ANALYSIS OF DIFFERENTIAL EXPRESSION OF GENES DETERMINING INFLORESCENCE ARCHITECTURE IN BLACK PEPPER (*Piper nigrum* L.) TYPE 'THEKKEN'

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(2014-11-257)

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DECLARATION

I, hereby declare that this thesis entitled **"Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me for any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellayani, **Hembade Vivekanand Laxman**

Date: 13/10/2016 (2014-11-257)

CERTIFICATE

Certified that this thesis entitled **"Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'"** is a record of bonafide research work done independently by Mr. Hembade Vivekanand Laxman (2014-11-257) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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We, the undersigned members of the advisory committee of Mr. Hembade Vivekanand Laxman (2014-11-257), a candidate for the degree of Master of Science in Agriculture with major in Plant Biotechnology, agree that the thesis entitled **"Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'"** may be submitted by Mr. Hembade Vivekanand Laxman, in partial fulfilment of the requirement for the degree.

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A goal is a milestone which a youngster dreams and successfully achieves only with the blessings and the guiding lights of ……

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Hembade Vivekanand Laxman

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ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AMV-RT	*Avian myeloblastosis virus* reverse transcriptase
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
G	Guanine
g	gram
g	standard acceleration due to gravity at the earth's surface
h	Hour
ha	Hectare
kbp	Kilo basepair
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram

nm	Nanometre
^{o}C	Degree celsius
OD	Optical density
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
qRT-PCR	Quantitative reverse transcriptase - polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcription - polymerase chain reaction
s	Second
SAM	Shoot apical meristem
T	Thymine
TAE	Tris-acetete EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (hydroxy methyl) aminomethane hydrochloride
U	Enzyme unit
V	Volt
v/v	volume/volume
w/v	weight/volume

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INTRODUCTION

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), often called as 'The King of Spices' is one of the most important and widely used spice in the world since prehistoric times. Black pepper is a flowering climber in the family *Piperaceae*, cultivated for its fruit which is usually dried and used as a spice and a very highly priced market commodity. India is the centre of origin of black pepper and lot of species diversity is reported in quantitative and qualitative traits.

Wild type inflorescence of black pepper is a slender spike which is unbranched. But black pepper type 'Thekken' reported by farmer T. T. Thomas from Idukki district, Kerala has branching inflorescences (Farm innovators, 2010). Number of branches per inflorescence increases the number of flowers and thereby the final berry yield. As the production and productivity of black pepper in India shows a drastic decline, the studies on the branching inflorescence trait can be useful in increasing production as well as productivity.

Exploitation of this economically important trait requires knowledge about mechanisms controlling inflorescence architecture and genes involved in different pathways controlling flowering in black pepper.

Molecular basis of inflorescence architecture has been extensively studied in model plants such as *Arabidopsis*, rice, maize, etc. Different genes (e.g. *PIN1*, *RAMOSA2*, *TFL1*, *BIF1*, etc.) have been reported to control the inflorescence architecture in different plants (Ohshima *et al*., 1997; Vernoux *et al*., 2000; Bortiri *et al*., 2006; McSteen *et al*., 2007).

Inflorescence branching appears to be largely regulated through the *RAMOSA* gene network, and the name '*ramosa*', originates from the Latin word '*ramus*' means branch. This reflects the phenotype of the *ra* mutants, which have a highly branched inflorescence. The *RAMOSA* family mainly involves three genes *viz*., *RAMOSA1* (*RA1*), *RA2* and *RA3*. *RA1* encodes a zinc-finger domain protein (Vollbrecht and Sigmons, 2005), *RA2* encodes lateral organ boundary domain protein (Bortiri *et al*., 2006) and *RA3* encodes a metabolic enzyme, a

trehalose-6-phosphate phosphatase (Satoh-Nagasawa *et al*., 2006). Mutation in any of the genes shows different patterns of inflorescence branching in different plants.

RAMOSA family genes have been reported in a wide range of plants (Kellogg, 2000; Vollbrecht and Sigmon, 2005; Bortiri *et al*., 2006; Satoh-Nagasawa *et al*., 2006; McSteen, 2006; Kellogg, 2007; Koppolu *et al*., 2013; Ishiai *et al*., 2016). It is possible that *RAMOSA* family genes may control inflorescence architecture in black pepper. Therefore, the objective of the present study was to detect the presence and differential expression of *RAMOSA* family genes (*RA1*, *RA2* and *RA3*) and analyse their influence in contributing to the branching trait in black pepper type 'Thekken'.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) (Family: *Piperaceae*) is a perennial, climbing vine. It is cultivated for the mature berries which are used as spice and in medicines (Karthikeyan and Rani, 2003). It is the most important spice crop due to its everyday use and its high market price. Black pepper requires tropical weather (i.e. high rainfall and humidity) therefore it is mostly cultivated in India and Southeast Asian countries such as Vietnam, Indonesia and Malaysia.

In India, hot and humid climate of sub mountainous regions of Western Ghats is found ideal for black pepper cultivation. Ideal range of temperature for growth of black pepper is 23 ^{o}C - 32 ^{o}C and the ideal range of relative humidity is 75-80%. Annual rainfall of 1250-2000 mm is considered ideal for black pepper (Thomas and Rajeev, 2015).

Black pepper is one of the most ancient crops cultivated in India. A lot of species diversity is reported in India in quantitative and qualitative traits of black pepper (Parthasarathy *et al*., 2006; Bhasi *et al*., 2010; Wu *et al*., 2016). *Piper* genus is reported with 700 species in American tropics, followed by 300 species in South Asia (Jaramillo and Manos, 2001). Black pepper (*Piper nigrum* L.) has originated in the hills of South-Western India i.e. from North Kanara to Kanyakumari (Pruthi, 1974; Ravindran, 2000a; Thangaselvabal *et al*., 2008; Wu *et al*., 2016).

Over 75 cultivars of black pepper are being cultivated in India. ‘Karimunda’ is the most popular cultivar in Kerala. The other important cultivars are Kottanadan (South Kerala), Narayakodi (Central Kerala), Aimpiriyan (Wayanad), Neelamundi (Idukki), Kuthiravally (Kozhikode and Idukki), Balancotta, Kalluvally (North Kerala), Panniyur (Kannur), Malligesara and Uddagare (Karnataka). Kuthiravally and Balancotta exhibit alternate bearing habit (Thomas and Rajeev, 2015).

Kerala alone accounts for 94 % of the total area and 96 % of the total production of pepper in India, followed by Karnataka with 3.5 % production. The rest is contributed by Tamil Nadu, Pondicherry and Andaman and Nicobar. Even though black pepper is cultivated all over Kerala, only four districts *viz*., Kozhikode, Kannur, Kottayam and Idukki account for 67 % of the total pepper area (Thomas and Rajeev, 2015).

Black pepper is used as a condiment, on its own and also in spice blends. Use of black pepper as a natural preservative for perishable foods has been known for centuries. It is one of important ingredients in Ayurvedic, Chinese, Unani and other traditional medicines. The main therapeutic use of pepper is for digestive purpose and is also used as a tonic (Thomas and Rajeev, 2015).

2.1 FLOWERING AND POLLINATION IN BLACK PEPPER

In India, flowering season of black pepper is during May to July monsoon season, generally about two to three years after planting. Flowers are staminate, pistillate, or hermaphrodite. Both male and female flowers can be present on a single spike. Based on the floral composition, a plant can be either pistillate or staminate. It takes about 11 to 37 days for complete emergence of spike depending on the cultivar (Menon, 1981). Flowering on spikes starts 10 to 15 days after spike emergence and will be completed in about 6 to 10 days. Protogyny of bisexual flowers is more pronounced in 'Karimunda'. In protogynous cultivars, male and female maturity phases are spaced by intervals of 1 to 14 day. Perianth is usually absent in flowers. Two small stamens are formed on each side of the ovary in bisexual flowers and are 0.1 cm long with small anthers having two sacs. The ovary is globose, one ovulated, surmounted by three to five lobed, star shaped stigma, covered with papillae, white when receptive, later turn brown (Ravindran *et al*, 2000b; Parthasarathy *et al*., 2008).

Black pepper is mainly self-pollinated (Sasikumar *et al*., 1992). But various levels of protogyny are also found. Selfing in black pepper spike is stimulated by positive geotropism, spatial arrangement of flowers, sequential

ripening of the stigma, and non-chronological dehiscence of anthers (Sasikumar *et al*., 1992; Ravindran, 2003). Anandan (1924) reported importance of splashing rain on pollination. Rain drops help in dispersal of pollen grains in all directions, either wash down the pollen grains to lower spikes or carry them to adjacent black pepper vines (Ravindran, 2003).

2.2 INFLORESCENCE OF BLACK PEPPER

The inflorescence of black pepper is a glabrous, filiform, pendulous spike borne opposite to the leaves on plagiotropic branches. Orientation of the spike is either pendulous or erect in the genus *Piper*. Species of *Piper* are diverse in spike shape and length (Amma *et al*., 2001). Spikes in black pepper can be straight or curved (Parthasarathy *et al*., 2007). In black pepper inflorescence, 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 inflorescence vary in number from 25 to 100, arranged in four to five rows (Parthasarathy *et al*., 2007). The apical buds of the plagiotropic branches transform into inflorescence. The apical meristem of the inflorescence grows in length before any organs are formed.

Mostly wild type black pepper species have unbranched spikes. Branching in spikes of black pepper is very rare. Varieties of black pepper 'Aimpiriyan' and 'Kathirinmelkkathir' show spike branching characteristic. But these branches are rudimentary and bear very few berries (Sasikumar *et al*., 2006).

2.3 'THEKKEN'- A MUTANT OF BLACK PEPPER

A pepper type *viz*., 'Thekken', a natural mutant of *Piper nigrum*, identified by T. T. Thomas in the forest area of Kanchiyar in Kattappana Panchayat, Idukki district, has been observed to have altered inflorescence architecture with remarkable spike branching character. Spike branching in 'Thekken' is more profuse and with more berry yield than 'Aimpiriyan' and 'Kathirinmelkkathir' (Sasikumar *et al*., 2006; Farm innovators, 2010).

In ‘Thekken’ both branched and unbranched type of spikes are seen. The proliferating spikes are of indeterminate growth habit. Some spikes of pepper plant appear as grape bunches and they are with persistent and large bracts (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 gives four times the reported yield of berries than from spikes of the highest yielding varieties, Panniyur-1, Panniyur-3 and Panniyur-5.

Branching spike of black pepper type ‘Thekken’

Yield is a quantitative trait and is directly dependent on number of flowers per spike (Mohsin *et al*., 2009). Spike branching trait is of great economic significance as it can contribute to high yield. Modifications in inflorescence architecture is reported in different crop species such as *Oryza sativa*, *Arabidopsis thaliana* and *Zea mays* due to mutations in different genes such as *LEAFY*, *APETALA1*, *TERMINAL FLOWER1*, *FLOWERING LOCUS T*, *RAMOSA* family genes etc (Gallavotti *et al*., 2010; Zhang *et al*., 2015; Wickland and Hanzawa, 2015; Ishiai *et al*., 2016).

2.4 GENES CONTROLLING INFLORESCENCE ARCHITECTURE

Environmental and genetic regulation of flowering time, branch complexity (e.g. number of iterations of branching prior to flowering), number of flowers per node and the extent of terminal meristem growth in the reproductive phase contribute to the overall pattern of an inflorescence. Inflorescence form varies enormously among different species and seems to play a determinant role in reproductive success as it has a strong effect on pollination and fruit set.

Inflorescence architecture is highly diversified among flowering plants. Diversity among inflorescences has been attributed to factors such as: extent of growth in each of the three dimensions of stem and stem-like structures,

determinacy or indeterminacy of meristems within the shoot system, specification of meristem identity and relative positions of lateral shoots and/or flowers (Ainsworth, 2006). These factors are controlled by genes performing different functions in tissues and individual cells.

From the studies on floral development and floral meristem identity genes in *Arabidopsis*, *Antirrhinum majus*, cauliflower, maize, rice, etc., it is now known that floral genes are highly conserved in the plant kingdom (Goto *et al*., 2001; Jack, 2004; Song *et al*., 2010).

After transition from vegetative phase to reproductive phase shoot apical meristem gives rise to different meristems *viz*., inflorescence meristem, branch meristem, spikelet meristem and floral meristem. These meristems form different architectures in the inflorescence. There are different levels of architecture in inflorescence, and each of them is formed from different meristems (Wu *et al*., 2009; Tanaka *et al*., 2013; Kyozuka *et al*., 2014).

2.4.1 Genes controlling inflorescence meristem formation

First meristem produced during transition from vegetative to reproductive phase is inflorescence meristem. The development of the *Arabidopsis* inflorescence meristem is controlled by mutual regulation of three genes *viz*., *TERMINAL FLOWER1* (*TFL1*), *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Liljegren *et al*., 1999; Blazquez *et al*., 2006; Kaufmann *et al*., 2010; Yoo *et al*., 2010). These genes maintain the balance between inflorescence meristem and floral meristem identity at the inflorescence apex (Ratcliffe *et al*., 1999; Blazquez *et al*., 2006; Benlloch *et al*., 2015).

Key elements of how the repressor of flowering and shoot meristem gene *TFL1* acts were tested by Baumann *et al.* (2015), by changing its spatiotemporal pattern and showed *TFL1* can act outside of its normal expression domain in leaf primordia or floral meristems to repress flower identity. Baumann *et al.* (2015) proposed that *TFL1* and other floral genes both can act and compete in the same

meristem. Kobayashi *et al.* (2012) proposed that *PANICLE PHYTOMER2* (*PAP2*) and the three *APETALA1* (*AP1*)*/FRUITFULL* (*FUL*)-like genes co-ordinately act in the meristem to specify the identity of the inflorescence meristem downstream of the florigen signal.

In *Arabidopsis*, *AGAMOUS* (*AG*) gene is also found to be regulating meristem determinacy (Barton, 2010; Sun and Ito, 2010). In *ag* mutants, a set of floral organs (sepal-petal-petal) are repeatedly formed (Bowman *et al*., 1989; Yanofsky *et al*., 1990). Expression of *WUSCHEL* (*WUS*) gene occurs in the floral meristem of the *ag* mutant at a late stage of flower development. But in wild type inflorescence, expression of *WUS* disappears after formation of the carpel (Lenhard *et al*., 2001, Lohmann *et al*., 2001). Therefore, in the floral meristem, *AG* regulates meristem determinacy by repressing *WUS*. Another gene *KNUCKLES* (*KNU*) encoding a transcriptional repressor, represses the expression of *WUS* by *AG* (Sun *et al*., 2009).

BARREN INFLORESCENCE2 (*BIF2*) gene encodes a maize ortholog of the *Arabidopsis* serine-threonine kinase *PINOID*. McSteen *et al*. (2007) found mutation in *BIF2* gene fails to initiate all axillary meristems of the inflorescence in maize indicating the role in determinacy of branch meristem.

2.4.2 Genes controlling branch meristem formation

Genetic control of branching is a primary determinant of yield, regulating seed number and harvesting ability, yet little is known about the molecular networks that shape grain bearing inflorescences of cereal crops.

In maize, inflorescence branching appears to be largely regulated through the *RAMOSA* gene network (*viz*., *RA1*, *RA2* and *RA3*) and mutations in these genes reflect highly branched inflorescences (Vollbrecht and Sigmon, 2005; Bortiri *et al*., 2006; Satoh-Nagasawa *et al*., 2006).

LAX1 gene from rice and *BARREN STALK1* (*BA1*) gene from maize are homologous genes. Mutation in these genes causes failure of initiation of axillary

meristems in both the vegetative and reproductive phases (Komatsu *et al*., 2003, Gallavotti *et al*., 2004; Gallavotti *et al*., 2008)**.**

Rice gene *TAWAWA1* (*TAW1*) encodes a nuclear protein with an ALOG domain. In *taw1-D* mutant, promotion of inflorescence meristem activity and delay in spikelet development was observed and this caused increased branching in rice inflorescence. In contrast, loss of *TAW1* function causes reduction in inflorescence meristem indeterminacy and small inflorescences (Yoshida *et al*., 2013).

2.4.3 Genes controlling spikelet meristem and spikelet pair meristem formation

Spikelet and spikelet pair meristem identity in maize is combinatorially regulated by *REVERSED GERM ORIENTATION1* and *INDETERMINATE SPIKELET* (*IDS1*) (Kaplinsky and Freeling, 2003; Chuck *et al*., 2007; Chuck *et al*., 2008).

PUCHI gene from *Arabidopsis* is the homology to maize *BRANCHED SILKLESS1* (*BD1*) and rice *FRIZZY PANICLE* (*FZP*). A partial conversion from floral meristem to inflorescence meristem is observed in the *puchi* mutant, in addition to other phenotypes (Karim *et al*., 2009).

