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Short Communication

Candidate markers assay for *Capsicum* pungency

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ABSTRACT

Effectiveness of candidate markers for differentiating the pungent, slightly pungent and non-pungent chilli lines and their use in marker assisted selection programmes was assessed. Two pungent *C. annuum*, one pungent *C. chinense*, two pungent *C. frutescens*, one non-pungent *C. annuum*, two non-pungent *C. annuum* var. *grossum* and two slightly pungent *C. annuum* var. *acuminatum* cultivars were analysed using MAP1 marker for locus *Pun1*, CS and B markers for locus *CS*, Pun1^1 marker for locus *Pun1* of *C. annuum* and Pun1^3 marker for locus *Pun1* of *C. frutescens*. This paper discusses the universal suitability of these candidate markers in MAS programmes for levels of pungency in *Capsicum*. Through sequencing of markers it is further shown that the proposed *CS* gene lies within the *Pun1* locus and since Pun1 markers are missing in non-pungent and slightly pungent lines, we propose that genes other than *Pun1* are involved in pungency mechanism in *Capsicum* spp.

Key words: Hot pepper, capsaicin, MAS, SCAR, *Pun1.*

Pungency offered by the alkaloid Capsaicin is characteristic to the genus *Capsicum*. Pungency varies from the very high levels in bird eye chillies (*C. frutescens*) to absence in paprikas (*C. annuum*). Initially, capsaicin synthesis was supposed to be coded by a single dominant gene *Capsaicin synthase*. Subsequent efforts to characterize this gene has failed and still it remains hypothetical (Prasad *et al*., 4). Alternatively, the candidate loci such as *CS*, *Pun1*, *Pun2*, *Catf-1*, *Catf-2* have been described which are associated with locus *C* in the second chromosome.

The first attempt to design marker for pungency was made by Blum *et al*. (1) when they have developed a CAPS marker linked to the proposed *C locus* using the sequence of fibrillin gene. Through sequence analysis, Lee *et al*. (3) reported that non-pungent peppers have a 2529 bp deletion in the 5′ upstream region of *Capsaicinoid synthetase* (*CS*) gene. Based on these deletions, CS and B series of markers were designed to differentiate the pungent and non-pungent lines. Rodríguez-Maza *et al*. (5) have identified a 15 bp deletion in *Pun1* locus in non-pungent pepper accessions. Allele-specific marker MAP1 generated fragments of 479 and 494 bp in non-pungent and pungent accessions, respectively. Wyatt *et al*. (7) developed sets of deletion based codominant markers for *Pun1^1* alleles of *Capsicum annuum* and *Pun1^3* alleles of *C. frutescens*. The present study was conducted with the objective to validate the suitability of molecular markers for use in molecular breeding of each *Capsicum* species, using accessions with different levels of pungency and belonging to different species.

Five pungent lines belonging to 3 species (*C. annuum*: Anugraha, Ujwala, *C. chinense*: Vellayani Thejus, *C. frutescens*: Vellayani Samrudhi, White Kandari) and five non-pungent *C. annuum* chilli lines (Kt-Pl-19, Byadagi Dabbi, Byadagi Kaddi, Arka Mohini and Arka Gaurav) were used in this study (Table 1).

Good quality DNA was isolated from 10 accessions using the CTAB protocol (Doyle and Doyle, 2). PCR mixture had 50 ng template DNA, 10 pM of each primer, 10 mM deoxyribonucleotide triphosphate, 10x *Taq* polymerase buffer, 10 mM $MgCl_2$ and 5 U *Taq* polymerase (Invitrogen, USA). Thermal cycling included pre-denaturation at 94°C for 5 min. followed by 35 cycles consisting 1 min. denaturation at 94°C, 1 min. annealing at 55-62°C and 2 min. extension at 72°C, followed by final extension for 10 min. at 72°C.

Fragments of *CS* gene amplified using BF7/BR9 primers from five pungent accessions and fragments of MAP1 marker amplified from five pungent and five non-pungent accessions, were eluted, sequenced on Sanger platform and sequences were analysed using BLASTn and Clustal Omega.

