MOLECULAR CHARACTERIZATION OF CANDIDATE GENE FOR PUNGENCY IN *Capsicum* spp.

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "Molecular characterization of candidate gene for pungency in *Capsicum* spp." 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 of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

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Date: 09.09.2016

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CERTIFICATE

Certified that the thesis entitled **"Molecular characterization of candidate gene for pungency in *Capsicum* spp."** is a record of research work done independently by **Miss. Anju Viswanath** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associate ship to her.

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Anju Viswanath

DEDICATED TO MY FAMILY AND MAJOR ADVISOR

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF ANNEXURES

Annexure No.	Title
1	Details of laboratory equipments used for study
2	Reagents of DNA isolation by CTAB method as per Rogers and Bendich (1994)
3	Composition of buffers and dyes used for gel electrophoresis

ABBREVIATIONS

ASTA	American Spice Trade Association
Bp	Base pair
β	Beta
CS	Capsaicin synthetase
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
°C	Degree Celsius
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene DiamineTetraAcetic Acid
HCl	Hydrochloric Acid
KAU	Kerala Agricultural University
L	Litre
M	Molar
MAS	Marker-Assisted Selection
ml	Millilitre
mg	Milligram
µl	Micro litre
µm	Micro molar

Mg	Magnesium
$MgCl_2$	Magnesium Chloride
mM	Milli mole
NaCl	Sodium Chloride
ng/µl	Nanogram per micro litre
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Percentage
PVP	Poly vinyl pyrrolidone
RNA	Ribonucleic Acid
RNase	Ribonuclease
Rpm	Revolutions per minute
SHU	Scoville Heat Unit
TAE	Tris Acetate EDTA
TE	Tris EDTA
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by volume

Introduction

1. Introduction

Chilli (*Capsicum* spp.) belonging to the family Solanaceae is one of the major vegetable cum spice crops. It was originated in tropical America and is also known as red pepper or chili pepper. Chillies were first introduced to the European Union in fifteenth century by Columbus and then spread along the spice trading routes to Africa, India, China and Japan to rest of the globe. The Portuguese introduced this wonderful crop in India in seventeenth century, (Bosland and Votava, 2000).

The world's largest producer, consumer and exporter of chillies are India. About 42.4 per cent of total Indian spices exports were contributed by chillies and earned a foreign exchange of Rs. 23.9 billion in 2015-16. Globally, India shares 40 per cent of area and 39 per cent of production in chilli. According to the statement of FAO stat (2012) the area under chilli in India is 8.93 lakh ha with a production of 13.0 lakh tones.

Capsaicin, a crystalline acrid volatile alkaloid present in the placenta of fruit is contributing for the pungency of chilli. Chilli oleoresin, the total flavor extract of dried and ground chillies has various importances in food processing and beverage industries. The nutraceutical applications of capsaicinoid are widely exploited and identified its medicinal properties like antioxidant, anticancer, antiarthritic, and analgesic (Prasad *et al.*, 2005).

In most of the developed countries, the natural color extract from chilli has replaced the artificial colors in food items. With the identification of paprika chillies which lack the pungency completely, the use of chillies as the best source for natural food colour was made possible.

A higher level of 4,000 American Spice Trade Association (ASTA) color units and 100,000–500,000 Scoville heat units *Capsicum* oleoresins are usually obtained from pungent chillies. Other than that, the paprika extract used in food

and cosmetics as a colorant should have 40,000 ASTA color units and zero pungency. Hence, regulation of capsaicin biosynthesis in *Capsicum* cultivars to meet the requirements of the food, pharmaceutical, and cosmetics industries were having a greater scope (Purseglove *et al.*, 1981).

The *Capsicum synthetase* gene, which was proposed to be responsible for the capsaicin synthesis, remains hypothetical. Alternatively, the candidate gene loci such as *Pun1*, *Pun2 etc.* which are proven to have direct association with capsaicin synthesis are proposed (Wyatt *et al.,* 2012). An understanding on the sequence variations at the pungency coding genomic regions, which may be revealed through the characterization of such loci, will be useful in the chilli breeding programmes aimed at altering the levels of pungency in the desired variety, especially for the industrial needs. So far *CS, AT3, Pun1, Catf-1* and *Catf-2* are the loci reported for the production of capsaicinoids in chilli. And thirteen SCARs were reported which can amplify the reported loci for pungency *viz;* CSF1/R2 and BF7/R9 for *CS* locus (Lee *et al.*, 2005), *Cen1* for *AT3* locus (Chakradhar *et al.*, 2013), MAP1, *Pun1*^{1}*fwd1, fwd2/rev, Pun1*^{2}*fwd/rev* and *Pun1*^{3}*fwd/rev1, rev2* for *Pun1* locus, U16F/U2R and U3, 1F/D3R, 14R, 1R for *Catf-1* locus and U25F/D25R and U22F/D22R for *Catf-2* locus. The information generated could be immediately used in the molecular and cisgenic breeding approaches to engineer the pungency levels.

Keeping the above in view, the present study on "Molecular characterization of candidate gene for pungency in *Capsicum* spp." was taken up at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, to characterize highly, mildly and non-pungent *Capsicum* spp. at molecular levels.

The objective of the study was to understand the molecular mechanisms behind the different levels of pungency in different species of *Capsicum.*

Review of literature

2. Review of literature

The study on "Molecular characterization of candidate gene for pungency in *Capsicum* spp." was conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur, with the objective to understand the molecular mechanisms behind the different levels of pungency in different species of *Capsicum*.

The investigation included the molecular characterization of ten chilli accessions with different levels of pungency viz. two lines of *C. frutescence* (White Khandari and Vellayani Samrudhi), one line from *C. chinense* (Vellayani Thejus), five lines from *C. annuum* var. *annuum* (Ujwala, Anugraha, Byadagi Dabbi, Byadagi Kaddi, paprika Kt-P1-19) and two lines from bell peppers (*C.annuum* var. *grossum*) (Arka Gaurav and Arka Mohini). The SCAR (Sequence Characterised Amplified Regions) markers reported specific for pungency responsible loci in Capsicum were used in the study. The relevant literatures on various aspects of the investigations are reviewed in this chapter.

Since time immemorial, chillies and other members of the genus *Capsicum* have been used to impart pungency to human foods. The nutraceutical application of capsaicinoid is widely exploited and identified its medicinal properties like antioxidant, anticancer, antiarthritic, and analgesic (Prasad *et al.*, 2005). Subsequently, the fruits of the crop have been identified as good source of vitamin C and the bell peppers which lack pungency have emerged as the major salad vegetable. With the identification of paprika chillies which lack the pungency completely, the use of chillies as the best source for natural food colour was made possible.

The most important characteristics features of chilli are the pungency for red peppers and the color value for paprika. Hot peppers derive their pungency from the compound capsaicin (8-methyl-N-vanillyl-6-enamide). It is a compound

which is present in the internal partitions of the fruit which has an acrid and burning taste. It has been first isolated in 1876 (Thresh, 1876), capsaicin has the ability to stimulate gastric secretions and in surplus it causes inflammation.

Capsaicin is a tasteless and odourless white crystalline substance. Its amount differs widely in capsicum species, from a minimum of 0.05 per cent in the less pungent types to a maximum of 1.3 per cent in the hot chillies. 100,000–500,000 Scoville heat units and a higher level of 4,000 American Spice Trade Association (ASTA) color units *Capsicum* oleoresins are commonly obtained from pungent chillies. Other than that, the paprika extract used in food and cosmetics as a colorant should have 40,000 ASTA color units and zero pungency. Hence, regulation of capsaicin biosynthesis in *Capsicum* genotypes to meet the requirements of the food, pharmaceutical, and cosmetics industries were having a greater scope (Purseglove *et al.*, 1981).

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2.1 Genetics of pungency

Zewdie and Bosland (2000) reported that pungency, caused by the presence of capsaicinoids, is a major quality-determining factor in chilli (*Capsicum* L. sp.). Interspecific hybridization of *C. annuum* L. × *C. chinense* Jacq the additive, dominance, and interaction effects were notable for capsaicin, dihydrocapsaicin, and isomer of dihydrocapsaicin was revealed by generations

mean analysis. Repeated backcrossing and selection will maximise and minimise the capsaicinoid content because of the significant additive gene effect and the tendency of the values of the capsaicinoids at backcross families to shift toward the recurrent parents. Different genes are controlling the synthesis of each capsaicinoid is proven by the different gene actions and a different number of effective factors involved in the capsaicinoids inheritance.

According to Zewdie *et al.* (2001), in the five *C. pubescens* Ruiz & Pav. genotypes the inheritance of capsaicinoid content was studied using diallel analysis. For all capsaicinoids studied general combining ability and specific combining ability effects were significant which indicates additive and non-additive gene actions are present. The predominance of additive gene action in capsaicinoid inheritance implies the association of high capsaicinoid contents with high positive general combining ability of the parents.

2.2 Genomics of pungency

Curry *et al.* (1999) stated that, the alkaloids answerable for pungency in chilli, Capsaicinoids are produced from condensation of phenylpropanoid compounds and short-chain branched-fatty acids. In the capsaicinoid pathway, the transcript levels of enzymes were observed in *C. annuum* and *C. chinense* fruits, based on the function of development, tissue type and genotype. From a cDNA library of habañero (*C. chinense*) placenta, clones for *Pal*, *Ca4h*, and *Comt* were secluded. These cDNA clones were used to measure transcriptome amounts in different fruit tissues throughout development in six cultivars varying in pungency. Degree of pungency in placental tissue were positively correlated with transcript levels for all three genes; habañero, the more pungent chilli fruit, had the maximum transcript levels, CalWonder, a non-pungent fruit, had the minimum levels. Using the transcript gathering model of the phenylpropanoid genes as a screening criterion, other cDNA clones have been selected. Clones for an aminotransferase, forecasted to metabolised vanillylamine, and for a 3-keto-acyl ACP synthase, foreseen to elongate branched-chain fatty acids, were observed.

These genes are expressed in a placental-specific manner, and fruit pungency is positively correlated with transcript levels.

Kim *et al.* (2001) stated that capsaicinoids answerable for pungency of chilli plants are exclusively produced in the placental tissue of the chilli. As a primary step in the molecular genetics study of capsaicinoid biosynthesis, using the suppression subtractive hybridization (SSH) a cDNA library was built from the placenta of a high pungent pepper, *C. chinense* cv. Habanero. From about 400 subtracted clones, thirty-nine cDNA clones were identified through dot blot analysis and according to their nucleotides sequence. By comparing it with DNA and protein databases, sequence information of the chosen clones were evaluated. Results revealed that the cDNA clones could be divided into four groups; cDNAs with identity in genes encoding metabolic enzymes including acyl transferase and fatty acid alcohol oxidase (Group I), putative cell wall proteins (Group II), biotic and abiotic stress-inducible proteins (Group III), and lastly cDNAs with no identity (Group IV). Northern blot analysis was conducted to confirm that these clones are differentially expressed in pungent chilli. The results shown that all cDNA clones were differentially expressed in pungent chilli. In addition, the cDNA clones of Groups I and IV were differentially or preferentially expressed in the placenta of pungent pepper.

According to Blum *et al.* (2002), the presence of capsaicinoids is the reason for the pungency in chilli. In the placenta of the fruit, the action of a single dominant gene, *C*, is required for the production of capsaicinoid in pungent genotypes has been known. In a cross between a pungent *C. frutescence* wild accession and a non-pungent *C. annuum* bell pepper they mapped *C* to the chilli chromosome 2. With the RFLP marker TG 205 *C* locus was co-segregated and within 1 cM, two additional RFLP markers were also located. The scientists focused on the recessive allele at the *C* locus for getting a diverse germplasm for the breeding programs in chilli around the world. So for marker-assisted selection any of these tightly linked markers may be useful. They developed a PCR-based

CAPS (cleaved amplified polymorphic sequence) marker to demonstrate this point that linked to *C* using the sequence of the *Capsicum* fibrillin gene located 0.4 cM from *C*.

Aluru *et al.* (2003) reported that, production of capsaicinoids in the placenta of chilli fruit is constructed to require the components of the fatty acid synthase (FAS) complex. The three genes for subunits in the complex are *Kas*, *Acl*, and *Fat*. It is then taken out based on differential expression. The transcription of these three genes which are placental-specific and RNA affluence was positively correlated with the level of pungency. The genes *Kas* and *Acl* were mapped to the linkage group 1 and *Fat* to the linkage group 6. None of these genes were linked to the pungency locus, *C*, on linkage group 2. Pungency was positively correlated with the KAS accumulation. By western blots of the placental extracts it was proven that the gathering of this enzyme was correlated with the pungency. Using crude cell extracts, enzyme activity of the recombinant form of the placental-specific KAS was proven. These genes are predicted to be linked with *Capsicum* fruit traits.

Lee *et al.* (2005) reported that the *capsaicinoid synthetase* (CS) gene cosegregated perfectly with the presence of pungency controlling *C* locus from a cross between *C. annuum* lines 'ECW123R' and 'CM334'. They concluded that *CS* and *C* are tightly linked. A deletion of 2,529 bp in the 5' upstream region of *CS* was found in non-pungent chillies after a sequence analysis of the genes of four pungent and four non-pungent chilli lines. They had developed molecular markers of the *C* locus to detect pungency at the seedling stage. They also developed five SCAR markers, two of them being codominant based on the deleted sequence. These SCAR markers will be useful for easy, accurate, and early detection of non-pungent individuals in breeding programs.

Stewart *et al.* (2005) identified that the acylation of an aromatic moiety, vanillylamine, with a branched-chain fatty acid results in the biosynthesis of capsaicin which is restricted to the genus *Capsicum*. Many of the enzymes

identified in the capsaicin production were not well identified and the regulation of the pathway is not completely studied. Based on the present pathway model, genes were identified from the databases and the literatures, and then it is mapped genetically. A published EST, *AT3* which is co-localized with the *Pun1* locus is needed for the presence of capsaicinoids. *AT3*, has been taken out and its nucleotide sequence has been identified. *AT3* showed significant similarity to acyltransferases. In every non-pungent accession studied, the recessive allele in this locus contains a deletion of exon of the predicted coding region and the promoter. The *AT*3 gene transcript and protein expression was developmentally regulated and tissue-specific. A phenotype consistent with *pun1* virus-induced gene silencing of *AT3* resulted in lowering the accumulation of capsaicinoids.

Lang *et al.* (2006) stated that the capsaicinoids in the fruit of *Capsicum* spp. resulted in pungency. From the placenta of the pungent species they isolated two genes characterized as putative *Capsicum* acyl-transferase genes (*Catf-1* and *Catf-2*). The accumulation of the capsaicinoids in pungent variety coincided with the expression pattern of *Catf-1*. This gene was only expressed in placenta of pungent fruits, but not in other organs like leaf, flower, seed and pericarp. With the varied developmental stage of pungent chilli fruits the accumulation of the mRNA of *Catf-1* were also varied. The presence of a sequence deletion at the 5' end of the *Catf-1* coding region in the non-pungent lines was revealed by genomic PCR and Southern blot studies. These results suggest that *Catf-1* is a candidate gene differentiating pungent from non-pungent chillies.

Stewart *et al.* (2006), identified that the production of the capsaicinoids is managed by the *Pun1* locus, which secreates a putative acyltransferase. In its homozygous recessive state, *pun1/pun1*, capsaicinoids will not be synthesized by the chilli fruits. Screening of non-pungent germplasm and genetic complementation tests demonstrated a previously unknown recessive allele of *Pun1*, named $pun1^2$. Sequence analysis of $pun1^2$ identified that a four base pair deletion cause a frameshift mutation and the expected synthesis of a truncated protein. The $pun1^2$ co-segregated absolutetly with the absence of blisters, non-

pungency, and a lower transcript accumulation of different genes present in capsaicinoid biosynthesis was revealed by genetic analysis. The overall results established that blister production needed the *Pun1* allele and that the *pun1*^{2} is a recessive allele from *C. chinense* that cause its non-pungency.

Garces-Claver *et al.* (2007) have designed 3 pairs of primers to study the *Pun1* gene and the *Catf2* partial sequence. Eight pungent *Capsicum* accessions did not amplify the corresponding *Pun1* allele, whereas the fragment corresponding to the *Catf2* sequence was obtained. On this case, an SNP associated with the pungent character was useful to characterize five genotypes as pungent.

Saritnum *et al.* (2008) have investigated the reason for low pungency in otherwise highly pungent *Capsicum frutescens*, using the F_2 and backcross populations of highly pungent and non-pungent lines. The segregation ratio indicated that low pungency was managed by a single recessive gene designated as the *cf* gene. Isolocus test between *cf* locus and *C* locus controlling capsaicinoid production was carried out using cleaved amplified polymorphic sequence (CAPS) marker SCY-800 linked to the *C* locus. CAPS pattern and low pungency were not related, so the *cf* locus is different from the *C* locus.

Variations at a single locus, *Pun1* are the reason for the loss of pungency in *Capsicum annuum* and *Capsicum chinense* (Stellari *et al.*, 2009). From a non-pungent accession of *Capsicum frutescens* an unreported null allele of *Pun1* was identified. The loss of pungency in *C. frutescens* maps to *Pun1* and co-segregates with a molecular marker developed to detect this allele of *Pun1, pun1*^{3}. Loss of pungency is correlated with loss of transcription of *pun1*^{3} . Although this mutation is allelic to *pun1* and *pun1*^{2}, the mutation causing loss of pungency in the undomesticated *Capsicum chacoense, pun2*, is not allelic to the *Pun1* locus as shown by mapping and complementation studies.

A group of non-pungent capsaicinoid analogues are produced in *Capsicum* fruits is called capsinoids. By loss-of-function of *putative aminotransferase* (*p-*

amt) forms of genes variations are occurring in the capsinoid biosynthesis (Tanaka et al., 2010). Three mildly pungent cultivars of *Capsicum chinense* contain high levels of capsaicinoid. This shows that these genotypes have new *p-amt* alleles, which is having variations that differ from those alleles of *C. annuum.* Sequence analysis of *p-amt* in mildly pungent cultivars had shown that a 5bp insertion (TGGGC) causes the frameshift mutation. A transposable element (*Tcc*) was identified in the *p-amt* of mildly pungent cultivars. An active p-AMT will not be produced from the *p-amt* harboring *Tcc*. These less pungent cultivars will provide a new natural source of capsinoids.

Naresh *et al.* (2012) have cloned and sequenced part of AT3 gene, an acyltransferase belonging to the BAHD family of acyltransferases. Abundant InDel and SNP events were identified at AT3 partial sequence. Both AT3-1 and AT3-2 are present in many cultivars that suggest paralogous gene lineages.

In the three developed Capsicum species, three particular alleles of *Pun1* is available (Wyatt *et al.*, 2012). A substantial accumulation of molecular markers for the arrangement of alleles are not distinguished despite the fact that these varieties have been earlierly reported. This has been forestalled with the nearness of atleast one paralogous locus that has a tendency to enhance inside the *Pun* 1. A suite of markers that can separate the four Pun 1 alleles and test them on different board of bean stew lines have additionally been concentrated on.

A DNA sequence with a high similarity to *Pun1* locus possibly related to pungency was sequenced. A 15 bp deletion in non-pungent chilli accessions was identified (Rodríguez-Maza *et al.,* 2012). An allele-specific pair of primers was designed and specific fragments of size 479 bp from non-pungent and 494 bp from pungent accessions were obtained. Polymorphism of this marker, MAP1, was tested in a multiple number of accessions, belonging to different *Capsicum* species, including pungent accessions of *C. chinense*, *C. baccatum*, *C. frutescens*, *C. pubescens*, *C. galapagoense*, *C. eximium*, *C. tovarii*, *C. cardenasii*,

and *C. chacoense* and pungent and non-pungent accessions of *C. annuum* L. All these *Capsicum* accessions were absolutely distinguised.

Chakradhar *et al.* (2013) have investigated a DNA sequence with high similarity to *Pun1/AT3* possibly related to pungency trait. Nucleotide alignment of the obtained sequences and corresponding fragments from the database have revealed a 16 bp deletion in non-pungent *C. annuum* 'Maor'. A multiplex agarose based co-dominant marker Cen1 was designed to detect the identified polymorphism. Different *Capsicum* species were correctly distinguished based on phenotype.

Through expansive transcriptional profiling of the placenta and pericarp of sharp pepper (*C. frutescens* L.) the qualities required in the biosynthesis of capsaicinoids were distinguished utilizing RNA-Seq approach (Liu *et al.,* 2013). The outcomes uncovered that 4,092 qualities were having altogether diverse expression levels between the placenta and pericarp. At the point when contrasted with those of the Pericarp, 2,049 qualities were up-controlled and 2,043 qualities were down-directed in the placenta. Many qualities which are conceivably directing capsaicinoid generation were distinguished from the up-controlled qualities. It was then checked by semi-quantitative RT-PCR and quantitative RT-PCR. Through quality cosmology and KEGG improvement investigation it was recognized that differentially communicated qualities were anticipated to be required in microbody, peroxisome, unsaturated fat synthase movement, CoA-ligase action, acyltransferase action, transaminase action, phenylalanine digestion system, and different procedures.

For the outflow of competitor translation elements placental RNAs from habanero organic product (*Capsicum chinense*) were screened with two applicant qualities with ERF group of interpretation components (Keyhaninejad *et al.*, 2013). A connection of expression with sharpness is exhibited with the portrayal of TFs, ERF and JERF, in nine stew cultivars with particular capsaicinoid substance. Amino corrosive variations were seen in both ERF and JERF from

various bean stew cultivars. Also, none of these progressions included the DNA restricting areas. In non-impactful *Capsium annuum* or *C. chinense* mutants gentle to no translation of Erf was recognized. This connection was described at an individual organic product level in an arrangement of jalapeño (*C. annuum*) lines again with unmistakable and variable capsaicinoid substance.

Utilizing Trinity programming RNA-seq for the blend of placenta and pericarp of impactful pepper (*Capsicum frutescens* L.) has yielded 54,045 excellent transcripts. The outcomes anticipated three new auxiliary qualities in particular DHAD, TD, PAT which filled the holes of the capsaicinoid biosynthetic pathway anticipated by Mazourek (2009). They additionally uncovered that taking into account KEGG (Kyoto Encyclopedia of Genes and Genomes) examination, the new competitor qualities were likewise required in capsaicinoid biosynthesis pathway. Countless and SNP markers were likewise anticipated in *C. frutescens* and *C. annuum* arrangements (Liu *et al.*, 2013).

