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Variability in Capsaicinoids content and phylogenetic analysis of *AT3*, an *Acyltransferase* gene in Chilli (*Capsicum annuum* L.)

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Abstract : Capsaicinoids are the alkaloids responsible for pungency in chilli fruits. Estimates of capsaicinoids obtained by colorimetric method and HPLC were highly correlated (R^2 =0.996). Data suggest that colorimetric method an effective means for estimating total capsaicinoids in extracts of fresh chilli fruits. Thirty accesions were screened using colorimetric method and considerable variability was observed for total capsaicinoids (0-1.65 %). In order to see sequence polymorphism associated with pungency level, we cloned and sequenced part of *AT3* gene, an acyltransferase belonging to the BAHD family of acyltransferases. Results revealed that abundant indel (insertion and deletion events) and SNP (single nucleotide polymorphisms) in the cloned *AT3* partial sequence. Both *AT3-1* and *AT3-2* are present in many cultivars that suggest paralogous gene lineages.

Introduction

Pungency (heat) is an important quality attribute of hot pepper besides colour (carotenoids). The nature of pungency has been established as a mixture of seven or more homologous braded-chain alkyl vanillylamides, named capsaicinoids (Torabi, 1997). Capsaicinoids are unique to the genus *Capsicum* and is produced in glands on the placenta of the fruit, they are odourless, colourless, flavourless, non-nutrient compounds. Capsaicin, a major alkaloid among capsaicinoids has wide applications in the food, medicine and pharmaceutical industries. As a medicine, capsaicin is known to kill some types of cancer cells (Min *et al*., 2004) and provide relief in arthritis and respiratory ailments (Mazzone and Geraghty, 1999). Chillies have been included in ayurvedic medicines and used as tonic toward off many diseases. The pharmaceutical application of capsaicinoids is attributed to its antioxidant, anticancer, antiarthritic and analgesic properties (Prasad *et al.,* 2006). Pungency in chilli is measured by Scoville scale which was named after Wilbur Scoville. One of the hottest chillies "Naga or Bhut Jolokia" (8, 55, 000 SHU) in the world is found in Tezpur India.

A single locus *C* has been reported to be essential for the control of production of capsacinoids, the pungent allele is dominant over the non-pungent allele (Ben chaim *et al*., 2001; Blum *et al*., 2002). Lang *et al. (*2006) isolated two genes characterized as putative *Capsicum* acyltransferase (*Catf-1* and *Catf-2*) from placenta of pungent pepper, expression of *catf-1* coincided with accumulation of capsaicinods suggesting that *catf-1* is a candidate gene differentiating pungent and non-pungent peppers. Through candidate gene analysis, Stellari *et al.* (2009) identified that the mutation results in the loss of pungency is a deletion in the gene *AT-3* (encodes an acyltransferase protein) which belongs to BAHD family. Fruit quality breeding is a major challenge to hot/ bell pepper breeders and to speed up the breeding process, molecular markers associated with quality traits in chilli could be effectively used to rapidly screen a large number of individual plants. With this objective, nucleotide variation at *AT3* (a candidate gene underlying the *Pun1 locus*) was studied to identify the possible sequence polymorphisms that could be associated with pungency level that can be used for development of molecular marker for pungency.

Materials and methods

Thirty pepper accessions with varied pungency level were selected from germplasm accessions for analysis of total capsaicinoids (Table 1).The plants were grown in field during 2009 *kharif* season (eastern dry zone of Karnataka state, at 12^0 58´ north latitude, 77^0 45´ east longitude and at an altitude of 930 meters above the mean sea level and average rainfall of this area is about 800mm)

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and all standard cultivation practices recommended for the locality were practiced. Fruit samples were harvested at full ripe stage and were dried in the oven at 60^0C for 36 h, ground in an electronic grinder, and passed through a 0.5mm sieve. Total capsaicinoids content of chilli lines (Table 2) was determined using colorimetric method as described by Bajaj *et al.*, (1980) and representative seven chilli lines was also determined using HPLC method (Suzuki, 1984) to compare sensitivity of colorometric method.

