2 and TFL1-R2 were designed specifically for the conserved sequences obtained using the multiple sequence alignment program (Clustal W) for the homologus sequences of the gene *TFL1*. GI: 30680238 (*Arabidopsis thaliana*), GI: 187761640 (*Malus domestica*), GI: 149250805 (*Picea abies*), GI: 82791228 (*Aquilegia formosa*) and GI: 83628279 (*Populus trichocarpa*) were used in CLUSAL W program. Repeated PCR reactions using the primers TFL1-F2 and TFL1-R2 did not yield any DNA amplification. It indicated that there is no homologus sequence in black pepper genomic DNA for the primers designed. As primers were designed for conserved sequences using cDNA of genes consisted of exonic sequences, possible intervention of lengthier intronic sequence might be a reason for non amplification. This may be the case for the primers FT-F2 and FT-R2, designed for the consensus sequence of GI: 30697201 (*Arabidopsis thaliana*), GI: 157429034 (*Chenopodium rubrum*), GI: 164457874 (*Citrus unshiu*) which did not yield any amplification. Lack of homologus sequences for the designed primers is evident, error in synthesis, problems with primer annealing and experimental errors are some possible reasons for non-amplification.

Morphological and molecular characterisation would be helpful in understanding the spike branching trait. In the present study, as an initial step towards molecular characterization, RAPD analysis was done for the spike branching type and seven other cultivars/varieties grown in the same farm, as farmer did not had any information about the origin of spike branching black pepper type. He has maintained a continuous vegetative propagation of many cultivars and varieties in his farm, over time. So, molecular marker analysis will be helpful in finding the closest relative of branching type present in his farm. Molecular markers, presently has become fundamental tools for finger printing varieties, establishing phylogenetics, tagging desirable genes, determining similarities among inbreds and mapping of plant genomes (Kang Fu Yu et al. 1993). Molecular characterization of black pepper cultivars using RAPD markers was reported by Pradeepkumar et al. (2001). According to George et al. (2005) RAPD analysis could be applied to a broad array of cultivars and wild accessions to get a more accurate picture of the genetic diversity within the genus *Piper*. This technique has been successfully utilized in identifying somaclonal variants of *P. longum* (Parani et al., 1997). Based on RAPD studies Hamrick and Godt (1989) reported that asexual species are as genetically diverse as sexual ones. Smitha Bhasi (2008) showed that RAPD profiles of 40 plants of Panniyur-1 had 66.3 per cent intraclonal variability.

In the present study, RAPD analysis was done for eight samples using eight primers, selected after screening. A total of 83 scorable bands were produced, out of which, 70 bands were polymorphic and 13 monomorphic. RAPD analysis yielded 83.57 per cent polymorphism, indicating intracultivar and intravarietal variation among the eight black pepper samples. Primers OPA-10 and OPU-13 produced specific band in the cultivar Vellamundi (300 bp and 250 bp, respectively) which were absent in other cultivars/varieties. Spike branching black pepper type has also shown specific band for OPA-10 (~200 bp) decamer primer. However, this band was different from that generated by OPA-10 in Vellamundi. Specific amplifications found for some primers need to be analysed further and confirmation tests using other molecular markers need to be done.

In the RAPD dendrogram, two big clusters are formed at the similarity index of 0.43, indicating only 43 per cent similarity among the cultivars/varieties analysed. Karimunda and Kumbakal Kodi formed separate cluster. However, both cultivars showed only 64.0 per cent intra-cluster similarity. First cluster had five mini clusters subdivided repeatedly at 0.57, 0.59, 0.61, 0.64 and 0.74 similarity indices. A maximum of 74.00 per cent similarity was obtained for the cultivar Vellamundi and spike branching type, indicating the highest genetic similarity among the cultivars/varieties studied. Use of more number of primers may give a clear picture of the molecular divergence among these genotypes.

Interestingly, the non-spike branching cultivar Vellamundi and spike branching black pepper have shown DNA amplification (~700 bp) for the primers TFL1-F1 and TFL1-R1. It indicated the possible sequence similarity in both the cultivars. Another non-spike branching cultivar the cultivar Karimunda has also shown DNA amplification for the specific primer, although it formed separate cluster at 0.43 similarity index in the RAPD dendrogram. The DNA amplification in Karimunda indicates possible conservation of *TFL1* locus in black pepper cultivars irrespective of the cultivar divergence.

Further advanced studies at transcript level might be helpful for assessing relative expression of different meristem identity genes, role of transcription factors (MADS-box genes) and their expression levels for better understanding of the conversion of floral meristem to inflorescence meristem.

Identification of gene pools related to yield traits in black pepper is important. Spike branching trait, as observed in the type from Idukki, district is significant in this context. The earlier reports on spike branching were on types showing only rudimentary branching of spikes. The black pepper type from Idukki has profuse branching of spikes and bears three to four times more berries. This is very significant. Detailed analysis of this trait at the molecular level, involving genes/ gene expression could be of great help in improving productivity of black pepper, involving biotechnological interventions. In future, it may be helpful in producing genetically modified black pepper with increased yield. This possibility is interesting, as there are no genetically modified crops, so far, transformed for higher yield. In

black pepper there are no reports so far on the molecular characterisation of the spike branching trait. The present study was an initial step towards molecular characterisation of spike branching and it could indicate the presence of *TFL1* gene, that has been reported to be involved in inflorescence branching in other crops and in the spike branching type of black pepper from Idukki.