Using allele specific MAP1 primer, Prasad *et al.* (2013) have amplified the *Pun1* locus of the genomic DNA extracted from zygotic embryos of seven chilly varieties. Markers with 479 and 494 bp, respectively were generated for non-pungent and pungent varieties.

In a differing gathering of *Capsicum annuum* SNPs connected with capsaicin pathway metabolites were distinguished through Association mapping (Reddy *et al.*, 2014). The qualities HCT, CCR, KAS and Pun were sequenced and coordinated with the entire genome arrangement draft of bean stew to distinguish SNP area. At that point the applicant quality affiliation mapping was led with the distinguished SNPs for every quality. Affiliation mapping results uncovered that a noteworthy controller of key metabolites in the capsaicin pathway which for the most part influences the capsaicinoids and antecedents for acyl moieties of capsaicinoids is Pun1. The capsaicin was discovered connected with six unique SNPs in the promoter grouping of Pun1. Their outcomes bolster that the control

point for the flux of p-coumaric corrosive to particular biosynthesis pathways is by CCR quality. KAS was found to manage the real forerunners for acyl moieties of capsaicinoids in this way assumes a key part in capsaicinoid creation.

According to Jun-Park and co-workers (2015), in the exon 7 of the *p-AMT* gene a 12 bp deletion mutation was observed on comparison of DNA coding sequences of pungent and low-pungent genotypes. In addition *p-AMT* gene transcript levels in placental tissue were positively correlated with the level of pungency in low pungent accessions. Low-pungency cultivars, exhibited no significant *p-AMT* transcript levels. On the other hand pungent genotypes displayed high transcript levels of this gene. Therefore they conclude that the loss of pungency in placental tissue is related to the deletion mutation in the *p-AMT* gene.

Sota *et al.* (2015) have recognized that in the phenylpropanoid-interceded capsaicinoid biosynthesis O-methyltransferase (COMT) will take part. An uncommon lessening in capsaicinoid collection will happen as a result of the quieting COMT expression. It was assumed that lost sharpness in Capsicum was brought on by COMT loss-of-capacity change. By cloning COMT1 and COMT2 from the placenta tissue of the sharp cultivar Habanero this speculation was tried. The two COMTs had high similitude with the COMTs of other plant species when it is subjected to the phylogenetic examination and correlation of basic amino-corrosive deposits for protein capacity. The two qualities could be possibility for capsaicinoid biosynthesis. COMT1 as the two COMTS were communicated in placenta tissue and communicated preceding the aggregation of capsaicinoids. A truncated COMT1 transcript was communicated in non-sharp mutant pepper which did not have His-265, and was totally essential for enzymatic action.

2.3 Proteins responsible for pungency in chilli

Capsaicin [(*E*)-*N*-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamide] and dihydrocapsaicin together contributes about more than 90% of the pungency (Cisneros-Pineda *et al.*2006).

According to Lee *et al.* (2006) in a proteome analysis, 37 protein spots were appeared only in pungent tissues but not in non-pungent tissues and it was analyzed by LC-ESI-MS/MS. The differential protein expression was further confirmed by Northern-blot analysis with RNA samples from placental tissues harvested from each chilli fruit at the same sampling intervals. The 22 identified proteins have revealed the levels of proteome and transcriptome form a complex biosynthesis network for capsaicinoids production in chilli.

In pungent and non-pungent chilli fruits by the establishment of cDNA libraries and comparative gene expression studies, Aza-González *et al.* (2011) have identified candidate genes involved in capsaicinoid biosynthesis.

Buildup of vanillylamine and 8-methyl nonenoic acylCoA by capsaicin synthase (*CS*) results in the biosynthesis of capsaicin. The genotype-particular capsaicin levels were corresponded with *CS* action (Prasad *et al.*, 2006a). The *CS* (≈35 kDa) protein was cleaned and described. Through immunolocalization, it was demonstrated that *CS* was particularly restricted to the placental tissues of Capsicum natural products. Western blot examination affirmed that the security change of *CS* levels and capsaicin accumulation during fruit development. They decided the N-terminal amino acid sequence of purified *CS*, cloned the *CS* gene (*csy1*) and sequenced full-length cDNA (981 bp). The deduced amino acid sequence of *CS* from full-length cDNA was 38 kDa. Functionality of *csy1* through heterologous expression in recombinant *Escherichia coli* was also revealed.

Pungency alkaloid, capsaicin of Capsicum natural products, is metabolized by enzymatic buildup of vanillylamine, a phenyl propanoid pathway item, and 8-

methyl-nonenoic acyl CoA, an unsaturated fat subsidiary from leucine/valine pathway by capsaicin synthase. Prasad *et al.* (2006b) reported that biotic elicitors, for example, watery mycelial concentrates of *Rhizopus oligosporus* and *Aspergillus niger* and abiotic lifts like salicylic acid and methyljasmonate, when included to cell-suspension culture of *Capsicum frutescens*, greatest rise of capsaicinoids happens. The phenyl propanoid intermediates were raised to the degree of 3-4 fold though the levels of 8-methyl-nonenoic acylCoA expanded to 6 folds in *R. Oligosporus* mycelial-extricate treated societies. The enzymatic examines of caffeic acid O-methyl transferases, keto acyl synthase and capsaicin synthase uncovered that these chemicals were over communicated in evoked and nonelicited cell-suspension culture. It was additionally since quite a while ago settled by opposite transcriptase polymerase chain response thinks about.

2.4 Environmental impact on pungency

Variety in pungency is specifically identified with the variety in the harm created by contagious pathogen in chilli seeds (Tewksbury *et al.*, 2008). Capsaicin in fruits' placenta need 3 mol of nitrogen for its production (Monforte-González *et al.*, (2010). Hence nitrogen availability may affect chilli pungency through its content in the fruit tissues. Potassium may also affect chilli pungency by given its positive effect on fruit development.

2.5 Development of molecular markers for pungency

In 2003, Blum *et al.* revealed that the difference in the level of pungency in fruit of chilli peppers is because of the quantitative variation in the accumulation of two major capsaicinoids, capsaicin and dihydrocapsaicin. It was analysed in a cross between the non-pungent *Capsicum annuum* parent cv. Maor and a pungent *Capsicum frutescences* parent, accession BG 2816. They screened the bulked DNA from F2 people with RAPD groundworks keeping in mind the end goal to distinguish the quantitative quality loci (QTL) for capsaicinoid content. They affirmed three polymorphic loci between the masses. These RAPD

markers were changed to SCARs and afterward mapped alongside extra RFLP markers to chromosome 7 of chilli organic product. Examination of the QTL interims for each and all out capsaicinoid content distinguished a noteworthy QTL called top which clarified around 34-38% of phenotypic variety for this attribute in two diverse developing situations. They found that for every one of the estimations the allele of the pungent parent give to the expanded level of pungency.

Ben-Chaim *et al.*(2006) uncovered that pungency in Capsicum organic product results from the biosynthesis and aggregation of alkaloid mixes known as capsaicinoids in the hindrances, for example, placental tissues. Pepper cultivars vary as for their level of pungency due to quantitative and subjective variety in capsaicinoid content. Investigation of isolation of three capsaicinoids: capsaicin, dihydrocapsaicin and nordihydrocapsaicin in F3 of between particular cross between a less impactful *Capsicum annuum* "NuMexRNaky" and the wild very sharp C. *frutescens* promotion BG2814-6 was finished. SSR based sub-atomic guide was inherent which Six QTL controlling capsaicinoid substance were recognized on three chromosomes. One quality from the capsaicinoid biosynthetic pathway, BCAT, and one arbitrary natural product EST, 3A2, co-restricted with the distinguished QTL on chromosomes 3 and 4. As a result of one jumbling variable in quantitative determination of capsaicinoid is natural product size, organic product weight estimations were taken in two trials. Two QTL controlling natural product weight were examined, and they didn't co-confine with QTL recognized for capsaicinoid content. The real grant to the phenotypic variety of capsaicinoid substance (24–42% of the aggregate variety) was allocated to a digenic cooperation between a fundamental impact QTL, cap7.1, and a marker present on chromosome 2 that did not majorly affect the quality. A second QTL, cap7.2 was started to compare to the QTL, top, distinguished in a past study as having proclaimed impact on capsaicinoid content.

2.6 SCAR (Sequence Characterized Amplified Region) markers designed for pungency

SCARs are DNA fragments amplified by the PCR using specific 15-30 bp primers, designed from nucleotide sequences established from cloned fragments linked to a gene of interest. By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encounters with RAPDs. Obtaining a codominant marker has been an additional advantage of changing RAPDs into SCARs, although SCARs may reveal dominance when one or both primers partly overlap the site of sequence variation.

Capsaicin and dihydrocapsaicin are the two noteworthy capsaicinoids in charge of the quantitative variety in the gathering of pungency in the products of chilli plants. It was examined in a cross between the non-pungent *Capsicum annuum* parent cv. Maor and an impactful *Capsicum frutescens* parent, increase BG 2816 (Blum *et al.*, 2003). Examination of built segregant was finished with 400 RAPD groundworks. Three polymorphic RAPD markers were changed to SCARs and after that mapped with extra RFLP markers to chromosome 7 of pepper. QTL interim investigation for each and complete capsaicinoid substance were recognized a noteworthy QTL, top, which clarified 34–38 for every penny of the phenotypic variety for this character in two developing situations.

The highly pungent variety "Yodson" (YS), was crossed with the low pungency variety YuYi (YY). The capsaicinoid content, broad-sense heritability (h_b^2), and DNA fingerprint by bulked-segregant analysis with UBC-20 SCAR primer were compared among F_1-hybrid and F_2 populations and their parents. The capsaicinoid contents of the F_1 and F_2 generations were high, similar to the high pungency parent (YS). Meanwhile, the h_b^2 in capsaicinoid content of the crosses was also quite high (~0.69). The SCAR primer for UBC-20 has amplified a common fragment of 1700 bp in both low and high pungency chilli plants of all generations (Techawongstien *et al.*, 2008).

Pungency in chillies is because of the nearness of capsaicinoid alkaloids, which are just delivered in capsicum species. The real quality *pun1* is required for the generation of capsaicinoid substance. Three particular mutant alleles of *pun1* have been found in three trained capsicum species. Despite the fact that these changes have been already reported, a strong gathering of molecular markers for the arrangement of alleles is not accessible. This has been discouraged by the presence of no less than one paralogous locus that has a tendency to intensify with *pun1*. They exhibit a suite of markers that can separate the three *pun1* alleles (wyatt *et al.*, 2012).

Materials and methods

3. Materials and methods

The research work on "Molecular characterization of candidate gene for pungency in *Capsicum* spp." was under taken at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during 2014 – 2016. The objective of the study was to understand the molecular mechanisms behind the different levels of pungency in different species of *Capsicum*. The experimental materials and methodology adopted for characterizing pungency at molecular level are presented in this chapter.

3.1 Materials

3.1.1 Plant materials

Ten capsicum accessions with different level of pungency were employed in the study. The accessions were White Khandari and Vellayani Samrudhi (both belonging to *C. frutescence*), Vellayani Thejus (*C. chinense*), Ujwala, Anugraha, (pungent cultivars of *C. annuum* var.*longum*), Byadagi Dabbi, Byadagi Kaddi(low pungent accessions of *C. annuum* var. *acuminatum*), Kt-Pl-19 (non-pungent paprika *C. annuum*) and Arka Gaurav and Arka Mohini (bell peppers *C. annuum* var. *grossum*). Seeds were collected from respective breeders/centres and pot raised seedlings were used for DNA extraction.

3.1.2 Laboratory chemicals, glassware and equipment

The chemicals used for the study were pure and of good quality obtained from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The Taq polymerase, dNTPs, Taq buffer and DNA ruler (Eco R1, 100 bp ladder) were supplied by Invitrogen. The plasticware were procured from Axygen and Tarson India Ltd. The SCAR and SSR primers were obtained from Sigma Aldrich Pvt. Ltd.

The equipment used for this investigation were high speed refrigerated centrifuge (KUBOTA 6500), NanoDrop ND-1000 spectrophotometer for

estimation of quality and quantity of DNA, horizontal gel electrophoresis system (BIO-RAD, USA) and Proflex thermal cycler for DNA amplification. Details of equipment are provided in Annexure 1.

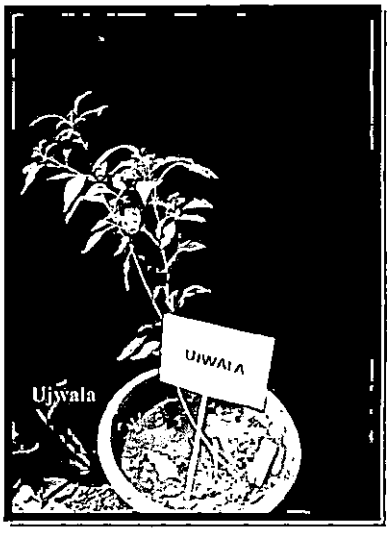
3.2 Methods

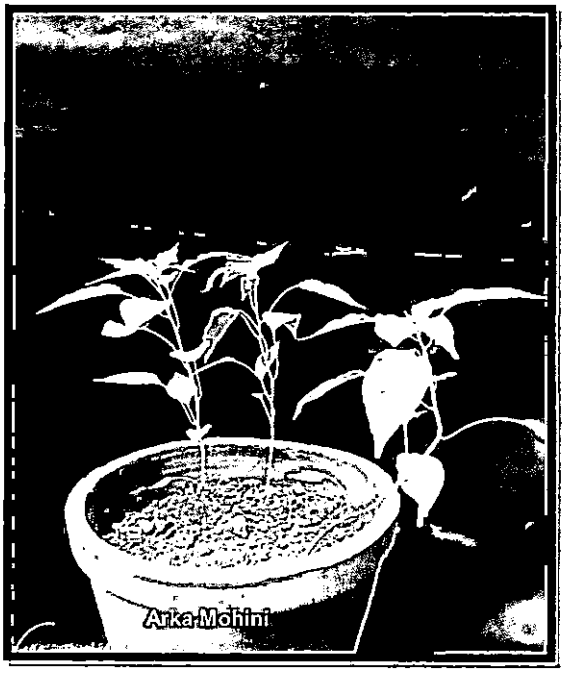
3.2.1 Raising the chilli cultivars

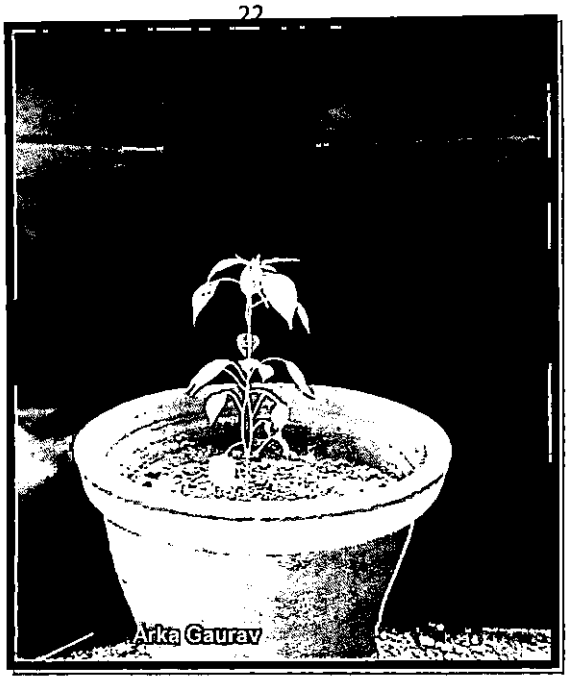
The cultivars used in this study were raised at CPBMB, KAU Vellanikkara. The plants were raised in pots with each plant in each pot, and five plants for each variety. The seed were sown on September 2014 and it was germinated within ten days after sowing. Fourteen days after germination the tender, immature leaves were collected for DNA isolation.

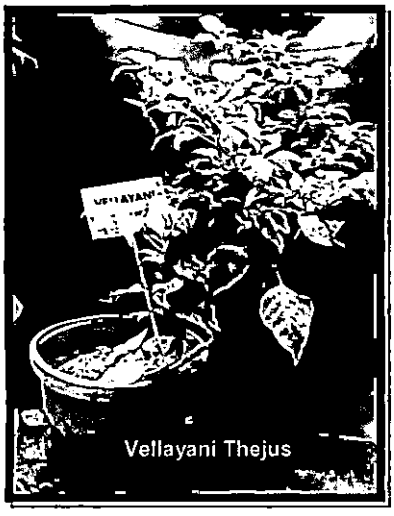

Kt-Pl-19

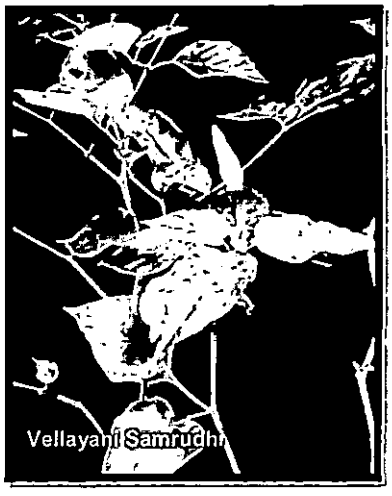

Byadagi Dabbi


Ujjwala


Arka Mohini







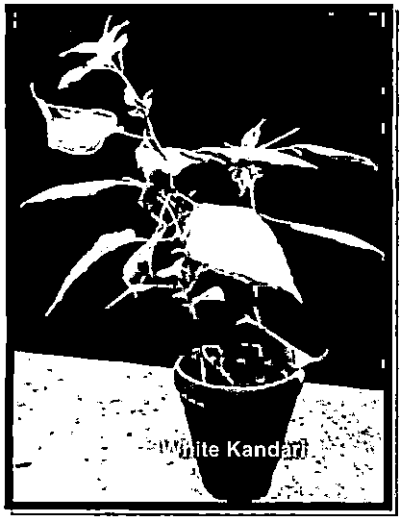


Plate 1. Chilli seedling raised in pots

3.2.2 Analysis of molecular markers for pungency

Molecular marker analysis on ten chilli genotypes was carried out using the SCAR marker system. The SCAR markers previously reported for the genes for pungency were used in the analysis.

Table 1. SCAR markers reported for pungency in chilli

Sl. No	SCARname/ Primers and sequences	Locus	Reference	Remarks
1	CSF1/R2	*CS*	Lee *et.al* (2005)	For all pungent varieties
2	CSF3/R4			
3	BF5/R8			
4	BF7/R9			
5	*Cen1*	*AT3*	Chakradhar *et.al* (2013)	For both pungent and non-pungent lines
6	MAP1		Rodriguez-Maza*et.al* (2012)	

7	*Pun1*[1]*fwd1,fwd2/rev*	*Pun1*	Wyatt *et.al* (2012)	Only for pungent *C. annuum*
8	*Pun1*[2]*fwd/rev*			Only for pungent *C. chinense*
9	*Pun1*[3]*fwd/ rev1,rev2*			Only for pungent *C. frutescens*
10	U16F/U2R	*Catf-1*	Lang *et.al* (2006)	Only in pungent and non-pungent *C. annuum*
11	U3F,1F/D3R,14R,1R			
12	U25F/D25R	*Catf-2*		
13	U22F/D22R			

3.2.2.1 Genomic DNA isolation

Young immature green leaves were collected in ice from individual plants of ten chilli cultivars. Leaves were surface sterilised using sterile distilled water and 70 per cent ethanol and ground to fine powder in liquid nitrogen along with β-

mercaptoethanol and PVP, using sterile ice cold mortar and pestle. DNA extraction was done using CTAB method (Rogers and Bendich, 1994).

Reagents

1. 2X CTAB buffer
2. 10% CTAB buffer
3. TE buffer
4. Chloroform : isoamyl alcohol (24:1v/v)
5. Isopropanol 80 per cent
6. Ethanol 70 per cent and glacial
7. Sterile distilled water

Chemical composition of reagents is provided in Annexure 2.

Procedure

One gram of cleaned leaf tissues were ground in a sterilized pre-chilled mortar and pestle using liquid nitrogen, β-mercaptoethanol (50 µl) and PVP. The ground sample was transferred to an autoclaved 50 ml centrifuge tube containing 3 ml pre-warmed 2X CTAB buffer. The contents were mixed well and incubated for 30 minutes at 65 °C, with occasional mixing by gentle inversions. Equal volume of chloroform: isoamyl alcohol (24:1) was added and again mixed by inversion. Centrifuged the tube with the mixture in a refrigerated centrifuge at 10,000 rpm for 15 minutes at 4°C. After centrifugation the contents in the centrifuge tube got separated into three distinct layers.

Aqueous topmost layer - DNA
Interphase - Fine particles, cell debris and emulsified protein
Lower layer - Chloroform and pigments

The topmost aqueous layer was transferred to a clean centrifuge and added $1/10^{th}$ volume of 10 per cent CTAB solution. Into the mixture equal volume of chloroform: isoamyl alcohol (24:1) was added after that the contents were mixed by gentle inversion. After the centrifugation, transferred the aqueous phase into a clean centrifuge tube and added 0.6 volume of chilled isopropanol. The contents

were mixed by gentle inversion till the DNA gets precipitated. For complete precipitation kept the tube at -20°C for half an hour. Gently poured off the supernatant after centrifugation. Seventy per cent ethanol was added for washing the DNA pellet. The ethanol was discarded after spun for 5 minutes at 8000 rpm. The pellet was air dried and dissolved in 50 µl of autoclaved distilled water and permanently stored at – 20 °C.

3.2.2.2 Purification of DNA

The RNA contamination was removed by treating the sample with RNase and the DNA was further precipitated.

Reagents

1. Chilled isopropanol
2. 70% ethanol
3. TE buffer
4. Chloroform : isoamyl alcohol (24:1, v/v)
5. 1% RNase

One per cent RNase (Sigma, USA) solution was formed by dissolving RNase in autoclaved distilled water. The solution was dispensed into aliquots and stored at -20 °C.

Procedure

RNase solution (2 µl) was added to 100 µl DNA sample and incubates at 37 °C in dry bath for 45 minutes. The contents were mixed gently, after added chloroform: isoamyl alcohol mixture (24:1) of equal volume. The aqueous layer after centrifugation was transferred to a fresh micro centrifuge tube and again added chloroform: isoamyl alcohol mixture (24:1). Again after centrifugation, the aqueous layer was transferred to a fresh micro centrifuge tube and added 0.6 volume of ice cold isopropanol. Mixed the contents by gentle inversion till the DNA got precipitated. Kept at the tube at -20 °C for half an hour for complete precipitation. The supernatant was gently removed and the DNA pellet was

washed with 70 per cent ethanol after centrifugation. DNA pellet was dried in air and dissolved in autoclaved distilled water and stored at -20 °C.