Colorimetric method: 0.5g dry chilli powder was weighed 10ml dry acetone was added into the glass stoppered test tube and kept overnight for extraction. Next day samples were centrifuged at 10000rpm for 10min and 1ml of the clear supernatant was taken into a test tube and evaporated to dryness in a hot water bath. The residue was dissolved in 5ml of 0.4% of NaOH solution and 3ml of 3% phosphomolybdic acid was added. The contents were shaken and left undisturbed for 1hr. After 1hr the solution was quickly transferred into centrifuge tubes to remove any floating debris, and then centrifuged at 5000rpm for 15min. The clear blue coloured solution was directly transferred into the cuvette and absorbance was read at 650nm along with a reagent blank. A standard graph was prepared using 0-200 µg pure capsaicin. Simultaneously 0.2, 0.4, 0.6, 0.8 and 1ml of working standard solution were taken into new test tubes and proceeded as mentioned above. Percent capsaicin calculated using the formula mentioned below;

$$\% \text{ capsaicin} = \frac{\mu g \text{ capsaicin X } 100 \text{ X } 100}{1000 \text{ X } 1000 \text{ X } 1 \text{ X } 0.5}$$

Percent capsaicin values were converted to SHU (Scoville Heat Units) multiplying the pepper dry weight concentrations by the coefficients of heat value 1,60,00,000 for capsaicin (Govindarajan *et al.,* 1977).

HPLC method: Two grams of dried powder was weighed and boiled with 95% ethanol for 30min for extraction; the final extract was made up to 50ml. The mixtures were filtered with filter paper, and then with a syringe driven filter (RanDisc Nylon 0.22µm). A 20µl aliquot of flow through solution (capsaicinoid extract) was analyzed by high performance liquid chromatography (HPLC) using a GEMINI 5µm C18 110A column (100mm (L) x 1.0mm (D) (Phenomenex Chemicals) with a mobile phase of 40% acetonitrile and 60% distilled water at a flow rate of 1.0ml/ min at 28^oC. The UV absorbance at 205nm was measured. The peak areas were converted to mg based on the standards.

Primers designing and PCR amplification: Genomic DNA was extracted from young leaves using CTAB method. The available gene sequences of *Capsicum acyltransferase* (*AT3-2*) were retrieved from the public databases, NCBI, multiple alignments of sequences has been done using CLUSTAL-W software, conserved regions were selected and based on which forward and reverse primers (C*atf*-F aaggacttgccttgggcgaa, C*atf*-R ctgtgttgcacatttgaaaaga) were designed manually to amplify 840bp of *AT3-2*. PCR analysis were performed in 25 µl reaction volumes containing 2.5µl of 10X PCR buffer, 1 unit Pfu Taq DNA polymerase, 3µl of 1mM dNTPs, 2.5µl of 5µM forward primer, 2.5µl of 5 µM reverse primer, 11.25µl of dH2O, and 3µl of 20ng/µl DNA template. The PCR profile comprised initial denaturation of 4 min at 95°C, followed by 35 cycles of 94°C for 1 min, 64°C for 1min, 72°C for 1 min and final extension of 5 min at 72°C. PCR products were separated on 2% agarose gel.

Cloning and sequencing: For sequence analysis, Bhut Jolokia (8,55,000 SHU), Habanero (3,00,000 SHU), EC631844 (1,60,000 SHU), PBC495 (1,10,000 SHU), PBC521 (1,15,000 SHU), PantC-1 (90,000 SHU), LCA 334 (95,000 SHU), Kashi Anmol (45,000 SHU), Arka Abhir (20,000 SHU), Arka Mohini (0 SHU) and PBC 1022 (0 SHU) were selected and amplified products were cloned. The DNA fragment were recovered using gel extraction kit (Cat# 28704, QIAGEN, GmbH, Hilden, Germany). The PCR amplified DNA fragment was cloned into the plasmid vector pTZ57R/T using InsT/A clone PCR Product cloning kit (Cat#K1214, MBI, Fermentas) following the manufacturer's instruction. The T/A cloning method is suitable for cloning of PCR fragments amplified with primers that carry dG or dC at their 5' ends. Ligation was carried out at 22^0C for 16 hrs. The ligation mixture was used for transformation. Plasmids were isolated from positive clone cultures and the plasmids were sent for sequencing (Eurofins Biotechnologies Pvt. Ltd. USA) and were sequenced in both directions with respective primers using ABI3700 DNA analyzer (Applied Biosystems, USA).