In *Arabidopsis*, *AGAMOUS* (*AG*), which encodes a C-class MADS-box transcription factor, is a very important gene responsible for determinacy of meristem. The *ag* mutants produce indeterminate flowers in which a set of sepal-petal-petal are repeatedly formed (Bowman *et al*., 1989; Yanofsky *et al*., 1990). *WUSCHEL* (*WUS*) expression persists in the floral meristem of the *ag* mutant at a late stage of flower development, whereas it disappears after formation of the carpel in the wild type (Lenhard *et al*., 2001; Lohmann *et al*., 2001). Therefore, in the floral meristem, *AG* regulates meristem determinacy by repressing *WUS*. *KNUCKLES* (*KNU*), which encodes a transcriptional repressor, has an important role to mediate the repression of *WUS* by *AG* (Sun *et al*., 2009).

Ikeda-Kawakatsu *et al*. (2011) characterised rice *aberrant panicle organization 2* (*apo2*) mutant. Map-based cloning showed that *APO2* is identical to *RFL* gene which is a rice ortholog of the *Arabidopsis LEAFY* (*LFY*) gene. The *apo2* mutant exhibits small panicles with reduced number of primary branches due to formation of spikelet meristems. The *apo2* mutants also displayed late flowering, aberrant floral organ identities and loss of floral meristem determinacy.

AP1 regulates cytokinin levels by directly suppressing the cytokinin biosynthetic gene *LONELY GUY1* and activating the cytokinin degradation gene *CYTOKININ OXIDASE/DEHYDROGENASE3* (Han *et al*., 2014). In *Arabidopsis*, Han *et al*. (2014) concluded that suppression of cytokinin biosynthesis and activation of cytokinin degradation mediates *AP1* function in establishing determinate floral meristem.

2.5 *RAMOSA* FAMILY GENES

Diversity in the patterns of inflorescence architecture is due to different gene combinations and expression patterns in the plant. Mutation in these genes can result in altered inflorescence architecture. Several genes affect these patterns of inflorescence architecture differently in different plants, allowing morphological differentiation that permits diversification. *RAMOSA* family genes are involved in maize inflorescence development. *RAMOSA* family genes (*viz*., *RAMOSA1*, *RAMOSA2* and *RAMOSA3*) have been cloned from maize and can form part of a network of genes that control the production of lateral branching (Tanaka *et al*., 2013).

2.5.1 Role of *RAMOSA* family genes

All three *RAMOSA* genes have been cloned and their protein products are studied. *RA1*, a member of *RAMOSA* family, encodes a zinc-finger domain protein, a presumed transcription factor (Vollbrecht and Sigmon, 2005). *RA2* is a LOB domain protein also presumed to be a transcription factor (Bortiri *et al*., 2006). *RA1* appears to act downstream of *RA2*. *RA3* encodes a metabolic protein, a trehalose-6-phosphate phosphatase, suggesting that trehalose-6-phosphate (T6P)

might have a role in the regulation of development (Satoh-Nagasawa *et al*., 2006). In *ra3* mutants, the expression of *ra1* is reduced, suggesting that *ra3* regulates *ra1* either directly or indirectly.

2.5.2 Occurrence of *RAMOSA* family genes

The paired spikelet of maize inflorescence is a feature of other members of the tribe Andropogoneae (e.g. sorghum and sugarcane), but do not occur in many other grasses, such as wheat or rice (Kellogg, 2000). Consistent with the taxonomic distribution of this feature, *ra1* and *ra3* are present in Andropogoneae, but both are missing in rice (although rice has a duplicate of *ra3*, namely *sister of ramosa3* [*sra*]) (Satoh-Nagasawa *et al*., 2006). Either these genes have been lost in rice or they were gained in the Andropogoneae by duplication after divergence from the common ancestor of rice and maize (Kellogg, 2007). Koppolu *et al*. (2013) reported *Six-rowed spike4* (*Vrs4*) ortholog of maize *RA2* in barley while Ishiai *et al*. (2016) reported presence of all *RAMOSA* family genes in *Vitis vinifera.*

2.5.3 *RAMOSA1*

Gallavotti *et al*. (2010) showed in maize (*Zea mays*), that the zinc (Zn)-finger transcription factor *RAMOSA1* interacts with the *TPL/TPR* factor *RAMOSA1 ENHANCER LOCUS2* (*REL2*) to repress indeterminate meristem fate. *REL2* was identified as a transcriptional corepressor of the indeterminant branching pathway, important in enforcing the differentiated fate of reproductive maize organs (Gallavotti *et al*., 2010).

Changing aspects of genes targeted *in vivo* by the transcription factor *RAMOSA1*, a key regulator of determinacy, revealed potential mechanisms for repressing branches in distinct stem cell populations, including interactions with *KNOTTED1* which is a master regulator of stem cell maintenance (Eveland *et al*., 2014).

RA1 encodes a transcription factor that regulates meristems to control the branching architecture of inflorescences. Another classical mutant, *ramosa2*, controls branching similarly, through the *ra1* genetic pathway. *RA1* acts by establishing a boundary between lower-order meristems and the principal inflorescence axis, thereby controlling the fundamental property of the duration of meristem activity. Reduced nucleotide diversity at *RA1* in modern maize implies that the gene was a target of selection during domestication or improvement. While the original loss-of-function mutant conferred extreme ear branching, intermediate levels of *ra1* gene activity led ears with crooked rows, suggesting selection for *ra1* forms that preserved straight rows in the massive ear of domesticated maize (Vollbrecht and Sigmon, 2005).

2.5.4 *RAMOSA2*

The *ramosa2* (*ra2*) mutant of maize results in increased branching, with short branches replaced by long indeterminate ones (Bortiri *et al*., 2006). Function analysis showed that *RA2* encodes the AS2/LOB domain transcription factor which controls the developments in stem cells of branch meristems in maize (Bortiri *et al*., 2006). Koppolu *et al*. (2013) reported ortholog of maize *RAMOSA2* in barley and it is named as *Six-rowed spike4* (*Vrs4*) gene. Genetic mapping and mutant analysis in barley showed that *Vrs4* controls spikelet determinacy and row type in barley.

2.5.5 *RAMOSA3*

Satoh-Nagasawa *et al*. (2006) showed that *RA3* encodes a trehalose-6-phosphate phosphatase expressed in discrete domains subtending axillary inflorescence meristems. Genetic and molecular data indicate that *RA3* functions through the transcriptional regulator *RA1*.

Satoh-Nagasawa *et al*. (2006) proposed that *RA3* regulates inflorescence branching by modification of a sugar signal that moves into axillary meristems. Apart from a potential metabolic role, mutant phenotype of *RA3* suggests that it is

involved in transcriptional regulation. Metabolic and transcriptional activity is evocative of other metabolic genes, such as *HEXOKINASE*, which has been shown to act as transcription factor as well as metabolic enzyme (Cho *et al*., 2006).

RAMOSA genes and *BARREN STALK1* (*BA1*) gene express in ring like domains at the base of the branch meristem. Location of ring like domain and mutant phenotypes of the genes suggests that the fate of branch meristem is controlled by a mobile signal whose import or export is regulated by the branching proteins. Candidates for such a signal could be carotenoid-derived molecules, which may have a role in auxin signaling (Booker *et al*., 2005; Snowden *et al*., 2005), and/or T6P produced by activity of *RA3*.

2.5.6 Expression related studies

Satoh-Nagasawa *et al*. (2006) selected ears with uniform size at a growth stage of 2 mm, where they found expression of *RA3* was the highest in the wild type and the very first signs of the mutant phenotype were visible as outgrowths of the spikelet pair meristems.

During early inflorescence development in maize, *RA3* gene was expressed in a narrow band subtending spikelet pair meristems (Satoh-Nagasawa *et al*., 2006). As trehalose-6-phosphate is a mobile signal, such as a sugar, it could be mediating RA3's control of development of axillary meristem (Rolland *et al*., 2006).

Eveland *et al*., (2010) found that down-regulated expression of *RAMOSA3* gene showed reduced production of trehalose phosphate synthase. In contrast to this, an uncharacterized gene was up-regulated in the mutant maize which showed sequence similarity to a trehalose-6-phosphate phosphatase of *Arabidopsis*, possibly as compensation for reduced *RA3* levels.

2.5.7 Mutant phenotypes of *RAMOSA* family genes

In the maize ear (female flower), there is no branching while, in maize tassel (male flower), branches are normally either long or short and there are no intermediate branches. In three classical mutants of maize, *ramosa1* (*ra1*), *ra2* and *ra3*, branching in ear and shift from long to short branches in tassel was disrupted, with branches being produced that are intermediate in length between the long and short branches (Heck *et al*., 1999). Specific function of *RAMOSA* family genes (*RA1*, *RA2* and *RA3*) is to maintain determinate identity of spikelet pair meristems (SPM), thereby limiting branch outgrowth (Vollbrecht and Sigmon, 2005; Bortiri *et al*., 2006; Satoh-Nagasawa *et al*., 2006). *RAMOSA1 ENHANCER LOCUS2* (*REL2*) is another gene, reported to interact with *RA1*, which regulates the determinacy of the spikelet pair meristem (Gallavotti *et al*., 2010).

Normal functioning of *RAMOSA* genes causes determinate spikelet pair meristem growth (Wu *et al*., 2009). But when any of the three *RAMOSA* genes are mutated, the spikelet pair meristems on both the tassel and the ear become more indeterminate (Vollbrecht and Sigmon, 2005; Bortiri *et al*., 2006; Satoh-Nagasawa *et al*., 2006). Spikelet pair meristems with altered *RAMOSA* genes shows a development more like branch meristems and forms highly branched inflorescences. So the different phenotypes of long branches and spikelet pairs may be seen as the consequences of different meristem identities. If meristems produced by the inflorescence meristem are determinate spikelet pair meristems, they will produce spikelet pairs and if they are indeterminate branch meristems, they will form lateral branches. *RAMOSA* family genes regulate the switch between spikelet pair meristems and branch meristems and control the meristem determinacy and identity. This suggests the *RAMOSA* genes promote the determinate fate of spikelet pair meristem (Tanaka *et al*., 2013).

2.5.8 QTL studies

QTL studies indicate that allelic variation in the *RAMOSA* genes might control diversity in inflorescence architecture within a species. Brown *et al*.

(2006) studied inflorescence development in sorghum. For this study they used population created by crossing a plant having spreading branches in inflorescence with one that had upright branches in inflorescence. One QTL corresponded to the region of *RA2*. Upadyayula *et al*. (2006) found a QTL in mapping population of maize, which was related to tassel branch number. This QTL corresponded to the location of *RA1* and other QTL in the region of *FASCIATED EAR2*.

2.5.9 Studies in black pepper type 'Thekken'

Vimarsha (2009) reported *TFL1* homologous band in black pepper and used RAPD primers to study relatedness and diversity among different black pepper varieties. He found that the variety Vellamundi depicted near relativeness (74%) with 'Thekken' compared to the wide cluster formed by famous local cultivar 'Karimunda'.

Subba (2014) performed molecular analysis of 'Thekken' using RAPD, SSR markers and SDS-PAGE. These studies showed no difference between branched and non-branched spikes of 'Thekken' at molecular level. Studies based on candidate genes showed difference in *PIN1* and *LOG1* profile between 'Thekken' and 'Karimunda' and absence of *BP* amplification in 'Thekken' and difference in *RA2* gene amplification.

2.6 COMPARATIVE GENOMICS FOR CANDIDATE GENE IDENTIFICATION

Candidate genes are genes with known biological function directly or indirectly regulating the developmental processes of selected trait which could be analysed in target organism by using molecular techniques (Tabor *et al*., 2002). Comparative genomics strategy can be used to identify and characterize the effects of selected candidate genes. It includes comparative functional genomics approach and comparative structural genomics approach, which results in comparative functional candidate gene approach and comparative positional candidate gene approach, respectively (Zhu and Zhao, 2007). In comparative

genomics approach, candidate gene selected may be functionally conserved or structurally homologous with genes identified in other plants (Phillips *et al*., 2002). Comparative genomics strategy can be more effectively used if candidate gene showing phenotypic variation of interest has already been confirmed in other plants (Ewart-Toland and Balmain, 2004).

Many of the genes governing inflorescence architecture have been found to be conserved in plant species (Ambrose *et al*., 2000; Ng and Yanofsky, 2001; Benlloch *et al*., 2007). Candidate gene approach could be advantageous in exploring the presence of these genes and their functions in different plant species using degenerate primers. Degenerate primers will be designed based on the conserved sequences among available variants of gene sequence (Garg *et al*., 2008).

2.6.1 Primer designing

A primer is a short oligonucleotide which is the reverse complement of a region of a DNA template. It would anneal to a DNA strand to facilitate the amplification of the targeted DNA sequence. Generally, two primers are used in PCR i.e. forward and reverse primer (Garg *et al*., 2008).

Degenerate primers are with degenerate bases which can base pair with other nucleotides. They are well-situated if the same gene is to be amplified from different organisms, as loci in genomes of different species are probably similar but not identical in different species (Patel and Prakash, 2013).

The primer sequence determines several parameters such as the length of the primer, its melting temperature, its annealing temperature and ultimately the yield (Wu *et al*., 1991; Wittwer and Schütz, 2001). If primers are not designed carefully, PCR result can be little or no product due to non-specific amplification. Primer dimer formation can suppress the PCR reaction with no amplification (Patel and Prakash, 2013). The sequences of the primers used for PCR

amplification can have a major effect on the specificity and sensitivity of the reaction.

The specificity is controlled by length of primer and annealing temperature of PCR reaction. Primer between 18-35 nucleotides is very specific for PCR (Garg *et al*., 2008). Primers with long runs of a single base should generally be avoided. In general, shorter the primer more quickly it will anneal to target DNA (Ahsen *et al*., 2001).

Primers longer than 30 bases do not show high specificity. GC content is an important feature of primer. Primers should have a GC contents between 50 and 60 %. GC content, melting temperature and annealing temperature of primer are dependent on each other (Rychlik *et al*., 1990; Wu *et al*., 1991; Garg *et al*., 2008). GC percentage present in primers is used to calculate their annealing temperature to the template DNA during PCR reaction. Melting temperature of primer will be higher if its GC content is higher, as the bond between G and C is bound by 3 hydrogen bonds. The 3' terminal position in PCR primers is necessary for reducing nonspecific bands (Kwok *et al*., 1994; Garg *et al*., 2008). Stronger hydrogen bonding of G and C bases helps to promote correct binding of primer if they are included at the 3′ end (Sheffield *et al*., 1989; Garg *et al*., 2008). This GC clamp reduces secondary bands on PCR reaction (Sheffield *et al*., 1989).

Difference between melting temperatures of both the forward and reverse oligonucleotide primers should be similar. Melting temperature of primers can be calculated by using formula given by Wallace *et al.* (1979). The stability of a primer template DNA duplex can be measured by its melting temperature.

2.6.2 DNA isolation

Isolation of DNA from leaf tissues with high levels of polysaccharide and polyphenol contents is very difficult. Method used for DNA extraction and quality of genomic DNA obtained could affect downstream analytical techniques (Boiteux *et al*., 1999; Fredricks *et al*., 2005). Several types of contaminants in the

DNA can reduce the activity of restriction endonucleases, polymerases and ligases (Shioda *et al*., 1987, Richards *et al*., 1988; Do and Adams, 1991; Maltas *et al*., 2011; Sahu *et al*., 2012). DNA extraction from black pepper is challenging due to the presence of large amount of polyphenolic compounds, polysaccharides and other secondary metabolites (Dhanya *et al*., 2007).

Antioxidants are used to reduce problems related to phenolic compounds during DNA isolation. Antioxidants like β-mercaptoethanol, ascorbic acid, bovine serum albumin (BSA), sodium azide and polyvinylpyrrolidone (PVP) are used in DNA isolation methods (Horne *et al*., 2004; Li *et al*., 2007). Oxidation of polyphenols causes browning of DNA samples which can be avoided by the use of PVP. It to removes polyphenols from mature, damaged and improperly stored leaf tissues (Doyle and Doyle, 1990; Howland *et al*., 1991; Li *et al*., 2007).

Presence of polysaccharides in DNA samples may become problematic and it may cause inhibition of enzymes like *Taq* polymerase (Fang *et al*., 1992) and restriction enzymes (Pandey *et al*., 1996). Lodhi *et al*. (1994) modified protocol reported by Doyle and Doyle (1990) including high concentration of NaCl in the buffer to remove polysaccharides. The addition of NaCl at concentrations higher than 0.5 M, along with CTAB, removes polysaccharides during DNA extraction (Paterson *et al*., 1993; Moreira and Oliveira, 2011; Sahu *et al*., 2012) Cetyltrimethylammonium bromide (CTAB) does not precipitate nucleic acids in high ionic strength solutions and form complexes with proteins. Therefore, CTAB is useful for purification of nucleic acid from plants with large quantities of polysaccharides (Sambrook and Russel, 2001; Tyagi and Sharma, 2013).