3.2.2.3 Quantification of DNA

The quantity and quality of isolated DNA was evaluated by agarose gel electrophoresis.

Materials used for agarose gel electrophoresis

1. Agarose
2. 50X TAE buffer (pH 8.0)
3. Tracking/loading dye
4. Ethidium bromide
5. Horizontal electrophoresis unit (BioRad), power pack, gel casting tray and comb
6. UV transilluminator
7. Gel documentation and analysis system

Composition of TAE buffer is provided in Annexure 3.

Procedure

The gel casting tray was prepared by wiping with absolute alcohol and sealed the ends with cello tape. Comb was also wiped with absolute alcohol and positioned in gel tray at about 1 inch from one end of the tray and placed it vertically so that the teeth are about 1-2 mm above the surface of the tray. Agarose (0.8g agarose in 100 ml 1X TAE buffer) was prepared in a conical flask. Microwaved for 45 to 60 seconds until the agarose has been completely dissolved and the solution was clear. The solution was kept in the room temperature so that the temperature has reduced to 40 - 45°C. At this stage, Ethidium Bromide was added to the solution at a concentration of 0.5 µg/µl. This warm solution was gently poured into the casting tray to a depth of about 5 mm and let it solidified for 35 – 40 minutes at room temperature. Slowly removed the comb and cello tape used for sealing after the gel gets solidified. Placed the tray in electrophoresis unit

and poured 1X TAE buffer into the unit until the gel is submerged and covered it. The samples were prepared for electrophoresis by adding 1 µl of 6X gel loading dye to 5 µl of DNA solution. Mixed it well and loaded 6 µl DNA per well with EcoR1 double digest (1000 bp) in one lane. Runned the gel at 100 volts until dye was migrated to two third of the gel. Intact DNA was appeared as orange fluorescent band when viewed under UV transilluminator. The degraded DNA appeared as smeared because of the presence of a large number of bands, which differ in one or two base pairs. The gel image was documented and saved in the gel documentation system.

3.2.2.4 Assessing the quality and quantity of DNA by NanoDrop spectrophotometer

The purity of DNA was again analysed using NanoDrop ND-1000 spectrophotometer. Nucleic acid shows maximum absorption at 260 nm whereas protein shows maximum absorption at 280 nm. The absorbance at both the wavelength was recorded and the purity of DNA was indicated by the ratio A_{260}/A_{280}. The OD values between 1.8 and 2.0 indicate pure DNA. The value more than 2.0 indicated RNA contamination and less than 1.8 indicated protein contamination. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} is equivalent to 50 µg/ml double stranded DNA/ml sample.Thus, OD_{260} x 50 gave the quantity of DNA in µg/ml.

Procedure for NanoDrop spectrophotometry

The NanoDrop spectrophotometer was connected to the system and the operating software ND-1000 was opened. The sample arm has been opened and Nucleic acid option was selected then 2 µl distilled water was pipetted onto the lower measurement pedestal. Sampling arm was closed and spectral measurement was initiated using the operating system on the PC. The spectral measurement was made by the sample column by automatically drawn between the upper and lower measurement pedestals. The reading was set to zero with blank sample. Pipetted 1 µl sample onto the measurement pedestal and selected measure. Opened the

sampling arm and wiped the sample from both the upper and lower pedestal using a soft laboratory wipe after the measurement was completed. Wiping prevented sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

3.2.3 Molecular marker used for study

SCAR (Sequence Characterized Amplified Regions) markers reported specifically for the pungency loci in chillies were used in the study "Molecular characterization of candidate gene for pungency in *Capsicum* spp.". Under each marker analysis, all the ten varieties were amplified separately with the selected primers. From the SCAR markers distinct band at the expected size was used for further characterization.

3.2.3.1 SCAR analysis using MAP1 markers for *Pun1* locus

Genomic DNA of good quality (30-40 ng/µl) was isolated from ten chilli genotypes using CTAB method and subjected to SCAR analysis as per the procedure reported by Rodriguez-Maza *et al.*, (2011). SCAR primer *viz* MAP1with good resolving power was used. The amplification was carried out in a Proflex Thermal Cycler. The PCR reactions were performed using 20 µl reaction mixtures.

Composition of the reaction mixture for PCR (20 µl)

Components	Quantity (µl)
1. Genomic DNA (30 ng/µl)	- 2.0
2. 10X Taq assay buffer B	- 2.0
3. $MgCl_2$	- 1.8
4. dNTPs (10 mM/µl)	- 1.8
5. Taq DNA polymerase	- 0.4
6. Primer (10 pM)	- 2.0
7. Autoclaved distilled water	- 10.0

Total - 20.0 µl

A master mix was prepared with all the components for the required number of reactions. The aliquots were then dispensed into the PCR tubes followed by the addition of template DNA in each tube. One tube without template DNA was kept as blank.

The PCR tubes were kept in the thermal cycler and the following programme was run:

Step1:	94°C for 2 minutes	-	Initial denaturation
Step2:	94°C for 30 seconds	-	Denaturation
Step3:	55°C for 1 minute	-	Primer annealing
Step4:	72°C for 2 minutes	-	Primer extension
Step5:	72°C for 2 minutes	-	Final extension
Step6:	4°C for infinity to hold the sample		

The amplified products were run on 2.0 per cent agarose gel using 1X TAE buffer stained with Ethidium bromide along with gene ruler (100 bp ladder). Electrophoresed at 70 volts for two hours, the gel was visualized under UV transilluminator and documented using gel documentation system. The single band which obtained in the gel was eluted carefully using the elution kit and send it to Sci-genome lab for direct sequencing.

3.2. 3.2 SCAR analysis using CS markers for *Capsaicinoid Synthetase* gene

Good quality genomic DNA from ten chilli genotypes were SCAR analysed as per the procedure reported by Jae-Lee *et al.*, (2004). SCAR primers viz.CSF1/CSR2 and BF7/BR9 with good resolving power were used. The amplification was carried out in a Proflex Thermal Cycler.

Composition of the reaction mixture was same as detailed previously.

A master mix was prepared with all the components for the required number of reactions. The aliquots are then dispensed into the PCR tubes followed by the addition of template DNA in each tube. One tube without template DNA was kept as blank.

The PCR tubes were kept in the thermal cycler and the following programme was run:

Step1:	94°C for 5 minutes	-	Initial denaturation
Step2:	94°C for 1minute	-	Denaturation
Step3:	62°C for 1 minute	-	Primer annealing
Step4:	72°C for 2 minutes	-	Primer extension
Step5:	72°C for 10 minutes	-	Final extension
Step6:	4°C for infinity to hold the sample		

For both the primers the PCR program was same.

3.2.3.3 SCAR analysis using *pun1*^{I} markers

Genomic DNA from ten chilli genotypes using CTAB method were subjected to SCAR analysis as per the procedure reported by Wyatt *et al.*, (2012). SCAR primers *viz pun1*^{I}fwd1/ *pun1*^{I}rev with good resolving power was used.

Composition of the reaction mixture was same as detailed previously.

A master mix was prepared with all the components for the required number of reactions. The aliquots are then dispensed into the PCR tubes followed by the addition of template DNA in each tube. One tube without template DNA was kept as blank.

The PCR tubes were kept in the thermal cycler and the following programme was run:

Step1:	94°C for 4 minutes	-	Initial denaturation
Step2:	94°C for 30seconds	-	Denaturation
Step3:	60°C for 1 minute	-	Primer annealing
Step4:	72°C for 2 minutes	-	Primer extension
Step5:	72°C for 10 minutes	-	Final extension
Step6:	4°C for infinity to hold the sample		

3.2.3.4 SCAR analysis using *pun1*[3] markers

Genomic DNA of good quality (30 to 40 ng/µl) was isolated from ten chilli genotypes using CTAB method were subjected to SCAR analysis as per the procedure reported by Wyatt *et al.*, (2012). SCAR primers *viz* *pun1*[3]fwd/ *pun1*[3]rev1with good resolving power was used. It is reported only in the *C. frutescens* species.

Composition of the reaction mixture was same as detailed previously.

A master mix was prepared with all the components for the required number of reactions. The aliquots are then dispensed into the PCR tubes followed by the addition of template DNA in each tube. One tube without template DNA was kept as blank.

The PCR tubes were kept in the thermal cycler and the following programme was run:

Step1:	94°C for 3 minutes	-	Initial denaturation
Step2:	94°C for 30seconds	-	Denaturation
Step3:	55°C for 30seconds	-	Primer annealing
Step4:	72°C for 1.30 minutes	-	Primer extension
Step5:	72°C for 15 minutes	-	Final extension

Step6: 4°C for infinity to hold the sample

The single band which obtained in the gel was eluted carefully using the elution kit and send it to Scigenom lab for direct sequencing.

3.2.4 DNA elution from agarose gel

Components in the elution kit (Company):

1. Miniprep column
2. 2 ml microfuge tube
3. 1.5 ml microfuge tube
4. Buffer DE-A
5. Buffer DE-B
6. Buffer W1
7. Buffer W2 concentrate
8. Eluent

Procedure for extraction of DNA from gel using kit.

Sliced the agarose gel containing the DNA fragment of interest and was excised using a clean, sharp scapel under ultraviolet illumination. The weight of the sliced gel was taken and was regarded as equivalent to the volume. The gel slice was transferred into a 1.5 ml microfuge tube. 3X sample volume of Buffer DE-Awas added and the gel was resuspended in buffer DE-A by vortexing. Heated the tube at 75°C until the gel is completely dissolved; intermittent vortexing was accelerated gel solubilisation. Added 0.5X Buffer DE-A volume of Buffer DE-B mix, if the DNA sample was less than 400 bp supplemented further with a 1X sample volume of isopropanaol. Placed a Miniprep column into a 2 ml microfuge tube (provided). Transferred the solubilized agarose into the column. Discarded the filtrate after centrifugation at 12,000g for 1 minute from the 2 ml microfuge tube. Returned the Miniprep column to the 2 ml microfuge tube and added 500 µl of Buffer W1. Repeat the same and added 700 µl of Buffer W2. Repeat the same and centrifuge at 12,000 g for 1 minute. Discarded the filtrate

from the 2 ml microfuge tube and placed the Miniprep column back into the 2 ml microfuge tube. Transferred the Miniprep column into a clean 1.5 ml microfuge tube after centrifugation. To elute the DNA added 25-30 µl of Eluent to the centre of the membrane and kept it for 1 minute at room temperature. To dissolved the eluted product centrifuge at 12,000 g for 1 minute.

3.2.5 Sequence analysis

After the sequence has been got from Sci-genom Biotech lab, Cochin it was analysed using Blastn and Clustal omega bioinformatics tools.

Results

4. Results

The study on "Molecular characterisation of candidate gene for capsaicin synthesis in *Capsicum* spp." was carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, KeralaAgriculturalUniversity, Vellanikkara during 2014 - 2016. The results of various aspects of the study are described in this chapter.

The research work included the molecular characterization of gene loci for pungency in ten *Capsicum* cultivars in which five highly pungent (Anugraha, Ujwala, Vellayani Thejus, Vellayani Samrudhi and White Khandari), two low pungent (Byadagi Dabbi and Byadagi Kaddi), one non-pungent (Kt-Pl-19) and two (Arka Mohini and Arka Gaurav) were included. SCAR molecular marker system was used in this study to characterize the gene loci for pungency.

4.1 Genomic DNA isolation

4.1.1 Source of DNA

For genomic DNA isolation, young, tender, green leaves were collected from the potted plants of all the cultivars maintained at CPBMB. Collection was made in the early morning hours and brought to the laboratory in ice.

4.1.2 Isolation and purification of DNA

The total genomic DNA was isolated by following the CTAB method (Roger and Bendich, 1994). The RNA contamination was removed by RNAse treatment.

4.1.3 Quantification of DNA

The quality and quantity of isolated DNA was analysed using both electrophoresis and NanoDrop^{R} ND-1000 spectrophotometer. The intact clear bands after electrophoresis have indicated that the isolated DNA is intact and of good quality. The A_{260}/A_{280} absorbance ratio of DNA in NanoDrop

spectrophotometer at 1.8 to 2.0 had indicated that the quality of DNA is good. After appropriate dilution to 40 ng/ µL, the isolated DNA was used as template for SCAR analysis.

Table 2. The quality and quantity of DNA estimated using NanoDrop spectrometer

Genotypes	Absorbance at 260 nm (A_{260})	Absorbance at 280 nm (A_{280})	$A_{260/280}$	Quantity(ng/µl)
Anugraha	86.33	48.77	1.81	4366.98
Ujwala	67.12	32.47	1.84	4876.32
VellayaniThejus	73.49	38.87	1.89	3457.32
VellayaniSamrudhi	82.76	47.65	1.79	2986.45
White Khandari	51.78	24.54	1.82	4623.98
Kt-Pl-19	63.12	30.32	1.84	3126.27
ArkaMohini	76.87	41.61	1.84	2134.76
ArkaGaurav	91.43	60.11	1.85	3198.23
ByadagiDabbi	87.41	49.03	1.78	2213.71
ByadagiKaddi	68.69	33.77	1.83	2098.11

4.2 Molecular characterization

SCAR marker system was used for molecular characterisation. SCAR analysis was done with the reported primers specific for the various genic loci responsible for pungency in different species of *Capsicum* (Table 1). Five primer sets *viz.*, MAPIF/R (Rodriguez-Maza *et.al.,* 2012), CSF1/R2 (Lee *et.al.,* 2005), BF7/BR9 (Lee *et.al.,* 2005), *Pun1*^{1}F1/R (Wyatt *et.al.,* 2012) and *Pun1*^{3} F/R1 (Wyatt *et.al.*, 2012) were selected for SCAR assay.

Table 3. List of SCAR primers used for characterizing chilly genotypes

Sl. No.	Primer	Sequence	Annealing temp. (°C)
1	MAP1 F	5'CCATTAGTCGTTCATTTTTGTTTG3'	55
	MAP1 R	5'TCTGCCCTTGTTGGATTTTC3'	
2	CSF1	5'ATGGCTTTTGCATTACCATCA3'	57
	CSR2	5'CCTTCACAATTATTCGCCCA3'	
3	BF7	5'GGGGTTGGGTAGAGGTTGTT3'	57
	BR9	5'GACAAACAATAATGGACGATG3'	
4	*Pun1*[1]F1	5'TCCTCATGCATCTCTTGCAG3'	60
	Pun1[1]R	5'CAAATGGCAGTTTCCCTTCTCTCATT3'	
5	*Pun1*[3] F	5'GTAGTTTTTCGGAAATGAAAAGTACT3'	55
	Pun1[3]R1	5'CACGCCTTGCCCAGCTTTGTAATCTT3'	

4.3 Identification of the genes for pungency

The objective of this study was to characterize the candidate gene/s for pungency in *Capsicum* spp. The study has targeted the candidate loci for pungency such as *Capsaicinoid Synthetase*, *Pun1*and *Pun3* loci, *AT3* locus, and *Catf*locus.

4.3.1 *Capsaicinoid Synthetase* (*CS*) gene

Capsaicinoid Synthetase (*CS*) gene in pungent chilli could be amplified using the primers CSF1/CSR2 and BF7/BR9 (Lee *et al.*, 2005). CSF1/CSR2 has generated a clear, distinct band of 434 bp in all the five pungent varieties and this band was absent in less pungent and non-pungent lines (Plate 2).

The primer combination BF7/BR9 has produced a clear, distinct amplicon of size 900 bp in all the five pungent lines and the corresponding banding was

missing in all other (Plate 3). It was clear that the (*CS*) gene is mandatory for high pungency in *Capsicum*. Further, there should be other genes which are contributing towards the mild pungency.

Plate 2. The amplification of *CS* gene locus using the CSF1/CSR2 primer combination

M- Marker (100 bp), K- Kt-Pl-19, A- Anugraha, Am- Arka Mohini, U- Ujwala, Ag- Arka Gaurav, T- Vellayani Thejus, Bd- Byadagi Dabbi, S- Vellayani Samrudhi, Bk- Byadagi Kaddi, W- White Kandari

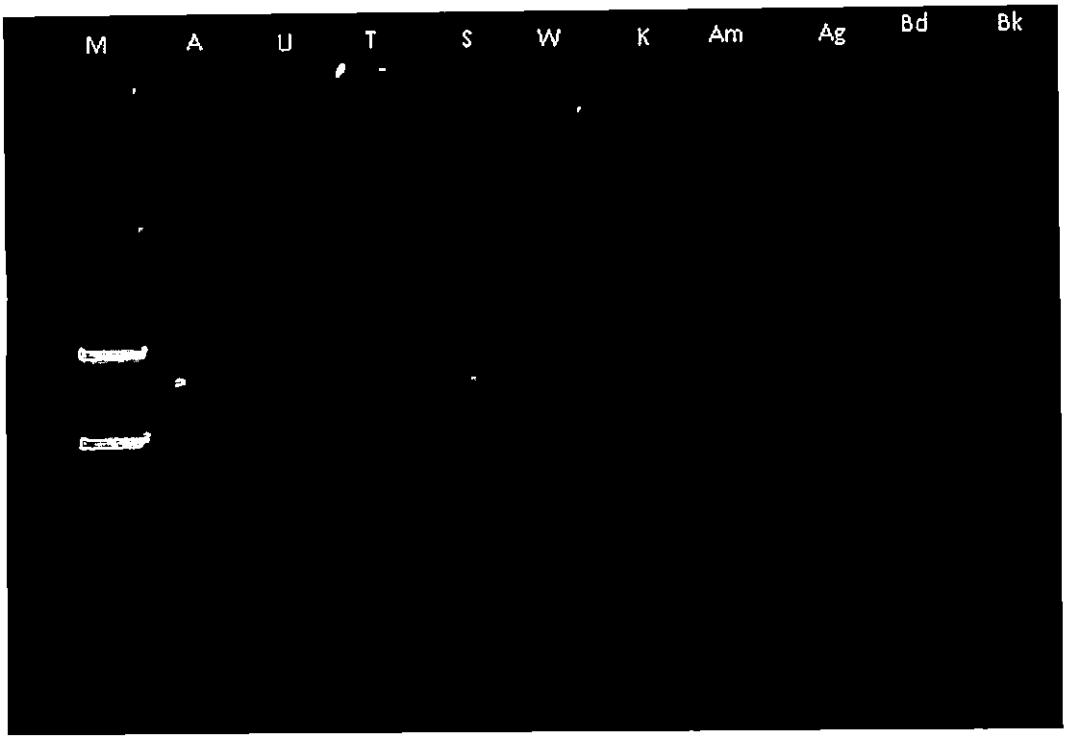


Plate 3. The amplification pattern of *CS* gene in chilli using the BF7/BR9 primer combination

M- Marker (100 bp), K- Kt-Pl-19, A- Anugraha, Am- Arka Mohini, U- Ujwala, Ag- Arka Gaurav, T- Vellayani Thejus, Bd- Byadagi Dabbi, S- Vellayani Samrudhi, Bk- Byadagi Kaddi, W- White Kandari

4.3.2 *Pun1*^{1} allele of *Pun1* locus

Pun1 is reported to be a major gene involved in the production of pungency principles in chilli. In different species of *Capsicum*, different loci of Pun1 are reported to act. In *C. annuum*, *Pun1*^{1} allele of *Pun1* is responsible for pungency, whereas, in *C. frutescens*, *Pun1*^{3} allele is acting. In this investigation, a primer set was analysed to understand the role of *Pun1*^{1}locus on pungency. The primer combination was *Pun1*^{1}F1/R (Wyatt *et al.*, 2012).

Pun1^{1}F1/R was *Capsicum annuum* specific *Pun1*^{1}locus primer set. The primer had given clear, intact single band of 850 bp only in *C. annuum* varieties (Anugraha and Ujwala) (Plate 4). In less-pungent and non-pungent *C. annuum* cultivars also this marker was absent.

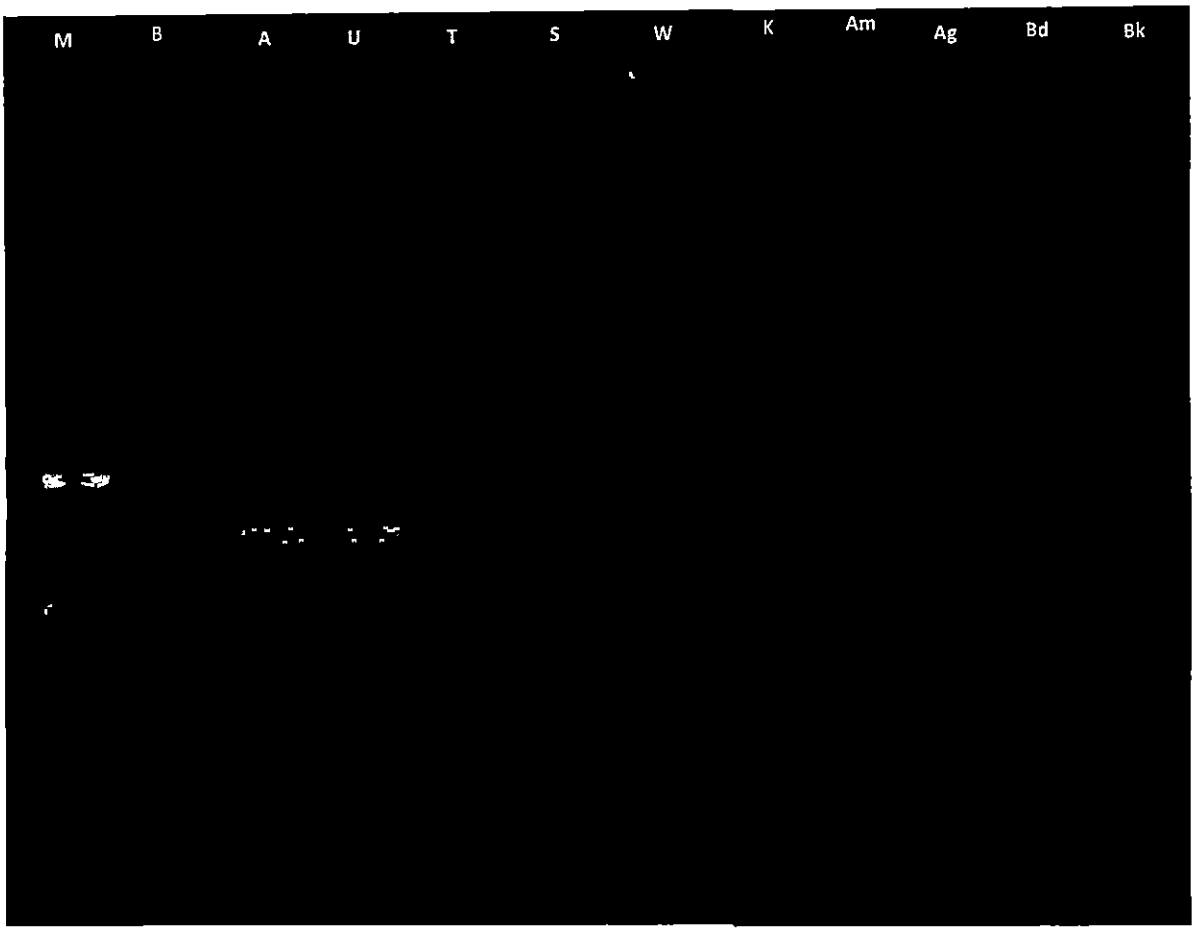


Plate 4. Amplification of *Pun1*^{I} in *C. annuum* cultivars using the *Pun1*^{I}F1/R primer set

M- Marker (100 bp), B- Blank, K- Kt-Pl-19, A- Anugraha, Am- Arka Mohini, U- Ujwala, Ag- Arka Gaurav, T- Vellayani Thejus, Bd- Byadagi Dabbi, S- Vellayani Samrudhi, Bk- Byadagi Kaddi, W- White Kandari

4.3.3 *Pun1*^{3} allele of *Pun1* locus

Pun1^{3}F/R1 primers were used to amplify the *Pun1*^{3}allele in *Capsicum* cultivars. This primer had given 1000 bp single, intact band in cultivars belonging to *C. frutescens* (White Kandari and Vellayani Samrudhi) (Plate 5). From these results it is clear that the *Pun1* locus in *Capsicum* carries different alleles in different species. The variations in degree of pungency among cultivars of *C. annuum* and *C. frutescens* may be due to the variation in the amino acids coded by the alleles of *Pun1* locus.