SNP identification and analysis: The contigs for each genotype were generated using both forward and reverse sequence chromatograms with the help of Bioedit programme. The contig sequences were subjected to BLAST in the NCBI website. Later, these sequences were used to develop multiple sequence alignment (MSA) using online ClustalW programme (http:// www.ebi.ac.uk/tools/ clustalw2/index.html). The MSA files were used to analyze the presence of SNP specific to genotype.

Results and Discussion

Variability of total capsaicinoids in Capsicum *accessions*

Estimates of capsaicinoids obtained by colorimetric method and HPLC were highly correlated (R^2 =0.996) (Table 1 and Fig. 1). The colorometric method using phosphomolybdic acid reagent showed consistent results, which is cheap, easy and can be effectively used for screening large samples. Bhut Jolokia and Habenaro fruits were not analyzed due to insufficient sample size. As expected there is considerable variability for levels of total capsacinoids (Table 1) ranged from 0% (Arka Mohini and PBC1022) to 1.65% (EC631817, a small fruited line of *Capsicum chinense*). Similar high variation was reported by Bajaj *et al.* (1980), Anu and Peter (2000), Sathiyamurthy *et al.,* (2001), Singh *et al.* (2003), Kaur and Singh (2008) and Thul *et al.* (2009). Further the pungent lines identified in this study can be used in breeding for high nutraceutical value.

Table 1. Mean value of total capsacinoids in chilli accessions

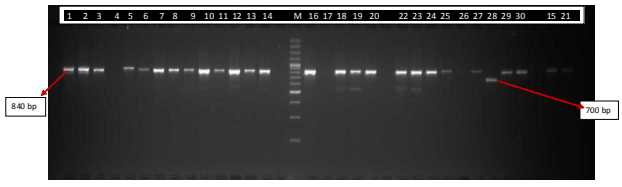
Accession	*Capsicum* spp.	Total capsaici-noids (%)	Total capsaici-noids (SHU))
Bhut Jolokia	*chinense*	NA	NA
Habanero	*chinense*	NA	NA
EC631803	*chacoense*	1.05	168000
EC631817	*chinense*	1.65	264000
EC631829	*chinense*	0.81	129600
EC631844	*frutescens*	1.34	214400
PBC495	*annuum*	0.675	108000
PBC 521	*annuum*	0.7	112000
PBC 569	*annuum*	0.659	105440
CHI Var 4	*annuum*	0.71	113600
Perennial	*annuum*	0.613	98080
Pant C1	*annuum*	0.57	91200
PMR14	*annuum*	0.56	89600
LCA334	*annuum*	0.652	104320
VN2	*annuum*	0.45	72000
ICPN11/7	*annuum*	0.324	51840
PBC 80	*baccatum*	0.208	33280
Kashi Anmol	*annuum*	0.26	41600
ICPN 11/2	*annuum*	0.255	40800
MS1(P)	*annuum*	0.235	37600
Byadagi Kaddi	*annuum*	0.19	30400
Byadagi Dabbi	*annuum*	0.18	28800
Arka Abhir	*annuum*	0.135	21600
EC631773	*annuum*	0.196	31360
GCVMV2	*annuum*	0.1	16000
Arka Mohini	*annuum*	0	0
PBC 1022	*annuum*	0	0
Ornamental	wild unknown	0.0034	544
EC631789	*annuum*	0.125	20000
EC631799	*annuum*	0.322	51520
Mean		0.465	
C.D1%		0.083	