Dhanya *et al.* (2007) isolated DNA from black pepper berries. They used potassium acetate (5M) which helped in removing most of secondary metabolites and polysaccharides from the DNA and use of PEG for precipitation of DNA resulted in comparatively purified homogenous DNA without proteins and polysaccharides.

Subba *et al.* (2014) isolated genomic DNA from mature leaves and spikes of black pepper by using modified CTAB method using higher concentrations of CTAB, NaCl, EDTA and PVP in DNA extraction buffer. Isolated DNA was of good quality and higher concentration.

2.6.3 RNA isolation

RNA degrading enzyme i.e. RNase is present ubiquitously. So, RNA is very unstable due to its presence (Buckingham and Flaws, 2007; Tadokoro and Kanaya, 2009). For success of RNA analysing techniques RNase enzymes are very critical factors.

Extracting a good quality RNA depends on good laboratory practices and RNase free technique. RNAse enzyme is thermo stable and renatures after heat denaturation. It is difficult to inactivate as it does not require cofactors (Doyle, 1996; Hongbao *et al*., 2008; Rezadoost *et al*., 2016). RNAses problem can be eliminated by using RNAse free equipment, glassware and chemicals used for RNA extraction. They should be maintained and stored separate from common lab equipment and treated with various harsh chemicals that destroy RNases (Kansal *et al*., 2008; Rezadoost *et al*., 2016).

Several RNA extraction protocols are based on the denaturation, separation and elimination of proteins, polyphenols, and polysaccharides (Hu *et al*., 2002; Vasanthaiah *et al*., 2008; Rai *et al*., 2010; Ghawana *et al*., 2011; Dash, 2013). Efficiency of RNA extraction depends on the type of reagents and homogenization procedures used (Portillo *et al*., 2006). Generally the extraction protocols use reagents such as acidic guanidinium thiocynate, cetyl- trimethyl ammonium bromide (CTAB), sodium dodecyl sulfate (SDS), phenol, chloroform, lithium chloride, sodium acetate, among other reagents, in order to obtain pure RNA samples free from proteins, polysaccharides, and polyphenolic compounds (Gesteira *et al*., 2003; Verica *et al*., 2004; Rezadoost *et al*., 2016). Various companies offer different kits for RNA extraction. Extraction of RNA from

different samples with extraction kits does not always result in satisfactory quality and quantity of RNA (Yu *et al*., 2012).

Strong denaturants used to inhibit endogenous RNases are mostly harmful and corrosive agents (Doyle, 1996). TRIzol reagent is a ready to use reagent for the extraction of RNA from different samples. TRIzol is a monophasic solution of phenol and guanidine isothiocynate and it is an upgrading of single-step RNA extraction method developed by Chomczynski and Sacchi (Gauthier *et al*., 1997; Hongbao *et al*., 2008; Rio *et al*., 2010).

Variety of RNA samples of various molecular sizes can be isolated by using TRIzol reagent (Ahmann *et al*., 2008). During sample homogenization, TRIzol reagent disrupts cells and dissolves cell components and maintains the integrity of RNA. The mixture separates into an aqueous phase and an organic phase. RNA remains in the aqueous phase after addition of chloroform followed by centrifugation. Aqueous phase is transferred to fresh tube and RNA can be precipitated with isopropanol (Chomczynski, 1993).

Another problem with RNA extraction is primary and secondary plant metabolites (e.g., phenolic compounds, polysaccharides). These metabolites vary within and between species, and can interfere with RNA extraction (Bilgin *et al*., 2009; Rajakani *et al*., 2013). Handayani *et al*. (2016) used polyvinyl pyrrolidone (PVP) in DNA extraction buffer to reduce phenolic compounds and polysaccharides from mature leaves of *Durio kutejensis*.

Birtic and Kranner (2006) isolated high quality RNA from seeds of five plant species by using standardised concentration of polyvinyl pyrrolidone with DNase and ethanol precipitation. Xie *et al*. (2013) developed modified RNA extraction methods to obtain high quality RNA from cotton roots. Siju *et al.* (2007) isolated RNA from berries of black pepper by modifying the standard RNA extraction protocol (Chomczynski and Sacchi, 1987) for detection of *Cucumber mosaic* virus in RT-PCR.

2.6.4 Quantity and purity of nucleic acids

Sambrook *et al*. (1989) used spectrophotometer to detect concentrations and purity of the DNA. This method of quantification and assessment of purity of DNA samples uses absorbance values at 260 nm and 280 nm (Glasel, 1995). Pure DNA would give concentration of 50 µg/ml and RNA of 40 µg/ml when the absorbance value at 260 nm is 1.0. The ratio of absorbance at 260 nm and 280 nm for DNA sample should be around 1.8 and for RNA it should be around 2.0 (Maniatis *et al*., 1982).

2.6.5 PCR technique

Polymerase chain reaction uses *in vitro* enzymatic reaction to amplify specific DNA fragment. This technique of DNA amplification uses multiple cycles of template denaturation, primer annealing, and primer extension. This process is an exponential amplification of DNA fragments because products from each cycle serve as templates for the next cycle (Mullis *et al*., 1986). It is a highly sensitive technique for the detection of target DNA fragment. After 20 to 40 cycles of PCR, enough amplified product is generated, so that it can be visualized on an agarose gel by using specific staining method (e.g. ethidium bromide). Components of PCR reaction are as follows: template, primers, magnesium ion, dNTPs, buffer for PCR reaction, and thermostable DNA polymerase enzyme (Mullis *et al*., 1986). The template used can be DNA; RNA or cDNA. Primers i.e. forward and reverse primers decide length of the amplicons (Saiki *et al*., 1985). *Taq* DNA polymerase is a most commonly used thermostable DNA polymerase and it is suitable for routine amplifications. The magnesium ion acts as cofactor for *Taq* polymerase. Its concentration affects enzyme activity, primer annealing, melting temperature of the template and the PCR product (Jordan *et al*., 2001; Zamft *et al*., 2012).

PCR is a very sensitive technique and it can be utilised for detecting and amplifying nucleic acids from any source, regardless of quantity and combination

(Joshi and Deshpande, 2010; Krohn-Molt *et al*., 2013; Johansen *et al*., 2013; Culley *et al*., 2014; McCall *et al*., 2015).

2.6.6 RT-PCR

The development of RT-PCR started from the discovery of enzyme reverse transcriptase, which is a RNA dependent DNA polymerase (Bustin, 2000; LeGrice and Gotte, 2013). With modifications in RT-PCR, it has become the standard technology for gene expression studies. Reverse transcriptase has revolutionized gene expression analysis. Qualitative and quantitative expression of RNA can be easily studied by using RT-PCR (Bustin *et al*., 2005; Arya *et al*., 2005; Maurya *et al*., 2015; Ravnikar *et al*., 2016).

First step in RT-PCR is the production of complementary copy of RNA using the reverse transcriptase enzyme (Freeman *et al*., 1999) followed by exponential amplification by PCR (Bustin, 2000; Bustin, 2002)

RT-PCR can be performed in single step or in two steps. In the one-step approach, the entire reaction occurs in a single tube under conditions optimized for both reverse transcription and PCR. As a disadvantage in one-step approach, reverse transcriptase could inhibit *Taq* polymerase at low concentrations of template in RT-PCR (Sellner *et al*., 1992; Chumakov, 1994; Chandler *et al*., 1998; Suslov and Steindler, 2005; Saunders *et al*., 2013). The sensitivity of RT-PCR can be improved by the use of non-homologous RNA in the reaction (Levesque-Sergerie *et al*., 2007), especially for templates in low concentration.

On the other hand, the two-step reaction requires that the cDNA synthesis and its PCR amplification be performed in different tubes. The one-step approach is thought to minimize experimental variation by containing all of the enzymatic reactions in a single environment (Wong and Medrano, 2005; Al-Shanti *et al*., 2009). Two-step RT-PCR is easy, popular and useful for detecting multiple genes expressed in the same sample, whereas one-step RT-PCR is more advantageous

with multiple samples, as carryover contamination is minimized (Arya *et al*., 2005; Al-Shanti *et al*., 2009; Ravnikar *et al*., 2016).

Gene expression can be studied at the level of single cell and can be performed on large number of samples and different genes in the same experiment (Wang and Brown, 1999). RT-PCR can be used to analyse differential gene expression or cloned cDNAs without constructing a cDNA library (Maurya *et al*., 2015; Frisen, 2015). RT-PCR is more sensitive and easier to perform than other RNA analysis techniques, including Northern blots, RNAse protection assays, *in situ* hybridization, and S1 nuclease assays (Okada *et al*., 1996; Preshaw *et al*., 1998; Garlet *et al*., 2003; Khodakov *et al*., 2008; Frisen, 2015).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2014-2016. Details regarding the experimental materials used and the methodology followed for various experiments are presented in this chapter.

3.1 COLLECTION OF SAMPLES

Leaf and non-branching spikes of 'Karimunda' variety were collected from College of Agriculture, Vellayani. Young leaves were collected for DNA isolation and different stages of non-branching spikes were collected for RNA isolation.

Leaves and branching spikes of type 'Thekken' were collected from farmer's field at Idukki district. All samples were collected in liquid nitrogen and stored at -80 ^{o}C (Panasonic - MDF U55V PE). Different stages were selected according to criteria given in Table 1.

Table 1. Criteria for selection of developmental stages of spikes

Stage	**Length of spikes (cm)**	**Days after emergence of bud**
Stage I	1 to 2	12 to 15
Stage II	6 to 8	22 to 25
Stage III	9 to 12	32 to 35

3.2 ISOLATION OF GENOMIC DNA

Genomic DNA was isolated from type 'Thekken' and 'Karimunda' leaves by using modified CTAB method of DNA extraction developed by Subba (2014) with a few modifications.

Young leaves were washed with tap water followed by distilled water and dried by wiping with blotting paper. Leaves were cut with sterile blade and crushed in mortar and pestle using liquid nitrogen. CTAB extraction buffer (Appendix I) was added to it (1 ml per 100 mg of plant sample) and 0.2% of β-mercaptoethanol and PVP were added and homogenised well with pestle and transferred to 2 ml centrifuge tubes. The samples were incubated in water bath at 60 ^{o}C for 30 min with intermittent shaking. After incubation, equal volume of chloroform/isoamyl alcohol in ratio 24: 1 (v/v) was added to it and mixed gently by inverting the tubes. The tubes were centrifuged at 8000 g for 15 min at 4 ^{o}C. The upper phase was transferred to a fresh centrifuge tube and equal volume of chloroform/isoamyl alcohol was added and centrifuged at 8000g for 15 min at 4 ^{o}C. Aqueous phase was taken in new tube and 0.7 volume of chilled isopropanol was added and mixed by inversion. Visible DNA was transferred to a fresh tube by using Pasteur pipette. The pellet was washed with 70% ethanol twice and dissolved in 100 µl of nuclease free water. The extracted DNA samples were stored at -20 ^{o}C (Samsung RS21HUTPN1).

3.3 ISOLATION OF RNA

Total RNA was isolated from spikes at different developmental stages using Trizol reagent (Invitrogen, USA). All the materials used for RNA extraction were treated with 3 per cent hydrogen peroxide overnight and autoclaved twice for sterilization.

The frozen samples (100 mg) were ground into a fine powder in liquid nitrogen using mortar and pestle. Trizol reagent was added to the powdered content (1 ml Trizol per 100 mg of plant sample) and samples were ground thoroughly. About 1 ml of homogenized mixture was then transferred to a two ml centrifuge tube. To this, 200µl of chloroform and 50 µl of β-mercaptoethanol were added and incubated for 2-3 min at room temperature after vigorous mixing. The samples were centrifuged at 9000 g for 15 min at 4 ^{o}C. The aqueous phase of the sample was transferred into a fresh tube. For RNA precipitation 0.7 volume of absolute isopropanol was added and content was mixed by inverting it slowly.

Visible RNA pellet was transferred to fresh tube by using Pasteur pipette. The pellet was washed with 1 ml of 70% ethanol. The sample was briefly vortexed and centrifuged at 9000 g for 10 min at 4ºC. RNA pellet was air dried for 20 min and suspended in 100 µl RNase free sterile water, followed by incubation in a water bath at 60 ºC for 10 min. Finally, RNA samples were stored at -20ºC until used.

3.4 QUALITY AND QUANTITY OF NUCLEIC ACIDS

Quality and quantity of nucleic acids were measured by using UV spectrophotometer. UV spectrophotometer was calibrated to blank at 260 nm and 280 nm wavelengths by using nuclease free water as blank. Absorbance at both wavelengths was measured by taking 2µl of respective nucleic acid (DNA or RNA) in 2 ml nuclease free water in quartz cuvette.

Quality of nucleic acids was determined by using ratio of absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), where a ratio of 1.8 denotes pure DNA and a ratio of 2.0 denotes pure RNA.

Formulae used for quantity determination of nucleic acids are as follows:

Amount of DNA = (A_{260} x 50 x Dilution factor) ng/µl

&

Amount of RNA = (A_{260} x 40 x Dilution factor) ng/µl

3.5 PRIMER DESIGNING

Degenerate primers were designed for *RAMOSA* family genes *viz*., *RA1*, *RA2* and *RA3* selected as candidate genes influencing inflorescence architecture. Bioinformatic tools i.e. Primer3 and Oligocalc were used for primer designing.

Sequences of selected candidate genes were retrieved from NCBI Nucleotide database from different organisms. Multiple sequence alignment of these sequences was performed using Clustal-Omega tool from EMBL database. Conserved regions spacing 200 bases to 600 bases were selected. Fragments of

approximately 20 bases were checked for ideal primer properties by using online software Oligocalc. Degenerate bases were used for less conserved bases in selected primers. The primers were synthesized from Sigma-Aldrich.

3.6 PCR AMPLIFICATION

PCR amplification of the genomic DNA was carried out using the degenerate primers designed. PCR master mix and conditions used for amplification are given in Table 2 and Table 3 respectively. PCR products were checked on agarose gel electrophoresis (1.5 % agarose) (Appendix II).

3.7 REVERSE TRANSCRIPTION- POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR was performed in two steps *viz*., cDNA synthesis and PCR with designed primers. cDNA was synthesized by using First strand synthesis kit (Invitrogen, USA) and PCR was performed as described above and PCR products were checked by using agarose gel electrophoresis.

3.7.1 Synthesis of cDNA

RNA samples stored at -80 ºC were taken out and incubated at 60 ºC in water bath for 5 min. 1000 ng of RNA from respective sample was transferred to the microfuge tube and volume made to 10 µl and incubated at 60 ºC for 10 min. Simultaneously, reaction mixture for reverse transcription was prepared as follows:

Rnasin	:	0.5 µl (20 U)
5 X Assay Buffer	:	4 µl
10 mM dNTP mix	:	2 µl
Oligo $d(T)_{18}$ primer	:	0.5 µl
AMV-Reverse Transcriptase	:	1 µl (20 U)
Nuclease Free Water	:	2 µl
Total Volume	:	10 µl

This reaction mixture was added to the RNA sample after 10 min incubation. The contents were mixed well and incubated at 42 ^{o}C for 1 h. The tubes were then incubated at 92 ^{o}C for 2 min and quickly placed the tubes on ice and spun briefly (for denaturation of RNA-cDNA hybrids). The cDNA samples were stored at -20 ^{o} C (Samsung RS21HUTPN1) until use.

Table 2. The components, volume and concentration of PCR reaction mixture

Sl. No.	**Components**	**Volume for one reaction**	**Final concentration**
1	10 X PCR buffer	2.5 µl	1X
2	dNTPs	0.5 µl	0.2 mM
3	*Taq* DNA polymerase	1.5 µl	1.5 U
4	$MgCl_2$	2.5 µl	2.5 mM
5	Primer (forward)	1.0 µl	70 pmol
6	Primer (reverse)	1.0 µl	70 pmol
7	Genomic DNA	1.0µl	50 ng
8	Sterile double distilled water	15 µl	
	Total	**25.0 µl**	

Table 3. Temperature profile used for DNA amplification

Step No.	**Temp (^{O}C)**	**Duration**	**Cycles**	**Function**
1	94	3 min		Initial denaturation
2	94	45 s	34	Denaturation
3	W.R.T. primer (annexure no. 2)	45 s		Annealing
4	72	1 min		Extension
5	72	5 min		Final extension
6	4	Hold		Final hold

3.7.2 PCR amplification of cDNA with designed primers

The cDNA samples were subjected to PCR with designed primers as given in Table 6. The PCR mixture of the total volume of 25 µl was prepared similarly as described in the Table 2, except that 1.5 µl of cDNA was used as template instead of 1µl of genomic DNA. The PCR programme was set as mentioned in Table 3.

3.7.3 Sequencing of amplicons

Amplicons obtained from RT-PCR were eluted from agarose gel and were sequenced at regional facility Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram (RGCB). And remaining quantity of eluted products was cloned using TA cloning kit and stored at -20 ^{o}C.