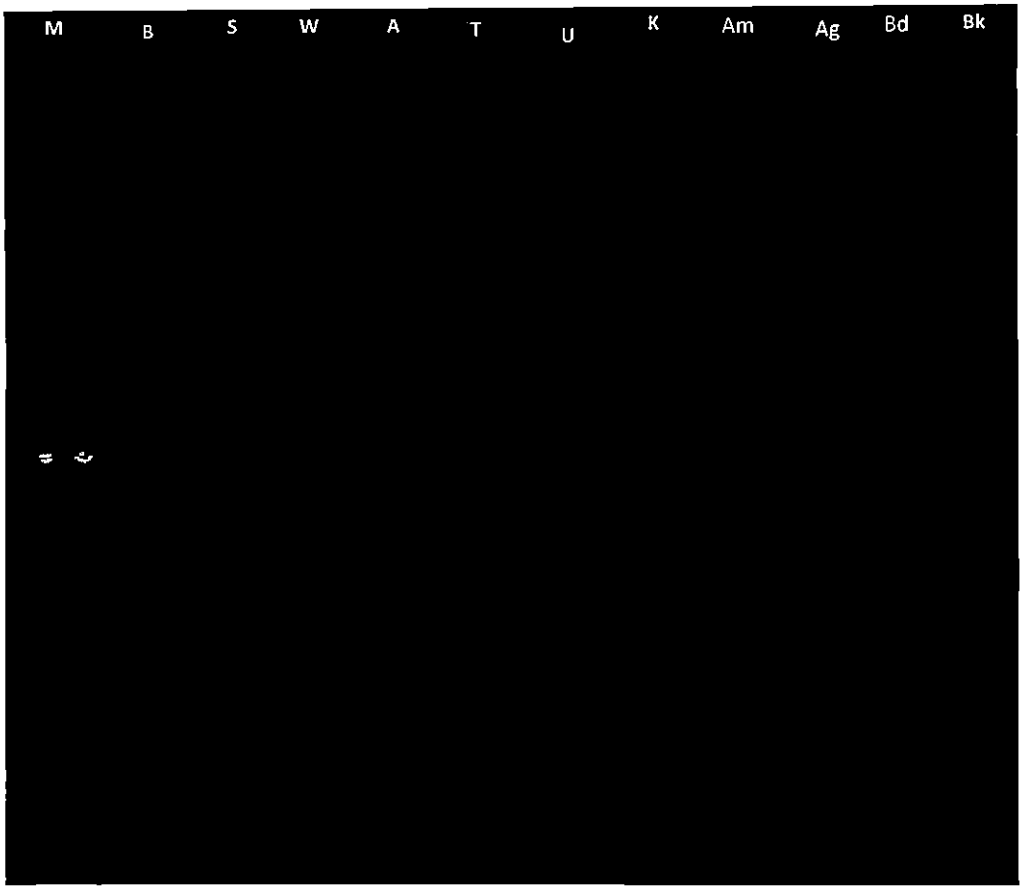


Plate 5. Amplification of *Pun1*^{3} allele of *Pun1* locus in cultivars belonging to *C.frutescens*using*Pun1*^{3}F/R1 primer set

M- Marker (100 bp), B- Blank, K- Kt-Pl-19, A- Anugraha, Am- Arka Mohini, U- Ujwala, Ag- Arka Gaurav, T- Vellayani Thejus, Bd- Byadagi Dabbi, S- Vellayani Samrudhi, Bk- Byadagi Kaddi, W- White Kandari

4.3.4 MAP1 primer for amplifying *Pun1* locus

MAP1F/MAP1R has given clear, single, intact band in all the ten chilli varieties but all the five pungent varieties were having higher size of 494 bp compared to the bands in the non-pungent ones 479 bp (Plate 6). These results had shown that there is deletion of 15 base pairs in the less pungent and non-pungent cultivars in comparison with the pungent cultivars.

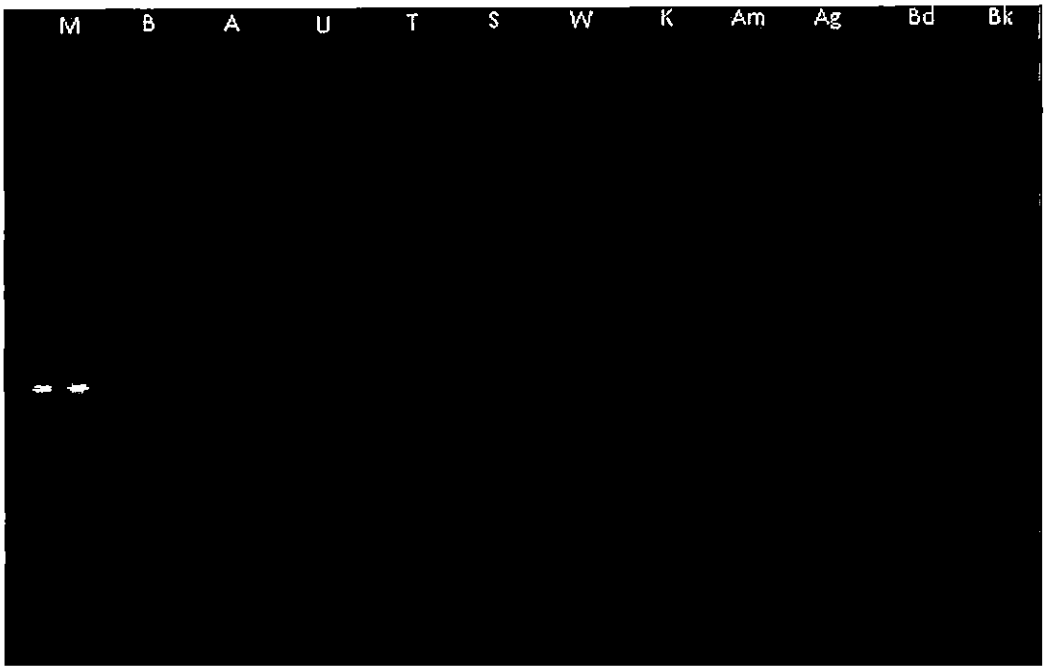


Plate 6. The amplification of *Pun1* locus in different chilli cultivars using MAP1F/R primer combination

M- Marker (100 bp), B- Blank, K- Kt-Pl-19, A- Anugraha, Am- Arka Mohini, U- Ujwala, Ag- Arka Gaurav, T- Vellayani Thejus, Bd- Byadagi Dabbi, S- Vellayani Samrudhi, Bk- Byadagi Kaddi, W- White Kandari

Table 4. Details of amplicon specific to different chilli genotypes in SCAR assay

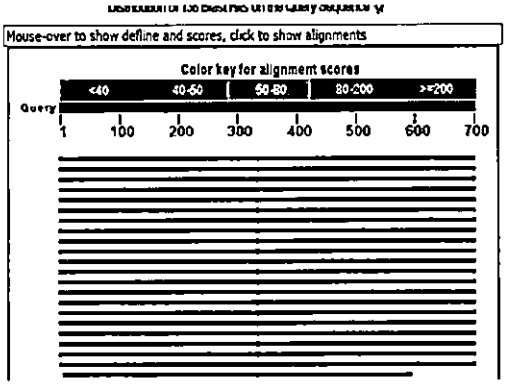
Genes	Primers	Pungent varieties					Non-pungent varieties					Reported Band size
		Anugraha	Ujwala	Vellayani Thejus	Vellayani Samrudhi	White Kandari	Kt-Pl-19	Arka Mohini	Arka Gaurav	Byadagi Dabbi	Byadagi Kaddi	
Capsaicinoid synthetase gene	CSF1 CSR2	+ (430 bp)	+ (430bp)	+ (430 bp)	+ (430 bp)	+ (430 bp)	-	-	-	-	-	434 bp
	BF7 BR9	+ (900 bp)	+ (900 bp)	+ (900 bp)	+ (900 bp)	+ (900 bp)	-	-	-	-	-	896 bp
Pun1 gene	*Pun1*^{1} fwd1 *Pun1*^{1} rev	+ (850 bp)	+ (850 bp)	-	-	-	-	-	-	-	-	850 bp
	Pun1^{3} fwd *Pun1*^{3} rev1	-	-	-	+ (1000bp)	+(1000bp)	-	-	-	-	-	1000 bp
	MAPF1 MAPR1	+ (494 bp)	+ (494 bp)	+(494b)	+ (494 bp)	+(494 bp)	+(479b)	+(479b)	+(479b)	+(479b)	+(479b)	479-494 bp

4.4 Molecular characterisation of the loci for pungency

The markers representing the various pungency loci in pungent, less pungent and non-pungent cultivars were eluted and sequenced to verify the loci and to understand the basis of different level of pungency.

4.4.1 Characterisation *Cs* gene using the marker from BF7/BR9 primers

The sequences were analysed using BLASTn and Clustal Omega bioinformatics tools. The BLASTn had shown that the sequences from all the pungent cultivars are 99 per cent identical to the *Pun1* locus (Plate 7).



Sequences producing significant alignments:

Select All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Capsicum frutescens cultivar shunla acyltransferase (Pun1) gene, Pun1-1 allele, complete cds	660	1276	100%	0.0	99%	HM554983.1
Nicotiana benthamiana acyltransferase (AT3-1) pseudogene, partial sequence	660	1270	100%	0.0	99%	EF155171.1
Solanum pennellii acyltransferase (AT3-1) gene, partial cds	660	1270	100%	0.0	99%	EF155155.1
Solanum lycopersicum cultivar Alicia Craig acyltransferase (AT3-1) gene, partial cds	660	1270	100%	0.0	99%	EF155156.1
Capsicum chinense cultivar NMCA30036 nonfunctional acyltransferase (Pun1) gene, Pun1-2 allele, complete sequence	660	1270	100%	0.0	99%	EF104913.1
Capsicum chinense cultivar habanero acyltransferase (Pun1) gene, complete cds	660	1276	100%	0.0	99%	AY819067.1

Plate 7.BLASTn of the Cs gene sequences showing 99 per cent identity with *Pun1/AT3* loci

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A       GGGGTTGGGTAGAGGTTGTTTAGCCAGTAACTCTATTATTTCATTTCCTTTTGTCTATAT
U       GGGGTTGGGTAGAGGTTGTTTAGCCAGTAACTCTATTATTTCATTTCCTTTTGTCTATAT
T       GGGGTTGGGTAGAGGTTGTTTAGCCAGTAACTCTATTATTTCATTTCCTTTTGTCTATAT
S       GGGGTTGGGTAGAGGTTGTTTAGCCAGTAACTCTATTATTTCATTTCCTTTTGTCTATAT
W       GGGGTTGGGTAGAGGTTGTTTAGCCAGTAACTCTATTATTTCATTTCCTTTTGTCTATAT

A       AAGTGTATCCATATATGCAAGAAAATGTCAACCGGCCAGCAG-ATATAATTTATTTGTTA
U       AAGTGTATCCATATATGCAAGAAAATGTCAACCGGCCAGCAG-ATATAATTTATTTGTTA
T       AAGTGTATCCATATATGCAAGAAAATGTCAACCGGCCAGCAG-ATATAATTTATTTGTTA
S       AAGTGTATCCATATATGCAAGAAAATGTCAACCGGCCAGCAG-ATATAATTTATTTGTTA
W       AAGTGTATCCATATATGCAAGAAAATGTCAACCGGCCAGCAG-ATATAATTTATTTGTTA

A       AATTAATTATGGCTTTTGCATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTA
U       AATTAATTATGGCTTTTGCATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTA
T       AATTAATTATGGCTTTTGCATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTA
S       AATTAATTATGGCTTTTGCATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTA
W       AATTAATTATGGCTTTTGCATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTA

A       TCAAACCTTCCTCTCTCACCCCCTCTAAACAAAGATTTCACAAGCTATCTTTCATCGATC
U       TCAAACCTTCCTCTCTCACCCCCTCTAAACAAAGATTTCACAAGCTATCTTTCATCGATC
T       TCAAACCTTCCTCTCTCACCCCCTCTAAACAAAGATTTCACAAGCTATCTTTCATCGATC
S       TCAAACCTTCCTCTCTCACCCCCTCTAAACAAAGATTTCACAAGCTATCTTTCATCGATC
W       TCAAACCTTCCTCTCTCACCCCCTCTAAACAAAGATTTCACAAGCTATCTTTCATCGATC

A       AATCTTTAA--AATATGTATATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGAC
U       AATCTTTAA--AATATGTATATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGAC
T       AATCTTTAA--AATATGTATATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGAC
S       AATCTTTAA--AATATGTATATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGAC
W       AATCTTTAA--AATATGTATATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGAC

A       TAGAAGACTCCAAAAATTCTGATGAGCTTTCCCATATAGCCCACTTGCTGCAAACATCTC
U       TAGAAGACTCCAAAAATTCTGATGAGCTTTCCCATATAGCCCACTTGCTGCAAACATCTC
T       TAGAAGACTCCAAAAATTCTGATGAGCTTTCCCATATAGCCCACTTGCTGCAAACATCTC
S       TAGAAGACTCCAAAAATTCTGATGAGCTTTCCCATATAGCCCACTTGCTGCAAACATCTC
W       TAGAAGACTCCAAAAATTCTGATGAGCTTTCCCATATAGCCCACTTGCTGCAAACATCTC

A       TATCACAAACTCTAGTCTCTTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTG
U       TATCACAAACTCTAGTCTCTTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTG
T       TATCACAAACTCTAGTCTCTTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTG
S       TATCACAAACTCTAGTCTCTTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTG
W       TATCACAAACTCTAGTCTCTTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTG

A       TTGACTGTAACGATATGGGAGCTGAGTTCTTGAGTGTTCGAATAAAATGTTCCATGTCTG
U       TTGACTGTAACGATATGGGAGCTGAGTTCTTGAGTGTTCGAATAAAATGTTCCATGTCTG
T       TTGACTGTAACGATATGGGAGCTGAGTTCTTGAGTGTTCGAATAAAATGTTCCATGTCTG
S       TTGACTGTAACGATATGGGAGCTGAGTTCTTGAGTGTTCGAATAAAATGTTCCATGTCTG
W       TTGACTGTAACGATATGGGAGCTGAGTTCTTGAGTGTTCGAATAAAATGTTCCATGTCTG

A       AAATTCTTGATCATCCTCGTGCTGCTCATGCAGAGGGCATAGTTTTTCCCAAGGATTTGC
U       AAATTCTTGATCATCCTCGTGCTGCTCATGCAGAGGGCATAGTTTTTCCCAAGGATTTGC
T       AAATTCTTGATCATCCTCGTGCTGCTCATGCAGAGGGCATAGTTTTTCCCAAGGATTTGC
S       AAATTCTTGATCATCCTCGTGCTGCTCATGCAGAGGGCATAGTTTTTCCCAAGGATTTGC
W       AAATTCTTGATCATCCTCGTGCTGCTCATGCAGAGGGCATAGTTTTTCCCAAGGATTTGC
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A       C--GGGCAAATAATTATGAAGGTGGTAATTTGCTTGTAGTTCAAGTAAGTAAGTTTGATT
U       C--GGGCAAATAATTATGAAGGTGGTAATTTGCTTGTAGTTCAAGTAAGTAAGTTTGATT
T       C--GGGCAAATAATTATGAAGGTGGTAATTTGCTTGTAGTTCAAGTAAGTAAGTTTGATT
S       C--GGGCAAATAATTATGAAGGTGGTAATTTGCTTGTAGTTCAAGTAAGTAAGTTTGATT
W       C--GGGCAAATAATTATGAAGGTGGTAATTTGCTTGTAGTTCAAGTAAGTAAGTTTGATT

A       GTGGAGGAATAGCCATTAGTATATGCTTTTCACACAACAGATCGGTGATGGTTGCTCTCT
U       GTGGAGGAATAGCCATTAGTATATGCTTTTCACACAACAGATCGGTGATGGTTGCTCTCT
T       GTGG-GGAATAGCCATTAGTATATGCTTTTCACACAACAGATCGGTGATGGTTGCTCTCT
S       GTGGAGGAATAGCCATTAGTATATGCTTTTCACACAACAGATCGGTGATGGTTGCTCTCT
W       GTGGAGGAATAGCCATTAGTATATGCTTTTCACACAACAGATCGGTGATGGTTGCTCTCT

A       ACTTAATTTCCTTAATGATTGGTCTAGCGTTACTCGTGATCATATGACGACA-CGACTCT
U       ACTTAATTTCCTTAATGATTGGTCTAGCGTTACTCGTGATCATATGACGACA-CGACTCT
T       ACTTAATTTCCTTAATGATTGGTCTAGCGTTACTCGTGATCATATGACGACAACGACTCT
S       ACTTAATTTCCTTAATGATTGGTCTAGCGTTACTCGTGATCATATGACGACAACGACTCT
W       ACTTAATTTCCTTAATGATTGGTCTAGCGTTACTCGTGATCATATGACGACAACGACTCT

A       AATTCCTTCTCCTAGGTTTATAGGAGATTCAATTTTCTCTACACAAAAATATGGCCCTCT
U       AATTCCTTCTCCTAGGTTTATAGGAGATTCAATTTTCTCTACACAAAAATATGGCCCTCT
T       AATTCCTTCTCCTAGGTTTATAGGAGATTCAATTTTCTCTACACAAAAATATGGCCCTCT
S       AATTCCTTCTCCTAGGTTTATAGGAGATTCAATTTTCTCTACACAAAAATATGGCCCTCT
W       AATTCCTTCTCCTAGGTTTATAGGAGATTCAATTTTCTCTACACAAAAATATGGCCCTCT

A       TATTACGCCACAAATAGTGTCCGATCTTAGTGAATGCGTACAGAAAAG-TTCATTTTTCC
U       TATTACGCCACAAATAGTGTCCGATCTTAGTGAATGCGTACAGAAAAG-TTCATTTTTCC
T       TATTACGCCACAAATAGTGTCCGATCTTAGTGAATGCGTACAGAAAAG-TTCATTTTTCC
S       TATTACGCCACAAATAGTGTCCGATCTTAGTGAATGCGTACAGAAAAG-TTCATTTTTCC
W       TATTACGCCACAAATAGTGTCCGATCTTAGTGAATGCGTACAGAAAAG-TTCATTTTTCC

A       TACAGACAAGTTAGATGCACTTCGAGCTAAGGTAAGACTACCATCACTCCATTATTGTCA
U       TACAGACAAGTTAGATGCACTTCGAGCTAAGGTAAGACTACCATCACTCCATTATTGTCA
T       TACAGACAAGTTAGATGCACTTCGAGCTAAGGTAAGACTACCATCACTCCATTATTGTCA
S       TACAGACAAGTTAGATGCACTTCGAGCTAAGGTAAGACTACCATCACTCCATTATTGTCA
W       TACAGACAAGTTAGATGCACTTCGAGCTAAGGTAAGACTACCATCACTCCATTATTGTCA

A       GTCTTTT-AGATTTTCTTTTGCAATACATTTTTGAAATATAATAATCCACCAAATTAAAT
U       GTCTTTT-AGATTTTCTTTTGCAATACATTTTTGAAATATAATAATCCACCAAATTAAAT
T       GTCTTTTAAGATTTTCTTTTGCAATACATTTTTGAAATATAATAATCCACCAAATTAAAT
S       GTCTTTTAAGATTTTCTTTTGCAATACATTTTTGAAATATAATAATCCACCAAATTAAAT
W       GTCTTTTAAGATTTTCTTTTGCAATACATTTTTGAAATATAATAATCCACCAAATTAAAT

A       TTGTATGGAAGAATGGTTTGATTTGACACTGGGTTTTAGATAGAACGGAAGTTTTTTAGG
U       TTGTATGGAAGAATGGTTTGATTTGACACTGGGTTTTAGATAGAACGGAAGTTTTTTAGG
T       TTGTATGGAAGAATGGTTTGATTTGACACTGGGTTTTAGATAGAACGGAAGTTTTTTAGG
S       TTGTATGGAAGAATGGTTTGATTTGACACTGGGTTTTAGATAGAACGGAAGTTTTTTAGG
W       TTGTATGGAAGAATGGTTTGATTTGACACTGGGTTTTAGATAGAACGGAAGTTTTTTAGG

