Current Trends in Biotechnology and Pharmacy
Vol. 7 (1) 544-551 January 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Identification and Validation of an Allele Specific Marker Associated with Pungency in *Capsicum* spp.

T. Chakradhar[1], B. Pradeep Reddy[1], N.Srinivas[1] and P.Chandra Obul Reddy[2]
[1] Centromere Biosolutions, Agri Incubation Park, International Crop Research Institute for Semi Arid Tropics (ICRISAT), Patancheru-502328, Hyderabad, India
[2]Department of Botany, Y.V. University, Kadapa-516003, Andhra Pradesh, India
*For Correspondence - coreddy@yogivemanauniversity.ac.in

Abstract

Pungency or heat in *Capsicum* spp. is due to the accumulation of unique secondary compounds known as capsaicinoids in their placental tissues. Detecting presence or absence of pungency at the nursery stage is a challenging task in CMS based hybrid pepper breeding programs. In this study a DNA sequence possibly related to pungency trait with high similarity to *Pun*1 or *At*3 gene was investigated. Nucleotide alignment of the obtained sequences and corresponding fragment from the data base has revealed a 16bp deletion in *C.annuum* 'Maor'. A multiplex agarose based co-dominant marker was designed to detect the identified polymorphism and named it as *Cen*1. This *Cen*1 marker is validated in a panel of 27 pepper genotypes belonging to *C.annuum*, *C.chinensis*, *C.frutescens* and *C.baccatum* for its wide utility. All these *Capsicum* accessions were correctly discriminated with phenotype. In addition, the ability of *Cen*1 marker to discriminate homozygous and heterozygous plants was demonstrated in F_1 hybrids crossed from a non pungent 'Maor' and a pungent 'Habanero'. The *Cen*1 marker was also associated with phenotypic character in the tested genotypes. Moreover the linkage association of *Cen*1 with *At*3 or *Pun*1 gene has also been discussed. Therefore the developed functional marker in this study will be highly useful in marker assisted selection (MAS) programmes, germplasam characterization and seed purity testing of chilli.

Key words: *Capsicum* spp. Marker assisted selection, *Pun1*, Capsaicinoids, Pungency, Allele.

Introduction

Pungency or heat is unique trait of *Capsicum* genus and is thought evolved to deter mammalian herbivores there by promoting seed dispersal by birds (1). Pungency in peppers is due to the production of group of alkaloids called 'capsaicinoids' in placental tissues of developing fruits approximately twenty days post anthesis. After biosynthesis they are secreted towards the outer epidermal cells and finally accumulated in structures called 'blisters' located on the placental surface (2, 3). Capsaicinoids are synthesized by condensation of branched chain fatty acids and vanillylamine, derived from phenylalanine, (4). Although more than ten different capsaicinoids structures exist (5), 'capsaicin' is responsible for sensation of pungency, accounting for almost 90% of all capsaicinoids (6, 7). In non-pungent pepper fruits vanillyl alcohol will be formed instead of vanillylamine which upon condensing branched chain fatty acid moiety, forms 'capsinoids' the non pungent alkaloids facilitating their application in human medicine as pain relievers (8, 9, 10).

It has been known for almost a century ago that mutations at single genetic locus,*Pun*1 (formerly known as *C*) is responsible for loss of pungency in sweet peppers (11, 12). Several studies with different *Capsicum* population could map *Pun*1 locus to pepper chromosome 2.

Vol. 7 (1) 544-551 January 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Recent studies by candidate gene approach concluded that *Pun*1 encodes for a 'putative acyltransferase AT3' that has a qualitative role in determining pungency (13). Later studies have identified another allelic series *Pun*2 in wild pepper *C.chacoense* and a quantitative role was attributed to it (14). Capsaicin biosynthesis is variable by environmental stress, fruit position and also by the height of the area (15, 16).

Phenotyping of pungency trait is an important task and can be a bottleneck in some pepper breeding programs. To facilitate this process, several methods have been developed from organoleptic tests well known as Scoville Test (17) to analytical methods based on separation techniques as gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled to detection techniques as ultraviolet (UV) or mass spectrometry (MS) (18). These methods have some advantages and/or disadvantages, and they share great requirements of time and labor.