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

Gel piece containing the amplicon was carefully cut and weighed and placed in a centrifuge tube. The gel was then crushed and 3 volume of gel solubilizer solution was added. This mixture was kept at 50 ^{o}C for 5 min to solubilize the gel. The dissolved gel was transferred to GeNei column with collection tube and centrifuged at 1000 g for 1 min. Column was transferred to new collection tube. 700 µl of was buffer was added and centrifugation was repeated. Finally, 50 µl of elution buffer was added to the column and centrifuged at 1000 g for 1 min. Eluent was cloned using TA cloning kit.

3.7.3.2 Cloning

For cloning of PCR products TA cloning kit (Invitrogen, USA) was used. Vial of pCR®2.1 vector was centrifuged to collect all the liquid in the bottom of the vial. Ligation reaction of 10 µl was prepared as follows:

Fresh PCR product	4 µl
10X Ligation Buffer	1 µl
pCR®2.1 vector	(25 ng/µl) 2 µl
Sterile water	2 µl
T4 DNA Ligase	1 µl
Final volume	10 µl

This reaction was incubated at 14°C for 4 hours. After incubation ligated PCR product was stored at -20 ^{o}C.

3.7.4 Sequence analysis

The resultant sequences of the amplicons were analyzed using bioinformatic tools *viz*., BLAST (TBLASTX and BLASTN), NCBI conserved Domain Search and Clustal omega. In TBLASTX, translated nucleotide query was used to search in translated nucleotide sequence database. NCBI conserved domain search uses nucleotide query and finds the conserved domain for which it belongs. Clustal omega was used as multiple sequence alignment tool and is available on EMBL database.

RESULTS

4. RESULTS

The study entitled "Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'", was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014-2016. The results related to the study are presented in this chapter.

4.1 DNA ISOLATION

Young leaves of control variety 'Karimunda' and branching type 'Thekken' were used for genomic DNA isolation. Plate 1 shows the agarose gel electrophoresis (1.0 %) of the extracted genomic DNA from 'Thekken' and 'Karimunda' leaves. The gel showed intact bands of genomic DNA. Lane 1, 2 and 3 shows the genomic DNA from young leaves from different 'Thekken' plant samples named A, B and C. Lane 4 and lane 5 shows the genomic DNA from different 'Karimunda' plant samples (D and E).

Further absorbance reading of the extracted genomic DNA by using spectrophotometric method revealed good quality and quantity of DNA (Table 4). Value of A_{260}/A_{280} for DNA samples ranged between 1.52 and 1.83. Yield of DNA ranged between 1300 ng/µl to 2800 ng/µl.

4.2 RNA ISOLATION

Different stages of spike development were used for extraction of total RNA. Plate 2 shows three intact bands of 28S, 18S and 5S RNA on agarose gel electrophoresis (1.8 %) of total RNA isolated from all stages of 'Thekken' and 'Karimunda'. There was no DNA contamination observed on agarose gel (Plate 2a and 2b).

Further quality and quantity of RNA was checked by using spectrometric method. Absorbance values at 260 nm and 280 nm and their ratio values of different RNA samples are mentioned in Table 5. For RNA, value of A_{260}/A_{280}

ranged between 1.76 and 2.27. The yield of RNA ranged between 1200 ng/µl and 2200 ng/µl.

Table 4. Quality and quantity of isolated genomic DNA

Sample	**Sl. No.**	**A_{260}**	**A_{280}**	**A_{260}/A_{280}**	**DNA yield (ng/µl)**
'Thekken'	1	0.026	0.016	1.625	1300
	2	0.032	0.021	1.523	1600
	3	0.044	0.024	1.833	2200
'Karimunda'	4	0039	0.023	1.695	1950
	5	0.056	0.036	1.555	2800

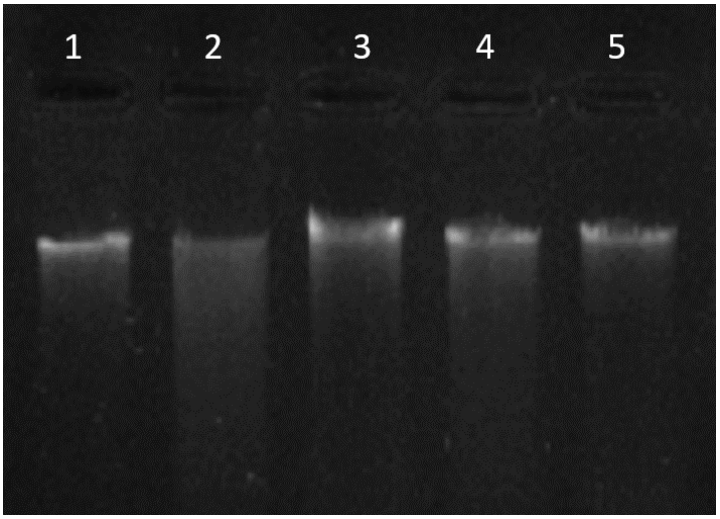
Table 5. Quality and quantity of isolated total RNA

Sample	**Sl. No.**	**A_{260}**	**A_{280}**	**A_{260}/A_{280}**	**RNA yield (ng/µl)**
'Thekken'	1	0.055	0.029	1.890	2200
	2	0.030	0.017	1.764	1200
	3	0.050	0.022	2.272	2000
'Karimunda'	4	0.044	0.021	2.095	1760
	5	0.050	0.026	1.923	2000
	6	0.035	0.018	1.944	1400

4.3 PRIMER DESIGNING

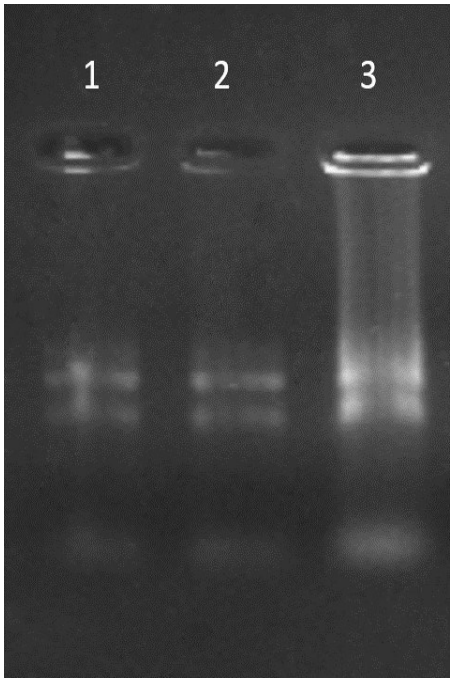
Primers for candidate genes i.e. *RAMOSA* family genes were designed based on the conserved regions shown by the Clustal Omega tool. The details of primers are given in Table 6. Properties of primers were analyzed by using online software Oligocalc.

The analysis of primers using Oligocalc tool revealed desirable GC content and annealing temperature, and also none of the designed primers exhibited any hairpin formation and 3' complementarity.



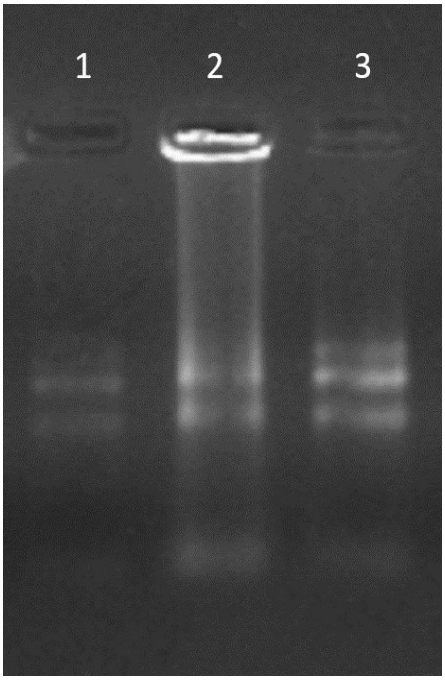
1- Thekken A
2- Thekken B
3- Thekken C
4- Karimunda A
5- Karimunda B

Plate 1 Agarose gel electrophoresis of genomic DNA from Thekken and Karimunda varieties



1- Thekken Stage-I
2- Thekken Stage-II
3- Thekken Stage-III

2a



1- Karimunda Stage-I
2- Karimunda Stage-II
3- Karimunda Stage-III

2b

Plate 2 Agarose gel electrophoresis of total RNA from spikes of different developmental stages

Table 6. Primers designed for *RAMOSA* family genes

Gene	Primer name	5'<-----Sequence----->3'	GC content (%)	Length (no. of bases)	Tm (^{o}C)
RAMOSA1 (*RA1*)	RA1F	CGCCRCAGRTAAGGTCGTC	58	19	59.5
	RA1R	ARCAGCGACGACAAGYTRAG	45-60	20	59.8
RAMOSA2 (*RA2*)	RA2F	GAGCACCAGCAACAACTCGG	60	20	56.5
	RA2R	GTGGTTCCGCATGAAGTAGC	55	20	53.8
RAMOSA3 (*RA3*)	RA3F	GSAAGCARATMGTGATGTT	40-50	19	51.8
	RA3R	GACCTGACCTCCTCGTTCA	58	19	54.6

4.4 PCR ANALYSIS OF GENOMIC DNA WITH DESIGNED PRIMERS

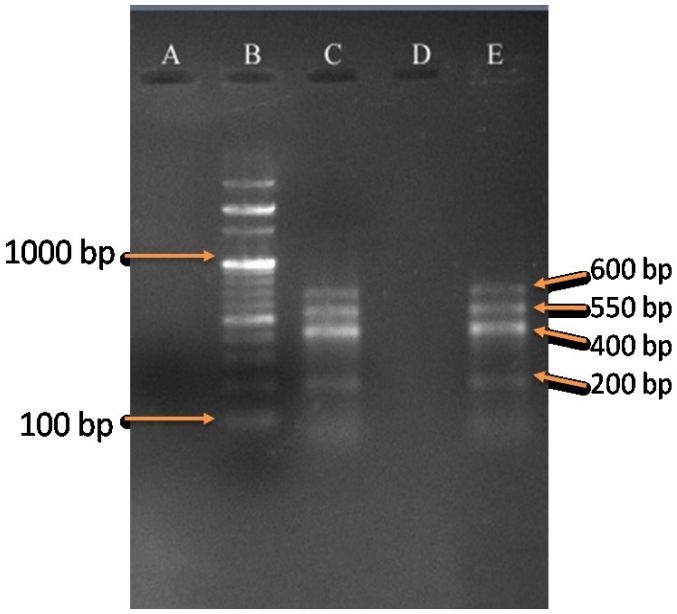
Genomic DNA from young leaves of 'Thekken' and 'Karimunda' were used as template for PCR. For *RA1* gene RA1F and RA1R primers were used for PCR analysis of genomic DNA. These primers amplified four bands of size 200 bp, 400 bp, 550 bp and 600 bp in both 'Thekken' and 'Karimunda' (Plate 3).

RA2 gene was analyzed using primers RA2F and RA2R in both black pepper varieties. These primers amplified fragment of 450 bp in both 'Thekken' and 'Karimunda' (Plate 4).

Two amplicons of 450 bp and 650 bp size were produced with *RA3* gene primers (RA3F and RA3R) in both 'Thekken' and 'Karimunda' (Plate 5). There was no variation in banding patterns for all genes screened in both varieties.

4.5 RT-PCR ANALYSIS

Presence of cDNA was confirmed by using *Ubiquitin* gene (housekeeping gene) specific primers. *Ubiquitin* gene specific primers amplified amplicon of size 99 bp in all cDNA samples. Plate 6 shows PCR amplification of cDNA of different stages of spike from both 'Thekken' and 'Karimunda'.



A:Template blank

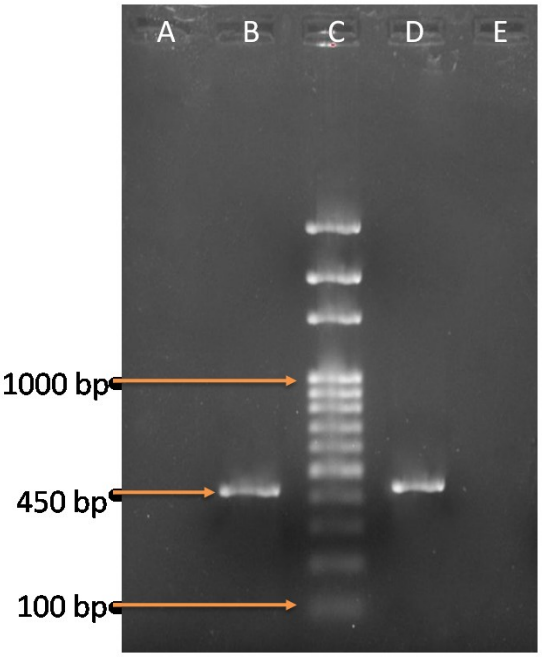
B: 100 bp ladder

C: Thekken

D: Primer blank

E: Karimunda

Plate 3 Agarose gel electrophoresis of PCR product from genomic DNA of Thekken and Karimunda using *RA1* primers



A- Template blank

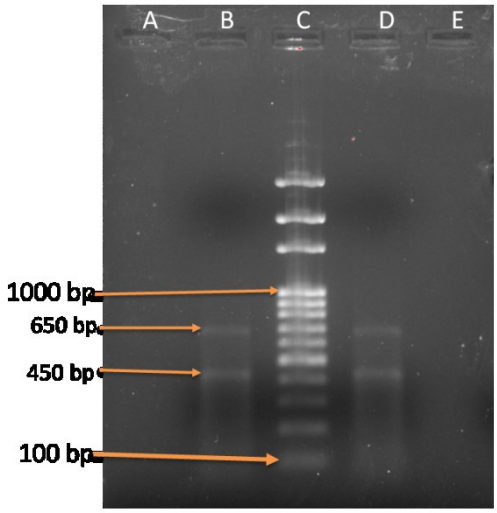
B- Thekken

C- 100bp ladder

D- Karimunda

E- Primer blank

Plate 4 Agarose gel electrophoresis of PCR product from genomic DNA of Thekken and Karimunda using *RA2* primers



A- Template blank

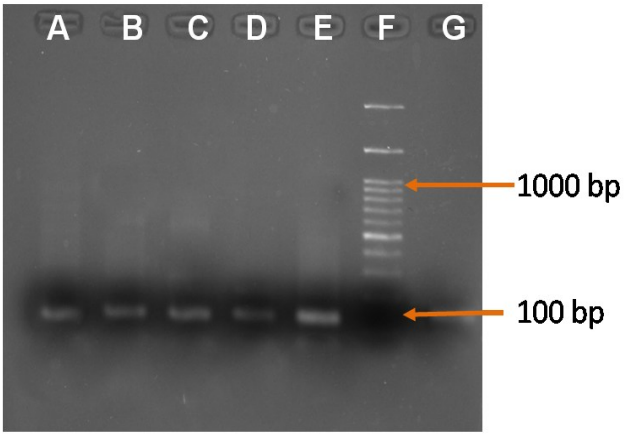
B- Thekken

C- 100bp ladder

D- Karimunda

E- Primer blank

Plate 5 Agarose gel electrophoresis of PCR product from genomic DNA of Thekken and Karimunda using *RA3* primers



A- Stage I (Karimunda)

B- Stage II (Karimunda)

C- Stage III (Karimunda)

D- Stage I (Thekken)

E- Stage II (Thekken)

F- 100 bp ladder

G- Stage III (Thekken)

Plate 6 Agarose gel electrophoresis of PCR product from cDNA of Thekken and Karimunda from different developmental stages using Ubiquitin gene primers

cDNA from different stages of spike development of 'Karimunda' and 'Thekken' showed no amplification with *RAMOSA1* gene specific primers (Plate 7). When PCR was performed using primers designed for *RAMOSA2* gene showed no clear amplification on agarose gel electrophoresis. Smear was obtained in stage II of 'Thekken' while other stages of 'Thekken' and all stages of 'Karimunda' showed no amplification (Plate 8).

RT-PCR produced a band of size of 450 bp in stage II of branching type of black pepper 'Thekken' with *RAMOSA3* gene specific primers. Plate 9 shows differentially expressed band (Lane- F) in stage II of 'Thekken'. There was no amplification in any other stages of 'Thekken' as well as 'Karimunda'.