A       ATTCATGCGTTGCCTTAAAT-TGCGTAAGATTTTAATTTGTCTTCCGGTATTTTTGGTAT
U       ATTCATGCGTTGCCTTAAAT-TGCGTAAGATTTTAATTTGTCTTCCGGTATTTTTGGTAT
T       ATTCATGCGTTGCCTTAAAT-TGCGTAAGATTTTAATTTGTCTTCCGGTATTTTTGGTAT
S       ATTCATGCGTTGCCTTAAAT-TGCGTAAGATTTTAATTTGTCTTCCGGTATTTTTGGTAT
W       ATTCATGCGTTGCCTTAAAT-TGCGTAAGATTTTAATTTGTCTTCCGGTATTTTTGGTAT
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A       TAACACTACCATTAGTCGTTCATTTTTGTTTGTC
U       TAACACTACCATTAGTCGTTCATTTTTGTTTGTC
T       TAACACTACCATTAGTCGTTCATTTTTGTTTGTC
S       TAACACTACCATTAGTCGTTCATTTTTGTTTGTC
W       TAACACTACCATTAGTCGTTCATTTTTGTTTGTC
```

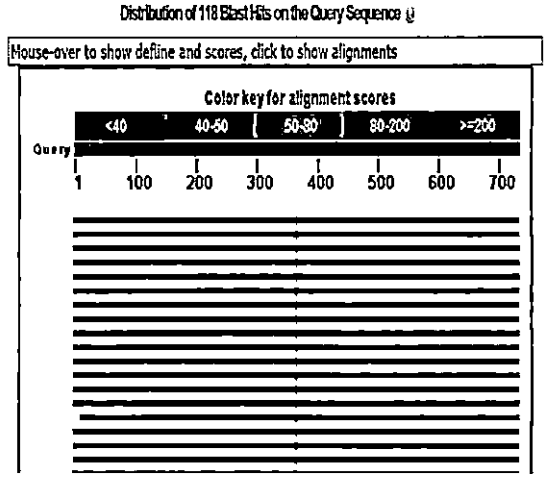
Plate 8. Sequence alignment of pungent varieties compare with sequence of *Pun1*

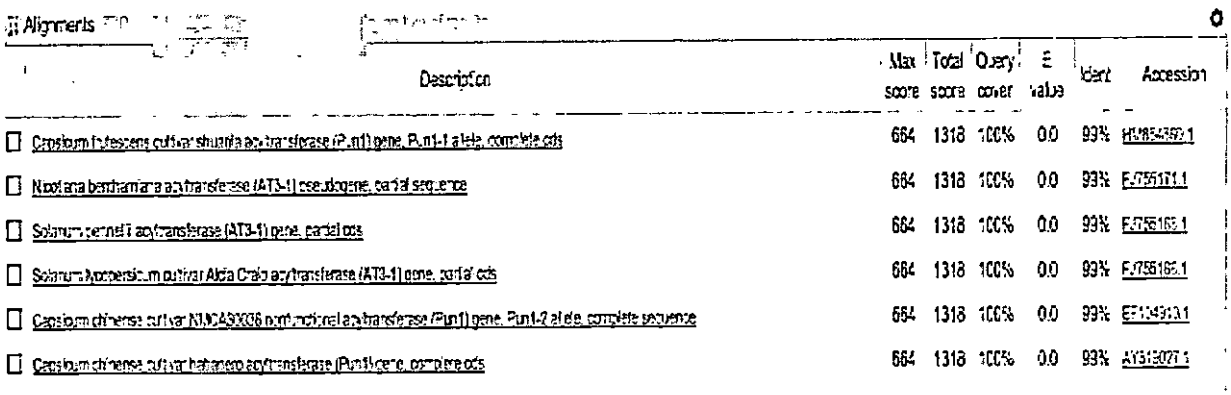
S- Vellayani Samrudhi, W- White Kandari, U- Ujwala, A- Anugraha, T- Vellayani Thejus

Thus it is shown that *Cs* gene is a part of *Pun1* locus and it was present at 1850 bp from 5' end of the *Pun1* locus.

4.4.2 Characterization of *Cs* using the marker derived from CSF1/CSR2 primer set

The BLASTn had shown that *Cs* is 99 per cent similar to *Pun1/AT3* locus (Plate 9).





Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Capsicum frutescens cultivar shumla acyltransferase (Pun1) gene, Pun1-1 allele, complete cds	664	1318	100%	0.0	99%	HM854397.1
Nicotiana benthamiana acyltransferase (AT3-1) pseudogene, partial sequence	664	1318	100%	0.0	99%	FJ755171.1
Solanum pennellii acyltransferase (AT3-1) gene, partial cds	664	1318	100%	0.0	99%	FJ755165.1
Solanum lycopersicum cultivar Alisa Craig acyltransferase (AT3-1) gene, partial cds	664	1318	100%	0.0	99%	FJ755166.1
Capsicum chinense cultivar NMCA59006 putative acyltransferase (Pun1) gene, Pun1-2 allele, complete sequence	664	1318	100%	0.0	99%	EF104913.1
Capsicum chinense cultivar habanero acyltransferase (Pun1) gene, complete cds	664	1318	100%	0.0	99%	AY519077.1

Plate 9.BLASTn of *Cs* shows 99 per cent identity with *Pun1/AT3* locus

Only the pungent cultivars have yielded the marker for *CS*. Alignment of these sequences with *Pun1* locus had shown that *CS* is located within the *Pun1* locus.

Plate 10 shows that there is only a small difference between the sequences of the cultivars belonging to different species. Vellayani Thejus belongs to the species *C.chinense*. This cultivar had shown a characteristic 10 bp loss for *CS* gene. In the cultivars belonging to *C. frutescence* (Vellayani Samrudhi and White Kandari) also, the characteristic deletion was observed. Thus to develop the specific *Cs* markers for these species these regions could be targeted in primer designing.

```
U          -ATGGCTTTTGC
A          -ATGGCTTTTGC
T          CATGGCTTTTGC
S          --TGGCTTTTGC
W          --TGGCTTTTGC


U          ATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTATTAAACCTTCCTCTCTCAC
A          ATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTATTAAACCTTCCTCTCTCAC
T          ATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTATTAAACCTTCCTCTCTCAC
S          ATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTATTAAACCTTCCTCTCTCAC
W          ATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTATTAAACCTTCCTCTCTCAC


U          CCCCTCTACACTTAGATTTCACAAGCTATCTTTCATCGATCAATCTTTAAGTAATATGTA
A          CCCCTCTACACTTAGATTTCACAAGCTATCTTTCATCGATCAATCTTTAAGTAATATGTA
T          CCCCTCTACACTTA-ATTTCACAAGCTATCTTTCATCGATCAATCTTTAAGTAATATGTA
S          CCCCTCTACACTTAGATTTCACAAGCTATCTTTCATCGATCAATCTTTAAGTAATATGTA
W          CCCCTCTACACTTAGATTTCACAAGCTATCTTTCATCGATCAATCTTTAAGTAATATGTA
```

173733

```
U       TATCCCTTGTGC-TTTTTTTACCCTAAAGTACAACAAAGACTAGAAGACTCCAAAAATTC
A       TATCCCTTGTGC-TTTTTTTACCCTAAAGTACAACAAAGACTAGAAGACTCCAAAAATTC
T       TATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGACTAGAAGACTCCAAAAATTC
S       TATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGACTAGAAGACTCCAAAAATTC
W       TATCCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGACTAGAAGACTCCAAAAATTC

U       TGATGAGCTTTCCCATATAGCCCACTTGCTACAAACATCTCTATCACAAACTCTAGTCTC
A       TGATGAGCTTTCCCATATAGCCCACTTGCTACAAACATCTCTATCACAAACTCTAGTCTC
T       TGATGAGCTTTCCCATATAGCCCACTTGCTACAAACATCTCTATCACAAACTCTAGTCTC
S       TGATGAGCTTTCCCATATAGCCCACTTGCTACAAACATCTCTATCACAAACTCTAGTCTC
W       TGATGAGCTTTCCCATATAGCCCACTTGCTACAAACATCTCTATCACAAACTCTAGTCTC

U       TTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTGTTGACTGTAACGATATGGG
A       TTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTGTTGACTGTAACGATATGGG
T       TTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTGTTGACTGTAACGATATGGG
S       TTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTGTTGACTGTAACGATATGGG
W       TTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACTGTTGACTGTAACGATATGGG

U       AGCTGAGTTCTTGAGTGTTCGTAATAAAATGTTCCATGTCTGAAATTCTTGATCATCCTC
A       AGCTGAGTTCTTGAGTGTTCGAAATAAAATGTTCCATGTCTGAAATTCTTGATCATCCTC
T       AGCTGAGTTCTTGAGTGTTCGATATAAAATGTTCCATGTCTGAAATTCTTGATCATCCTC
S       AGCTGAGTTCTTGAGTGTTCGA-ATAAAATGTTCCATGTCTGAAATTCTTGATCATCCTC
W       AGCTGAGTTCTTGAGTGTTCGA-ATAAAATGTTCCATGTCTGAAATTCTTGATCATCCTC

U       ATGCATCTCTTGCAGAGAGCATAGTTTTGCCCAAGGATTTGCCTTGGGCGAATAATTGTG
A       ATGCATCTCTTGCAGAGAGCATAGTTTTGCCCAAGGATTTGCCTTGGGCGAATAATTGTG
T       ATGCATCTCTTGCAGAGAGCATAGTTTTGCCCAAGGATTTGCCTTGGGCGAATAATTGTG
S       ATGCATCTCTTGCAGAGAGCATAGTTTTGCCCAAGGATTTGCCTTGGGCGAATAATTGTG
W       ATGCATCTCTTGCAGAGAGCATAGTTTTGCCCAAGGATTTGCCTTGGGCGAATAATTGTG

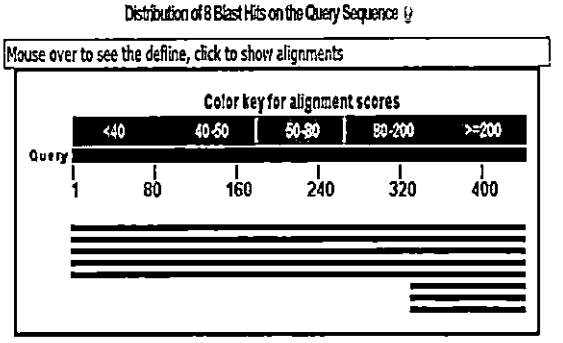

U       AAGGA-
A       AAGGA-
T       AAGGAA
S       AAGGA-
W       AAGGA-
```

Plate 10. Sequence alignment of *Cs* gene in pungent varieties

A- Anugraha, T- Vellayani Thejus, U- Ujwala, S- Vellayani Samrudhi, W- White Kandari

4.4.3 Characterization of *Pun1* locus using the marker from MAP1F/R primer set

BLASTn of the marker sequence from the pungent cultivars had shown 98 per cent identity with the *Pun1* locus (Plate 11). Sequence of non-pungent lines had only 95 per cent identity with the *Pun1/AT3* locus (Plate 12).

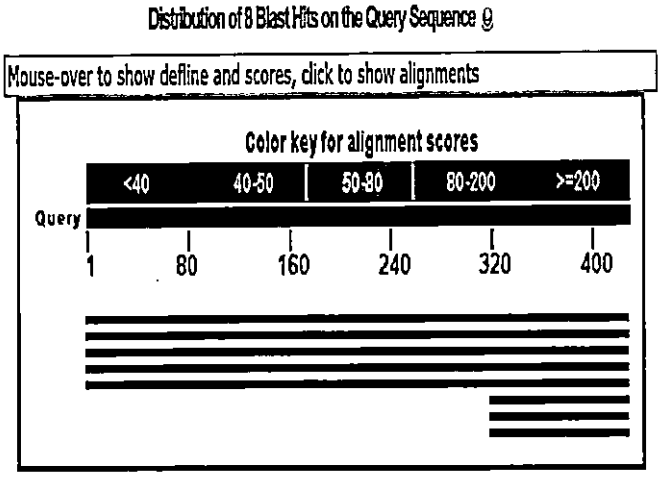


Select: All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Capsicum chinense putative ketoacyl-synthase (Pun1) gene, complete cds	778	778	100%	0.0	96%	AY619697.1
Capsicum frutescens cultivar BG2814-6 acyltransferase (Pun1) gene, Pun1-1 allele, complete cds	765	765	100%	0.0	96%	AY619705.1
Capsicum annuum cultivar Thai Hot acyltransferase (Pun1) gene, complete cds	737	737	100%	0.0	97%	AY619729.1
Capsicum annuum cultivar Hot 1403 acyltransferase (Pun1) gene, complete cds	736	736	100%	0.0	97%	AY619694.1
Capsicum frutescens BAMD acyltransferase (AT3-1) pseudogene, AT3-1-pun1-3 allele, complete sequence	725	725	100%	0.0	96%	EU571995.1
PREDICTED: Capsicum annuum uncharacterized LOC107854548 (LOC107854548), transcript variant X5, misc_RNA	139	139	25%	5e-29	89%	XR_001670941.1
PREDICTED: Capsicum annuum uncharacterized LOC107854548 (LOC107854548), transcript variant X5, misc_RNA	139	139	25%	5e-29	89%	XR_001670940.1
PREDICTED: Capsicum annuum uncharacterized LOC107854548 (LOC107854548), transcript variant X1, mRNA	139	139	25%	5e-29	89%	XM_016970754.1

Plate 11.BLASTn of MAP1F/R marker of pungent lines sequence showing 98 % identity with *Pun1/AT3* locus



Sequences producing significant alignments:

Select: All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Capsicum chinense putative pepper acyltransferase (Pun1) gene, complete cds	699	699	100%	0.0	95%	AY819727.1
Capsicum frutescens cultivar BG2814-6 acyltransferase (Pun1) gene, Pun1-1 allele, complete cds	680	680	100%	0.0	95%	AY819726.1
Capsicum annuum cultivar Hot 1493 acyltransferase (Pun1) gene, complete cds	662	662	100%	0.0	94%	AY819728.1
Capsicum annuum cultivar Thai Hot acyltransferase (Pun1) gene, complete cds	658	658	100%	0.0	94%	AY819729.1
Capsicum frutescens BAHD acyltransferase (AT3-1) pseudogene, AT3-1-pun1-3 allele, complete sequence	652	652	100%	0.0	94%	FJ871985.1
PREDICTED: Capsicum annuum uncharacterized LOC107654546 (LOC107654546), transcript variant X6, misc_RNA	132	132	25%	8e-27	65%	XR_001670041.1
PREDICTED: Capsicum annuum uncharacterized LOC107654546 (LOC107654546), transcript variant X5, misc_RNA	132	132	25%	8e-27	68%	XR_001670042.1
PREDICTED: Capsicum annuum uncharacterized LOC107654546 (LOC107654546), transcript variant X1, mRNA	132	132	25%	8e-27	88%	XM_016572664.1

Plate 12.BLASTn results showing identity of non-pungent lines with *Pun1/AT3* locus

Alignment had shown that the *Pun1/AT3* region is present inside the *Pun1* locus and there is a difference of 15 bp between the pungent and non-pungent lines. The electrophoresis results also have confirmed these results (Plates 11 and 12).

```
A       CCATTAGTCGTT
U       CCATTAGTCGTT
T       CCATTAGTCGTT
S       CCATTAGTCGTT
W       CCATTAGTCGTT
K       CCATTAGTCGTT
AM      CCATTAGTCGTT
AG      CCATTAGTCGTT
BD      CCATTAGTCGTT
BK      CCATTAGTCGTT
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A     CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
U     CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
T     CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
S     CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
W     CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
K     CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
AM    CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
AG    CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
BD    CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT
BK    CATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGAGAT

A     ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
U     ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
T     ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
S     ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
W     ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
K     ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
AM    ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
AG    ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
BD    ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG
BK    ATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATG

A     ATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCTCAATTCGACA
U     ATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCTCAATTCGACA
T     ATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCTCAATTCGACA
S     ATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCTCAATTCGACA
W     ATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCTCAATTCGACA
K     ATCATATACT------------G---CACATGCAGCAGGCTAGAGGTCCTCAATTCGACA
AM    ATCATATACT------------G---CACATGCAGCAGGCTAGAGGTCCTCAATTCGACA
AG    ATCATATACT------------G---CACATGCAGCAGGCTAGAGGTCCTCAATTCGACA
BD    ATCATATACT------------G---CACATGCAGCAGGCTAGAGGTCCTCAATTCGACA
BK    ATCATATACT------------G---CACATGCAGCAGGCTAGAGGTCCTCAATTCGACA

A     TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
U     TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
T     TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
S     TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
W     TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
K     TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
AM    TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
AG    TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
BD    TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG
BK    TGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAG

A     TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
U     TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
T     TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
S     TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
W     TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
K     TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
AM    TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
AG    TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
BD    TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
BK    TTAATAGCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATA
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A       ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
U       ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
T       ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
S       ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
W       ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
K       ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
AM      ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
AG      ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
BD      ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA
BK      ACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATATAAAAGAATGCAGGAGGAACAA

A       ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
U       ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
T       ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
S       ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
W       ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
K       ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
AM      ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
AG      ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
BD      ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT
BK      ATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAATAAATTCAGTTTAT

A       AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
U       AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
T       AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
S       AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
W       AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
K       AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
AM      AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
AG      AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
BD      AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA
BK      AATTTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGAAA

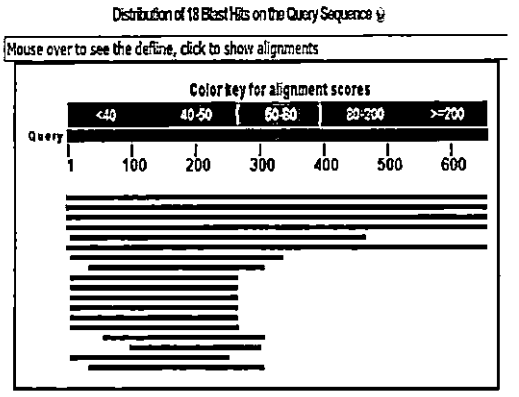
A       ATCCAACAAGGGCAGA
U       ATCCAACAAGGGCAGA
T       ATCCAACAAGGGCAGA
S       ATCCAACAAGGGCAGA
W       ATCCAACAAGGGCAGA
K       ATCCAACAAGGGCAGA
AM      ATCCAACAAGGGCAGA
AG      ATCCAACAAGGGCAGA
BD      ATCCAACAAGGGCAGA
BK      ATCCAACAAGGGCAGA
```

Plate 13.Clustal omega result showing *Pun1* gene alignment of pungent and non-pungent varieties

Kt- Kt-Pl-19, Am- Arka Mohini, Ag- Arka Gaurav, Bk- Byadagi Kaddi, Bd- Byadagi Dabbi, A- Anugraha, U- Ujwala, T- Vellayani Thejus, S- Vellayani Samrudhi, W- White Kandari

4.4.4 Characterisation of *Pun1*^{I} locus

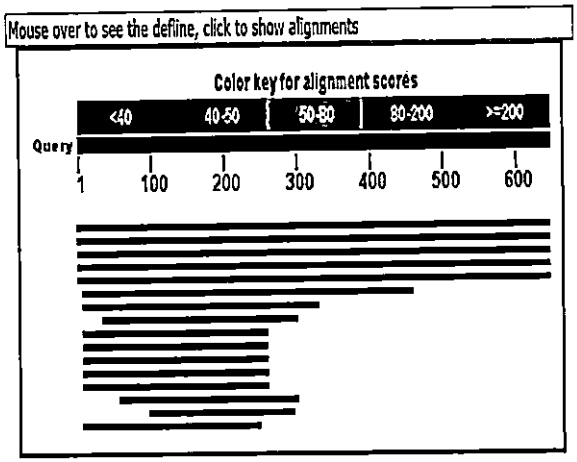
The marker with the *Pun1*^{I}F1/R primer was developed only in the pungent cultivars of *C. annuum*. BLASTn of the sequence had shown that the locus lies within the *Pun1/AT3* locus. Highly pungent cultivar Ujwala and medium pungent Anugraha had shown 96 and 95 per cent identity with the *Pun1* locus, respectively (Plate 14, 15).



Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Capsicum chinense cultivar habanero acyltransferase (Pun1) gene, complete cds	1090	1090	100%	0.0	96%	AY819027.1
Capsicum frutescens cultivar BG2814-6 acyltransferase (Pun1) gene, Pun1-1 allele, complete cds	1068	1068	100%	0.0	96%	AY819026.1
Capsicum annuum cultivar Thai Hot acyltransferase (Pun1) gene, complete cds	970	970	100%	0.0	93%	AY819029.1
Capsicum annuum cultivar Hot 1493 acyltransferase (Pun1) gene, complete cds	898	898	100%	0.0	92%	AY819028.1
Capsicum frutescens BAC 215H17, complete sequence	689	689	70%	0.0	93%	EF517792.1
Capsicum frutescens BAHD acyltransferase (AT3-1) pseudogene, AT3-1-pun1-3 allele, complete sequence	682	682	100%	0.0	86%	FJ871955.1

Plate 14.BLASTn of *Pun1*^{I} allele of Ujwala shows 96 per cent identity with the *Pun1/AT3* locus



Select: All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Capsicum chinense cultivar habanero acyltransferase (Pun1) gene, complete cds	1031	1031	100%	0.0	95%	AY819027.1
Capsicum frutescens cultivar BG2814-6 acyltransferase (Pun1) gene, Pun1-1 allele, complete cds	1014	1014	100%	0.0	94%	AY819026.1
Capsicum annuum cultivar Thai Hot acyltransferase (Pun1) gene, complete cds	922	922	100%	0.0	92%	AY819029.1
Capsicum annuum cultivar Hot 1493 acyltransferase (Pun1) gene, complete cds	857	857	100%	0.0	91%	AY819028.1
Capsicum frutescens BAHD acyltransferase (AT3-1) pseudogene, AT3-1-pun1-3 allele, complete sequence	640	640	100%	2e-179	85%	FJ871955.1
Capsicum frutescens BAC 215H17, complete sequence	638	638	70%	6e-179	92%	EF517792.1

Plate 15.BLASTn of *Pun1*[1] allele of Anugraha show 95 per cent identity with the *Pun1/AT3* locus

```
A          TCATTAGAAGGTCATACCGCTCCACGAAAATGCACCTTGAAAGATATGGCACGGC--AAC
U          TCATTAGAAGGTCATACCGCTCCACGAAAATGCACCTTGAAAGATATGGCACGGC--AAC


A          AATCATATTCCCCATCATCACTATTACTCCCACTTCCCTCGCACTCTTCACTGC--CACA
U          AATCATTATCCCCATCATCACTATTACTCCCACTTCCCTCGCACTCTTCACTGC--CACA


A          TGACACTCCGCTTGGCAACATCTTTACTAGAATCGACGTAGTCGCTTATCTCCTTTAACT
U          TGACACTCCGCTTGGCAACATCTTTACTAGAATCGACGTAGTCGCTTATCTCCTTTAACT
           ********************  ** ************************************


A          CCGAATCTGATTCGGACACCGACTCTTCAATTTTCTTTCTTTTTGACACTTTTTCAATTG
U          CCGAATCTGATTCGGACACCGACTCTTCAATTTTCTTTCTTTTTGACACTTTTTCAATTG