Biased selection pressure for sweet pepper to be used as vegetable during domestication has resulted in large deletions of *At*3 or *Pun*1 gene. So far three different allelic mutations were observed in *At*3 or *Pun*1 gene (19). Among them most prominent and widely exploited one is *pun*1^1 which involves a 2.5 kb deletion spanning the putative promoter region extending through 5'UTR and first exon of coding region (20). The *pun*1^1 allele is specific to *C. annuum* derived cultivars and several markers were identified that co-segregated with this allele (21-24). Another recessive allele of *Pun*1 is *pun*1^2 identified in *C.chinensis* with 4bp deletion in the first exon of *At*3 gene associated phenotypically with absence of blisters in placental epidermal cells (3). A tightly linked marker for *pun*1^2 using florescent primer was developed by Wyatt et al., (19). The third allele *pun*1^3 was reported from *C.frutescens* with 210bp deletion including the stop codon (14).

In this paper, we report a new genetic source of allele that controls pungency. We designed a novel co-dominant functional marker for the identified null allele and attempted to associate with phenotype in *Capsicum* spp. The versatility of developed marker was demonstrated among the panel of pepper genotypes and also in F_1 hybrids.

Material and Methods

For sequencing analysis of candidate gene *At*3 four different pepper accessions were selected based on the differential banding pattern observed in one of the selected plants while genotyping pungency trait using ARMS-PCR marker (25). The samples include the non-pungent *C.annuum* 'Maor', *C.frutescens* 'Grif' and pungent *C.annum* 'SCM-334', *C.chinensis* 'Orange Habanero'. The developed marker was tested in a wide range (twenty seven) of pungent and non pungent pepper genotypes belonging to various species of *Capsicum*. The details of species and cultivar of each genotype is given in Table-1. The *capsicum* accessions studied were obtained from Vibha seeds Ltd, India and the seeds were selfed in the green house conditions between 20-25^{0}C at Yogi Vemana University, Kadapa, A.P, India, using standard horticultural practices. The co-dominant nature of the marker was demonstrated on F_1 hybrids obtained by a cross between *C.annuum* 'Maor' X *C.chinensis* 'Orange Habanero'

Organoleptic test was performed to score the pungency of genotypes under study. At least two persons tasted the matured fruits randomly from three plants per accession. A sample was declared non pungent when all samples tasted were non-pungent. If at least one among the tasted fruit is happened to be pungent then that accession was considered as pungent. However capsaicinoid content was also quantified using ELISA (enzyme linked immunosorbent assay) kit from Beacon Analytical (Portland, ME,USA) according to manufacturer's protocol taking five mature dried fruits from three replicates per accession.

Genomic DNA from young leaf tissue was extracted as per the modified protocol of Doyle

and Doyle (1990) with minor changes (26). The obtained PCR products were gel purified using QIAquick Gel Extraction Kit (Qiagen, USA) and cloned into pJET1.2 vector according to manufacturer's protocol (Thermo scientific, USA). Sequencing of cloned fragments was done with MWG (Eurofins Genomic India Pvt Ltd). A contig was created with the overlapping PCR clones using Geneious v5.3 software (27). Primers used in this study were designed using fast PCR (http://fastpcr.software.informer.com/).

To amplify the *At*3 gene, sequence information already submitted to gene bank was used to design gene specific primers (F.P 5'-CCATGGATTGTTGCTCGGGCCTCC-3' and R.P 5'-CCGTACCGCCCCATTGCGATTCC-3'). Using these primers *At*3 or *Pun*1 gene amplified from selected pepper accessions and sequenced in both direction using the same primers. A co-dominant marker designed specific to the newly identified genetic locus consists of three primers Cen1F.P (5'-CCATTAGTCGT TCATTTTTG TTTG-3'), Cen1 R.P_1(5'-TCTGCCCTTGTT GGATTTTA-3') and Cen1R.P_2 (3'-GCATG TGGTATCATGCATG-5'). The PCR analysis was carried out in 20μl reaction system consisting of 50-100ng of template DNA, 0.5μl of 20μM each primer, 1μl of 20mM dNTPs, 2μl of 10X PCR buffer and 1U of taq polymerase (NEB, USA). A 20mM TMAC (Tetramethyl ammonium chloride) was used for reaction used with *Cen*1 marker. PCR was performed in Bio-rad thermal cycler system1000 (Bio-rad, USA) for 35 cycles and conditions for *At*3 gene amplification include an initial denaturation of 4 min at 94^{o}C, followed by 35 cycles of 94^{o}C/1min, 58^{o}C/1min, 72^{o}C/2min with final extension of 5 min.