4.6 SEQUENCING

Amplicons produced by the degenerate primers were eluted from the agarose gel and purified using Gel Extraction Kit, the eluted products were cloned and sequenced at RGCB, Thiruvananthapuram. Three sequences were obtained; two sequences were obtained from genomic DNA with *RA2* primers (RA2F and RA2R) and one sequence from cDNA using *RA3* forward primer).
Sequences of the amplicons are given below:

1. Sequence of genomic DNA fragment amplified with RA2F primer

>GDNA-RA2F-Forward primer (5' to 3')
AGGGGGTAATTTTTTTACTCCCCAGAGAGTGAGGGAGGTGAAAAGGG
GGGCGACTTACCATTGGACTACATCCATCCAACCAAAAAAGTCACCCT
TTTCTTGTTCGTTCTTCGAATTACGCTGTGGAGACTAATTCCTTCCGGC
TAATGAAGAGACTACTCCAGTCTTCCCATTCATTCCAAGTGGATCCTTC
TTATTCCTAAGAATTGAACAAACCAAGTTCACCTATACGAGAGTGAGG
AAGACATTTCCAACAAGAAACTTCCTTTGATGTCCCGTTTGACGATTCG
ATTCCGCTAGTTTATAGAAACTCAGGAGAAGGACAGGGGTCGTCGCTA
GGTCAGAAAAGCGATAACTAAAAAATCTCCCCAAGTAGGATTTGAAC
CTACGACCAGTCAGTTAACAGCCGACCGCTCTACCACTGAGCTACTTC
ATGCGGAACCACCAAA

2. Sequence of genomic DNA fragment amplified with RA2R primer

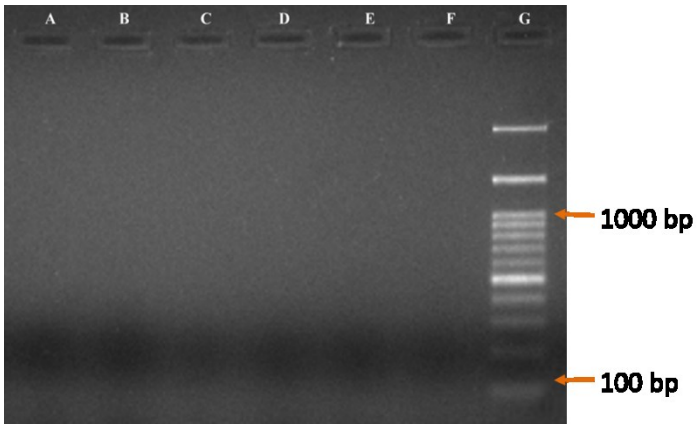
>GDNA-RA2R-Reverse primer (5' to 3')

CGGATTGGGTTGGCTGTTACTGACTGGTCGTAGGTTCAAATCCTACTTG
GGGAGATTTTTTAGTTATCGCTTTTCTGACCTAACGACGACCCCTGTCC
TTCTCCTGAGTTTCTATAAACTAGCGGAATCGAATCGTCAAACGGGAC
ATCAAAGGAAGTTTCTTGTTGGAAATGTCTTCCTCACTCTCGTATAGGT
GAACTTGGTTTGTTCAATTCTTAGGAATAAGAAGGATCCACTTGGAAT
GAATGGGAAGACTGGAGTAGTCTCTTCATTAGCCGGAAGGAATTAGTC
TCCACTAGCGTAATTCGAAGAACGAACAAGAAAAGGGTGACTTTTTTG
GTAGGATGGATGTAGTCCAATGGCTAAAGCTCTGCCAGCTTCTTGTAG
ACTGGCAGTCTCCGAGTTGTTGCTGGTGCTCCAAAA

3. Sequence of cDNA fragment amplified with RA3F primer

>cDNA-RA3F-forward primer (5' to 3')

ATGAAATTTTGCTTTTCATCCTCTCCTTAACTTGCTTATGTAACTTCTTC
ACCAAATCTGCCTTAGAATTAGCATCAACACTCACAAAATTGTTAGGT
GGCAATGGCAATAAATCAATAGGGGTGAGTGGATTAAAACCATAAAC
AATCTCAAATGGAGAACAATGAGTAGTGCTATGAATAGCCCTATTGTA
AGCAAACTCCACAAATGGTAAACAATCCTCCCATGTCCTAATGTTCTTT
TCTATGATTGCACTAAGCAAAGTAATCAAAGTCCTATTAACTACTTCA
GTTTGCCCATCTGTTTGAGGGTGACAAGTAGTTGAAAATAATAGCTTA
GTTCCTAACTTTCCCCACAACACACGCCAAAAATGACTCAAAAACTTA
ACATCACTATCTGCTTCCCACAGG



A- Stage I (Karimunda)

B- Stage I (Thekken)

C- Stage II (Karimunda)

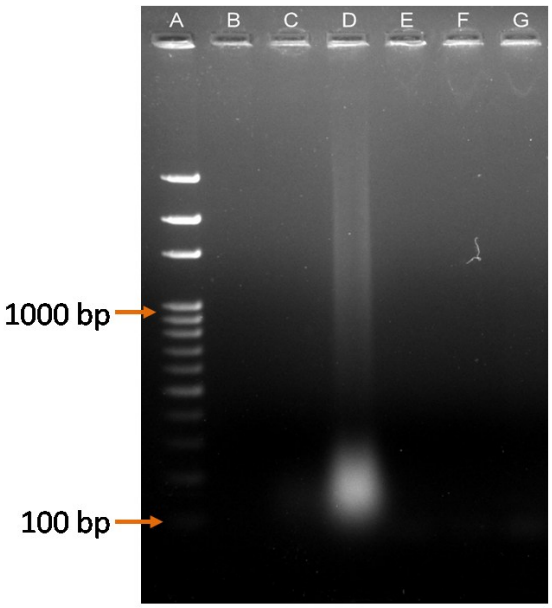
D- Stage II (Thekken)

E- Stage III (Karimunda)

F- Stage III (Thekken),

G- 100 bp ladder

Plate 7 Agarose gel electrophoresis of PCR product from cDNA of Thekken and Karimunda from different developmental stages using *RA1* gene primers



A- Stage I (Karimunda)

B- Stage I (Thekken)

C- Stage II (Karimunda)

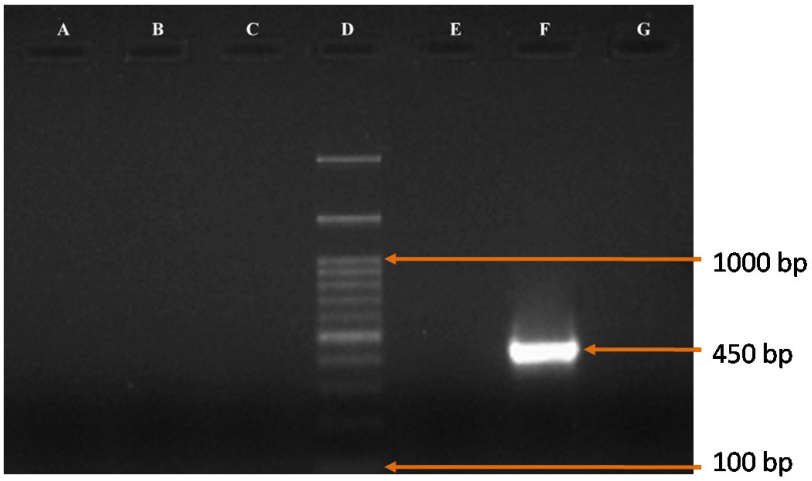
D- Stage II (Thekken)

E- 100 bp ladder

F- Stage III (Karimunda)

G- Stage III (Thekken)

Plate 8 Agarose gel electrophoresis of PCR product from cDNA of Thekken and Karimunda from different developmental stages using *RA2* gene primers



A- Stage I (Karimunda)
B- Stage II (Karimunda)
C- Stage III (Karimunda)
D- 100bp ladder
E- Stage I (Thekken)
F- Stage II (Thekken)
G-Stage III (Thekken)

Plate 9 Agarose gel electrophoresis of PCR product from cDNA of Thekken and Karimunda from different developmental stages using *RA3* gene primers

4.7 ANALYSIS OF THE AMPLIFIED SEQUENCE

The sequences of the amplicons obtained with *RA2* and *RA3* primers were analysed using bioinformatic tools *viz*., TBLASTX, BLASTN, Clustal Omega and NCBI Conserved Domain Search.

4.7.1. Sequences obtained from genomic DNA with *RA2* primers

Two sequences (GDNA-RA2Fand GDNA-RA2R) obtained from genomic DNA were analysed (Forward and reverse primer) using above mentioned tools.

1. **TBLASTX**

TBLASTX is a BLAST program which searches translated nucleotide database using a translated nucleotide query. Amplicon of *RA2* with RA2F primer gave 2031 hits and with RA2R gave 2487 hits (Plate 10a and 10b) when searched against non-redundant database. The results for RA2F primer showed best match with sequence from chromosome 3B of *Triticum aestivum* with E-value of 1e-31 and 94% query cover. For sequence obtained with RA2R primer best match was with mitochondrial genome of *Phoenix dactylifera* with 98% query cover and E-value of 2e-31.

2. **BLASTN**

Sequence obtained using RA2F primer showed from 121 hits and sequence obtained with RA2R primer showed 109 hits when searched using BLASTN tool. Best hit result for both sequences was NADH dehydrogenase subunit 2 (*nad2*) gene from *Parinari campestris* with 83% query cover and E-value of 2e-93 reported at NCBI database. Output files of BLASTN are shown in Plate 11a, 11b and Plate 12a, 12b.

3. **NCBI Conserved Domain Search**

This uses nucleotide query and finds the conserved domain for which the query sequence belongs to. No conserved domains were found in both sequences obtained using *RA2* primers.

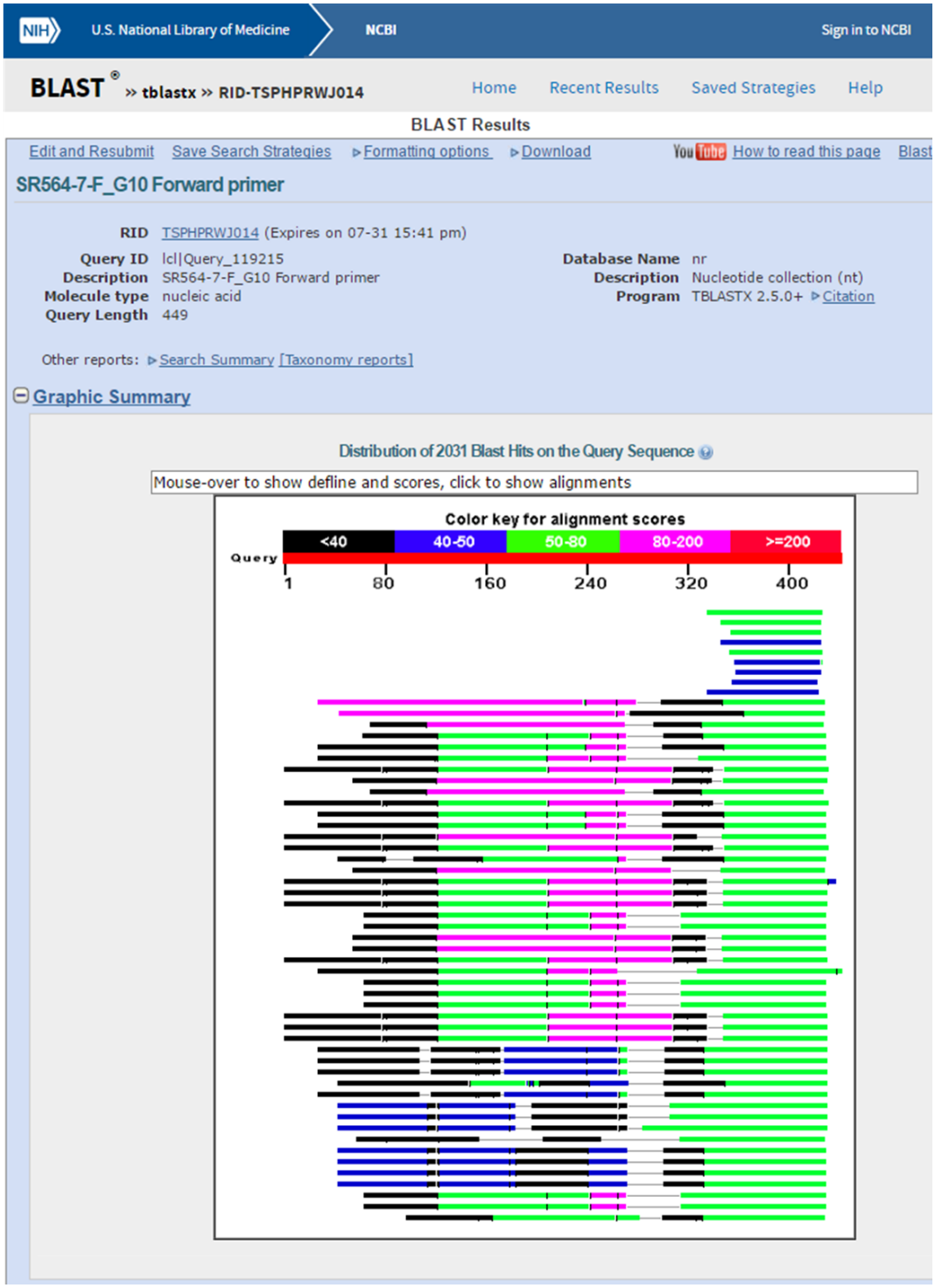
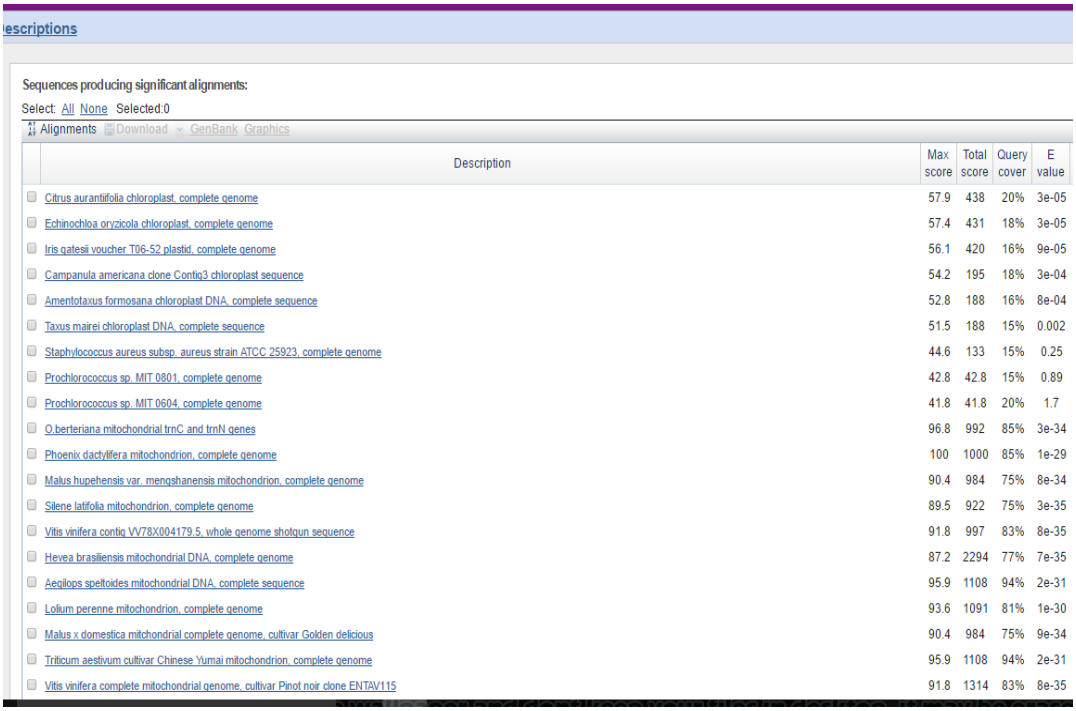


Plate 10a Screenshot showing the result of TBLASTX of sequence obtained with primer RA2F of *RA2* gene



escriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics

Description	Max score	Total score	Query cover	E value
Citrus aurantiifolia chloroplast, complete genome	57.9	438	20%	3e-05
Echinochloa oryzicola chloroplast, complete genome	57.4	431	18%	3e-05
Iris gatesii voucher T06-52 plastid, complete genome	56.1	420	16%	9e-05
Campanula americana clone Contig3 chloroplast sequence	54.2	195	18%	3e-04
Amentotaxus formosana chloroplast DNA, complete sequence	52.8	188	16%	8e-04
Taxus mairei chloroplast DNA, complete sequence	51.5	188	15%	0.002
Staphylococcus aureus subsp. aureus strain ATCC 25923, complete genome	44.6	133	15%	0.25
Prochlorococcus sp. MIT 0801, complete genome	42.8	42.8	15%	0.89
Prochlorococcus sp. MIT 0604, complete genome	41.8	41.8	20%	1.7
O.berteriana mitochondrial trnC and trnN genes	96.8	992	85%	3e-34
Phoenix dactylifera mitochondrion, complete genome	100	1000	85%	1e-29
Malus hupehensis var. mengshanensis mitochondrion, complete genome	90.4	984	75%	8e-34
Silene latifolia mitochondrion, complete genome	89.5	922	75%	3e-35
Vitis vinifera contig VV78X004179.5, whole genome shotgun sequence	91.8	997	83%	8e-35
Hevea brasiliensis mitochondrial DNA, complete genome	87.2	2294	77%	7e-35
Aegilops speltoides mitochondrial DNA, complete sequence	95.9	1108	94%	2e-31
Lolium perenne mitochondrion, complete genome	93.6	1091	81%	1e-30
Malus x domestica mitchondrial complete genome, cultivar Golden delicious	90.4	984	75%	9e-34
Triticum aestivum cultivar Chinese Yumai mitochondrion, complete genome	95.9	1108	94%	2e-31
Vitis vinifera complete mitochondrial genome, cultivar Pinot noir clone ENTAV115	91.8	1314	83%	8e-35