Table 2. Total capsaicinoides and its components of chilli accessions

Accession	Total capsaicinoids (%)		Components of capsaicinoids measured through HPLC (%)		
	Colori-metric method	HPLC	Capsiacin	Nordihy dro-capsaicin	Dihydro-capsaicin
Kashi Anmol	0.26	0.238	0.024123	0.144603	0.07005
Ornamental Chilli	0.003	0.007	0.001203	0.002635	0.003753
PBC 495	0.675	0.618	0.072198	0.309088	0.236578
PBC 521	0.699	0.656	0.060329	0.41435	0.181766
Arka Abhir	0.135	0.138	0.010218	0.08282	0.045063
LCA 334	0.65	0.603	0.044454	0.356367	0.201588
Pant C1	0.567	0.563	0.032828	0.267965	0.262288

Allelic variation at AT3 *gene*

The 5' 3' primers amplified a single, specific band of 840bp size in all the lines without showing any polymorphism on agarose gel between pungent and non-pungent genotypes. EC631817 (*C. chinense*) and PBC 80 (*C. baccatum*) did not get amplified. Interestingly only ornamental chilli (a wild species) showed 700 bp amplified product, which is a non-pungent (sweet). The gel picture showing the amplification was presented in Fig.1. PCR amplified products of the selected 12 genotypes with varied level of pungency were cloned and sequenced. The sequences showed maximum similarity (99%) to *AT3-2* gene, when the sequences were subjected to BLAST at NCBI but ornamental chilli (wild species) shown similarity to AT3-1. Further, already available nucleotide sequences for *AT3* gene in public domain were also composed. Multiple alignments of the nucleotide sequences were performed and the presence of structural changes in the sequences was found. Several SNPs were found scattered throughout the cloned sequence (Table 3). Stellari *et al.* (2009) also reported that *Pun1* alleles of *C. annuum*, *C. chinense* and *C. frutescens* differed by single nucleotide

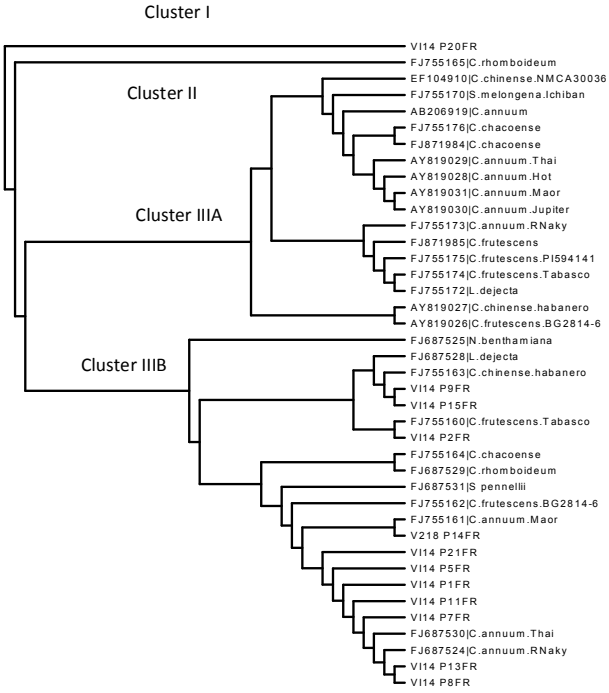


1) Bhut Jolokia, 2) Habanero, 3)EC631803, 4)EC631817, 5)EC631829, 6)EC631844, 7)PBC 495, 8)PBC 521, 9) PBC 569, 10)CHIVAR 4, 11)PERENNIAL 12) PANT C 1, 13) PMR14, 14) LCA334, 15) VN2, 16) ICPN11-7, 17) PBC80, 18)Kashi Anmol, 19) ICPN 11-2, 20) MS1(P), 21) Byadagi Kaddi, 22) Byadagi Dabbi 23) Arka Abhir, 24) EC631773, 25)GCVMV2, 26) Arka Mohini, 27)PBC 1022, 28) Ornamental Chilli, 29)EC631789, 30)EC631799. M-100bp ladder

Fig 1: Gel profile of *Acyltransferase* gene specific primers in 30 chilli accessions with varied level of pungency

polymorphisms scattered throughout the gene and each mutation has an independent origin predating the diversification of the species complex. Bhut Jolokia showed 99% similarity to the Habanero cultivar in the sequenced 840bp except for a single nucleotide change at 769 bp *i.e.* instead of guanine (G), thiamine (T) was present, indicating that Bhut Jolokia and Habanero are derived from *C.chinense*.