PCR cycles for Cen1 marker includes 94^{o}C for 5 min, with 35 cycles of 94^{o}C/1 min, 60^{o}C / 1.30 min, 72 ^{o}C/2min min and a final extension of 8 min at 72 ^{o}C. The amplified products were resolved on 1.5% agarose gel (Lonza,USA) with added ethedium bromide (50ng/ul) in electrophoresis system (Hoefer, Inc. USA) at 80volts containing 1X TAE buffer. The gels were visualized under UV light with an image analysis system GelDoc XR^{+} (Bio rad, USA).

Results and Discussion

We hypothesized that there would be a new source of allele that could be the source of genetic basis for loss of pungency in *Capsicum* spp. other than reported ones (19). To execute this, *Pun*1 or *At*3 was amplified from three *C.annuum* genotypes and one *C.chinensis* using gene specific primers and analysed their sequences. *Pun*1 or *At*3 which encodes for acyltransferase-3 is the only locus that has qualitative role on pungency in domesticated peppers (28). By sequence analysis it come to know that we could amplify another gene '*catf*-2' which has high homology (87%) with *At*3 or *Pun*1 gene (29). It was known from literature that *catf*-2 also belongs to acyltransferase proteins and co-expressed in placental tissue of capsicum along with other gene involved in capsaicinoids biosynthesis (13). Hence we further analysed the obtained sequences by multiple alignment using clustalW program (Fig. 2) and a comparative study with other deposited pepper accessions of *catf*2 and *At*3 from GeneBank (# AB206920, # AY819027.1, # AY819031.1, # AY819030.1 and # EF104910.1) was also done. Alignment of the consensus sequences of different genotypes revealed several SNPs and indels. A 16bp deletion was found in *C.annuum* 'Maor' a non-pungent type between 2,644bp-2,659bp (Fig. 2). Multiple alignment of deduced amino acid sequence of the obtained sequence with other acyltransferases showed highly conserved motif (LVSYYPYAG), characteristic of genes belonging to BAHD super family of acyltrasferases (data not shown) (30). A three primer PCR marker was developed based on identified polymorphism and named it as *Cen*1. While designing the primers a second reverse primer was designed to complement the deleted region so that a 166bp & 494bp bands were observed only in pungent types and 478bp was observed in non pungent types (Fig. 1a & b). The *Cen*1 marker was validated in accessions used for sequencing and also tested on panel of 27 genotypes of pepper

belonging to *annum-chinensis-frutesense* complex of *Capsicum* to reliably differentiate pungent and non pungent types (Table1).

During phenotyping by taste, 9 among 16 *C.annuum*, 4 in *C.chinensis*,3 of 4 in *C.frutescens*, 2 in *C.baccatum* and the single