Plate 10b Screenshot showing the result of TBLASTX of sequence obtained with primer RA2F of *RA2* gene

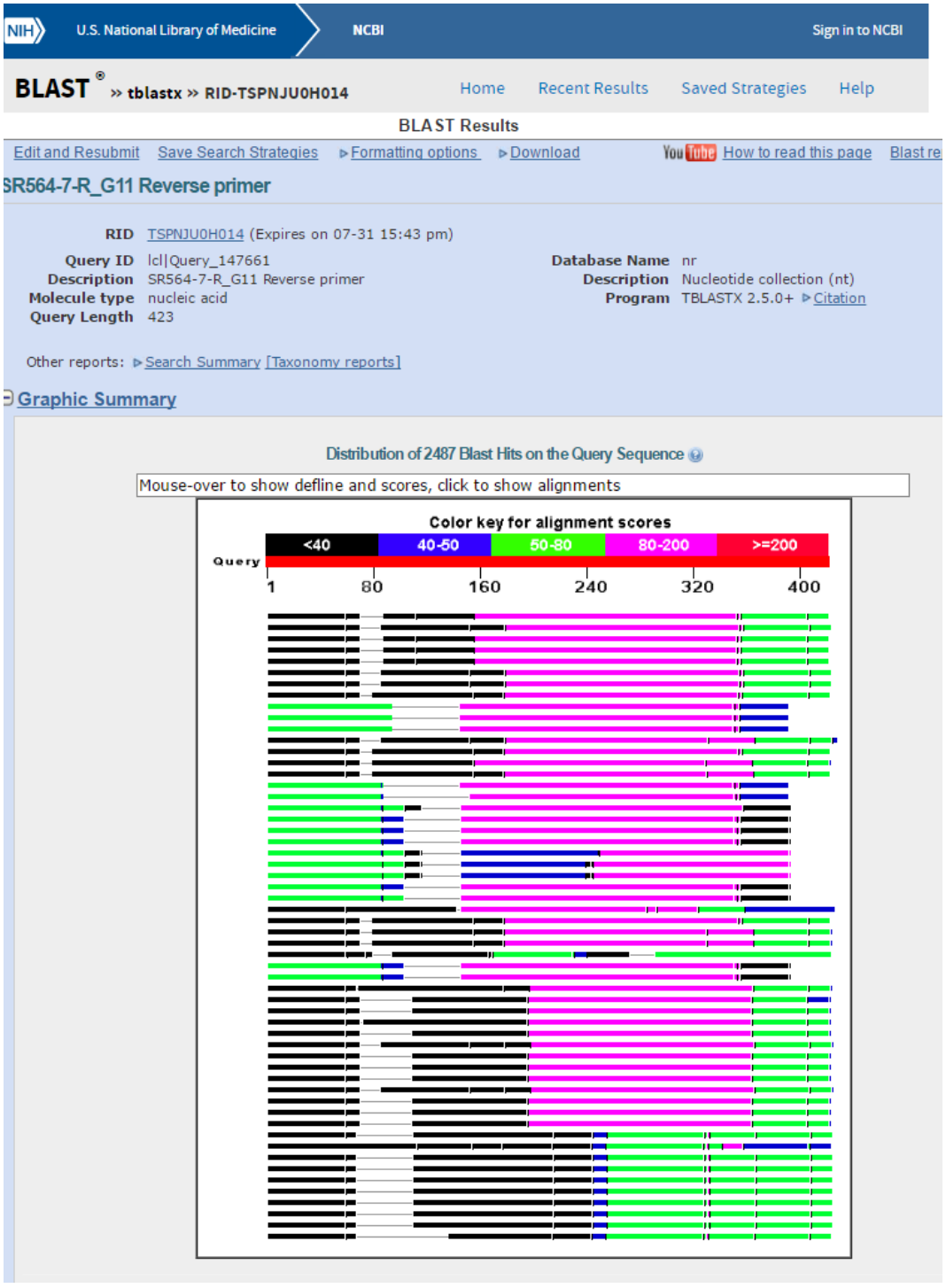
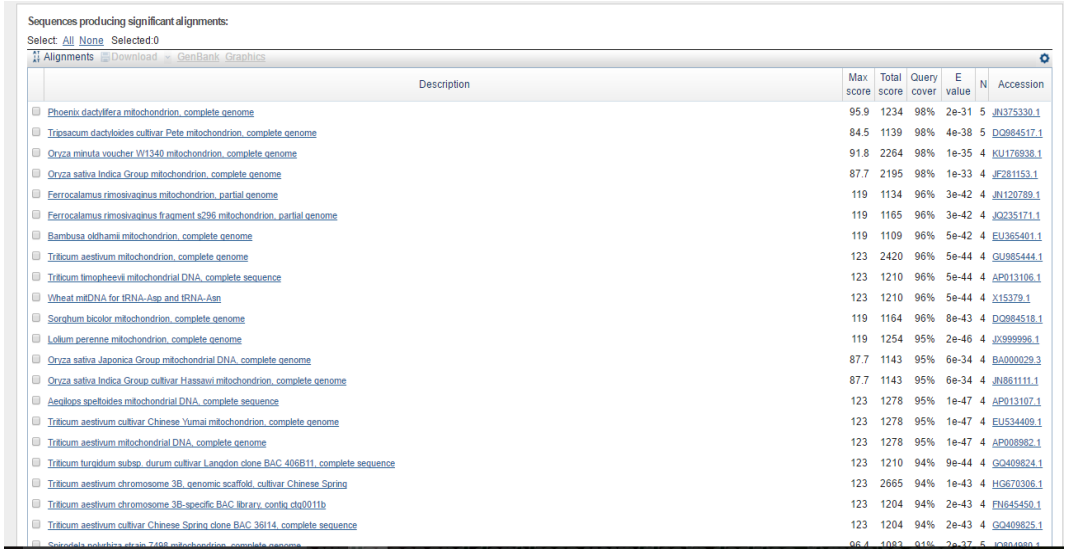


Plate 11a Screenshot showing the result of TBLASTX of sequence obtained with primer RA2R of *RA2* gene



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics

Description	Max score	Total score	Query cover	E value	N	Accession
Phoenix dactylifera mitochondrion, complete genome	95.9	1234	98%	2e-31	5	JN375330.1
Tripsacum dactyloides cultivar Pete mitochondrion, complete genome	84.5	1139	98%	4e-38	5	DQ984517.1
Oryza minuta voucher W1340 mitochondrion, complete genome	91.8	2264	98%	1e-35	4	KU176938.1
Oryza sativa Indica Group mitochondrion, complete genome	87.7	2195	98%	1e-33	4	JF281153.1
Ferrocalamus rimosivaginus mitochondrion, partial genome	119	1134	96%	3e-42	4	JN120789.1
Ferrocalamus rimosivaginus fragment s296 mitochondrion, partial genome	119	1165	96%	3e-42	4	JQ235171.1
Bambusa oldhamii mitochondrion, complete genome	119	1109	96%	5e-42	4	EU365401.1
Triticum aestivum mitochondrion, complete genome	123	2420	96%	5e-44	4	GU985444.1
Triticum timopheevii mitochondrial DNA, complete sequence	123	1210	96%	5e-44	4	AP013106.1
Wheat mtDNA for tRNA-Asp and tRNA-Asn	123	1210	96%	5e-44	4	X15379.1
Sorghum bicolor mitochondrion, complete genome	119	1164	96%	8e-43	4	DQ984518.1
Lolium perenne mitochondrion, complete genome	119	1254	95%	2e-46	4	JX999996.1
Oryza sativa Japonica Group mitochondrial DNA, complete genome	87.7	1143	95%	6e-34	4	BA000029.3
Oryza sativa Indica Group cultivar Hassawi mitochondrion, complete genome	87.7	1143	95%	6e-34	4	JN861111.1
Aegilops speltoides mitochondrial DNA, complete sequence	123	1278	95%	1e-47	4	AP013107.1
Triticum aestivum cultivar Chinese Yumai mitochondrion, complete genome	123	1278	95%	1e-47	4	EU534409.1
Triticum aestivum mitochondrial DNA, complete genome	123	1278	95%	1e-47	4	AP008982.1
Triticum turgidum subsp. durum cultivar Langdon clone BAC 406B11, complete sequence	123	1210	94%	9e-44	4	GQ409824.1
Triticum aestivum chromosome 3B, genomic scaffold, cultivar Chinese Spring	123	2665	94%	1e-43	4	HG670306.1
Triticum aestivum chromosome 3B-specific BAC library, contig ctg0011b	123	1204	94%	2e-43	4	FN645450.1
Triticum aestivum cultivar Chinese Spring clone BAC 36I14, complete sequence	123	1204	94%	2e-43	4	GQ409825.1
Spirodela polyrhiza strain 7498 mitochondrion, complete genome	96.4	1083	91%	2e-37	5	KP894980.1

Plate 11b Screenshot showing the result of TBLASTX of sequence obtained with primer RA2R of *RA2* gene

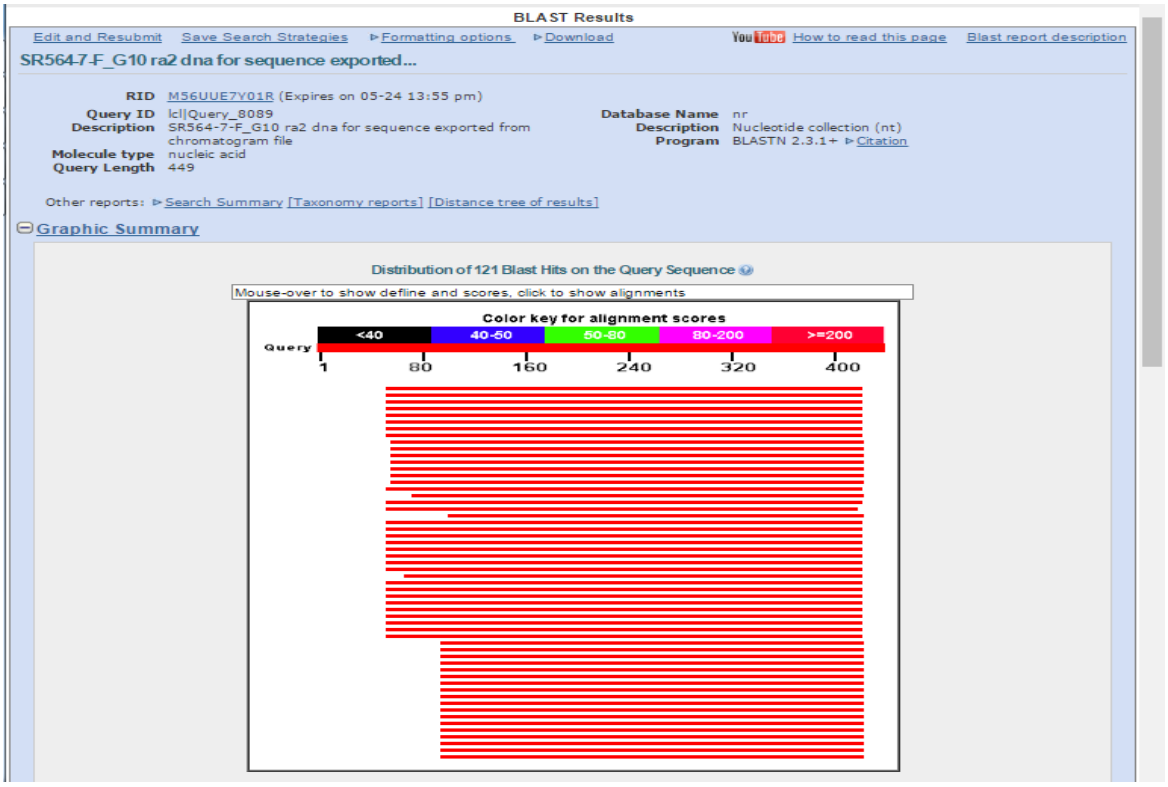


Plate 12a Screenshot showing the result of BLASTN of sequence obtained with primer RA2F of *RA2* gene

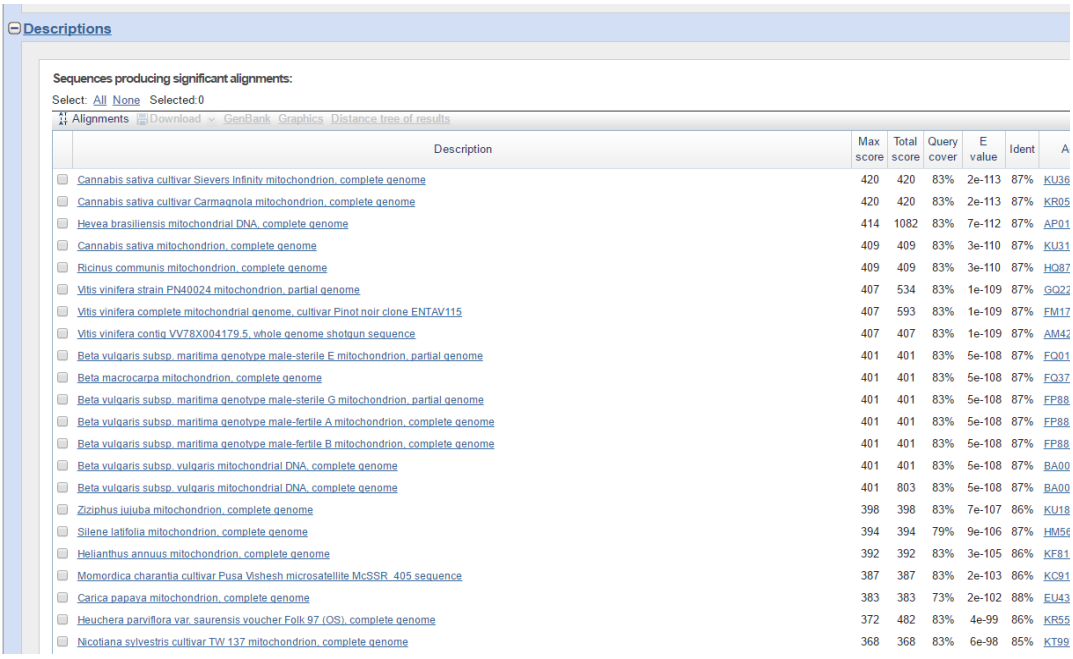


Plate 12b Screenshot showing the result of BLASTN of sequence obtained with primer RA2F of *RA2* gene

4.7.2 Sequence obtained using *RA3* primer- RA3F (cDNA)

1. **TBLASTX**

Amplicon of *RA3* primers gave 3574 hits (Plate 13a and 13b) when searched against non-redundant database. The results show the uncharacterised protein. The best match was with chromosome 8 of *Cucumis melo* with 99% query cover and E-value of 1e-56.

2. **BLASTN**

With BLASTN, 52 hits (Plate 14a and 14b) were obtained and best hit was contig sequence from *Vitis vinifera* with 70% query cover and E-value of 7e-52. Sequence showed similarity to uncharacterized mRNA sequence from *Brassica napus* with 60% query cover and E-value of 3e-41.

3. **CLUSTAL OMEGA**

Clustal Omega analysis showed 39.21 and 40.94 percent identity (Table 7) with the reported sequences of *RA3* of *Zea mays* and *Vitis vinifera* in the NCBI database, and these two reported sequences among themselves showed 61.30 percent identity. An alignment of sequences is shown on Plate 15.

Table 7 Percent Identity Matrix - created by Clustal2.1

SR564-8-F_H10	100.00	39.21	40.94
gi|162459858|ref|NM_001112394.1|	39.21	100.00	61.30
gi|787035541|dbj|LC037416.1|	40.94	61.30	100.00

4. **NCBI Conserved Domain Search**

On CDD search, the presence of an integrase core domain was revealed in the sequence obtained (Plate 16). The specific hit obtained was located at 269 and 382 bases interval near to the 3' end. CDD search showed E-value of 3.06e-05 for integrase core domain obtained.