MAP1F/MAP1R primer set has given single, intact marker in all accessions. Markers were 494 and 479 bp in sizes in pungent and non-pungent accessions, respectively (Fig. 1A), showing 15 bp deletion in less pungent/ non-pungent ones. Though codominant in nature, the fibrillin gene based CAPS marker reported by Blum *et al*. (1) involves the additional steps of eluting and purifying the PCR product from the gel, digesting it using the restriction enzyme, followed by a second electrophoresis. This long protocol makes

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Table 1. *Capsicum* accessions used in the study

Sl. No.	Accession	Specie	Pungency status	Source
1	Anugraha	*C. annuum* var. *annuum*	Highly pungent	Kerala Agricultural University, Thrissur
2	Ujwala	*C. annuum* var. *annuum*	Highly pungent	
3	Vellayani Thejus	*C. chinense*	Highly pungent	
4	Vellayani Samrudhi	*C. frutescens*	Highly pungent	
5	White Kandari	C. frutescens	Highly pungent	
6	Kt-Pl-19	*C. annuum* var. *annuum*	Non-pungent	IARI, RS-Katrain
7	Arka Mohini	*C. annuum* var. *grossum*	Non-pungent (bell pepper)	IIHR, Bangalore
8	Arka Gaurav	*C. annuum* var. *grossum*	Non-pungent (bell pepper)	
9	Byadagi Dabbi	*C. annuum* var. *acuminatum*	Slightly pungent under hot and humid conditions	
10	Byadagi Kaddi	*C. annuum* var. *acuminatum*	Slightly pungent under hot and humid conditions	

this marker necessary only if the zygosity of the chosen plants with respect to pungency has to be tested. Since the PCR products can not be directly used for MAS, this marker was not included in the present study. However, MAP1 marker is proven to be a good alternative since this act like a codominant marker to differentiate the different alleles, similar to CAPS markers.

Capsaicinoid synthetase (*CS*) gene in pungent chilli was amplified using the primers CSF1/CSR2 and BF7/BR9 (Lee *et al*., 3). CSF1/CSR2 has generated a distinct band of 434 bp in all pungent accessions and this band was absent in less pungent/ non-pungent accessions (Fig. 1B). The primer combination BF7/BR9 has produced distinct amplicon of 900 bp in pungent accessions only (Fig. 1C). The capsaicin, a capsaicinoid compound contributing about 69 per cent of pungency, is synthesised with the help of Capsaicin synthase enzyme from the *CS* gene. Markers with their forward primer landing in region of

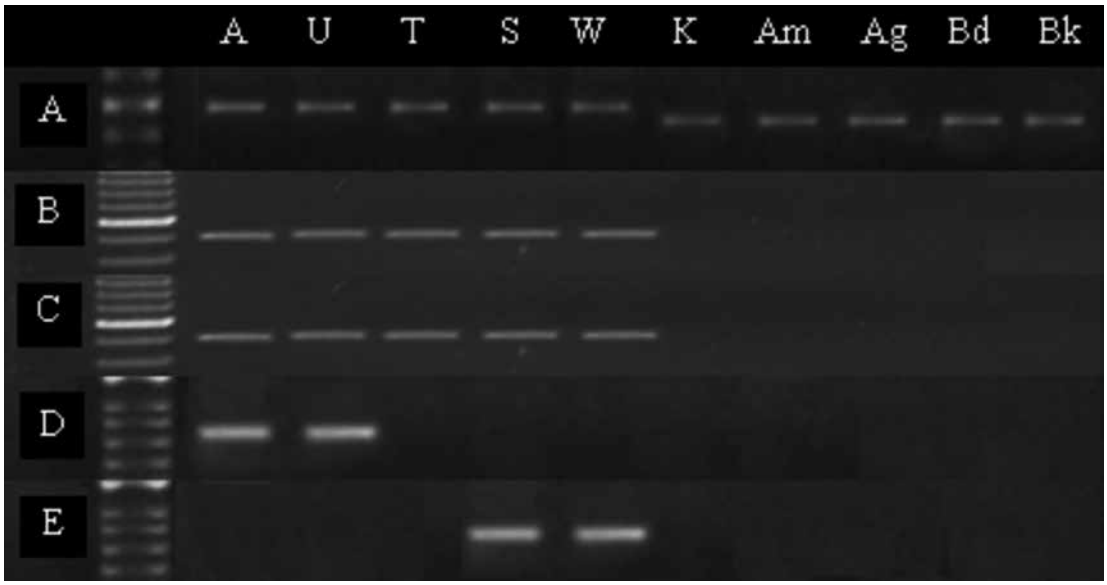

Fig. 1. A - MAP1 marker at 494 bp in pungent and 479 bp in non-pungent or mild-pungent accessions, B - CSF1/R2 marker at 430 bp in pungent lines, C - BF7/R9 marker at 900 bp in pungent lines, D - Pun1^{1} F1/R1 marker at 850 bp in pungent *C. annuum* accessions, E - Pun1^{3} F1/R1 marker at 1000 bp in pungent *C. frutescens* accessions. A - Anugraha, U - Ujwala, T - Vellayani Thejus, S - Vellayani Samrudhi, W - White Kandari, K - Kt-Pl-19, Am - Arka Mohini, Ag - Arka Gaurav, Bd - Byadagi Dabbi, Bk - Byadagi Kaddi

Table 2. SCAR primers used and corresponding markers amplified.