Summary

6. SUMMARY

The study entitled "Molecular analysis of spike branching observed in black pepper (*Piper nigrum* L.) type from Idukki" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2006-2008. The objective of the study was to analyse the presence of inflorescence architecture gene *TFL1* and flowering gene *FT* in spike branching black pepper type collected from Idukki, using polymerase chain reaction technique.

Observations were made on morphological parameters of the plants showing spike branching, in the farmer's field in Idukki. The plants exhibited profuse spike branching. More than 22 proliferating branches of varying length were observed per spike. Leaf samples were collected and preserved at -80°C for molecular analysis. Modified CTAB method was used for isolation of genomic DNA from the samples. The procedure was optimised to yield good quality DNA.

As an initial step towards molecular analysis, two sets of primers were designed for the two genes, *TFL1* and *FT* that have been reported to be important in determining inflorescence architecture in other plants. Gene sequences of *Arabidopsis thaliana* was used as a standard reference, as there was no reported sequence for black pepper genes. Each gene had four exons, with conserved exon/intron boundaries. Fourth exon of both the genes was the longest and conserved for which primers TFL-F1, TFL-R1 and FT-F1, FT-R1 were designed. The primers TFL-F2, TFL-R2 and FT-F1, FT-R2 were designed for consensus sequences obtained from multiple sequence alignment of homologus sequences. PCR was carried out using these primers. Reaction conditions for PCR were standardised. PCR analysis of genomic DNA using TFL1-F1 and TFL1-R1 yielded an single amplicon of size approximate 700 bp. In spite of varied PCR conditions other primer pairs did not yield any amplification. Amplified product of TFL-F1 and TFL-R1 primer was visualised on 1.4 percent agarose gel. These primer pair produced an amplicon of similar size in spike branching black pepper type and Vellamundi. However, a similar faint band was also produced by the cultivar Karimunda.

Presence of sequence homology for the gene *TFL1* indicates the possible involvement of *TFL1* gene, which had been reported to be associated with inflorescence branching in *Arabidopsis thaliana,* in the spike branching trait of the black pepper type. This result is significant, as five out of the eight cultivars tested did not give any positive response for the primer pair designed based on *TFL1* gene. However, two non-spike branching varieties including Karimunda and Vellamundi have also shown amplification for the TFL1 primer pair.

Random amplified polymorphic DNA analysis was done involving spike branching type and seven non spike branching cultivars/varieties to find out the closest relative. Eight primers were screened for RAPD analysis which produced about 83 scorable bands (average of 10.37 bands per primer). Thirteen bands were monomorphic and remaining 60 bands showed polymorphism. An average of 83 per cent polymorphism per primer was observed. Specific bands were observed in Vellamundi and spike branching type.

Dendrogram generated using RAPD data showed divergence for all the cultivars/varieties at 0.43 similarity index. Two of the varieties; Karimunda and Kumbakal Kodi formed separate big cluster, indicating the wide divergence compared to the other six varieties. First cluster was subdivided into five sub-clusters. Frequent cluster divergence among sub-clusters started within the similarity index ranging from 0.56 to 0.59, indicating the initiation of divergence for five of the cultivars/varieties belonging to the same cluster. The spike branching type and Vellamundi recorded the highest similarity index of 0.74. However, there is no report of spike branching in Vellamundi. The findings of the present study needs further confirmation using other molecular markers.

Molecular studies at transcript level for the meristem identity genes and regulatory transcription factors (MADS-box genes) are essential to characterise spike branching black pepper type. As spike branching found in black pepper has impact on yield, which is economical, isolating and characterising the genes responsible for such a morphological change can be useful in producing genetically modified black pepper varieties, which in turn can improve productivity of black pepper.

References

7. REFERENCES

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. Science 309: 1052–1056

Abo-elwafa, A., Murai, K. and Shimada, T. 1995. Intra and inter specific variations in *Lens* revealed by RAPD markers. Theor. Appl. Gen. 90: 335 340

Ahearn, K. P., Johnson, H. A., Weigel, D. and Wagner, D. R. 2001. *NFL1*, a *Nicotiana tabacum LEAFY*-like gene, controls meristem initiation and floral structure. Plant Cell Physiol. 42:1130-1139

Ajith, A. 1997. Marker aided studies for screening genetic stability in micropropagated plants of *Piper* sp. Biotechnology of Spices, Medicinal and Aromatic plants. Indian Soc. for Spices, Calicut p. 94-97

Altschul, Stephaen, F., Madden, T. L., Schaffer, A. A., Zhang, J., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programmes. Nucleic Acid Res. 25: 3389-3402

Alvarez, J., Guli, C. L., Yu, X. and Smyth, D. R. 1992. Terminal flower: a gene affecting inflorescence development in *Arabidopsis thaliana*. Plant J. 2: 103–116

Amaya, I., Ratcliffe, O. and Bradley, D. 1999. Expression of *CEN* and *CEN*-like genes in tobacco reveals a conserved mechanism controlling phase change in diverse species. Plant Cell 11: 1405–1417