Neighbour-Joining phylogenetic tree constructed from the ClustalW alignment of the partial nucleotide sequences of *AT3* gene (Fig. 2) showed three major clusters; Ornamental chilli (a wild species) getting into completely different cluster *i.e.* cluster I. Cluster II included *Capsicum rhomboideum* (GenBank acc. No. FJ755165) in which recombination has occurred between AT3-1 and AT3-2 (Stellari *et al.,* 2009), cluster III contained *Pun1* alleles. The *Pun1* locus was the only locus identified that had a qualitative effect on pungency (Blum *et al.,* 2002). Two sub-clusters were found in cluster III i.e cluster III A included *AT3-1* sequences and cluster III B included *AT3-2* sequences of different lines and showed clear demarcation of *AT3-1* from *AT3-2* (*Pun 1* encodes *AT3*, an acyltransferase belonging to the BAHD family of *acyltransferases*. A tandem duplication of this gene, designated as *AT3-1* and *AT3-2*, respectively). Phylogenetic analysis demonstrated that the paralogous gene lineages of *AT3-1* and *AT3-2* from well supported phylogenetic clades (Stellari *et al.*, 2009). Further, non pungent alleles are scattered and not clustered together into a single clade suggesting multiple origins of the loss of pungency. This result sheds light on *AT3-2,* as only part of the gene sequence was studied further characterization of full length sequence and understanding the *AT3-2* will undoubtedly provide a valuable resource for marker-assisted selection and other manipulations of capsaicin biosynthesis for human use. Our work now focuses on a more detailed understanding of the role of *AT3-2* in capsaicinoid biosynthesis and on the elucidation of the genetic, biochemical and other events that underlie the acquisition,



V14 P20 FR-Ornamental Chilli, V14 P9 FR-Bhut Jolokia, V14 P15 FR-Habanero, V14 P2FR-EC631844, V14 P14 FR-PBC 1022, V14 P21 FR-PBC 521, V14 P5 FR-Arka Mohini, V14 P1 FR-PBC 495, V14 P11 FR-LCA 334, V14 P7 FR- Arka Abhir, V14 P13 FR-PANT C-1, V14 P8 FR- Kashi Anmol.

Fig 2: Neighbour-Joining phylogenetic tree constructed from the ClustalW alignment of the partial nucleotide sequences of *AT3* gene of 12 genotypes cloned with corresponding published sequences from NCBI data base.

Table 3. Identified Single Nucleotide Polymorphisms and InDels in amplified 840bp of AT3-2 gene sequences

Genotype	Nucleotide position (bp)																
	61-64	72	80	108	121	124	125	138	163	209	254	308	328	333	339	344	446-470
Bhut Jolokia	AAGT	T	C	G	T	T	C	C	G	A	T	A	G	A	T	A	25bp InDel
Habanero	AAGT	G	C	G	T	T	C	C	G	A	T	A	G	A	T	A	25bp InDel
EC631844	-	T	C	G	T	C	C	C	G	A	A	A	T	A	T	A	-
PBC495	AAGT	T	T	T	T	T	C	T	A	A	A	G	G	A	T	A	-
PBC521	AAGT	T	T	T	C	T	C	T	A	A	A	G	G	A	C	A	-
Pant C-1	AAGT	T	T	T	T	T	C	T	A	A	A	G	G	A	T	G	-
LCA 334	AAGT	T	T	T	T	T	C	T	A	A	A	G	G	A	T	A	-
Kashi Anmol	AAGT	T	T	T	T	T	C	T	A	G	A	G	G	A	T	A	-
Arka Abhir	AAGT	T	T	T	T	T	C	T	A	A	A	G	G	A	T	A	-
Arka Mohini	AAGT	T	T	T	T	T	C	T	A	A	A	G	G	A	T	A	-
PBC 1022	AAGT	T	T	T	T	T	A	T	A	A	A	G	G	G	T	A	-
FJ687524	-	T	T	T	T	T	C	T	A	A	A	G	G	A	T	A	-
FJ755161	-	T	T	T	T	T	C	T	A	A	A	G	G	A	T	A	-
FJ755163	-	T	C	G	T	T	C	C	G	A	T	A	G	A	T	A	25bp InDel
FJ755160	-	T	C	G	T	T	C	C	G	A	A	A	T	A	T	A	-