Sl. No.	Primer name	Primer sequence	Annealing temperature (°C)	Marker size (bp)	Target	Target locus	Reported by
1	MAP1 F	5'CCATTAGTCGTTCATTTTTGTTTG3'	55	494 - pungent lines, 479 - non-pungent lines	Pungent and non-pungent lines in all species	*Pun1*	(Rodríguez-Maza *et al*., 6)
	MAP1 R	5'TCTGCCCTTGTTGGATTTTC3'					
2	CS F1	5'ATGGCTTTTGCATTACCATCA3'	57	430	Pungent lines in all species	Capsaicinoid synthetase (*CS*)	Lee *et al*., (3)
	CS R2	5'CCTTCACAATTATTCGCCCA3'					
3	B F7	5'GGGGTTGGGTAGAGGTTGTT3'	57	900			
	B R9	5'GACAAACAATAATGGACGATG3'					
4	Pun1[1] F1	5'TCCTCATGCATCTCTTGCAG3'	60	850	Pungent lines in *C. annuum*	*Pun1*	Wyatt *et al*., 7
	Pun1[1] R1	5'CAAATGGCAGTTTCCCTTCTCTCATT3'					
5	Pun1[3] F1	5'GTAGTTTTTCGGAAATGAAAAGTACT3'	55	1000	Pungent lines in *C. frutescens*		
	Pun1[3] R1	5'CACGCCTTGCCCAGCTTTGTAATCTT3'					

deletion in *CS* gene in non-pungent lines, result the amplification in pungent accessions only. This result revealed that nucleotide deletion within *CS* gene is the reason for the loss of pungency. Absence of this marker in slightly pungent lines shows that the (*CS*) gene is mandatory for high level pungency but there should be other genes which are contributing towards the mild pungency.

The primer combination of *Pun1[1]* F1/R1 detailed by Wyatt *et al.* (7) specifically for *Pun1[1]* locus in *Capsicum annuum* had given intact band of 850 bp, only in *C. annuum* varieties (Anugraha and Ujwala). In less-pungent/ non-pungent *C. annuum* cultivars also this marker was absent (Fig. 1D). *Pun1[3]*F/R1 primers amplified a 1000 bp band, only in *Capsicum frutescens* accessions (White Kandari and Vellayani Samrudhi) (Fig. 1E). Results of SCAR marker evaluation are summarized in Table 2. From these results, it is clear that the *Pun1* locus in *Capsicum* carries different alleles in different species. The difference in pungency levels between *Capsicum annuum* and *Capsicum frutescens* may be due to the variation in the amino acids coded by *Pun1[1]* and *Pun1[3]* alleles.

Analysis of sequence of MAP1 marker fragment from pungent and non-pungent accessions had shown that the 15 bp deletion in non-pungent lines lies in ORF3 of *Pun1* locus. Similarly, using a genome walking strategy, Rodríguez-Maza *et al*. (6) have identified a characteristic 51 bp deletion in the non-pungent *Capsicum* accessions. The amplicon sequences of *CS* gene had shown that this gene, whose location was not yet confirmed, also resides within the *Pun1* locus.

This investigation had shown that irrespective of species, deletions in the coding regions of *Pun1* locus leads to variation of pungency levels. Different alleles of *Pun1* act in different species; in *C. annuum*, *Pun1[1]* and in *C. frutescens, Pun1[3]*. All the five markers studied could be used to distinguish the pungent and non-pungent lines even in the seedling stage and hence in marker assisted selection (MAS). Identification that the *CS* gene resides within the *Pun1* locus is an important finding of this study. All the reported loci for pungency are located within *Pun1* locus and the SCAR primers amplify different regions of *Pun1*. Thus, it can be inferred that *Pun1* locus, which is in the chromosome 2 of chilli is the major deciding locus for the production of capsaicinoids.

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