A          CCGCAGCTCTTCTTTTTCTACTACTACCAGCG--ATCGTCTTTTTTTTTTAGGAATAACAT
U          CCGCAGCTCTTCTTTTTCTACTACTACCAGCG--ATCGTCTTTTTTTTTTAGGAATAACAT
```

```
A       TTTTTCTACCCATTTTCAACACAGTCTACACCTAAAGAACAAATCTCCCATTTTCAGTTC
U       TTTTTCTACCCATTTTCAACACAGTCTACACCTAAAGAACAAATCTCCCATTTTCAGTTC

A       ATAA-CCACAAGTCTATCAACAGAAATAACTCAATGATCAAATGAACCCCCCCCCCCCA-
U       ATAA-CCACAAGTCTATCAACAGAAATAACTCAATGATCAAATGAACCCCCCCCCCCCA-

A       -CACAAAAAAAAAAAAAATTAACAAACACCCCACCATTAAACAGTTCACTACACAAACATA
U       -CACAAAAAAAAAAAAAATTAACAAACACCCCACCATTAAACAGTTCACTACACAAACATA

A       CAATAATTGACCAA-AATCGAACATGCAAAATATCAAAACACAA-CAATGCTAAAAATCA
U       CAATAATTGACCAA-AATCGAACATGCAAAATATCAAAACACAA-CAATGCTAAAAATCA

A       AACTAGTGCACCTAATTAAACTAATTAGTTATTAATATTTTAATTTC-ACTATTTCAGCA
U       AACTAGTGCACCTAATTAAACTAATTAGTTATTAATATTTTAATTTC-ACTATTTCAGCA

A       ATCATGTT-TTAAAAAATTTCATACGTCTGAAAATTGAAATATATCTAGGGCATTCTCAT
U       ATCATGTT-TTAAAAAATTTCATACGTCTGAAAATTGAAATATATCTAGGGCATTCTCAT