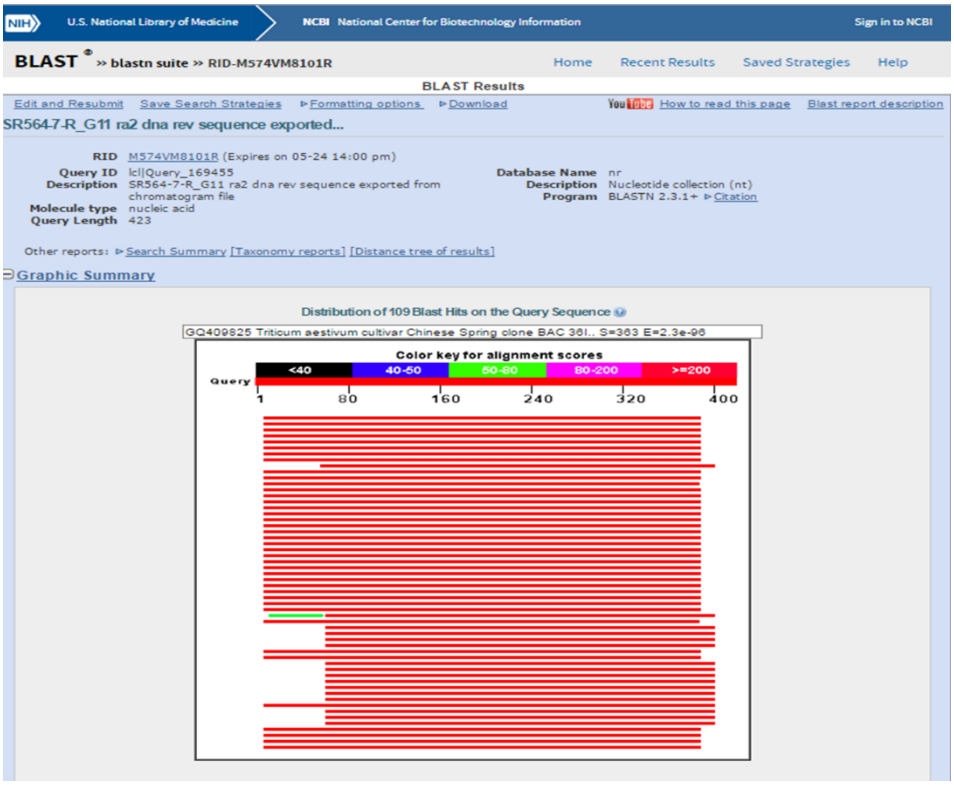


Plate 13a Screenshot showing the result of BLASTN of sequence obtained with primer RA3F of *RA3* gene

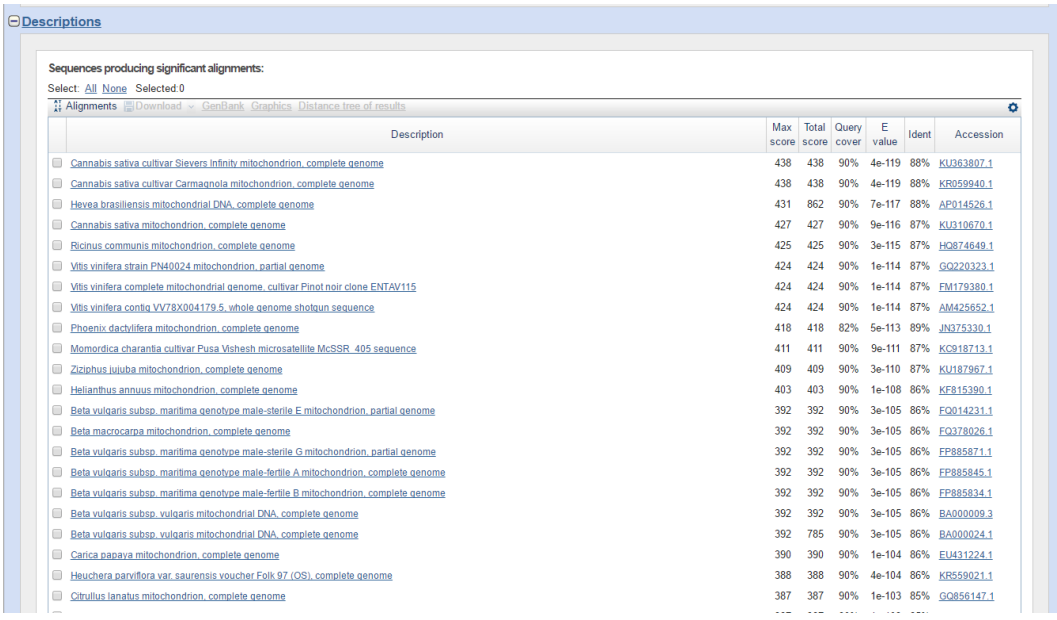


Plate 13b Screenshot showing the result of BLASTN of sequence obtained with primer RA3F of *RA3* gene

BLAST Results

SR564-8-F_H10 sequence exported from chromatogram...

RID	MWYEWH0E015 (Expires on 06-02 13:59 pm)		
Query ID	lcl|Query_97195	**Database Name**	nr
Description	SR564-8-F_H10 sequence exported from chromatogram file	**Description**	Nucleotide collection (nt)
		Program	TBLASTX 2.3.1+
Molecule type	nucleic acid		
Query Length	410		

Graphic Summary

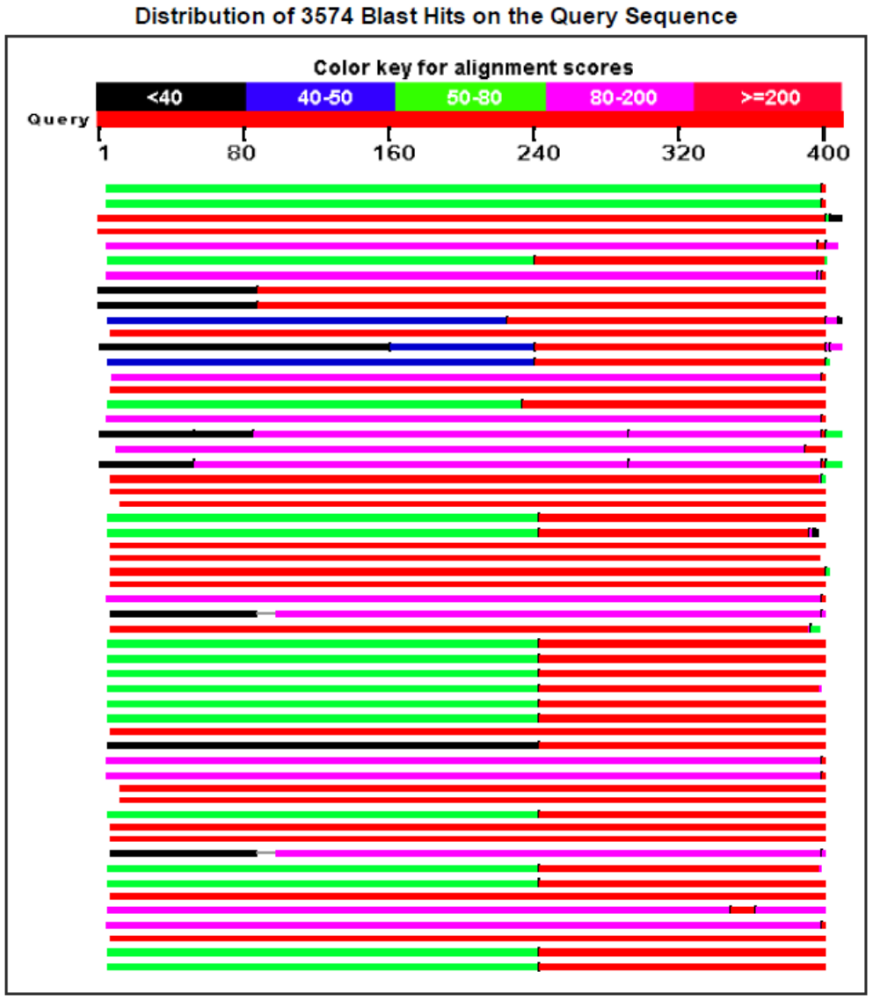


Plate 14a Screenshot showing the result of TBLASTX of sequence obtained with primer RA3F of *RA3* gene

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	N	Accession
Cucumis melo genomic chromosome, chr_7	228	15685	96%	7e-57	Sumn1	LN713261.1
Cucumis melo genomic scaffold, anchoredscaffold00027	228	2134	96%	7e-57	Sumn1	LN681873.1
Cucumis melo genomic chromosome, chr_8	228	21210	99%	1e-56	Sumn1	LN713262.1
Cucumis melo genomic scaffold, anchoredscaffold00036	228	4418	97%	1e-56	Sumn1	LN681878.1
Cucumis melo genomic chromosome, chr_4	220	14179	98%	2e-54	Sumn1	LN713258.1
Cucumis melo genomic scaffold, anchoredscaffold00076	220	2121	96%	2e-54	Sumn1	LN681883.1
Cucumis melo genomic scaffold, anchoredscaffold00032	220	3457	96%	2e-54	Sumn1	LN681826.1
Cucumis melo genomic chromosome, chr_12	214	16945	97%	1e-52	Sumn1	LN713266.1

Plate 14b Screenshot showing the result of TBLASTX of sequence obtained with primer RA3F of *RA3* gene

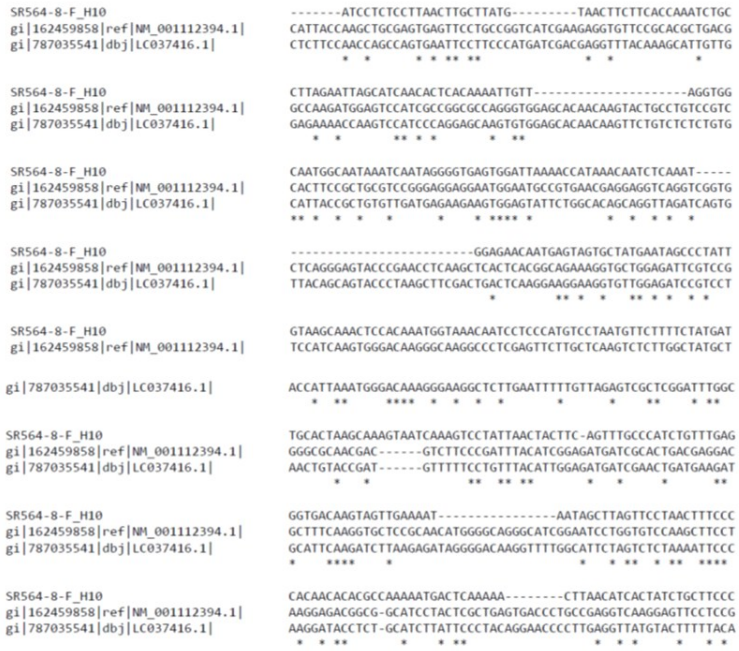


Plate 15 Screenshot showing the result of Multiple sequence alignment of sequence obtained with primer RA3F of *RA3* gene and *RA3* gene sequences available in database

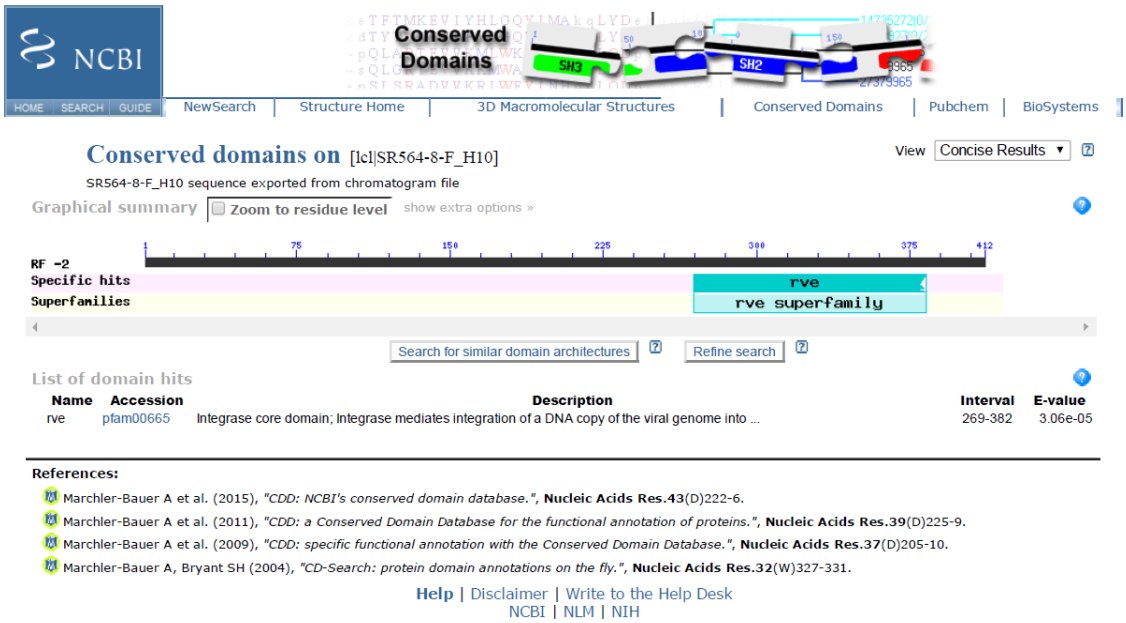


Plate 16 Screenshot showing the result of Conserved domain database search of sequence obtained with primer RA3F of *RA3* gene

DISCUSSION

5. DISCUSSION

A mutant of black pepper (*Piper nigrum* L.) type 'Thekken' reported from Idukki district of Kerala shows altered inflorescence architecture with remarkable spike branching character. As branching is a trait of high economic value that can contribute to increase in yield, the study entitled "Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'" was carried out to analyze the trait at the genomic level and at different stages of spike development at the transcriptome level in 'Thekken' and a non-branching variety 'Karimunda'. The study was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014-2016. Discussions pertaining to this study are discussed in this chapter.

5.1 DNA ISOLATION

Genome is specific to respective organism and DNA-based techniques are used in differentiating the varieties, identifying genes and for marker assisted selection (Kaur *et al*., 2015). Quality of isolated DNA is an important factor, as it can affect molecular techniques (Henderson *et al*., 2013). Isolating good quality DNA from black pepper is challenging due to the presence of a large amount of polyphenolic compounds, polysaccharides and other secondary metabolites (Dhanya *et al*., 2007).

In the present study, DNA was isolated from young leaves of black pepper using modified CTAB extraction protocol reported by Subba *et al*. (2014). Plate 4 shows the 1 % (w/v) agarose gel electrophoresis of isolated DNA. Gel picture showed no contamination of RNA. Any degradation occurring during the DNA preparations can be easily viewed as smearing or indistinct bands. Presence of single distinct DNA bands in Plate 4 indicated good quality DNA.

Smith *et al*. (1991) reported that increase in CTAB and NaCl concentrations increase the yield of cellular DNA. CTAB is generally used as a detergent to separate out polysaccharides with higher concentration of NaCl (Fang *et al*., 1992). In the present study, increased concentrations of CTAB (2.5% to

3%) and NaCl (1.5 M to 2.5 M) showed increase in DNA quantity. George *et al*. (2005a) isolated DNA from black pepper leaves by using the protocol reported by Doyle and Doyle (1990) with increased concentrations of CTAB (4%) and β-mercaptoethanol (0.5%). Table 1 shows the spectrophotometric evaluation of DNA samples. The DNA yield ranged from 1300 ng/µl to 2800 ng/µl in different samples.

Vimarsha *et al*. (2014a) isolated genomic DNA from eight black pepper varieties by using protocol reported by Doyle and Doyle (1990) and the absorbance ratio A260/A280 ranged between 1.71 and 1.88 indicating good quality of DNA. Subba *et al*. (2014) modified this protocol (Doyle and Doyle, 1990) to isolate genomic DNA from mature spikes and berries of black pepper and the absorbance ratio of A260/A280 ranged between 1.7 and 2.2. In the present study, DNA was isolated by using protocol reported by Subba *et al*. (2014) with minute modifications and the absorbance ratio, A260/A280 of the DNA samples from 'Thekken' and 'Karimunda' ranged between 1.63 and 1.83 which indicated good quality DNA.

In this study, the DNA was hooked out using wide bore pipette after addition of isopropanol. This step reduced the contaminants precipitating along with DNA during centrifugation. The present protocol used for DNA isolation from leaves of black pepper also yielded good quantity of DNA.

5.2 RNA ISOLATION

In the present study, high quality total RNA was successfully isolated from different developmental stages of spikes of black pepper ('Karimunda' and 'Thekken') by using Trizol reagent. Plate 5 shows the 1.8 % (w/v) agarose gel electrophoresis of isolated RNAs. Distinct 28S, 18S and 5S eukaryotic ribosomal RNA bands were viewed on ethidium bromide stained agarose gel. Ribosomal RNA represents more than 90% of the total RNAs (Asif *et al*., 2000). Any degradation occurring during the RNA preparations can be easily viewed as

smearing or indistinct bands. Presence of distinct ribosomal RNA bands in Plate 5 indicated good quality of RNA without any degradation.

In the present study, use of PVP and β-mercaptoethanol during extraction process increased the yield of RNA. Table 2 shows the spectrophotometric evaluation of RNA samples. The RNA yield ranged from 1200 ng/µl to 2200 ng/µl in different samples. George *et al*. (2005b) reported that use of PVP in strong denaturing buffer containing guanidinum thiocyanate yielded good quality RNA suitable for reverse transcription experiments.