Anandaraj, M., Devasahayam, S., Iswar Bhat, A., Zachariah, T. J., Sannthosh, J., Eapan, B., Sasikumar, B. and Thankamani, C. K. 2005. Black pepper (Exiension Pamplet). Indian institute of Spices Research. Calicut p. 20

Bamnolker, P. T. and Samach, A. 2005. The Flowering Integrator FT Regulates SEPALLATA3 and FRUITFULL Accumulation in Arabidopsis Leaves. The Plant Cell 17: 2661–2675

Banfield, M. J. and Brady, R. L. 2000. The structure of Antirrhinum CEN protein (*CEN*) suggests a role as a kinase regulator. J. Mol. Biol. 297: 1159–1170

Bavappa, K. V. A., Peter, K. V., Thomas, J., Edison, S., Sarma, Y. R. and Parthasarathy, V. A. 2007. Guidelines for the conduct of test for distinctiveness, uniformity and stability on black pepper (*Piper nigrum* L.) (Draft). Protection of Plant varieties and Farmer's Right Authority, GOI p.15

Blazquez, M. A., Green, R., Nilsson, O., Sussman, M. R. and Weigel, D. 1998. Gibberlins promote flowering of Arabidopsis by activating the *LEAFY* promoter. Plant Cell 10: 791-800

Boss, P. K., Vivier, M., Matsumoto, S., Dry, I. B. and Thomas, M. R. 2001. A cDNA from grapevine (*Vitis vinifera* L.), which shows homology to *AGAMOUS* and *SHATTERPROOF*, is not only expressed in flowers but also throughout berry development. Plant Mol. Biol. 45:541-553

Boutros, P. C. and Okey, A. B. 2004. PUNS: transcriptomic and genomic *in silico* PCR for enhanced primer design. Bioinformatics 20: 1534-1538

Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M. and Smyth, D. R. 1993. Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. Development 119: 721-743

Bradley, D., Carpenter, R., Copsey, L., Vincent, C., Rothstein, S. and Coen, E. 1996. Control of inflorescence architecture in Antirrhinum. Nature 379: 791–797

Bradley, D., Ratcliffe, O. J., Vincint, C., Carpenter, R. and Coen, E. S. 1997. Inflorescence commitment and architecture in Arabidopsis. Science 275: 80-83

Brown, P. T. H., Lang, F. D., Kranz, E. and Lorz, H. 1993. Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol. Genet. 237: 311-31

Brown, P. J., Klein, P. E., Bortiri, E., Acharya, C. B., Rooney, W. L. and Kresovich, S. 2006. Inheritance of inflorescence architecture in Sorghum. Theor. Appl. Genet. 113:931–942

Brunner, A. M., Rottmann, W. H., Sheppard, L. A., Krutovskii, K., DiFazio, S. P., Leonardi, S. and Strauss, S. H. 2000. Structure and expression of duplicate *AGAMOUS* orthologus in poplar. Plant Mol. Biol. 44:619-634

Carpenter, R., Copsey, L., Vincent, C., Doyle. S., Magrath, R. and Coen, E. 1995. Control of flowering development and phyllotaxy by meristem identity genes in Antirrhium. The Plant Cell 7: 2001-2011

Coa, Y., Wang, L., Xu, K., Kou, C., Zang, Y., Wei, G., He, J., Wang, Y. and Zhoa, L. 2005. Informtion-theory based algorithem for *in silico* prediction of PCR products with whole genomic sequences as templates. BMC Bioinformatics 6 : 190

Coen, E. D., Bradley, R., Elliott, R., Simon, J., Romero, S., Hantke, S., Doyle, M., Mooney, D., Luo, P., McSteen, L., Copsey, C., Robinson and Carpenter. 1994. Genes controlling flower development in Antirrhium. Development 6: 233-239

Coen, E. S. and Nugent, J. M. 1994. Evolution of flowers and inflorescences. Development (Supplement) 6: 107-116

Colsanti, J. H., Yuan, Z. and Sundaresan, V. 1998. The indeterminate gene encodes a zinc finger protein and regulates a leaf-generated signal required for the transition to flowering in maize. Cell 93: 593-603

CPCRI. 1979. Annual Report of Central Plantation Crops Research Institute, Kasargod. Kerala p.142-144

Das, A. 2005. Studies on genetic fidelity of plants regenerated from somatic embryos of black pepper (*Piper nigrum* L.) using RAPD polymorphism (PR 9). Abstracts of M. Sc. and Ph. D. Dissertations on spice crops. IISR

Datta, P. C. and Dasgupta, A. 1979. Comparision of vegetative anatomy of Piperales III. Vascular supplies of leaves. Act. Bot. India 7:39- 46

De Candolle, C. 1923. Piperacearum Clavis Anylitica. Candollea, Je. Cramer, Germany 1:65-415

De Folter, S., Immink, R. G. H., Kieffer, M., Parenicova, L., Henz, S. R., Weigel, D., Busscher, M., Kooiker, M., Colombo, L., Kater, M. M., Davis, B. and Angenent, G. C. 2005. Comprehensive interaction map of the Arabidopsis MADS box transcription factors. Plant Cell 17: 1424-1433

Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K. and Mattick, J. S. 1991. Touchdown PCR to circumvent spurious priming during gene amplification. Nuclic Acids Res. 19: 40-48

Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15

Espinosa-Soto, C., Padilla-Longoria, P., and Alvarez-Buylla, E. R. 2004. A gene regulatory network model for cell-fate determination during *Arabidopsis thaliana* flower development that is robust and recovers experimental gene expression profiles. The Plant Cell 16: 2923–2939

Ezhova, T. A. and Sklyarova, O. A. 2001. Genes Controlling Inflorescence Structure and Their Possible Role in Evolution. Russian Journal of Developmental Biology 32(6): 388-396

Farm guide. 2008. Government of Kerala p. 23

Faure, S., Higgins, J., Turner, A. and Laurie . D. A. 2007. The FLOWERING LOCUS T-Like Gene Family in Barley (*Hordeum vulgare*). Genetics 176: 599–609

Ferranndiz, C., Gu, C., Martienssen, R. and Yanofsky, M. F. 2000. Redundant regulation of meristem identity and plant architecture by FRUITFUL, APETALA1 and CAULIFLOWER. Development 127: 725-734

George, P. S., Nair, K. N. and Pushpangadan, K. 1989. The Pepper Economy of India. Oxford and IBH publishing Co. Pvt. Ltd. New Delhi

George, J. K., Ganga, G., Varma, S. R., Kumar, S. B., Saji, K. V. 2005. Identification of hybrids in black pepper using male-specific RAPD markers. Curr. Sci. 88: 216-218

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

Hamrick, J. L., Godt, H. J. W. 1989. Allozyme diversity in plant species. Plant Population Genetics, Breeding and Genetic Resources, Sinauer, Sunderland p.43-63

Hanzawa, Y., Money, T. and Bradley, D. 2005. A single amino acid converts a repressor to an activator of flowering. PNAS 102: 7748-7753

Hayama, R., Yokoi, S., Tamaki, S., Yano, M. and Shimamoto, K. 2003. Adaptation of photoperiodic control pathways produces short-day flowering in rice. Nature 422: 719–722

Hecht, V., Foucher, F., Ferrandiz, C., Macknight, R., Navarro, C., Morin, J., Vardy, M. E., Ellis, N., Beltran, J. P., Rameau, C. and Weller, J. L. 2005. Conservation of Arabidopsis Flowering Genes in Model Legumes. Plant Physiology 137: 1420–1434

Hooker, J. D. 1886. The Flora of British India, Todays and Tomorrows. Printers and Publishers, New Delhi p. 331–333

Ibrahim, K. K., Pillay, V. S. and Sasikumaran, S. 1984. Indian Spices 21: 3-9

Ibrahim K. K., Pillai, V. S and Sasikumar, S. 1985a. Genotypic and phenotypic correlations among yield and its components in black pepper (*Piper nigrum* L.). Agric. Res. J. Kerala 23: 150-154

Ibrahim, K. K., Pillai, V. S and Sasikumar, S. 1985b. Path coefficient analysis for some yield components in black pepper *(Piper nigrum* L.). Indian Spices 22(3): 21-25

Ibrahim K. K., Pillai, V. S and Sasikumar, S. 1985c. Variability, heritability and genetic advance for certain quantitative charectors in black pepper. Agric. Res. J. Kerala 23:45-48

Ibrahim, K. K., Pillai, V. S and Sasikumar, S. 1986. Comparative genetic variability with in the open pollinated seedlings of certain varieties of black pepper (*Piper nigrum* L.). Agric. Res. J. Kerala 214: 74-76

Ibrahim, K. K., Pillai, V. S and Sasikumar, S. 1987. Correlated responses in yield and certain component characters in pepper (*Piper nigrum* L.). Agric. Res. J. Kerala 25: 263-264

Ibrahim, K. K., Pillai, V. S and Sasikumar, S. 1988. Expression and association of spike characters in black pepper. Agric. Res. J. Kerala 26: 127-129

IISR. 1996. Ann.Rep. 1995-1996, Indian Institute of Spice Research , Calicut

Ikedia, K., Sunohara, H. and Nagato, Y. 2004. Developmental courses of inflorescence and spikelet in rice . Breed. Sci. 54:147-156

Innis, M. A., Gelfland, D. H., Sninsky, J. J. and White, T. J. 1990. *PCR Protocols: A Guide to Methods and applications*. Academic Press, San Diego, California p. 1560

IPC. 2006. Focus on Pepper (*piper nigrum* L.). Journal of the Pepper Industry. International Pepper Community, Jakartha, Indonasia 3(1): 43-58

Irish, V. F. and Sussex, I. M. 1990. Function of the *apetala-1* gene during Arabidopsis floral development. Plant Cell 2: 741–753

Jack, T. 2004. Molecular and genetic mechanism of floral control. The Plant Cell (Suppl.) 16: 1-17

Jacobsen, S. E., and Olszewski, N. E. 1993. Mutations at the SPINDLY locus of Arabidopsis alter gibberllin signal transduction. Plant Cell 5: 887-896

Jaramillo, M. A. and Manos, P. S. 2001. Phylogeny and patterns of floral diversirsity in the genus Piper (Piperaceae). American J. botany 88 (4): 706-716

Jarvis, E. E., Clark, K. L. and Sprague, G. F. 1989. Disruption of and RNA helicase/RNAse III gene in Arabidopsis cause unregulated cell division in floral meristems. Development 126: 5231-5243