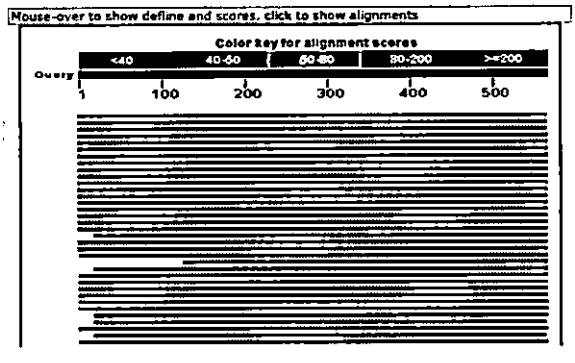
A       TTCATAG
U       TTCATAG
```

Plate 16. *Pun1*[1] allele sequence variation among *C. annuum* cvs.Anugraha and Ujwala

U- Ujwala, A- Anugraha

4.4.5 Characterisation of *Pun1*[3] allele

BLASTn of *Pun1*[3]F/R1 marker sequence had shown 99 per cent identity with the *Pun1/AT3* locus (Plate 17).



4.5 Mapping all the loci for pungency

The different loci identified so far as responsible for various levels of pungency in *Capsicum* are mapped in the *Pun1* locus of the negative strand in the second chromosome.

5'TCATTAGAAGGTCATACCGCTCCACGAAAATGCACCTTGAAAGATATAACACGGACAACGAATCATTATCC
CCATCATCACTATTACTCCCACTTCCCTTGCACTCTTCACTGTCACCACTGACACTCCGCTTGGCAACATTTTC
ACTAGAATCGACGTAGTCGCTTATCTCCTTTAACTCCGAATCTGATTCGGACACCGACTGTTCAATTTTCTTTC
TTTTTGAGACTTTTTCAACTGCTTCAGTTCTTCTTTTTCTACTGTTACCAGCGGTACCGGCTTTGCGTTTAGGA
ATGATGTTTTTTTTTACCCATTTTCAACACAATCTACACCTAAAGAACAAATCTCCCATTTTTAGTTCATAGACG
ACAAGTCTATCAACAGAAAAACTTAATGATCAAATGAACACCACCCCCCCCCCCCCCAAAAAAAAAAAATT
AACAAACACCCCATCATTAAACAGTTCACTACACAAACATACAATAATTGAACCAAAATCGAACATGCAAA
ATATCAAAACACAACAATTGCTAAAAATCAAACTAGTGCACCTAATCAAACTAATTAGCTATTAATATTTCA
ATTTTCACTATTTCAGCAATCATGTTTTAAAAGAATTTCATACGTCTGAAAATTGATATATATCTAGGGCATT
CTCATTTCATAGACCACGGGTCTACGGATAGACCTCGGGTCTACGAACAGAAATAGGTCGCTGTTCAAATCA
AAATGCCAAAATAACTCTTCAAACAACTATTATCCCACCATTCAACACTTCGTTGCTAAAAAAATCACAACT
AAACCAAAACATTAAATTCGAAGAAAAAATTTCTACATCACTACGAATTGATTAGCAAAAAAAAAACATTTA
AATGGATCTAGAAATGATCGAAACTTGATTGTAACTAACCTTGTAAAGCAGCAACAACCCCTTAGCAGCTGG
AGAAGAAGACGAAATGAAAATGGCATTTTTGAGAGAAGTAGTTTCAAAAGCAGGAGTTGGAAATTGAAGAG
GAGAGAGAGGGTGGGTTTTTTTAAATATTGGAATAATTGGAGGGTGTTAGGTGTATTATATTAAATTTGTAA
AGTTGTAAAAATGATGAATTGGTCCCTTGGCCGACGCGTGGGCCCCACTTTTTCCACTTTTTCGTAAAAAATA
AATTAAAAAGAAATTAAGTAGGTATTTGACAAATTAATTTTGGAGGGTTCCTTCTTTGCCAATTATTCCCCAC
TAAGCTACTCTCATTCACTCTTATATTATAGATTATAGTATAAAGTAATACAAACTATGAATTGTTTTTATATT
TTATTTTACAAGTTATGAATAGTGTTTATATAGGTCTCTATTTCCATACAATCAACATTTTGTGGGCAGTTTTT
TTGGGATTGTCACGAAGGCGAGGTTTGTTCATTTTGTGGAAAGAGAATTGGATTTCTACATTTTTATCATCTT
CTAGGTGTGATGTTGATACTACTATTTGCCCAAATATTTGTTTTAAACATATTAATATTATGTATCAAAATGT
GTACAATATAATTTAACACACGTGCAGTATGCATGTATCGCGAAACTAGTTAATTACATGCATCACATGTAA
TAGCAATAGTATTATTGTACGACGTACTAATATATTAGTATCTATTCTAGCTACTAATTTCCTCTTAACCGTCT
CCATGCTGAAAACAACGCCACAGTGCAACGAGCCTTCTATAAAAGTTGAATTATATAAAAATAAGGTACAGT
TTAGAAATAAAACTAACAAAAAGGTAACCTATAGTTTGGGGGTTGGGTAGAGGTTGTTTAGCCAGTAACTCT
ATTATTTCATTTCCTTTTGTCTATATAAGTGTATCCATATATGCAAGAAA**ATGTCAACCGGCCA**
GCAGCATATAATTTATTTGTTAAATTAATTATGGCTTTTGCATTAC
CATCATCACTTGTTTCAGTTTGTGACAAATCTTTTATCAAACCTTC
CTCTCTCACCCCCTCTAAACAAAGATTTCACAAGCTATCTTTCATC
GATCAATCTTTAAGTAATATGTATATCCCTTGTGCATTTTTTTACC
CTAAAGTACAACAAAGACTAGAAGACTCCAAAAATTCTGATGAGC
TTTCCCATATAGCCCACTTGCTGCAAACATCTCTATCACAAACTCT
AGTCTCTTACTATCCTTATGCAGGAAAGTTGAAGGACAATGCTACT
GTTGACTGTAACGATATGGGAGCTGAG*TTCTTGAGTGTTCGAATAAA*
ATGTTCCATGTCTGAAATTCTTGATCATCCTCGTGCTGCTCATGCAGAG
GGCATAGTTTTTCCCAAGGATTTGCCTTGGGCAAATAATTATGAAGGTG
GTAATTTGCTTGTAGTTCAAGTAAGTAAGTTTGATTGTGGAGGAATAGC
CATTAGTATATGCTTTTCACACAACAGATCGGTGATGGTTGCTCTCTACT
TAATTTCCTTAATGATTGGTCTAGCGTTACTCGTGATCATATGACGACAA
CGACTCTAATTCCTTCTCCTAGGTTTATAGGAGATTCAATTTTCTCTACA
CAAAAATATGGCCCTCTTATTACGCCACAAATAGTGTCCGATCTTAGTG
AATGCGTACAGAAAAGATTCATTTTTCCTACAGACAAGTTAGATGCACTT
CGAGCTAAGGTAAGACTACCATCACTCCATTATTGTCAGTCTT*TTAAGATTTTC*
TTTTGCAATACATTTTTGAAATATAATAATCCACCAAATTAAATTTGTATGGAAGAATGGTTTGATTTGACACTGGGTTT
TAGATAGAACGGAAGTTTTTTAGGATTCATGCGTTGCCTTAAATATGCGTAAGATTTTAATTTGTCTTCCGGTATTTTTG
GTATTAACACTACCATTAGTCGTTCATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGTTTATTAGTCTTGTTGA
GATATAATAATCAAGAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAATGAGATGATCATATACTTCCAACAT
*GCATGATACCACATGCAGCAGGCAGAGGTCCTCAATTCGACA***TGTTAACAAGCCTGGGCTAT**
TAGTCTATTTGTAGAGACTACTCTTAAACGCCTCACTTAGTTAATA
GCACTCCACTTATTGGTGTCAAAAACTACTCTTGGACATGTCATTT

ACTTAATAACACTCCACTTAATTATCGAACAGTAAAGTGGAAAATA
TAAAAGAATGCAGGAGGAACAAATACTTCTTATTTTTTTAAGACGA
CAAAGAATTAAATATTTTGAAATAAATTCAGTTTATAATTTAGTTT
GGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGT
AGAAAATCCAACAAGGGCAGAAGTTGTTAGCGCTCTTCTTTTCAA
ATGTGCAACAAAGGCATCATCAAGTACAACTACTTCAATGCCTAT
GCGCCCATCAAAGTTGGTTCACTTCTTAAACATACGTACAATGATC
AAACCTCGTCTACCCCGAAATACCATTGGAAATATCTTGTCCATGT
TCTCCACAGCAGCAACTAACGAGCAGGACATTGAGTTGCCAAGTT
TGGTTCGTAATTTGAGGAAGGAAGTTGAGGTGGCGTACAAGAAAG
ACCAAGTCGAACAAAATGAACTGATCCTAGAAGTAGTAGAATCCA
TAAGAAAAGGTAAAATGCCTTTTGAAAATAAGGATGGATATCATG
AGAATGTTTATATTTGCAGTAATCTTTGCAAATACCCATACGACAC
TGTAGATTTTGGATGGGGAAGACCTGAAAGTGTGTGTATAGCAAA
TGGTCCCTTCAAGAATGCCTTCTTCTTGAAAGATTACAAAGCTGG
GCGAGGTGTG*GAGGCGCGGGTGATGTTGCACAAGCAACAAATGTCTGAATTTGAACGCAATGAGGAACT*
CTTTGAATTTATTGCCTAATTAATTCCAAGTTTGGGAGTAATTGGATGTCATTTCCAAGTCTTTTGTGGTGTTTGATTGA
AGAGAGAGGGATCTTACGAAATAAAGGAATACTTTTGAAACTTACGAAACAAAGGTAGGAATACTTTTGTAATTGTTGT
*GTGTTTCATCAATATAATTTCAACGGAAGTTTACGGTCAACAA*3'

Pun 1[1]fwd1/rev CSF1/R2 + BF7/R9

MAP1F/R *Pun1[3]fwd/rev1* + BF7/R9 BF7/R9 + CSF1/R2 + *Pun1[3]fwd/rev1*

Pun1 locus Yellow marked region - BF7/R9

Bold large fond – *AT3* locus

Italics – *Caft1/2*

4.6 Open Reading Frames in the *Pun1* sequence

When the sequence was analysed for the ORFs, it was found that the region contains 11 ORFs.

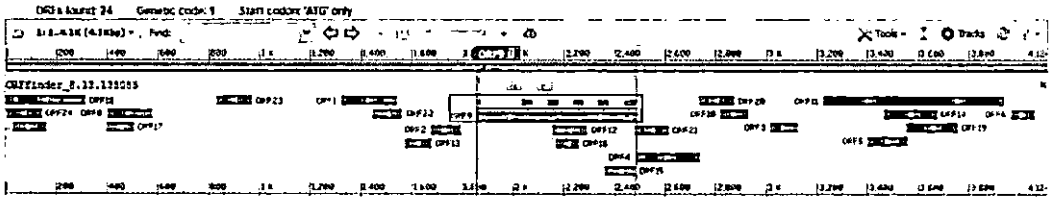


Plate 19. ORFs identified from the sequence of the *Pun1* locus

4.6.1 ORF11

Among them the ORF11was the longest, coding for 238 aminoacids. The ORF11 spanned from 3225 to 3941 bp (717bp). The amino acid sequences coded by this ORF is given below

MQEEQILLIFLRRQRIKYFEINSVYNCSLVDINLGLKVAVESGVENPTRAEV
VSALLFKCATKASSSTTTSMPMRPSKLVHFLNIRTMIKPRLPRNTIGNILSM
FSTAATNEQDIELPSLVRNLRKEVEVAYKKDQVEQNELILEVVESIRKGMP
FENKDGYHENVYICSNLCKYPYDTVDFGWGRPESVCIANGPFKNAFFLKD
YKAGRGVEARVMLHKQQMSEFERNEELFEFIA

BLASTp had shown that this sequence stands for the *AT3* protein. The reverse primer MAP1R was bind in this ORF for amplification.

4.6.2 ORF 9

ORF 9 was the second longest ORF in this sequence. This has started from nucleotide 1857 to 2486 (630 bp), coding for 209 amino acids. The sequence coded is given below

MQENVNRPAAYNLFVKLIMAFALPSSLVSVCDKSFIKPSSLTPSKQRFHKL
SFIDQSLSNMYIPCAFFYPKVQQRLEDSKNSDELSHIAHLLQTSLSQTLVSY
YPYAGKLKDNATVDCNDMGAEFLSVRIKCSMSEILDHPRAAHAEGIVFPK
DLPWANNYEGGNLLVVQVSKFDCGGIAISICFSHKIGDGCSLLNFLNDWS
ALLVII

BLASTp had shown that the protein is AT3. The *Pun1*[3]*fwd/rev* primer which is exclusively amplify in *C. frutescence* and forward primer CSF1 specific for *CS* gene were bind in this ORF9.

4.6.3 ORF4

ORF4 was 255 bp long (2483 to 2737 nucleotides), coding for 84 amino acids. The sequence coded was

MTTTTLIPSPRFIGDSIFSTQKYGPLITPQIVSDLSECVQKRFIFPTDKLDALR
AKVRLPSLHYCQSFKIFFCNTFLKYNNPPN

BLASTp has shown that the protein belongs to *AT3*.

4.6.4 ORF1

ORF1 was 222 bp long (1321 to 1542 nucleotides), coding for 73 amino acids. The sequence coded was

MNSVYIGLYFHTINILWAVFLGLSRRRGLFILWKENWISTFLSSSRCDVDT
TICPNICFKHINIMYQNVYNII

4.6.5 Other ORFs

ORF 8 had fallen between 402 and 575 bp (174 bp), coding for 57 amino acids. ORF5 had fallen between 3401 to 3556 bp (156bp within ORF11), coding for 51 amino acids. ORF7 was 132 bp long (30 to 161 bp), coding for 43 amino acids. ORF 2 was 120 bp long (1675 to 1794 bp), coding for 39 amino acids. ORF 10 had fallen between 2820 and 2927 bp, ORF 3 had fallen between 3016 to 3123 bp, and ORF 6 had fallen between 3968 to 4060 bp and BLASTp had shown similarity to 30s ribosomal protein which was not related to pungency. Thus it is shown that the pungency related pun1 locus is completed at 3941 bp.

The forward primer of BF7 specific for *CS* gene is located in the region of ORF2. The binding of forward primer MAP1F and reverse primer BR9 were identified in ORF 10. The deletion of 15 bp in non-pungent varieties using MAP1F/R primer is located in this ORF3.

4.7 Regions of deletion in relation to pungency

The ORFs present in the *Pun1* locus are presented in italics

TCATTAGAAGGTCATACCGCTCCACGAAAA*TGCACCTTGAAAGATATAACACGGACAACGAATCATTATCCCC* 73

ATCATCACTATTACTCCCACTTCCCTTGCACTCTTCACTGTCACCACTGACACTCCGCTTGGCAACATTTTCA 146

*CTAGAATCGACGTAG*TCGCTTATCTCCTTTAACTCCGAATCTGATTCGGACACCGACTGTTCAATTTTCTTTC 219

TTTTTGAGACTTTTTCAACTGCTTCAGTTCTTCTTTTTCTACTGTTACCAGCGGTACCGGCTTTGCGTTTAGG 292

AATGATGTTTTTTTTACCCATTTTCAACACAATCTACACCTAAAGAACAAATCTCCCATTTTTAGTTCATAGA 365

CGACAAGTCTATCAACAGAAATAACTTAATGATCAA*ATGAACACCACCCCCCCCCCCCCCAAAAAAAAAAAT* 438

TAACAAACACCCCATCATTAAACAGTTCACTACACAAACATACAATAATTGAACCAAAATCGAACATGCAAAA 511

*TATCAAAACACAACAATTGCTAAAAATCAAACTAGTGCACCTAATCAAACTAATTAGCTATTAA*TATTTCAAT 584

TTTCACTATTTCAGCAATCATGTTTTAAAAGAATTTCATACGTCTGAAAATTGATATATATCTAGGGCATTCT 657

CATTTCATAGACCACGGGTCTACGGATAGACCTCGGGTCTACGAACAGAAATAGGTCGCTGTTCAAATCAAAA 730

TGCCAAAATAACTCTTCAAACAACTATTATCCCACCATTCAACACTTCGTTGCTAAATAAACCACAACTAAAC 803

CAAAACACCAAATTCGAAGAAAAAATTTCTACATCACTACGAATTGATTAGCAAAAAAAAAACGTTTAAATGG 876

ATCTAGAAATGATCGAAACTTGATTTTAACTAACCTTGCAAAGCAGCAACAACCCCTTAGTAGCTGGAGAAGA 949

AGACGAAATGAAAATGGCATTTTTGGAAGAAGTAGTTTCAAAAGCAGGAGTTGGGAATTGAAGAGGAGAGAGA1022

GGGTGGGTTTTTTTAAATATTGGAATAATTGGAGGGTGTTAGGTGTATTATATTAAATTTGTAAAGTTGTAAA 1095

AATGATGAATTGGTCCCTTGGCCGACGCGTGGGCCCCACTTTTTCCACTTTTTCGTAAAAAATAAATTAAAAA 1168

GAAATTAAGTAGGTATTTGACAAATTAATTTTGGAGGGTTCCTTCTTTGCCAATTATTCCCCACTAAGCTACT 1241

CTCATTCACTCTTATATTATAGATTATAGTATAAAGTAATACAAACTATGAATTGTTTTTATATTTTATTTTA 1314

CAAGTT*ATGAATAGTGTTTATATAGGTCTCTATTTCCATACAATCAACATTTTGTGGGCAGTTTTTTTGGGAT* 1387

TGTCACGAAGGCGAGGTTTGTTCATTTTGTGGAAAGAGAATTGGATTTCTACATTTTTATCATCTTCTAGGTG 1460

TGATGTTGATACTACTATTTGCCCAAATATTTGTTTTAAACATATTAATATTATGTATCAAAATGTGTACAAT 1533

*ATAATTTAA*CACACGTGCAGTATGCATGTATCGCGAAACTAGTTAATTACATGCATCACATGTAATAGCAATA 1606

GTATTATTGTACGACGTACTAATATATTAGTATCTATTCTAGCTACTAATTTCCTCTTAACCGTCTCC*ATGCT* 1679

GAAAACAACGCCACAGTGCAACGAGCCTTCTATAAAAGTTGAATTATATAAAAATAAGGTACAGTTTAGAAAT 1752

*AAAACTAACAAAAAGGTAACCTATAGTTTGGGGGTTGGGTAGAGGTT*GTTTAGCCAGTAACTCTATTATTTCA 1825

TTTCCTTTTGTCTATATAAGTGTATCCATAT*ATGCAAGAAAATGTCAACCGGCCAGCAGCATATAATTTATTT* 1898

GTTAAATTAATTATGGCTTTTGCATTACCATCATCACTTGTTTCAGTTTGTGACAAATCTTTTATCAAACCTT 1917

CCTCTCTCACCCCCTCTAAACAAAGATTTCACAAGCTATCTTTCATCGATCAATCTTTAAGTAATATGTATAT 2044

CCCTTGTGCATTTTTTTACCCTAAAGTACAACAAAGACTAGAAGACTCCAAAAATTCTGATGAGCTTTCCCAT 2117

ATAGCCCACTTGCTGCAAACATCTCTATCACAAACTCTAGTCTCTTACTATCCTTATGCAGGAAAGTTGAAGG 2190

ACAATGCTACTGTTGACTGTAACGATATGGGAGCTGAGTTCTTGAGTGTTCGAATAAAATGTTCCATGTCTGA 2263

AATTCTTGATCATCCTCGTGCTGCTCATGCAGAGGGCATAGTTTTTCCCAAGGATTTGCCTTGGGCAAATAAT 2336

TATGAAGGTGGTAATTTGCTTGTAGTTCAAGTAAGTAAGTTTGATTGTGGAGGAATAGCCATTAGTATATGCT 2409

TTTCACACAAGATCGGTGATGGTTGCTCTCTACTTAATTTCCTTAATGATTGGTCAGCGTTACTCGTGATCAT 2482

ATGACGACAACGACTCTAATTCCTTCTCCTAGGTTTATAGGAGATTCAATTTTCTCTACACAAAAATATGGCC 2555

CTCTTATTACGCCACAAATAGTGTCCGATCTTAGTGAATGCGTACAGAAAAGATTCATTTTTCCTACAGACAA 2628

GTTAGATGCACTTCGAGCTAAGGTAAGACTACCATCACTCCATTATTGTCAGTCTTTTAAGATTTTCTTTTGC 2701

*AATACATTTTTGAAATATAATAATCCACCAAATTAA*ATTTGTATGGAAGAATGGTTTGATTTGACACTGGGTT 2774

TTAGATAGAACGGAAGTTTTTTAGGATTCATGCGTTGCCTTAAA*TATGCGTAAGATTTTAATTTGTCTTCCGG* 2847

TATTTTTGGTATTAACACTACCATTAGTCGTTCATTTTTGTTTGTCTAATGGTATTTTTGAAAAGAAAAACGT 2920

*TTATTAG*TCTTGTTGAGATATAATAATCAAGAAAAAAAAACACGTGTTTTTTATAACATTATAATCTCTTAAAT 2993

GAGATGATCATATACTTCCAAC*ATGCATGATACCACATGCAGCAGGCAGAGGTCCTCAATTCGACATGTTAAC* 3066

*AAGCCTGGGCTATTTAGTCTATTTGTAGAGACTACTCTTAAACGCCTCA*CTTGTTAATAGCACTCCACTTATT 3139

GGTGTCAAAAACTACTCTTGGACATGTCATTTACTTAATAACACTCCACTTAATTATCGAACAGTAAAGTGGA 3212

AAATATAAAAGAATGCAGGAGGAACAAATACTTCTTATTTTTTTAAGACGACAAAGAATTAAATATTTTGAAA 3285

TAAATTCAGTTTATAATTGTAGTTTGGTTGACATTAATTTGGGTTTGAAGGTGGCAGTAGAATCAGGAGTAGA 3358

AAATCCAACAAGGGCAGAAGTTGTTAGCGCTCTTCTTTTCAAATGTGCAACAAAGGCATCATCAAGTACAACT 3431

ACTTCAATGCCTATGCGCCCATCAAAGTTGGTTCACTTCTTAAACATACGTACAATGATCAAACCTCGTCTAC 3504

CCCGAAATACCATTGGAAATATCTTGTCCATGTTCTCCACAGCAGCAACTAACGAGCAGGACATTGAGTTGCC 3577

AAGTTTGGTTCGTAATTTGAGGAAGGAAGTTGAGGTGGCGTACAAGAAAGACCAAGTCGAACAAAATGAACTG 3650

ATCCTAGAAGTAGTAGAATCCATAAGAAAAGGTAAAATGCCTTTTGAAAATAAGGATGGATATCATGAGAATG 3723

TTTATATTTGCAGTAATCTTTGCAAATACCCATACGACACTGTAGATTTTGGATGGGGAAGACCTGAAAGTGT 3796

GTGTATAGCAAATGGTCCCTTCAAGAATGCCTTCTTCTTGAAAGATTACAAAGCTGGGCGAGGTGTGGAGGCG 3869

CGGGTGATGTTGCACAAGCAACAAATGTCTGAATTTGAACGCAATGAGGAACTCTTTGAATTTATTGCCTAAT 3942

TAATTCCAAGTTTGGGAGTAATTGG*ATGTCATTTCCAAGTCTTTTGTGGTGTTTGATTGAAGAGAGAGGGATC* 4015

*TTACGAAATAAAGGAATACTTTTGAAACTTACGAAACAAAGGTAG*GAATACTTTTGTAATTGTTGTGTGTTTC 4088

ATCAATATAATTTCAACGGAAGTTTACGGTCAACAA 4124

Italics – ORF ------ - BF7 ----- MAP1 deleted portion in non-pungent

------ - MAP1F/R ----- CSF1/R2 ----- *Pun1[3] fwd/rev* ------ *Pun1[1] fwd/rev*

Discussion

5. DISCUSSIONS

The 'Wonder Spice', chilli, is a valuable and economically important condiment which comes under the genus *Capsicum*. It is also known as hot pepper or cayenne pepper which can able to grow throughout the world. The primary centre of origin of the crop is Tropical America. The first ever domesticated crop in America is said to be chilli. The common ancestor of the three chilli species *C. annuum, C. frutescens* and *C. chinense* was evolved from the North of the Amazon basin (NW-Brazil, Columbia). Thereafter, both *C. annuum* and *C. frutescens* were domesticated to Central America; from there they finally brought to Mexico and Panama respectively, whereas *C. chinense* moved to the West and was first put to cultivation in Peru. *C. baccatum* and *C. pubescens* are the other two species which were first cultivated in Western South America in the Peruvian low lands and at the higher elevations in the Andes (Peru, Bolivia, Ecuador) respectively (Manju and Sreelathakumary, 2002). In Kerala, hot chilli locally known as Kandari (*C. frutescens*) is a traditional crop grown in the homesteads for domestic purposes.

A very wide range of natural variability is available in *Capsicum* spp. As a result of selection and habituation a large number of cultivars were evolved and about 400 such genotypes are available with farmers for cultivation. Using molecular tools, many works have been conducted to characterize these cultivars and varieties (Bhadragouder and Patil, 2011; Patel *et al.*, 2011; Thul *et al.*, 2012).

In chillies enormous works have been carried out to develop high yielding and high quality varieties through selection and hybridisation. Kerala Agricultural University has so far released seven varieties namely Jwalamukhi, Jwalasakhi, Anugraha, Ujwala, Vellayani Athulya, Vellayani Thejus and Vellayani Samrudhi. Among these Ujwala, Vellayani Thejus and Vellayani Samrudhi are highly pungent.

In this study, Anugraha, Ujwala, White Kandari, Vellayani Thejus and Vellayani Samrudhi were pungent cultivars, Byadagi Dabbi and Byadagi Kaddi

were slightly pungent and Kt-Pl-19 and bell peppers Arka Mohini and Arka Gaurav were the non-pungent. Anugraha is a high yielding chilli variety suitable for growing in Kerala conditions with attractive long green medium pungent fruits, which turn deep red on ripening. Ujwala is a high yielding bacterial wilt resistant variety suitable for Kerala conditions. It is having dark green fruits that turn dark brown on ripening, suitable for making green chilli and for drying purpose. It's a highly pungent variety, suitable for extracting oleoresins and colouring pigments. Both belong to the species *C. annuum*. White Khandari and Vellayani Samrudhi are two important varieties included under the species *C. frutescens*. Both are highly pungent varieties having white colour fruits. Vellayani Thejus is a shade tolerant chilli variety with highly pungent round shaped fruits, belonging to *C. chinense*.

Kt-Pl-19 is a non-pungent paprika chilli variety belonging to *C. annuum*. It is commercially used as a natural colourant next to turmeric. Because of the absence of pungency, Kt-Pl-19 fruits are used for extracting capsanthin, a natural colourant. Byadagi is an important *C. annuum* chilli type known for its deep red colour and negligible pungency. It is mainly cultivated in the different parts of Karnataka state and exported as oleoresin which serves as a substitute to paprika oleoresin. In Byadagi chilli, the reports on characterization using molecular markers are scanty even though germplasm collection and evaluation studies were reported by Sandeep (2007). Arka Mohini and Arka Gaurav are two bell pepper released from IIHR, Bangalore.

Pungency is a specific trait which is present only in the species coming under genus *Capsicum*. It imparts colour and aroma to the human foods, other than that it is having some pharmaceutical properties like antiarthritic, antioxidant, anticancer and analgesic. Because of the above reason it has become one of the most valuable and important alkaloid in the production of drugs for several diseases. Since the production of pungency is restricted to *Capsicum* sp. the amount produced in the plant is much less when compared to its demand. So it is necessary to isolate the genes which are responsible for pungency in chilli and

further used it in secondary metabolite production by various techniques and also to produce highly pungent chilli varieties by transferring the genes. Thus for isolating the genes for pungency, the marker specific for that trait is very important. Since the pungency trait is a quantitative character which is governed by several genes, it is complicated to identify the exact genes responsible for the trait. Hence, in the present study, the available SCAR markers are used to characterise candidate gene for pungency at molecular level in chilli genotypes.

5.1 Molecular characterization of genes for pungency in Capsicum

5.1.1 Isolation, Purification and Quantification of DNA

Young, immature leaves were used as a sample for isolating the DNA from ten chilli genotypes. CTAB method reported by Roger and Bendich (1994) was used to extract the DNA from the samples. This method had yielded sufficient quantity of good quality DNA. The electrophoresed DNA had shown distinct bands without shearing.

Grinding in liquid nitrogen was found to improve the quality of isolated DNA. For preventing the phenolic oxidation, the addition of antioxidants like β-mercaptoethanol during DNA isolation was found effective.

The disruption of the cell membrane of the sample is done with the help of the detergents which were used in the extraction buffer of CTAB (Cetyl Trimethyl Ammonium Bromide). As a result of the action of this detergent the nucleic acid inside the cell was released into the extraction buffer and prevented the co-precipitation of the polysaccharides with nucleic acid by acting as a selective precipitant of nucleic acid. CTAB being a cationic detergent had solubilised the cell membranes and formed a complex with DNA (Sghaier and Mohammed, 2005).

As reported by Gallego and Martinez (1996) and Sreenath *et al.* (1992), using the CTAB along with PVP was having an advantageous effect on the quality of DNA. It acted along with the NaCl which effectively disrupted the cell

membrane and separated the polysaccharides. The EDTA in the extraction buffer was chelated with the Mg^{2+} ions of DNA, protecting the DNA from endonucleases. In the DNA extraction protocol, double treatment with chloroform: isoamylalcohol mixture and centrifugation had removed the pigments and proteins from the nucleic acid. The DNA got precipitated with the addition of chilled isopropanol and the pellet obtained after the centrifugation was washed with 70 per cent alcohol followed by absolute alcohol to clear the traces of CTAB. TE buffer rehydrated the DNA and dissolved it (Wettasinghe and Peffley, 1998; Babu, 2000).

According to Wettasinghe and Peffley (1998), a DNA sample was reported to be in high quality if it had a band of high molecular weight with little smearing and a low amount of RNA. As per the findings of Wettasinghe and Peffley (1998) and Gallego and Martinez (1996), to remove the RNA contamination from the isolated DNA samples, RNase treatment was given. In the present study, on electrophoresis, the RNase treated DNA samples had shown a high molecular weight, forming a single band just below the well. This indicated that the DNA is of good quality.

For checking the quality of DNA, the absorbance ratio was calculated as OD at 260/280, using spectrophotometer. Those samples which were having the ratio ranging 1.8–2.0 were considered to be of high quality DNA. With RNA contamination, the absorbance ratio had gone beyond 2.0 and with protein contamination, the ratio had gone below 1.8. High quality DNA was used in the molecular analyses.

5.1.2 SCAR analysis of pungency loci

Molecular marker technology provides novel tools for characterization studies. Most of the molecular markers are developed by the PCR (Polymerase Chain Reaction) technology and amplifies unique regions of the genomic DNA based on primer designed for DNA amplification. One such marker system

(SCAR) was utilized in this study on molecular characterization of candidate gene for pungency in ten chilli genotypes.

Five SCAR primers used in this study for characterising the candidate gene for pungency in chilli were MAPIF/R, CSF1/R2, BF7/BR9, *Pun1*[1] fwd1/ rev and *Pun1*[3] fwd/rev1.

5.1.2.1 Analysis of *CS* locus using CSF1/R2 primers

PCR amplification with primer CSF1/R2 generated single, clear, intact amplicons of about 400 bp in pungent varieties and neither fragments in the non-pungent varieties. The primer specifically amplified the *Capsaicinoid synthetase* (*CS*) region of the *C* locus in the genome of the *Capsicum* (Lee *et al.*, 2005). Absence of fragments in the non-pungent lines had shown that the primer binding position of the *C* locus is absent or having characteristic variation in the non-pungent lines. It is evident that *Cs* is an essential locus needed for the development of pungency in chilli. Since this portion was absent in slightly pungent chillies also, it also shows that there should be some other regions in chilli genome which further contributes to the pungency. But for a variety to be pungent, this locus is necessary. These results were in accordance with the findings by Lee and co-workers who have reported this primer combination to differentiate the pungent and non-pungent cultivars.

5.1.2.2 Analysis of *CS* locus using BF7/BR9 primers

When BF7/BR9 primer combination reported by Lee *et al.* 2005, were used to screen the chilli accessions which differed in the levels of pungency, it had given distinct bands at 900 bp in pungent varieties and no fragments were generated in the non-pungent lines. As reported earlier this primers will specifically amplify the *Capsaicinoid synthetase* (*CS*) region of the *C* locus in the genome of the *Capsicum.* The deletion when using the other primer proves the deletion/ characteristic variation at primer binding region in the *C* locus of the non-pungent lines. This variation in the genome of the chilli cultivars might be a reason for the loss of pungency in chilli. As it is already discussed, this primer

also differentiates the pungent and non-pungent accessions based on the variations in *CS* locus. However, it points that there should be some other loci involved in the pungency in chillies since this marker has failed to differentiate the non-pungent and slightly pungent cultivars.

5.1.2.3 Characterization of *Pun1* locus

MAPIF/R marker which amplifies the *Pun1* locus in the genome of the chilli cultivars (Rodriguez-Maza *et al.*, 2012). The MAPI marker precisely identified pungency of accessions from 10 chilli lines used in this study. In the pungent lines, the primer set has generated the characteristic bands of 497bp whereas, in non-pungent lines, the banding was generated at 479bp. When compared to the pungent lines there was a deletion of 15 bp in the non-pungent lines in the *Pun1* region. The speciality about this primer is that it can easily discriminate the deletion or the difference in the targeted genic region of both pungent and non-pungent lines. The results were similar to what was reported by Rodriguez-Maza *et al.* (2012) but this primer also had failed to represent the slight pungency compared to the absence of pungency and hence some more loci also should be involved in the pungency mechanism.

5.1.2.4 Characterizing the *C. annuum* specific *Pun1*^{1} locus

Wyatt *et al.* (2012) have shown that the pungency contributing locus *Pun1* is species specific by designing the primer set which amplifies only the *Pun1* locus in pungent *C. annuum* cultivars, whereas, in pungent cultivars belonging to other species, no banding pattern was generated. These points to the fact that different species of chillies have evolved independently and the mechanisms of pungency in each species could be varying. *Pun1*^{1} fwd1/ rev and *Pun1*^{3} fwd/rev1 amplifed the regions of the *Pun1* locus in the *C. annuum* only. In the pungent lines of *C. annuum*, the primer has generated bands of 850bp as reported by Wyatt and co-workers in 2012. The regions showing characteristic variations for the species in the *Pun1* locus were used for placing the primers.

5.1.2.5 Characterization of *C. frutescens* specific *Pun1*^{3} locus

Specific primers to *C. frutescens* species was designed by Wyatt and co-workers (2012). The primers specific for the *Pun1* locus in *C. frutescens* have generated bands in pungent lines of this species at 1000 bp. This difference in amplification of the above two primers in the same locus proves that there were some difference in the nucleotide sequence between the varieties. These mutations which were occurring at the *Pun1* locus in the *Capsicum* lines may be a reason for the variation of degree of pungency in different species of *Capsicum*. For characterizing the chilli genotypes into various species also this region is playing a major role. *Pun1*^{1} and *Pun1*^{3} are the two alleles of the *Pun1* gene, where *Pun1*^{1} will be present exclusively in pungent *C. annuum* and *Pun1*^{3} were present only in *C. frutescens*. Because of the above reasons both the primers were given amplicons only in their respective species.

Pun1^1 allele was absent in the non-pungent varieties of *C. annuum* such as Kt-Pl-19, Byadagi varieties and bell peppers. These deletions at many of these loci might be the reason behind the low level or no of production of capsaicin in low pungent varieties of *C. annuum*.

As reported till now, the major gene which plays an important role in production of capsaicinoid in chilli is *Pun1* (Wyatt *et al.*, 2012). The other loci which are contributing for pungency *viz. AT3* and *Catf1* and *Catf2* were located inside the *Pun1* locus. In that *AT3* gene is present only in the wild cultivars of the capsicum species such as *C. chacoense* and *C. rhomboideum* (Stellari, 2009), *Catf1* and *Catf2* were reported only in pungent and non-pungent varieties coming under *C. annuum* (Lang *et al.*, 2006). The *Pun1* gene is having three allelic forms *viz. Pun1*^{1}, *Pun1*^{2} and *Pun1*^{3} which were seen exclusively in the pungent varieties of *C. annuum, C. chinense* and *C. frutescens* respectively (Wyatt *et al.*, 2012). The difference among the nucleotide sequences of genes inside the *Pun1* locus of the chilli species is the main reason behind the variation of production of capsaicinoids for pungency in chilli varieties.

5.2 Sequence analysis for characterizing the pungency loci in chillies

5.2.1 Sequence analysis of *CS* gene amplified through BF7/BR9 primer set