Siju *et al*. (2007) isolated RNA from black pepper samples by using modified acid guanidium thiocyanate-phenol-chloroform protocol and found that use of sodium sulphite in RNA extraction buffer increases the quality and yield of total RNA and also the sensitivity of virus detection by RT-PCR.

The purity of isolated RNAs was determined by the absorbance ratio A260/A280 (Logemann *et al*., 1987; Manning, 1990) and it ranged between 1.7 and 2.2. This ratio suggested less contamination of protein substances during RNA preparations (Gehrig *et al*., 2000). RNA was hooked out using wide bore pipette after addition of isopropanol. This step reduced the contaminants precipitating with RNA during further centrifugation.

5.3 PCR ANALYSIS

PCR was performed with genomic DNA to detect the presence of *RAMOSA* family genes in black pepper. DNA isolated from leaves of black pepper mutant type ‘Thekken’ and control variety ‘Karimunda’ was used as template for PCR.

When PCR was performed using degenerate primers designed for *RA1* and *RA3* genes, multiple bands were obtained on agarose gel electrophoresis. *RA1* gene primers amplified four bands, while *RA3* gene primers amplified two bands in black pepper genome in both ‘Thekken’ and ‘Karimunda’. Non-specific bands can sometimes occur when degenerate primers are used in PCR (Nix *et al*., 2006).

Multiple bands obtained may be due to degeneracy of the primers or presence of primer homologous sequences in black pepper genome.

When PCR was performed using primers designed for *RA2*, only a single band was obtained. This primer pair was without any degeneracy. The single band obtained was cloned and sequenced for further analysis by assuming it as a *RA2* gene specific amplicon.

Sequences obtained with *RA2* gene primers showed best match with sequence from chromosome 3B of *Triticum aestivum* on BLASTN analysis and sequence from mitochondrial genome of *Phoenix dactylifera* on TBLASTX analysis. BLAST analysis of sequence of the amplicon did not showed any direct hit with *RA2* sequences available at NCBI database. This can be due to diversity of *RA2* gene sequence over different plants or lower specificity of primers to the gene.

Vimarsha *et al.* (2014b) performed PCR with primers designed for *TFL1* gene of *Arabidopsis* and reported homolog of *TFL1* gene in black pepper type 'Thekken' and 'Karimunda'.

Vimarsha *et al.* (2014a) studied diversity among different black pepper varieties. In this study 'Karimunda' formed a separate cluster indicating the genomic diversity from 'Thekken'. However, in present the study all genes showed no polymorphism in both varieties of black pepper i.e. all bands were present in both varieties with same molecular size. This indicates the relatedness of 'Karimunda' and 'Thekken' at amplified loci with gene specific primers.

5.4 RT-PCR ANALYSIS

Total number of genes in the genome of plant shows diversity in terms of expression levels, both temporally and spatially. Different genes controlling inflorescence development express at different developmental stages (Tanaka *et al*., 2013). In the present study, three stages of spike development were selected with 10 days interval and with specific length. Respective stages selected from

'Thekken' and 'Karimunda' were at same developmental stage by considering days after emergence of bud and length of spike at the time of collection.

In the present study, we used RT-PCR to detect the presence and differential expression of *RAMOSA* family genes (*viz*., *RA1*, *RA2* and *RA3*) in the spikes of black pepper 'Thekken' and 'Karimunda'. RT-PCR was performed in two steps. Two step RT-PCR is easier to perform and has been used to detect multiple genes expressed in the same sample (Ravnikar *et al*., 2016). RT-PCR can be used for gene identification, quantitative expression of particular gene, tissue specific expression studies, and differential gene expression analysis (Simpson and Brown, 1995).

Housekeeping genes are usually used to check intactness of cDNA. Housekeeping genes express continuously in the cell and these could be detected easily at any time and in any tissue of the plant. Different housekeeping genes (e.g. *Ubiquitin*, *Actin*, *18S rRNA* etc.) have been used for confirming intactness of cDNA (Bansal and Das, 2013; Hemanth, 2014; Rayani and Nayeri, 2015).

In the present study, intactness of cDNA was confirmed by using housekeeping gene *Ubiquitin* specific primers which yielded a PCR product of expected size (99 bp).

5.4.1 Expression of *RA1* and *RA2* genes in black pepper

RA1 and *RA2* gene specific primers showed no amplification in cDNA of all stages of both 'Thekken' and 'Karimunda'. Absence of amplification may indicate the absence of expression of *RA1* and *RA2* genes in selected stages. *RAMOSA* genes are expressed usually at very early stage during development of inflorescence in ring like domain at the base of the branch meristem of maize (Booker *et al*., 2005; Snowden *et al*., 2005). Satoh-Nagasawa *et al*. (2006) found very high expression of *RAMOSA* genes in ears with uniform size at a growth stage of 2 mm length. In black pepper, *RAMOSA* family genes may express very early during development of inflorescence. Another reason for no amplification of *RA1* and *RA2* in cDNA can be absence of homologous regions selected for primer

designing. McSteen (2006) reported absence of *RA1* in *Arabidopsis* plant, whereas Kellogg (2007) reported absence of *RA1* and *RA3* but presence of *SRA* in rice. Koppolu *et al*. (2013) found that ortholog or homolog of *RA1* is missing in barley. In *Vitis vinifera*, Ishiai *et al*. (2016) reported the presence of all the *RAMOSA* homolog genes and their expression in grape inflorescence. These reports may indicate the diversity and evolution of *RAMOSA* genes over different plant groups and their involvement in the evolution of inflorescence morphogenesis.

5.4.2 Differential expression of *RA3* specific sequence in type 'Thekken'

RT-PCR yielded an amplicon of size 450 bp in stage II of mutant black pepper type 'Thekken' and it was absent in all stages of control variety 'Karimunda'. *RA3* gene in maize and homolog of *RA3* (*OsSRA*) in rice is reported to show highly localized expression pattern at the base of inflorescence branches (Satoh-Nagasawa *et al*., 2006). The differential expression pattern in the present study may indicate the involvement of *RA3* amplicon in inflorescence development in black pepper. Expression of the amplicon needs to be checked in different stages of spike development in black pepper control variety 'Karimunda' to confirm the actual time of expression in normal spike development.

In the present study, amplicon obtained in branching inflorescence of black pepper type 'Thekken' indicate a possible role of *RA3* in spike branching in black pepper.

5.4.3 Diversity of *RA3* gene

BLAST analysis of sequence of the *RA3* specific amplicon did not showed any direct hit with *RA3* sequences available at NCBI database. BLASTN analysis of the sequence obtained showed similarity with uncharacterized mRNA sequence from *Brassica napus* while TBLASTX analysis showed similarity with uncharacterised sequence from *Cucumis melo*. This can be due to diversity of *RA3* gene sequence over different plants or lower specificity of primers to the gene.

To know the diversity among *RAMOSA3* gene sequences available at NCBI database, we compared two *RAMOSA3* gene sequences of *Zea mays* and *Vitis vinifera* by using clustal omega and found that these sequences contain only 61.30 percent identity. Sequence obtained with *RA3* primers showed 39.21 and 40.94 percent identity with the reported sequences of *RA3* of *Zea mays* and *Vitis vinifera* respectively. Further sequence analysis with CDD search showed presence of integrase core domain which is absent in *RA3* gene sequences available at NCBI database. Presence of integrase core domain can be a possible reason for reduced percent identity of the amplicon obtained.

A homolog of *RA3*, named *SISTER OF RAMOSA3* (*SRA*), has been identified in barley (*HvSRA*) (Koppolu *et al*., 2013) and related grasses (Satoh-Nagasawa *et al*., 2006). This may indicate *RA3* gene sequence is widely diversified in different plants.

5.4.4 Presence of integrase core domain in *RA3* primer amplicon

Presence of integrase core domain in the sequence obtained with *RA3* gene primers is another important finding from the present study. Integrase core domain is important domain of Integrase enzyme, which carries out integration of a DNA copy of the viral genome into the host chromosome (Dyda *et al*., 1994). These domains are particularly abundant in plant genomes and are intimately involved in the evolution of genome structure and size (Feschotte *et al*., 2002; Vitte and Panaud, 2005).

Presence of integrase core domain has been reported in transposable elements. Every prokaryotic and eukaryotic genome contains transposable elements and which exert a complex mutagenic activity that leads to changes in chromosome architecture, generation of new regulatory networks and increases in the protein collection (Feschotte, 2008; Bourque, 2009; Shapiro, 2010). On the basis of structural-functional characteristics, transposable elements have been separated into two major classes each comprising subclasses or orders, superfamilies, families and subfamilies (Kapitonov and Jurka, 2008; Wicker *et al*.,

2010). Among the most widespread retrotransposons are the LTR superfamilies Gypsy and Copia, which include two ORFs coding for the structural virus-like protein GAG and the reverse transcriptase (RT)/integrase (INT) enzyme POL (Neumann *et al*., 2003; Havecker *et al*., 2004; Amyotte *et al*., 2012; Santana *et al*., 2014).

Retroviral integration of integrase core domain in differentially amplified band obtained with *RAMOSA3* gene primers can be a possible reason for the altered inflorescence architecture in the novel mutant variety of black pepper 'Thekken'.

SUMMARY

6. SUMMARY

The project entitled "Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014-2016, with the objective to detect the presence and differential expression of *RAMOSA* family genes (*RA1, RA2* and *RA3*) and analyse their influence in contributing to the branching trait in black pepper (*Piper nigrum* L.) type 'Thekken'.

A mutant of black pepper (*Piper nigrum* L.) type 'Thekken' reported from Idukki district of Kerala shows altered inflorescence architecture with remarkable spike branching character. As branching is a trait of high economic value that can contribute to increase in yield, a study was carried out to analyze the trait at molecular level. Screening using degenerate primers designed for *RAMOSA* family genes, reported to contribute to branching trait in other crops, was carried out in 'Thekken' and a non-branching variety 'Karimunda' at the genomic level and at different stages of spike development at the transcriptome level.

Samples used in the study *viz*., leaves and spikes of black pepper type 'Thekken' and the control non branching variety 'Karimunda' were collected from farmer`s field. Genomic DNA was extracted from the leaf samples and RNA was extracted from the spikes at three different stages of development (Stage I - 1 cm; Stage II - 4 cm and Stage III - 9 cm). Methods used for nucleic acid extractions yielded good quality DNA and RNA. Value of A_{260}/A_{280} for DNA samples ranged between 1.52 and 1.83 and the yield of DNA ranged between 1300 ng/µl to 2800 ng/µl. For RNA, value of A_{260}/A_{280} ranged between 1.76 and 2.27 and the yield of RNA ranged between 1200 ng/µl and 2200 ng/µl

Degenerate primers were designed for *RA1*, *RA2* and *RA3* genes using Primer3 and Oligocalc tools and these primers were used for screening at the genome and transcriptome level by PCR and RT-PCR. The amplicons obtained were resolved on agarose gel. At genomic level, a band of size 450 bp was

obtained for *RA2* primers, whereas *RA1* primers produced four bands (600 bp, 550 bp, 400 bp and 200 bp) and *RA3* primers produced two bands (650 bp and 450 bp). There was no difference in the banding profile in 'Thekken' and 'Karimunda'. The *RA2* specific band (450 bp) obtained in 'Thekken' was sequenced and analysis with TBLASTX showed similarity with sequence from chromosome 3B of *Triticum aestivum* with E-value of 1e-31 and 94% query cover. When sequence was analysed using BLASTN it showed similarity with NADH dehydrogenase subunit 2 (*nad2*) gene of *Parinari campestris* with 83% query cover and E-value of 2e-93. On CDD search, no conserved domains were found in both sequences obtained using *RA2* primers.

On screening the cDNA, the primers designed for *RA1* and *RA2* gene showed no amplification in both 'Thekken' and 'Karimunda'. However, the primers designed for *RA3* gene showed differential expression in Thekken and 'Karimunda'. A band of size 450 bp was obtained in stage II of the spike of 'Thekken', whereas no amplification was obtained in the 'Karimunda' variety. The amplicon obtained using *RA3* primer was cloned and sequenced.

Analysis of the *RA3* specific sequence using TBLASTX showed best match with chromosome 8 of *Cucumis melo* with 99% query cover and E-value of 1e-56 and BLASTN analysis showed similarity to uncharacterized mRNA sequence from *Brassica napus* with 60% query cover and E-value of 3e-41. Clustal Omega analysis showed 39.21 and 40.94 percent identity with the reported sequences of *RA3* of *Zea mays* and *Vitis vinifera* while these two sequences among themselves showed 61.30 percent identity. Conserved domain database (CDD) search revealed the presence of an integrase core domain in this sequence.

All the three sequences obtained with *RA2* and *RA3* primers have been deposited in the NCBI database as 'Floral architecture related sequence isolated from branching type black pepper' (Accession numbers: KX518738, KX518739 and KX518740).

The present study is the first report of the presence of an integrase core domain in the genome of black pepper. Differential amplification of cDNA of stage II from 'Thekken' and 'Karimunda' with *RA3* primers suggests that altered expression of the region under study may play a role in the induction of spike branching in 'Thekken'. The presence of the integrase core domain also suggests a possible role of retroviral integration in differential expression.

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7. REFERENCES

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APPENDICES

APPENDIX I

CTAB Extraction Buffer

C-TAB	3 %	
Tris- HCl (pH 8.0)	150 mM	
EDTA	50 mM	
NaCl	2.5 M	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA extraction
PVP	3 % (w/v)	

APPENDIX II

TAE Buffer (5X) for 1 liter solution

Tris base	242 g
Acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

ABSTRACT

ANALYSIS OF DIFFERENTIAL EXPRESSION OF GENES DETERMINING INFLORESCENCE ARCHITECTURE IN BLACK PEPPER (*Piper nigrum* L.) TYPE 'THEKKEN'

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ABSTRACT

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ABSTRACT

The project entitled "Analysis of differential expression of genes determining inflorescence architecture in black pepper (*Piper nigrum* L.) type 'Thekken'" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014-2016. The objective of the study was to detect the presence and differential expression of *RAMOSA* family genes (*RA1, RA2* and *RA3*) that determine the inflorescence architecture and to analyse their influence on the branching trait in black pepper (*Piper nigrum* L.) type 'Thekken'.

Samples used in the study *viz*., leaves and spikes of black pepper type 'Thekken' and the control non branching variety 'Karimunda' were collected from farmer`s field. Genomic DNA was extracted from the leaf samples and RNA was extracted from the spikes at three different stages of development (Stage I - 1 cm; Stage II - 4 cm and Stage III - 9 cm). Methods used for nucleic acid extractions yielded good quality DNA and RNA.

Degenerate primers were designed for *RA1*, *RA2* and *RA3* genes using Primer3 and Oligocalc tools and these primers were used for screening at the genome and transcriptome level by PCR and RT-PCR respectively. The amplicons obtained were resolved on agarose gel. At genomic level, a band of size 450 bp was obtained for *RA2* primers, whereas *RA1* primers produced four bands (600 bp, 550 bp, 400 bp and 200 bp) and *RA3* primers produced two bands (650 bp and 450 bp). There was no difference in the banding profile in 'Thekken' and 'Karimunda'. The *RA2* specific band (450 bp) obtained in 'Thekken' was sequenced and showed similarity with NADH dehydrogenase subunit 2 (*nad2*) gene of *Parinari campestris*.

On screening the cDNA, the primers designed for *RA1* and *RA2* genes showed no amplification in both 'Thekken' and 'Karimunda'. However, the primers designed for *RA3* gene showed differential expression in 'Thekken' and 'Karimunda'. A band of size 450 bp was obtained in stage II of the spike of

'Thekken', whereas no amplification was obtained in the 'Karimunda' variety. The amplicon obtained using *RA3* primer was cloned and sequenced.

Analysis of the *RA3* specific sequence using tBLASTx showed best match with fragment of chromosome 7 of *Cucumis melo* and BLASTn analysis showed similarity to uncharacterized mRNA sequence from *Brassica napus*. Clustal Omega analysis showed 39.21 and 40.94 percent identity with the reported sequences of *RA3* of *Zea mays* and *Vitis vinifera* while these two sequences among themselves showed 61.30 percent identity. Conserved domain database (CDD) search revealed the presence of an integrase core domain in this sequence.

All the three sequences obtained with *RA2* and *RA3* primers have been deposited in the NCBI database as 'Floral architecture related sequence isolated from branching type black pepper' (Accession numbers: KX518738, KX518739 and KX518740).

The present study is the first report of the presence of an integrase core domain in the genome of black pepper. Differential amplification of cDNA of stage II from 'Thekken' and 'Karimunda' with *RA3* primers suggests that altered expression of the region under study may play a role in the induction of spike branching in 'Thekken'. The presence of the integrase core domain also suggests a possible role of retroviral integration in differential expression.