Jenson, C. S., Salchert, K. and Nielsen, K. K. 2001. A TERMINAL FLOWER 1 like gene from perennial ryegrass involved in floral transition and axillary meristem identity. Plant physiology, Rockvilles 125:1517-1528

Joseph, T. S. and Skaria, B. P. 2001. *Pepper*: A medicinal genus. Indian J. Arecanut Spices Med. Pl. (3): 21-23

Joy, N., Abraham, Z. and Soniya, E. V. 2007. A preliminary assessment of genetic relationships among agronomically important cultivars of black pepper. BMC Genetics 8: 42-49

Kang Fu Yu, Deynze, A. V. and Peter Pauls, K. 1993. Random Amplified Polymorphic DNA (RAPD) Analysis. Methods in Plant Molecular Biology and Biotechnology (Eds. Bernand, R.G. and John, E.T). CRC Press. Inc. Boca Raton, Florida. pp 287-301

K.A.U. 1978. Annual report (1977-1978) of Kerala Argi. Univ., Vellanikara, Thrissur p. 122-123

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer FT. Science 286: 1962–1965

Kaundun, S. S., Zyvoloup, A. and Park, Y. G. 2000. Evaluation of the genetic diversity among elite tea (*Camellia sinensis* var. *sinensis*) accessions using RAPD markers. Euphytica 115: 7-16

Kellogg, E. A. 2000. A Model of Inflorescence development. In Monocots: systematics and evolution . Victoria: CSIRO publishing p. 84-88

Kempin, S., Savidge, B., Yanofsky, M. F. 1995. Molecular basis of the *CAL* phenotype in Arabidopsis. Science 267: 522-525

Kishore, K. K. 2005. Genetic analysis of black pepper (*Piper nigrum* L.) hybrids and its parents based on Random Amplified Polymorphic DNA (RAPD) markers (PR 4). Abstracts of M. Sc. and Ph. D. Dissertations on spice crops. Indian Institute of Spices Research, IISR

Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T (1999) A pair of related genes with antagonistic roles in mediating flowering signals. Science 286: 1960–1962.

Komeda, Y. 2004. GENETIC REGULATION OF TIME TO FLOWER IN *Arabidopsis thaliana*. Annu. Rev. Plant Biol. 55: 521–535

Koshay, M., Prasead, T. G. and Pillai, V. S. 1989. Influence of severe reduction of source area of yield attributes and photosynthetic accumulation in black pepper (*Piper nigrum* L.). J. Plant Physiol. 32:248-250

Kumar, P. H. and Sreedharan, A. 1984. Effect of source removal on developing spike in black pepper (*Piper nigrum* L.). Turrialba 33:343-347

Larsson, A. S., Landberg, K. and Meeks-Wagner, D. R. 1998. The TERMINAL FLOWER2 (TFL2) Gene Controls the Reproductive Transition and Meristem Identity in *Arabidopsis thaliana*. Genetics 149:597–605

Levy, Y. Y. and Dean, C. 1998. The transition to flowering. Plant Cell 10: 1973-1989

Li, W., Wang. B. and Wang, J. 2006. Lack of genetic variation of an invasive clonal plant *Eichhornia crassipes* in China revealed by RAPD and ISSR markers. Aq. Bot. 84: 176-180

Liljegren, S. J., Gustafson-Brown, C., Pinyopich, A., Ditta, G. S. and Yanofsky., M. F.1999. Interactions among *APETALA1*, *LEAFY*, and *TERMINAL FLOWER1* Specify Meristem Fate. Plant Cell 11: 1007-1018

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

Manoj, K. A. 2005. Variation in yield and growth performance of cuttings derived from top, middleand bottom nodal explants of different varieties of black pepper under nursery condition (PR 1). Abstracts of M. Sc. and Ph. D. Dissertations on spice crops. IISR

Mathai, C. K. 1986. Growth and yield analysis in black pepper varieties (*Piper nigrum* L.) under different light conditions. Mysore J. Agric. Sci. 18: 317-319

Mathai, C. K., Prasead, T. G. and Pillai, V. S. 1989. Influence of severed reduction in source area of yield attributes and photosynthate accumulation in black pepper (*Piper nigrum* L.) laterals. Indian J. of Plant Physiology 32: 248-250

Mathai, C. K. and Nair, B. P. 1990. Biomass production levels in relation to economic yield in black pepper varieties. J. Plantation Crops 18:125-128

Mathew, P. J., Mathew, P. M. and Kumar, V. 2006. Multivariate analysis in fifty cultivars / landraces of 'black pepper' (*Piper nigrum* L.) occurring in Kerala, India. Rev. Bras. Pl. Med., Botucatu 8: 180-185

McSteen, P., Laudencia-Chingcuanco, D. and Colasanti, J. 2000. A floret by any other name: Control of meristem identity in maize. Trends in plant Science 5: 61-66

Meeks-Wagner, S. 1991. A mutation in the *Arabidopsis TFL1* gene affects inflorescence meristem development. Plant Cell 3:87–92

Menachery, A. J. 1993. Comparative anatomical studies on five species of *Piper* L.(Piperaceae). M. Phil. thesis, Univ. Calicut. Kerala

Mondal, T. K., Singh, H. P. and Ahuja, P. S. 2000. Isolation of genomic DNA from tea and other phenol rich plants. J. Plantn. Crops 28: 30-34