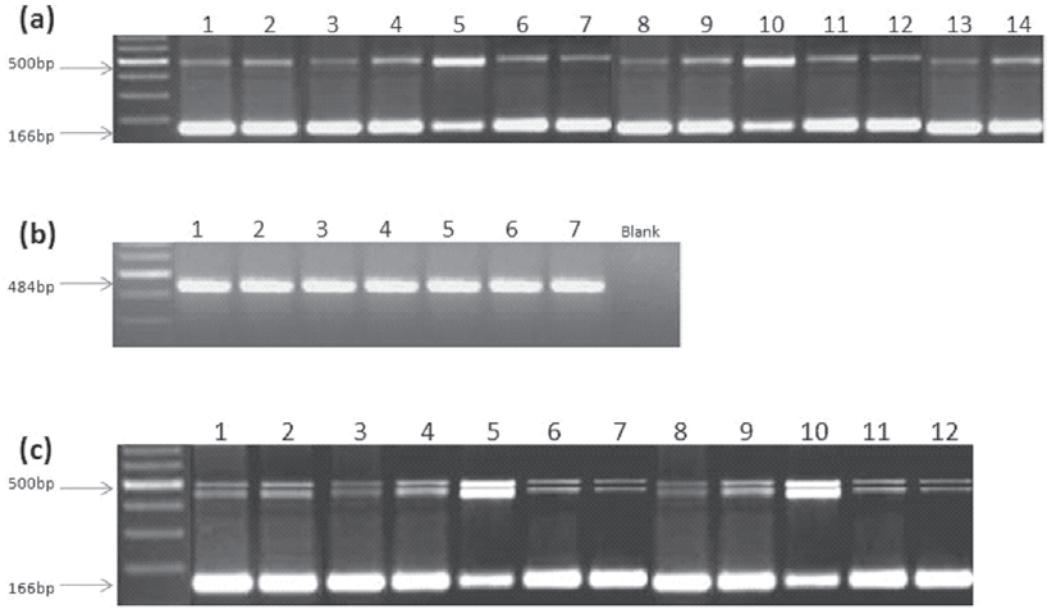

Fig. 1. a) Agarose gel showing genotypic data with *Cen*1 marker .1: 'Jalapeno'2: 'Asain'3: 'CH-19'4: 'SCM334' 5: 'Bukesh' 6: 'BG 2816' 7: 'Habanero' 8: 'NMCA30036' 9: 'Bhut Jolokia' 10: 'C-238' 11: 'Aji' 12: 'Manzano' 13: 'PI 355819' 14: 'Red Savina' **b)** Genotyping data with non pungent accessions 1: 'Maor' 2: 'Bell' 3. 'Paprika' 4: Aleppo' 5: 'Yolo Wondor' 6: 'New Mexico' 7: 'Grif 9182'. **c)** co-dominant genotyping of F1 hybrids developed from a non pungent 'Maor'x 'Habanero.

```
Maor        AAATGAGATGATCATATACT----------------CCACATGCAGCAGGCAGAGGTCCT
Grif9185    AAATGAGATGATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCT
SCM334      AAATGAGATGATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCT
Habanero    AAATGAGATGATCATATACTTCCAACATGCATGATACCACATGCAGCAGGCAGAGGTCCT

Maor        CAATTCGACATGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACG
Grif9185    CAATTCGACATGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACG
SCM334      CAATTCGACATGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACG
Habanero    CAATTCGACATGTTAACAAGCCTGGGCTATTAGTCTATTTGTAGAGACTACTCTTAAACG
```

Fig. 2. Multiple sequence alignment of four selected genotypes: C. annuum "Maor', C. annuum 'SCM334' C.chinensis "Orange Hebenero' and C.frutescens 'Grif9185'. The part dotted denote the deletion portion.

genotype of *C.pubescens* turned out to be pungent both in organoleptic as well ELISA based tests. During organoleptic test heat sensation was observed in pungent accessions 10 days after pollination (DAP) and found decreased gradually 20 days after DAP, indicating strict developmental regulation of genes involved in capsaicinoid biosynthesis. A further study is required to identify the gene that play qualitative role in pungency. *Pun*1 ARMS-PCR marker identified previously by Garcer-Claver was used to screen the same genotypes to know the accuracy of the markers in predicting the phenotype (25). Few of the accessions which assessed as pungent by both organoleptic and HPLC were genotyped as non pungent with ARMS-PCR marker (data not shown) where as the *Cen*1 marker developed in this study revealed perfect association with the phenotype with two exceptions, one in *C.frutescens* and another in *C.baccatum* 'C-238'. The result implies that the deletion may be unique to *C.annuum* spp occurred as result of duplication in a recent evolutionary event. Domestication of *Capsicum*

Table 1. Capsicum spp. Genotypes and their respective phenotyping for pungency obtained with *Cen*1 marker