The sequence of the amplicons of BF7/BR9 primer set had shown 99 per cent identity with the *Pun1* locus. When the sequence was aligned in Clustal Omega with the sequence of *Pun1* gene, it is shown as the *CS* gene is resides inside the *Pun1* gene. The primer will give amplification only in the pungent lines of Capsicum. The SNPs were identified. This shows that the specific deletions in the nucleotides in this locus directly lead to the loss of pungency and this is the most deciding locus on the levels of pungency in chillies.

5.2.2 Sequence analysis of *CS* gene amplified through CSF1/CSR2 primer set

The sequence analysis of the bands generated using CSF1/CSR2 primer set had shown that, it resides inside the region amplified by the BF7/BR9 primer in the pungent varieties. For every pungent variety, the SNPs were identified. This shows that the specific deletions in the nucleotides in this locus directly lead to the loss of pungency and this is the most deciding locus on the levels of pungency in chillies.

5.2.3 Sequence analysis on *Pun1* locus

Amplicons sequences obtained for both pungent and non-pungent varieties were analysed after doing PCR with MAP1F/R primer. The sequence of the pungent lines shows 98 per cent identity with *Pun1* locus and non-pungent lines were shown only 95 per cent similarity with *Pun1* locus. It is because of the deletion of 15 bp in the non-pungent lines compared to pungent lines. These deletions in the *Pun1* locus can be a reason for the low production of capsaicinoids in non-pungent lines. Since it is a co-dominant specific marker, variation in both the pungent and non-pungent lines can be easily distinguishable. This study was successful to obtain the characteristic deletions in this locus in nonpungent cultivars as detailed by (Rodriguez Maza *et al.*, 2012).

5.2.4 Sequence analysis of *Pun1*^{1} locus in pungent *C. annuum*

The primer set *Pun1*^{1} fwd1/ rev has amplified the bands with 850 bp size only in the pungent lines of *C. annuum*. This observation was exactly as reported by Wyatt et al., 2012. Among the pungent lines of *C. annuum,* both the pungent lines Ujwala and Anugraha have shown sequence variation. Please explain the variations Because of these variations Anugraha is showing only 95 per cent similarity with *Pun1* locus when compared with Ujwala which is showing a slightly more similarity of 96 per cent. It lies in the starting region of the *Pun1* locus. This region is specific only for pungent lines of *C. annuum* and not for other species of *Capsicum* spp. This may be the reason for the difference in the levels of pungency in various species of *Capsicum.*

5.2.5 Sequence analysis of *Pun1*^{3} locus in pungent *C. frutescens*

The sequence of the specific marker generated by primer set *Pun1*^{3} fwd/rev1 had shown that the locus lies within the *Pun1* locus itself. This was as per the reports given by Wyatt *et al.*, 2012. The results had shown that both are having a sequence identity of 99 per cent with the *Pun1* locus. It lies at the 2500 bp of the *Pun1* locus. The sequence variation existing in the primer landing position of this marker could be a reason for the difference of level of pungency in *C. frutescens* species compared to other species of *Capsicum* spp.

The Byadagi varieties which come under slightly pungent category are showing a higher pungency when grown in Kerala conditions. As reported by Monforte-González *et al.* (2010) capsaicinoids, which is produced mainly in the fruit's placenta, requires 3 mol of nitrogen to be formed and further, the climatic conditions might influence the pungency. Hence nitrogen availability may affect chilli pungency through its content in the fruit tissues. On the other hand, potassium may also affect pepper pungency given its positive effect on fruit development. This can led to the up-regulation of the region in the *Pun1* locus which is amplified by the MAP1F/R primer.

5.3 Mapping the various loci on the chromosome

The *Pun1* locus also known as *AT3* locus is present on the second chromosome of *Capsicum* (Blum *et al.*, 2003). The different loci identified so far as responsible for various levels of pungency in *Capsicum* were mapped in the *Pun1* locus of the negative strand in the second chromosome. The $Pun1^{I}$ region present exclusively in the *C. annuum* is located in the starting region of 5' end from the *Pun1* locus. The Cs gene starts from the 1800 bp from the 5' end of *Pun1* locus. *AT3* gene strats from the 1850 bp from the 5' end of the *Pun1* locus and *Catf1* and *Catf2* genes were located at the 2050 bp from the 5' end of the *Pun1* locus.

In the *Pun1* locus a total of 11 ORFs were identified. The deletion of 15 bp in non-pungent varieties identified using MAP1F/R primer is located in this ORF3. Since it is a coding region, the deletion in the coding region will significantly affect the pungency protein formation (Rodriguez Maza *et al.*, 2012). This can be the main reason for the absence of pungency protein in non-pungent lines.

In case of the forward *CS* primer BF7, it is located on the ORF2. The deletion of the specific region for the binding of this primer is the reason for the absence of amplification in the non-pungent lines. The deletion in the coding region of the non-pungent lines will affect the production of pungency proteins. So this can be the reason for the absence of pungency in non-pungent lines. Similarly, the forward primer and reverse primer of CSF1/CSR2 are located in the ORFs 9 and 11 respectively. So when the non-pungent lines are not giving any amplification the reason is that that specific coding region is absent in the non-pungent lines. This deletion can be another reason for the absence of pungency in non-pungent lines. From the above, it is clear that there are several deletions in the coding regions of the *Pun1* locus which affect the level of pungency in *Capsicum* spp. significantly.

In this study, it is very clear that though many workers report many loci which are responsible for pungency, all these loci are lying within the *Pun1* locus. Pungency in chillies is due to the presence of some compounds called capsaicinoids. The capsaicinoids are produced in chillies as a result of the combination of products which are formed in the phenyl propanoid pathway and fatty acid pathway. Phenyl alanine is the precursor for phenyl propanoid pathway and Valine or Leucine is the precursor for fatty acid pathway. Vanillyl amine is the product formed after phenyl propanoid pathway and acyl CoA moieties were the product formed as a result of fatty acid pathway. When both the vanillyl amine and acyl CoA moieties were combined in the presence of putative acyltransferase enzyme formed from the genes present on the *Pun1* locus *viz. AT3* gene, *Catf* gene and *CS* gene, capsaicinoid compounds will be formed. Capsaicin and several other related compounds such as dihydrocapsaicin, nordihydrocapsaicin, homohydrocapsaicin and homocapsaicin were contributed to the production of pungency in chillies. In that capsaicin contribute more for pungency principle and its contribution is about 87 per cent of total pungency. This particular capsaicinoid is produced when vanillylamine combines with a specific acyl CoA moiety called 8-methyl 6-enoyl CoA with the help of capsaicin synthase putative acyltransferase from *CS* gene (Sukrasno *et al.*, 2003).

In this experiment, the primers CSF1/R2 and BF7/R9 were amplifying the *CS* gene and among this, CSF1/R2, which resides inside the sequence amplified by the BF7/R9 primer is much more specific and it is giving single, clear, intact band in all the pungent varieties and neither band is present in the non-pungent ones. From this it is concluded that the *CS* gene, which is responsible for the production of major compound in the pungency that is capsaicin, present only in the pungent varieties. The sequence variations in this particular gene can also be a reason for low pungency in chilli which is not producing the capsaicinoid called, capsaicin.

The most striking feature of the present investigation is that after the alignment of all the amplicons produced from all primers in this study, it is

concluded that all pungency related SCAR primers have amplified the sequences coming inside the *Pun1* locus. So, this locus which is present in the chromosome 2 of all the chilli varieties plays a major role in the variation degree of pungency in every line. In the *Pun1* locus itself the region of *CS* gene has a significant influence in the regulation of pungency.

In depth investigation on locus other than *Pun1* are needed for identifying more genes involved in the pungency. Detailed studies based on pungency markers specifically for Vellayani Samrudhi like *Pun1*^{3} fwd/rev1 were helpful for isolation of genes involved in pungency of chilli and can be used in other fields of biotechnology like secondary metabolite production and genetic engineering.

In 2014, whole genome sequencing of the hot chilli was completed by the combined effort of 28 scientists from various institutes (Kim *et al.* 2014). The genome size is approximately four times larger than tomato and consists of 34,903 genes. Their study strongly suggests that chilli pungency originated through the evolution of new genes by unequal duplication of existing genes and owing to changes in gene expression in fruits after speciation. The hot chilli genome provides a strong foundation for further studies using comparative genomics, metabolic engineering and transgenic approaches to unveil the complete pathway of capsaicinoid biosynthesis in *Capsicum* species.

Summary

6. Summary

The study entitled "Molecular characterization of candidate gene for pungency in *Capsicum* spp." was under taken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture during the period from November 2014 - June 2016.

The research work included the objective to understand the molecular mechanisms behind the different levels of pungency in different species of *Capsicum*. Ten *Capsicum* accessions with different levels of pungency were employed in this study. These included two lines of *C. frutescens* (White Kandari and Vellayani Samrudhi), one line from *C. chinense* (Vellayani Thejus) and seven lines of *C. annuum* (Ujwala, Anugraha, Byadagi Dabbi, Byadagi Kaddi, paprika Kt-Pl-19, bell peppers Arka Gaurav and Arka Mohini). SCAR molecular markers specific to pungency character in chilli were used to characterize all the ten chilli genotypes.

The salient findings of the present study are as follows:-

1. The protocol suggested by Rogers and Bendich (1994) was found good for DNA isolation from young and immature leaves of chilli genotypes. The RNA contamination was completely removed through RNase treatment.

2. The quality and quantity of DNA were analysed by NanoDrop^{R} ND-1000 spectrophotometer. The absorbance ratio ranged from 1.78-1.85, which indicated good quality DNA. The recovery of DNA was high and was suitable for SCAR analysis.

3. Five chilli gene specific SCAR primers were subjected to the SCAR analysis (Lee *et.al.*, 2005; Wyatt *et.al.*, 2012; Rodriguez-Maza *et.al.*, 2012).

4. The *Pun1* specific primers MAP1F/R, *Pun1*^{1} fwd1/rev, *Pun1*^{3}fwd/rev1 and CS specific primers CSF1/R2, BF7/R9 were successful to differentiate the pungent and non-pungent genotypes.

5. All the pungency specific markers used in this study were amplified the regions inside the *Pun1* locus because all the loci previously reported for pungency resides in the *Pun1* locus.

6. The markers for pungency from these SCARs were characterized by sequencing.

7. *Pun1* locus present in the chromosome 2 of chilli species is the major player in regulating the production of capsaicinoids.

8. *CS* gene located inside the *Pun1* locus is the gene which produce capsaicinoid synthetase enzyme needed for the production of capsaicin, a key content in capsaicinoid compounds.

9. Deletion and variation of the sequences inside the *Pun1* locus is the reason for varying degree of pungency in different chilli genotypes.

10. Even though the Byadagi varieties are showing deletions in the *Pun1* locus the fruits are showing very mild pungency when growing in Kerala condition because of the influence of medium to high range of Potassium in Kerala soils.

References

REFERENCES

Aluru, M. R., Mazourek, M., Landry, L. G., Curry, J., Jahn, M., and O'Connell' M. A. 2003. Differential expression of fatty acid synthase genes, *Acl*, *Fat* and *Kas*, in *Capsicum* fruit . *J. Exp. Bot.* 54(388):1655-1664.

Aza-Gonazalez, C., Nunez-Palenius, H. G., and Ochoa-Alejo, N. 2011. Molecular biology of capsaicinoid biosynthesis in chili pepper (*Capsicum* spp.). *Plant Cell Rep.* 30:695-706.

Babu, T. P. H. 2000. RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Pipernigrum* L.) plant. M.Sc. (Ag.) Thesis, Kerala Agricultural University, Thrissur, 83p.

Ben-Chaim, A., Borovsky, Y., Falise, M., Mazourek, M., Kang, B. C., Paran, I. and Jahn, M. 2006. QTL analysis for capsaicinoid content in *Capsicum*. *Theor. Appl. Genet.* 113:1481-1490

Bhadragoudar, M. R. and Patil, C. G. 2011. Assessment of genetic diversity among *Capsicum annuum* L. genotypes using RAPD markers. *Afr. J. Biotechnol.* 10(76): 17477-17483

Blum, E., Liu, K., Mazourek, M., Yoo, E. Y., Jahn, M. M., and Paran, I. 2002. Molecular mapping of the *C* locus for presence of pungency in *Capsicum*. *Genome* 45:702-705.

Blum, E., Mazourek, M., O'Connell, M., Curry, J., Thorup, T., Liu, K., Jahn, M., and Paran, I. 2003. Molecular mapping of capsaicinoid biosynthesis genes and quantitative trait loci analysis for capsaicinoid content in *Capsicum*. *Theor. Appl. Genet.* 108:79-86

Bosland, P. W. and Votava, F. J., 2000. *Pepper, Vegetable and Spice Capsicums.* CABI Publishing, p.204.

Chakradhar, B. Pradeep Reddy, N.Srinivas and P.Chandra Obul Reddy (2013) Identification and Validation of an Allele Specific Marker Associated with Pungency in Capsicum spp. T. *J. Biotech. Pharmacy* 7(1): 544-551

Cisneros-Pineda, O., Torres-Tapia, L. W., Gutiérrez-Pacheco, L. C., Contreras-Martín, F., González-Estrada, T., and Peraza-Sánchez, S. R. 2006. Capsaicinoids quantification in chili peppers cultivated in the state of Yucatan, Mexico. *j.food chem.* 10:762-774

Curry, J., Aluru, M., Mendoza, M., Nevarez, J., Melendrez, M., and O'Connell' M. A. 1999. Transcripts for possible capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent *Capsicumspp. Plant Sci.* 148(1): 47–57

FAO. 2012. *Statistics* [*online*]. Available: http:// www.fao.org/statistics. [July 2014].

Gallego, F. J. and Martinez, I. 1996. Molecular typing of rose cultivars using RAPD. *J. Hort. Sci.* 71(6): 901-908

Garces-Claver, A., Fellman, S. M., Gil-Ortega, R., Jahn, M., and Arnedo-Andres, M. S. 2007. Identification, validation and survey of a single nucleotide polymorphism (SNP) associated with pungency in *Capsicum* spp. *Theor. Appl. Genet.* 115:907-916

Keyhaninejad, N., Curry, J., Romero, J., and O'Connell, M. A..2013. Fruit specific variability in capsaicinoid accumulation and transcription of structural and regulatory genes in *Capsicum* fruit. j. Plant Sci. 215: 59-68

Kim, M., Kim, S., Kim, S., and Kim, B. 2001. Isolation of cDNA Clones Differentially Accumulated in the Placenta of Pungent Pepper by Suppression Subtractive Hybridization. *Molecules & Cells* 11 (2):213-219

Lang, Y., Yanagawa, S., Sasanuma, T., and Sasakuma, T. 2006. A Gene Encoding a Putative Acyl-transferase Involved in Pungency of *Capsicum. Breed. Sci.* 56(1): 55-62

Lee, C. J., Yoo, E.Y., Shin, J. H., Lee, J., Hwang, H. S., and Kim, B. D. 2005. Non-pungent Capsicum contains a deletion in the capsaicinoid synthetase gene, which allows early detection of pungency with SCAR markers. *Mol. Cells* 19(2): 262-267

Lee, J. M., Kim, **S.**, Lee, J. Y., Yoo, E. Y., Cho, M. C., Cho, M. R., Kim, B., and Bahk, Y. Y. 2006. A differentially expressed proteomic analysis in placental tissues in relation to pungency during the pepper fruit development. *Proteomics*. 6(19): 5248-5259

Liu, S., Li, W., Wu, Y., Chen, C., and Lei, J. (2013) De Novo Transcriptome Assembly in Chili Pepper (Capsicum frutescens) to Identify Genes Involved in the Biosynthesis of Capsaicinoids. Plos one. 8(1). Available: http://www.plos.org/dx.doi.org/10.1371/journal.pone.0048156.pdf.[272 Jan.2013]

Liu, S., Chen, C., Chen, C., Cao, B., Chen, Q. and Lei, J .2013. RNA-sequencing tag profiling of the placenta and pericarp of pungent pepper provides robust candidates contributing to capsaicinoid biosynthesis. *Plant Cell Tiss. Organ Cult.*110(1): 111-121

Monforte-González, M., Guzmán-Antonio, A., Uuh-Chim, F., and Vázquez-Flota, F .2010. Capsaicin accumulation is related to nitrate content in placentas of habanero peppers (*Capsicum chinense* Jacq.). *j. Sci. Food Agric.* 90(5): 764-768

Naresh, P., Reddy, K. M., Reddy, M. K., and Ravishankar, K. V.2012. Variability in Capsaicinoids content and phylogenetic analysis of AT3, and Acyltransferase gene in Chilli (Capsicum annuum L.). *Veg. Sci.* 34(8):45-54

Park Y. J., Nishikawa, T., Minami, M., Nemoto, K., Iwasaki, T., and Matsushima, K.2015. A low-pungency S3212 genotype of*Capsicum frutescens* caused by a mutation in the *putative aminotransferase* (*p-AMT*) gene. Mol. Genet. Genomics 290(6): 2217-2224

Patel, A. V., Sasidharan, N., Vala, A. G. and Kumar, V. 2011. Genetic relation in *Capsicum annuum* L. cultivars through microsatellite markers: SSR and ISSR. *Electr. J. Plant Breed.* 2(1): 67-76

Prasad, B. C. N., Gururaj, H.B., Kumar, V., Giridhar, P., and Ravishankar, G. A. 2006._Valine Pathway Is More Crucial than Phenyl Propanoid Pathway in Regulating Capsaicin Biosynthesis in *Capsicum frutescens* Mill. *J. Agric. Food Chem.* 54 (18): 6660–6666

Prasad, B. N., Kumar, V., Gururaj, H. B., Parimalan, R., Giridhar, P., and Ravishankar, G. A. 2006. Characterization of capsaicin synthase and identification of its gene (*csy1*) for pungency factor capsaicin in pepper (*Capsicum* sp.). *Proc. Natl. Acad. Sci. USA* 103:13315-13320

Prasad, N. B. C., Shrivastava, R. and Ravishankar, G. A. 2005. Capsaicin. *Evidence Based Integrative Med.* 2:147-166

Purseglove, J. W., Brown, E. G., Green, C. L., and Robbins, S. R. J. 1981. *Spices* Vol.1, Longman Scientific and Technical, New york.

Reddy, U. K., Almeida, A., Abburi, V. L.,Alaparthi, S. B., Unselt, D., Hankins, G., Park, M., Choi, D., and Nimmakayala, P.2014. Identification of Gene-Specific Polymorphisms and Association with Capsaicin Pathway Metabolites in Capsicum annuum L.Collections. Plos one. 9(1). Available: http://www.plos.org/dx.doi.org/10.1371/journal.pone.0086393.pdf.[27 Jan.2014]

Rodriguez-Maza, M. J., Garces-Claver, A., Park, S. W., Kang, B. C., and Arnedo-Andres, M. S. 2012. A versatile PCR marker for pungency in *Capsicum* spp. *Mol. Breed.* 30:889-898.

Rogers, S. O. and Bendich, A. J.1994. *Plant Molecular Biology Manual* (2nd Ed.). Springer Publishing, Netherlands, pp 183-190.

Sandeep, 2007. Genetic variability, correlation, morphological and Molecular diversity in byadagi kaddi and byadagi dabbi Chillies (*Capsicum annuum* L.) Accessions. M.Sc. Thesis. University of Agricultural Sciences, Dharwad, Karnataka. 1-110

Saritnum, O., Minami, M., Matsushima, K., Minamiyama, Y., Hirai, M., Baba, T., Bansho, H., and Nemoto, K. 2008. Inheritance of Few-pungent Trait in chili pepper 'S3212' (*Capsicum frutescens*). *J. Jpn. Soc. Hortic. Sci.*77(3): 265-269

Sghaier, Z. A. and Mohammad, C. 2005. Genomic DNA extraction method from pearl millet (*Pennisetum glaucum*) leaves. *Afr. J. Biotechnol.* 4(8): 862-866

Sota, K., Kosuke, S., Yuri, T., Rihito, T., and Akira, K. 2015. A Comt1 Loss of Function Mutation Is Insufficient for Loss of Pungency in Capsicum. Am.*J. Plant Sci.* 6(8): 1243-1255

Sreenath, H. L., Mniswamy, B. and Naidu, M. M. 1992. Embryo cultureof three interspecific crosses in coffee. *J. Plant Crops* 20: 243-247

Stellari, G. M., Mazourek, M., and Jahn, M. M. 2010. Contrasting modes for loss of pungency between cultivated and wild species of *Capsicum. Nature* 34: 56-60

Stewart, C., Kang, B. C., Liu, K., Mazourek, M., Moore, S. L., Yoo, E. Y., Kim, B. D., Paran, I. and Jahn, M. m. 2005. The *Pun1* gene for pungency in pepper encodes a putative acyl-transferase. *Plant J.* 42:675-688

Stewart, C., Mazourek, M., Stellari, G. M., O'Connell' M. A., and Jahn, M. 2005. Genetic control of pungency in *C. chinense* via the*Pun1* locus. *J. Exp. Bot.* 58(5): 979-991

Tanaka, Y., Hosokawa, M., Miwa, T., Watanabes, T., and Yazawa, S. 2010. Newly Mutated *putative-aminotransferase* in Nonpungent Pepper (*Capsicum annuum*) Results in Biosynthesis of Capsinoids, Capsaicinoid Analogues. *J. Agric. Food Chem. 58* (3): 1761–1767

Techawongstien, S., Khemnak, S., and Theerakulpisut, P.2008. Etermination of the inheritance of pungency in *capsicum* by molecular techniques. *Acta Hortic.*765:133-145

Tewksbury, J. J., Reagan, K. M., Machnicki, N. J., Carlo, T. A., Haak, D. C., Calderon-Penaloza, A. L., and Levey, D. J. 2008. Evolutionary ecology of pungency in wild chilli . *Proceeding of the National Acadamy of Science*, 29-31 Jan. 2008, USA. International Institute of Tropical Agriculture, USA, pp. 27-36.

Thul, S. T. Darrokar, M. P., Shaseny, A. K.,A and Khanuja, S. P. S. 2011. Molecular profiling of genetic variability in *Capsicum* species based on ISSR and RAPD markers. *Mol. Biotechnol.* 22: 429-435

Wettasinghe, R. and Peffley, E. B. 1998. A rapid and efficient extraction method for onion DNA. *Plant Breed.* 117: 588-589

Wyatt, L. E., Eannetta, N. T., Stellari, G. M., and Mazourek, M. 2012. Devolopment and application of a suite of non-pungency markers for the *Pun1* gene in pepper (*Capsicum* spp.). *Mol. Breeding* 30:1525-1529

Zewdie, Y. and Bosland, P. W. 2000. Capsaicinoid Inheritance in an Interspecific Hybridization of *Capsicum annuum* × *C. chinense*. *J. Am. Soc. Hortic. Sci.*125 (4): 448-453

Zewdie, Y., Bosland, P. W., and Steiner, R. 2001. Combining Ability and Heterosis for Capsaicinoids in *Capsicum pubescens*. *HortSci.*36(7): 1315-1317

Annexures

ANNEXURE I

Details of laboratory equipments used for the study

1. High speed refrigerated centrifuge : Kubota 6550, Japan
2. Horizontal electrophoresis system : BIO-RAD, USA
3. Thermal cycler : Proflex thermal cycler
4. NanoDrop^{R} ND-1000 spectrophotometer : NanoDrop^{R} Technologies Inc. USA
5. Gel documentation : Gel Documentation System, BIORAD, USA
6. Water purification system : Millipore, Germany
7. Ice flaking machine : F100 Compact, Ice matics
8. Laminar Air Flow : HML-104, Thermadyne

ANNEXURE II

Reagents for DNA isolation by CTAB method as per Rogers and Bendich (1994)

Reagents:

A. 2X CTAB Buffer:

- 2% CTAB (w/v)
- 100 mM Tris (pH 8)
- 0.5 M EDTA (pH 8)
- 1.4 M NaCl

B. 10% CTAB solution:

- 10 % CTAB (w/v)
- 0.7 M NaCl

C. TE buffer:

- 10 mM Tris (pH 8)
- 1 mM EDTA (pH 8)

ANNEXURE III

Composition of buffers and dyes used for agarose gel electrophoresis

1. **50X TAE buffer (pH 8):**
    - 242g Tris base
    - 57.1 ml glacial acetic acid
    - 100 ml 0.5 mM EDTA (pH 8)

2. **Tracking/ loading dye (6X):**
    - 0.25% Bromophenol blue
    - 0.25% Xylene cyanol
    - 30% Glycerol in water

3. **Ethidium bromide:**
    - The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in dark bottle.

MOLECULAR CHARACTERIZATION OF CANDIDATE GENE FOR PUNGENCY IN *Capsicum* spp.

By

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ABSTRACT OF THESIS

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ABSTRACT

Chilli, also known as "Wonder spice", has been cultivated since 3000 BC. Out of the 21 identified species of chilli, *C. annuum, C. chinense, C. frutescens, C. baccatum* and *C. pubescens* are the domesticated species. It is a major vegetable cum spice crop which can impart pungency, colour and aroma to the human foods. Pungency is one of the most important and peculiar character of all the species belonging to the genus *Capsicum.* Capsaicinoids are the alkaloid compounds which are responsible for pungency in chilli. Because of the nutraceutical properties possessed by these capsaicinoids, it has much importance in manufacturing several drugs. Though so many studies are conducted to understand the genetic mechanisms behind pungency, the gene action responsible for its production is still an enigma.

This experiment was undertaken with the objective to assess the molecular mechanisms behind different levels of pungency in different species of *Capsicum.* The investigations were carried out in ten chilli genotypes namely, Ujwala, Anugraha, Byadagi Dabbi, Byadagi Kaddi, paprika Kt-Pl-19 and bell peppers Arka Gaurav and Arka Mohini (*C. annuum*), Vellayani Thejus (*C. chinense*) and White Khandari, Vellayani Samrudhi (*C. frutescence*). Among the genotypes Anugraha, Ujwala, Vellayani Thejus, Vellayani Samrudhi and White Kandari are pungent lines and Kt-Pl-19, Byadagi Dabbi, Byadagi Kaddi, Arka Mohini and Arka Gaurav are non-pungent lines. Good quality genomic DNA has been extracted from all the genotypes with an absorbance ratio ranging from 1.79 - 1.85 and concentration more than 1000 ng/µl. The DNA was screened with five pungency specific SCAR (Sequence Characterized Amplified Region) primers. Among the five SCAR primers used, three were specific for *Pun1* locus (MAP1F/R, *Pun1*[1]fwd1/rev, *Pun1*[3]fwd/rev1) and two were specific for *CS* (*Capsaicinoid synthetase*) gene (CSF1/R2, BF7/R9). *Pun1* and *CS* are the loci responsible for the synthesis of putative acyl-transferase and capsaicin synthase enzymes leading to the synthesis of capsaicinoids.

The results revealed that MAP1F/R is the most significant primer which gave distinct amplifications in both pungent lines and non-pungent lines. A 15 bp deletion was clearly identified in the non-pungent lines compared to the pungent lines. This result

revealed that the 15 bp deletion in the non-pungent lines is the reason for the absence of pungeny in them. The other two primers *Pun1*^{1} fwd1/rev, *Pun1*^{3} fwd/rev1 gave amplification only for pungent lines in *C. annuum* and *C. frutescence* respectively since *Pun1*^{1}and *Pun1*^{3} are the mutant alleles of *Pun1* locus present in the respective species.

The capsaicin, which is a capsaicinoid compound contributing about 69 per cent of pungency, is produced with the help of the capsaicin synthase enzyme produced from the *CS* gene. The primers specific for the *CS* gene have amplified only in the pungent lines. This result revealed that the nucleotide change in the primer binding region is the reason for the absence of pungency in them. The amplicon sequences of *CS* gene was subjected to *insilico* analysis such as BLASTn and Clustal Omega, which identified that the CS gene whose location was not yet confirmed also resides within the *Pun1* locus. The *insilico* analysis has also proven that the 15 bp deletion identified in the non-pungent lines were located at the ORF3 in the *Pun1* locus. This deletion in the coding region significantly affects the capsaicinoid formation for the pungency.

Irrespective of the species, the deletions occurring in the coding regions of the *Pun1* locus and *CS* gene, are the reasons for the variation of pungency levels in chillies. All the five primers attempted were promising and can be utilized to distinguish the pungent and non-pungent lines even in the seedling stage and hence in marker assisted selection (MAS). Identification of the location of *CS* gene inside the *Pun1* locus is the most striking finding of this study. From this it can be infered that *Pun1* locus, which is in the chromosome 2 of chilli is the major deciding locus for the production of capsaicinoids.