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. 1986. Specific enzyme amplification of DNA *in vitro*: The Polymerase Chain Reaction. Cold spring Harb. Symp. Quant. Biol. 51: 256-273

Munthali, M. T., Newbury, H. J. and Ford Lioyd, B. V. 1996. The detection of somaclonal variants of beet using RAPD. Pl. Cell Rep. 15: 474-478

Nazeem, P. A., Babu, T. D., Kesavachandran, R., Achuthan, C. R., Girija. D., Sureshkumar, P and Peter, K. V. 2003. Detection of genetic diversity in Piper species using RAPD and AFLP markers. National seminar on New Perspective in Spices, Medicinal and Aromatic Plants, 27-29 November 23, Goa

Nirmal Babu, K., Naik, V. G. and Ravindran, P. N. 1993. Two new taxa of *Piper* (*Piperaceae*) from Kerala, India with a note on their origin and inter relationships. J. Spices and Aromatic Crops 2: 26-33

Ohshima, S., Murata, M., Sakamoto, W., Ogura, Y. and Motoyoshi, F. 1997. Cloning and molecular analysis of the *Fdopsis* gene *terminal flower 1*. Mol. Gen. Genet. 254:186–94

Olsen, K. M., Womack, A., Garrett, A. R., Suddith, J. I. and Purugganan, M. D. 2002. Contrasting Evolutionary Forces in the *Arabidopsis thaliana* Floral Developmental Pathway. Genetics 160: 1641–1650

Palacios, C. and Gonzales, F. 1997. Lack of genetic variability in the rare and endangered *Limonium cavanillesii* (Plumbaginaceae) using RAPD markers. Mol. Ecol. 6: 671-675

Parani, M., Anand, A. and Parida, A., Curr. Sci., 1997, 73, 81–83.

Parthasarathy, U., Saji, K. V., Jayarajan. K. and Parthasarathy, V. A. 2006. Biodiversity of Piper in South India – application of GIS and cluster analysis. CURRENT SCIENCE 91(5): 652-658

Parthasarathy, V. A., Sasikumar, B., Nair, R. R. and George, J. K. 2007. Blackpepper-Botany and Horticulture . Hort. Review 33 (In press)

Perry, S. E., Lehti, M. D. and Fernandez, D. E. 1999. The MADS-domain protein *AGAMOUS* like 15 accumulates in embryonic tissues with diverse origins. Plant Physiol. 120:121-129

Petersen, K., Kolmos, E., Smyth, D. R., Bowman, J. and Meyerowitz, E. M. 2006. Early flower development in Arabidopsis. Plant Cell 2: 755-767

Pillai, K. S. 1977. Studies on the comparative performance of the different kinds of planting materials on the yield of pepper. Agric. Res. J. Kerala 15:87-88

Pillai, K. S., Ibrahim, K. K. and Sasikumar, S. 1987. Heterosis in Panniyur 1 black pepper (*Piper nigrum* L.). Agric. Res. J. Kerala 25: 116-118

Pnueli, L., Carmel-Goren, L., Hareven, D., Gutfinger, T., Alvarez, J., Ganal, M., Zamir, D. and lifschitz, E. 1998. the SELF-PRUNING gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog od CEN and TFL1. Development 125: 1979-1989

Pnueli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N. and Lifschitz, E. 2001. Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. Plant Cell 13: 2687–2702

Pradeepkumar, T., Karihaloo, J. L. and Sunil, A. 2001. Molecular characterization of *Piper nigrum*.L. cultivars using RAPD markers. Curr. Sci. 81: 246–248

Pradeepkumar, T., Babu, S. D., Aipe, K. C. and Mathew, S. 2003. Clonal variability in black pepper hybrid Panniyur-1. Journal of Spices and Aromatic crops 12: 154-157

Prasannakumari Amma, E., Nybe, V., Sujatha, V. S. and Prabhakaran, P. V. 2001. Survey, evaluation and identification of black pepper cultivars. Journal of Tropical Agriculture 39: 9-12

Rahiman, B. A and Subbaiah, C. E.1984. Flavanoid analysis in eight species of black pepper from Western Ghats. Pl. Physiol. Biochem. 11: 26-32

Raji, K. 2005. Random Amplified Polymorphic DNA (RAPD) analysis of selected Piper species of South India (PR 120). Abstracts of M. Sc. and Ph. D Dissertations on spice crops. IISR

Ratcliffe, O. J., Amaya, I., Vincent, C. A., Rothstein, S., Carpenter, R., Coen, E. S. and Bradley, D. J. 1998. A common mechanism controls the life cycle and architecture of plants. Development 125: 1609–1615

Ravindran, P. N., Nair, M. K. and Nair, R. A. 1987. Two taxa of *Piper* (Piperaceae) from Silent Vally Forests, Kerala. J. Eco. Tax. Bot. 10: 167-169

Ravindran, P. N. and Nirmal Babu, K. 1988. Black pepper varieties sutable for various regions . Indian Cocoa, Arecanut and Spices J. 11: 110-112

Ravindran, P. N., Ashokan, N. R., Nirmalbabu, K., Chandran, K. and Nair, M. K. 1990. Ecological and morphological notes on Piper species from the silent valley forest, Kerala. J. Bombay Nat. Hist. Society 87(3): 421-426