S.No	Samples of germplasam and F1 hybrids of *C.annuum* 'Maor' X *C.chinensis* 'Habanero'	Phenotype	Genotype
1	*C. annuum var. annuum* cv.'Aleppo'	Non pungent	B
2	*C. annuum var. annuum* cv.'New Mexico'	Non pungent	B
3	*C. annuum var. annuum* cv. 'Jalapeno'	Pungent	A
4	*C. annuum var. annuum* cv. 'Bell'	Non pungent	B
5	*Capsicum annuum* var. *glabriusculum*	Pungent	A
6	*C. annuum* cv. 'Asain'	Pungent	A
7	*C. annuum* cv. 'Paprika'	Non pungent	B
8	*C. annuum* cv. 'CH-19'	Pungent	A
9	*C. annuum* cv. 'Maor'	Non pungent	B
10	*C. annuum* cv. 'SCM334'	Pungent	A
11	*C. annuum* cv. 'Yolo Wondor'	Non pungent	B
12	*C. annuum* cv. 'Cal wonder orange'	Non pungent	B
13	*C. annuum* cv. 'Bukesh'	Pungent	A
14	*C. annuum* cv. 'Agridulce'	Pungent	A
15	*C. annuum* cv. 'Aviculare'	Pungent	A
16	*C. annuum* cv. 'czech Black'	Pungent	A
17	*C. pubescens* cv. 'Manzano'	Pungent	A
18	*C. frutescens* cv. 'Wild'	Pungent	A
19	*C. frutescens* cv. 'BG 2816'	Pungent	A
20	*C. frutescens* cv. 'Grif 9182'	Non pungent	B
21	*C. frutescens* cv. 'PI 355819'	Pungent	A
22	*C. chinense* cv. 'Orange Habanero'	Pungent	A
23	*C. chinense* cv. 'Red Savina'	Pungent	A
24	*C. chinense* cv. 'NMCA30036'	Pungent	A
25	*C. chinense* cv. 'Bhut Jolokia'	Pungent	A
26	*C. baccatum* var *pendulum* cv. 'C-238'	Pungent	A
27	*C. baccatum* cv. 'Aji'	Pungent	A

species happened independently early during evolution and hence each taxa appear to carry different mutations for non-pungency (16, 31). The reason for not detecting the trait in *C.frutescens* and in *C.baccatum* could be because of existence of different source of alleles that could be responsible for loss of pungency (14). The *Cen*1 marker developed in this study was also used to demonstrate zygosity status of the allele i.e. to distinguish between homozygous and heterozygous plants. The results using the parental lines, 'Moar and 'Habanero' and their corresponding pungent F_1 hybrids revealed the presence of three fragments in F_1 heterozygous plants with amplicons of pungent allele specific 500 & 166 bp and non-pungent allele specific 484bp. (Fig.1 c). All F_1 hybrids exhibited a pungent phenotype indicating the dominant nature of the allele.

Conclusions

Identification of pungent and non pungent genotypes at the seedling stage much earlier than fruit setting is a challenge in pepper breeding programs. This study provides a new allele specific versatile marker (*Cen*1) for the identification of the pungent trait in capsicum. The results, after assessing a large number of capsicum genotypes, have clearly demonstrated the feasibility of this allele specific deletion based co-dominant marker in breeding applications like germplasam screening, genetic purity test and marker assisted back crossing.

References

1. Tewksbury, J. and Nabhan, G.P. (2001). Directed deterrence by capsaicin in chillies. Nature, 412: 403–404.
2. Suzuki, T., Fujiwake, H. and Iwai, K. (1980). Intracellular localization of capsaicin and its analogues, capsaicinoid, in *Capsicum* fruit: Microscopic investigation of the structure of the placenta of *Capsicum annum* var. *annum* cv. Karayatsubusa. Plant Cell Physiol, 21: 839–853.
3. Stewart, C., Mazourek, M., Stellari, G.M., O'Connell, M. and Jahn, M. (2007). Genetic control of pungency in *C.chinensis* via the *Pun*1 locus. Journal of Experimental Botany, 58: 979–991.
4. Curry, J., Aluru, M., Mendoza, M., Nevarez, J., Melendrez, M. and O'Connell, M.A. (1999). Transcrips for possible capsaicinoids biosynthetic genes are differentially accumulated in pungent and non pungent *Capsicum* spp. Plant Sci, 148: 47-57.
5. Mazourek, M., Pujar, A., Borovsky, Y., Paran, I., Mueller, L. and Jahn, M.M. (2009). A dynamic interface for capsaicinoid systems biology. Plant Physiol, 150: 1806–182.
6. Kozukue, N., Han, J.S., Kozukue, E., Lee, S.J., Kim, J.A., Lee, K.R., Levin, C.E. and Friedman, M. (2005). Analysis of eight capsaicinoids in peppers and pepper-containing foods by high-performance liquid chromatography and liquid chromatography-mass spectrometry. J Agric Food Chem, 53: 9172–9181.
7. Choi, S.H., Suh, B.S., Kozukue, E., Kozukue, N., Levin, C.E. and Friedman, M. (2006). Analysis of the contents of pungent compounds in fresh Korean red peppers and in pepper-containing foods. J Agric Food Chem, 54: 9024–9031.
8. Kobata, K., Kawaguchi, M. and Watanabe, T. (2002) Enzymatic synthesis of a capsinoid by the acylation of vanillyl alcohol with fatty acid derivatives catalyzed by lipases. Biosci Biotechnol Biochem, 66:319–327.
9. Luo, X.J., Peng, J. and Li, Y.J. (2011) Recent advances in the study of capsaicinoids and capsinoids. Eur J Pharmacol, 650: 1-7.
10. Sasahara, I., Furuhata, Y., Iwasaki, Y., Inoue, N., Sato, H., Watanabe, T. and Takahashi, M. (2010). Assessment of the biological similarity of three capsaicin