continued

Genotype	Nucleotide position (bp)																		
	471	483	477	486	492	548	553	567	585	612	641	645	650	661	741	756	758	769	782
Bhut Jolokia	T	A	A	-	G	G	C	C	T	C	T	A	A	G	T	C	C	T	T
Habanero	T	A	A	-	G	G	C	C	T	C	T	A	A	G	T	C	C	G	T
EC631844	-	C	A	-	T	G	G	C	T	C	T	A	G	G	-	A	G	G	C
PBC495	-	A	A	A	T	T	C	C	T	C	C	A	A	T	-	A	G	G	T
PBC521	-	A	A	A	T	T	C	C	C	C	T	A	A	T	-	A	G	G	T
Pant C-1	-	A	A	A	T	T	C	C	T	C	T	A	A	T	-	A	G	G	T
LCA 334	-	A	A	A	T	T	C	C	T	C	T	A	A	T	-	A	G	G	T
Kashi Anmol	-	A	A	A	T	T	C	C	T	C	T	A	A	T	-	A	G	G	T
Arka Abhir	-	A	A	A	T	T	C	C	T	C	T	A	A	T	-	A	G	G	T
Arka Mohini	-	A	G	A	T	T	C	C	T	C	T	G	A	T	-	A	G	G	T
PBC 1022	-	A	A	A	T	G	C	T	T	T	T	A	A	T	-	A	G	G	T
FJ687524	-	A	A	A	T	T	C	C	T	C	T	A	A	T	-	A	G	G	T
FJ755161	-	A	A	A	T	G	C	C	T	T	T	A	A	T	-	A	G	G	T
FJ755163	T	A	A	-	G	G	C	C	T	C	T	A	A	G	T	C	C	G	T
FJ755160	-	C	A	-	T	G	G	C	T	C	T	A	G	G	-	A	G	G	C

FJ687524- Rnaky, FJ755161-Maor, FJ755163-Habanero, FJ755160-Tabasco

evolution and regulation of this unique biosynthetic capacity in *Capsicum.*

सारांश

कैप्सेइसिनॉएड्स मिर्च के फल में तीखेपन के लिए जिम्मेदार एल्केलॉएड्स हैं। वर्णमापी तरीका और एच.पी.एल.सी. से प्राप्त कैप्सेइसिनॉएड का आकलन बहुत सह–संबंधित (R^2 =0.996) था। आँकड़े सुझाव देते हैं कि वर्णमापी युक्ति मिर्च के ताजे फलों के अर्क में कुल कैप्सेइसिनॉएड का आकलन करने का एक प्रभावी साधन है। वर्णमापी युक्ति का प्रयोग करके तीस एसेसन परखे गये और कुल कैप्सेइसिनॉएड्स की उल्लेखनीय परिवर्तिता (0-1.65%) अवलोकित की गयी। तीखेपन के स्तर के साथ सम्बद्ध अनुक्रम बहुरूपता को देखने के लिए हमने *AT3* जीन, एक एसाइल ट्रान्सफेरेज के बी.एच. डी. परिवार से सम्बन्धित है, का एक भाग क्लोन और अनुक्रमित किया। परिणाम प्रदर्शित करते हैं कि क्लोन किए हुए ।ज3 का आंशिक अनुक्रम में प्रचुर एस.एन.पी. (एकल न्यूक्लियोटाइड बहुरूपता) प्राप्त किया गया। *AT3 -1* और *AT3 -2* दोनों कई किस्मों में उपस्थित हैं जो इनके तकामासिक जीन वंशों का सुझाव देता है।

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