Ravindran, P. N. 1991. Studies on black pepper and some of its wild relatives. Ph.D thesis, Univ. Calicut

Ravindran, P. N., Balakrishnan, R and Nirmal Babu, K. 1992a. Numerical taxonomyof South Indian Piper L.(piperaceae) cluster analysis. Rheedea 2(1): 55-61

Ravindran, P. N., Sasikumar, B. and Nirmal Babu, K. 1992b. Genetics of shoot tip colour in black pepper. J. Plantation Crops. 20: 76-78

Ravindran, P. N. and Nirmal Babu, K. 1994. Genetic resources of black pepper. In: Chanda, K.L. and Rethinam, P. (eds.). *Advances in Horticulture.* Malhotra publishing House, New Delhi 9:99-120

Ravindran, P. N., Nirmal Babu, K. 1996. Numerical taxonomy of South Indian Piper L. II Principal component analysis of the major taxa. Rheedea 6: 75-86

Ravindran, P. N., Balakrishnan, R. and Nirmal Babu, K. 1997. Morphometric studies on black pepper . I. Cluster analysis of black pepper cultivars. J.Spices and Aromatic Crops 6: 9-20

Ravindran, P. N. 2000. Black Pepper (*Piper nigrum* L.). Harward publication, Singapore p 34-58

Ravindran, P. N., Nirmal Babu, K. and Shiva, K. N. 2005 . Genetic resources of spices and their conservation. Advances in Spices Research. History and achievements of spices research in India since independence. Ed. Ravindran, P.N., Nirmal Babu, K. and Shiva, K.N. and Kallupurakel, J.A. AGRIBIOS (INDIA) p. 63-91

Renuka, M. 2005. ISSR and RAPD markers in diversity analysis of Piper species (PR 112). Abstracts of M.Sc.and Ph.D Dissertations on spice crops. IISR

Riechmann, J. L., Krizek, B. A. and Meyerowitz, E. M. 1996. Dimerization specificity of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLA, and AGAMOUSE. Proc. Natl. Acad. Sci. USA 93:4793-4798

Rohlf, F. J. 1998. NTSYS pc Numerical taxonomy and multivariate Analysis Systems. Exeter Software

Saji, K. V and Sasikumar, B. 2006. genetic resources and varieties of black pepper. Spice India pp. 2-7

Sasikumar, B., Saji, K. V. and George, J. K. 2006. Spike proliferation in black pepper (*Piper nigrum* L.)-A new variation. Journal of the Pepper Industry 3 (1): 11-13

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

Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H. and Sommer, H. 1990. Genetic control of flower development by homeotic genes in *Antirrhinum majus*. Science 250:931-936

Sebastian, A. and Sujatha, V. S. 1996. Isoenzyme variation and species relationship in the genus Piper. J. Tropical Agric. 34: 136-137

Sebastian, A., Sujatha, V. S, Nybe, E. V., Sreekandan, N. G. and Mallika, V. K. 2000. Isoenzyme variation in *Piper nigrum* L. J. Tropical Agric. 38: 9-14

Shannon, S. and Meeks-Wagner, D. R. 1991. A mutation in the Arabidopsis TFL1 gene affects inflorescence meristem development. Plant Cell 3: 877-892

Sharma, A. K. and Bhattacharya, N. K. 1959. Chromosome studies on two genera of family Piperaceae. Genetica 29: 256-289

Shore, P. and Sharrocks, A. D. 1995. The MADS-box family of transcription factors. Eur. J. Biochem. 229:1-13

Smitha Bhasi. 2008. M. Sc. Thesis. Kerala Agricultural University. College of Agriculture, Vellayani

Sreedevi, M. 2005. Molecular and morphological characterization of black pepper lines (PR 50). Abstracts of M. Sc. and Ph.D Dissertations on spice crops. IISR

Stayan, P. and Drev, T. K. 1979. Physiology of mineral nutrition in pepper. Fizol. Rest.(Sofia) 5: 88-96

Sujatha, R and Namboodiri, K. M. N. 1995. Influence of plant characters on yield in black pepper (*Piper nigrum* L.) J. Trop. Agri. 33:11-15

Taylor, S., Hofer, J., Murfet, I., Sollinger, J., Singer, S., Knox, M. and Ellis, N. 2002. PROLIFERATING INFLORESCENCE MERISTEM, a MADS-box

gene that regulates floral meristem identity in pea. Plant Physiol. 129:1150-1159

Tebbs, M. 1989. The climbing species of new world piper (Piperaceae). Wilenowia 19:175-189

Theissen, G., Becker, A., Di Rosa, A., Kanno, A., Kim, J. T., Munster, T., Winter, K.U. and Saedler, H. 2000. A short history of MADS-box genes in plants. Plant Mol. Biol. 42:115-149

Treu, R., Holmes, D. S., Smith, B. M., Astley, D., Johnson, M. A. T and Trueman, L. J. 2001. *Allium ampeloprasum* var. babingtonii (Alliaceae): an isoclonal plant found across a range of habitats in S.W. England

Tucker, S. C. 1982. Inflorescence and flower development in Piperacaeae.II. Inflorescnce development of Piper. Amer. J. Bot. 69: 1389-1401