analogs (capsinoids) found in non-pungent chili pepper (CH-19 Sweet) fruits. Biosci Biotechnol Biochem, 74:274–278.

11. Webber, H. (1911). Preliminary notes on pepper hybrids. Am Breeders Assoc Annu Report, 7:188–199.

12. Deshpande, R.B. (1935). Studies in Indian chillies: Inheritance of pungency in *Capsicum annuum* L. Indian J Agric Sci, 5:513–516.

13. Lang, Y., Yanagava, Y., Sasakuma, T. (2006). A gene encoding a putative Acyltransferase involved in pungency of *Capsicum*. Breeding Science, 56: 55-62.

14. Stellari.G.M., Mazourek, M. and Jahn, M.M. (2010). Contrasting modes for loss of pungency between cultivated and wild species of Capsicum. Heredity,104:460-471.

15. Zewdie, Y. and Bosland, P.W. (2000). Evaluation of genotype, environment, and genotype-by-environment interaction for capsaicinoids in *Capsicum annuum* L. Euphytica, 111:185–190.

16. Tewksbury, J.J., Reagan, K.M., Machnicki, N.J., Carlo, T.A., Haak, D.C., Penaloza, A.L.C. and Levey, D.J. (2008). Evolutionary ecology of pungency in wild chilies. Proc Natl Acad Sci, 105: 11808–1181.

17. Scoville, W. L. (1912). Note Capsicum. J. Am. Pharm. Assoc, 1: 453.

18. Wall, M.M. and Bosland, P.W. (1998). Analytical methods for color and pungency of chiles (Capsicum). In: Wetzel D, Charalambous G (eds) Instrumental methods in food and beverage analysis. Elsevier, Amsterdam, pp 347–373.

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

20. Stewart, C., Kang, B.C., Liu, K., Mazourek, M., Moore, S.L., Yoo, E.Y., Kim,B.D .and Paran, I. (2005). The *Pun*1 gene for pungency in pepper encodes a putative acyltransferase. The Plant Journal, 42: 675–688.

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

22. Minamiyama, Y., Kinoshita, S., Inaba, K. and Inoue, M. (2005). Development of a cleaved amplified polymorphic sequence (CAPS) marker linked to pungency in pepper. Plant Breeding, 124: 288—291.

23. 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 synthase gene which allows early detection of pungency with SCAR marker. Mol. Cells, 19: 262-267.

24. Truong, H.T.H., Kim, K.T., Kim, S., Kim, H.R., Cho, M-C. and Woo, J-G. (2009). Development of gene-based markers for the Pun1pungency gene in pepper (*Capsicum* spp.) for marker assisted selection. Hortic Environ Biotechnol, 50: 358–365.

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

26. Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. Focus, 12: 13–15.

27. Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field,

M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T. and Wilson, A. (2010). Geneious v5.3. Available at http://www.geneious.com.

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

29. Kim, M., Kim, S., Kim, S. and Kim, B.D. (2001). Isolation of cDNA clones differentially accumulated in the placenta of pungent pepper by suppression subtractive hybridization. Mol. Cell, 11: 213–219.

30. Pierre,St, B. and De Luca, V. (2000). Evolution of acyltransferase genes: origin and diversiûcation of the BAHD superfamily of acyltransferases involved in secondary metabolism. Recent Adv. Phytochem. 34, 285–315.

31. Pickersgill, B. (2007). Domestication of plants in the Americas: insights from Mendelian and molecular genetics. Ann Bot, 100: 925–940.