Vega, K. G., Chavira, M. G.,Vega, O. M., Simpson, J., and Vandermark, G. 2001.Analysis of genetic diversity in *Agave tequilana* var. Azulusing RAPD markers. Euphytica 119:335-341

Wang, B., Li, B. and Wang, J. 2005. Genetic diversity of *Alternanthera philoxeroides* in China. Aq. Bot. 81: 277-283

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

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

Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J. L. and Meyerowitz, E. M. 2006. Genome-wide analysis of gene expression during early Arabidopsis flower development. PLoS Genet. 2: 1012-1024

William, D. A., Su, Y. H., Smith, M. R., Lu, M., Baldwin, D. A. and Wagner. D. 2004. Genomic identification of direct target genes of *LEAFY*. Proc. Natl. Acad. Sci. U. S. A 101:1775-1780

Wilson, R. N. and Somerville, C. R. 1995. Phenotypic suppression of the Gibberlin-Insensitive Mutant (gai) of Arabidopsis. Plant Physiol. 108: 495-502

Yanofsky, M. F. 1995. Floral meristems to Floral organs: Genes controlling early events in Arabidopsis flower development in Arabidopsis flower development. Annu Rev. Plant Physiol. Plant Mol. Biol. 46: 167-188

Zhu, Q-H, Hoque, M. S. Dennis, E. S. and Upadhyaya, N. M. 2003. Ds tagging of BRANCHED FLORETLESS 1 (*BFL1*) that mediates the transition from spikelet to floret meristem in rice (*Oryza sativa* L). BMC Plant Biology 3: 6-19

http://www.indianetzone.com

http://www.fokker.wi.mit.edu/primer3/

http://www.Soe.ucsc.edu/kent.scr/unzipped/isPcr

http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/

http://www.Soe.ucsc.edu/kent.scr/unzipped/isPcr

http://www.nmce.com/commodityStudy/pepper_study.jsp

http://www.Basic.northwester.edu/bitools/oligocalc.html

http://www.pitt.edu/Kaliszlab/Protocols/DNAExtraction.doc

http://align.genome.jp

Appendices

APPENDIX I

CTAB extraction buffer

C-TAB	2 % w/v
NaCl	3 M
Tris- HCl (pH 8.0)	100 mM
EDTA	0.5 M
PVP	4 % w/v
β-mercaptoethanol	0.5 % v/v

APPENDIX II

50 X TAE buffer

Tris Acetate	242.0 g
EDTA (0.5 M)	100 ml
Glacial Acetic Acid	57.1 ml

Make up volume to 1000 ml

APPENDIX III

TE buffer

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

APPENDIX IV

Gel loading buffer

Bromophenol blue	0.25 % w/v
Glycerol	30 %
Sterile water	70 %

MOLECULAR ANALYSIS OF SPIKE BRANCHING OBSERVED IN BLACK PEPPER (*Piper nigrum* L.) TYPE FROM IDUKKI

VIMARSHA, H.S.

Abstract of the
thesis submitted in partial fulfilment of the requirement
for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2009

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Abstract

ABSTRACT

The study entitled "Molecular analysis of spike branching observed in black pepper (*Piper nigrum* L.) type from Idukki" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2006-2008. The objective of the study was to analyse the sequence homology for the inflorescence architecture gene (*TFL1)* and flowering gene (*FT)* in spike branching black pepper type from Idukki, using polymerase chain reaction technique.

Spike branching in black pepper is very rare phenomenon. Profuse spike branching was observed in an uncharacterized black pepper type from Idukki. Single spike had three to four times more berry yield, compared to improved varieties. This type needed to be characterized at morphological and molecular levels to know its parental descendent and possible involvement of gene regulation responsible for spike branching. As an initial approach, molecular analysis was done to find out the presence of *TFL1* and *FT* genes, which have been reported to be involved in inflorescence branching in modal plants.

TFL1 primer pair (TFL-F1 and TFL-R1), designed for the fourth exonic sequence of *TFL1* gene in *Arabidopsis thaliana,* could amplify a 700 bp DNA sequence in the spike branching type, as well as in Karimunda and Vellamundi, indicating homology for the gene sequence and possible conserved exonic sequence in black pepper. Presence of sequence homology for the gene *TFL1* indicated the possible involvement of *TFL1* gene, which had been reported to be associated with inflorescence branching in *Arabidopsis thaliana,* in the spike branching trait of the black pepper type. This result is significant, as five out of the eight cultivars tested did not give any positive response for the primer pair designed based on *TFL1* gene. However, two non-spike branching varieties including Karimunda and Vellamundi have also shown amplification for the TFL1 primer pair.

As an initial step towards characterisation of the spike branching type, RAPD analysis of it along with seven other cultivars/varieties was done. This analysis revealed variability, accounting for 83 per cent polymorphism. In the dendrogram, at

a similarity index of 0.43 the plants grouped into two big clusters, indicating 67 per cent dissimilarity. All the eight plants under study formed individual clusters at similarity index 0.74, except the spike branching type and Vellamundi.

Results of gene specific PCR which yielded single amplicon can be hypothecated for the presence of sequence homology for the gene *TFL1* and has conserved fourth exon in black pepper cultivars and varieties. Further transcript level and expression level studies are essential to find the specific role of the *TFL1* gene in spike branching black pepper.