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Allelic variation at *capsanthin capsorubin synthase* gene for ripening fruit color in chilli (*Capsicum annuum* L.)

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Abstract

Twenty six accessions of *Capsicum annum* with varied ripening color were screened for total carotenoids (0.004-0.47%), red carotenoids (0-0.27%) and yellow carotenoids (0.004-0.2%) and also to obtain some new or useful alleles associated with ripening color in chilli, using gene-specific marker. Considerable genetic variability for carotenoids was observed along with the allelic variations of candidate gene Capsanthin capsorubin synthase (Ccs). The results revealed that *Ccs* gene coding region was present in yellow pepper line LCA 1068 (Aparna) suggesting *Ccs* gene deletion is not a prerequisite for change in color from red to yellow. *Ccs* coding region contained no introns but did exhibit polymorphism among chilli lines studied resulting in amino acid changes. Novel allelic variants were found in Byadagi Dabbi (dark red) and LCA1068 (yellow) in the study, which could provide candidate/allele specific molecular markers for selection of lines that may be used in chilli quality breeding for varied colors.

Key words: Allelic variants, *Capsicum*, *Capsanthin capsorubin synthase,* SNP

Introduction

The diverse and brilliant colors i.e. green, yellow, orange and red of hot/bell pepper fruits originate from the carotenoid pigments present in the thylakoid membranes of the chromoplasts produced in the fruit during ripening stage. More than 30 different pigments have been identified in pepper fruits [1]; including the green chloroplasts (a & b); the yellow-orange (lutein, zeaxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin and β-carotene) and the red pigments (capsanthin, capsorubin and crytocapsin), which are found only in pepper fruits. The red color in pepper comes from the carotenoides, capsanthin and capsorubin, while the yellow-orange is from β-carotene and violaxanthin. Capsanthin, the major carotenoid in ripe fruits contributing up to 60% of the total carotenoids. Capsanthin and capsorubin increase proportionally with advanced stages of ripeness, with capsanthin being the more stable of the two [2]. These carotenoids act as dietary precursors of vitamin A, which plays an important role in the regulation of vision, growth and reproduction [3]. High β-carotene peppers, *Capsicum* spp. could be an alternate solution in the battle to fight vitamin A deficiency [4].

For pepper breeding, the demand for fruits with various colours, besides red, such as yellow and orange, has increased as the carotenoids from pepper are used as natural colorants in the industry. To meet this demand, it is necessary to breed and select lines of peppers objectively and efficiently. Expression levels of the carotenoid biosynthetic genes are directly linked to high levels of total carotenoid accumulation in *Capsicum* [5]. Eight phenotypes in the F_2 segregation of a cross of white with red fruited lines have earlier been reported [6]. There are three independent loci determining fruit color, which are known as *c1*, *c2* and *y*. To chilli breeders, these loci have high economic value since they determine the mature red, orange, and yellow color of commercial pepper cultivars. The last step of the carotenoid biosynthetic pathway in pepper fruits is the conversion of antheraxanthin to capsanthin and violaxanthin to capsorubin and is catalysed by the bifunctional enzyme Capsanthin-Capsorubin synthase [7]. The gene for capsanthin-capsorubin synthase

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(CCS), which plays a role in the conversion, has been considered as a candidate gene for the *y* locus [8] and a genomic clone of the *Ccs* gene was isolated using the *Ccs* cDNA and its sequence was devoid of introns [9]. Although the cDNA sequences for *Ccs* gene is available in GenBank, it is only available for one red bell pepper Yolo Wonder cultivar. In view of this, the objectives of the present study were to characterize the alleles associated with different fruit ripening color and to identify possible sequence polymorphisms in candidate gene *Capsanthin capsorubin synthase*, which could provide candidate molecular markers. The results obtained are presented here under.

Material and methods

Twenty six pepper accessions (Table 1) with varied ripened color were selected from germplasm accessions screened for ripened color. The plants were grown in field during 2009 *kharif* season (eastern dry zone of Karnataka state, India, at 12^{o} 58´ north latitude, 77^{o} 45´ east longitude and at an altitude of 930 meters above the mean sea level and average rainfall of this area is about 800mm) in three replications and all standard cultivation practices recommended for the locality were followed. Fruit samples were harvested at full ripe stage and were dried in the oven at 60^{o}C for 36h, ground in an electronic grinder, and passed through a 0.5mm sieve.

Biochemical analysis

Total red (C_R; capsanthin, capsorubin and capsanthin-5, 6-epoxide) and yellow/orange (C_Y; zeaxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, β-carotene and cucurbitaxanthin A) carotenoid isochromic fractions were estimated following protocol of spectrophotometric method [10]. Carotenoids were extracted from dried samples by placing 100 mg of dried fine powder in 25ml acetone until the complete exhaustion of all colour in the dark. The optical density of samples was measured at two wavelengths, 472 and 508nm using UV/V Spectrophotometer. Following formulae were used.

$$C_R\ (\mu g/\ ml) = \frac{A_{508} \times 2144.0 - A_{472} \times 403.3}{270.9}$$

$$C_Y\ (\mu g/\ ml) = \frac{A_{472} \times 1724.3 - A_{508} \times 2450.1}{270.9}$$

Spectrophotometery, was used as an alternative to HPLC as it is cheap, fast and so can be effectively used to screen larger population. In fact, difference below 5% between the estimates in carotenoids by HPLC and spectrophotometric method were reported.

Gene specific primers, PCR amplification and cloning

Genomic DNA was extracted from young leaves using CTAB method. Ccs gene specific primers obtained from literature (7) i.e CCS-F (ccttttccatctcctttccat) CCS-R (aaggctctctattgctagattgcccag) were used. 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 94°c, followed by 35 cycles of 94°c for 1 min, 60°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 and selected lines products were cloned into the plasmid vector pTZ57R/ T. Plasmids were isolated from positive clone's culture and were sequenced in both directions with respective primers at Eurofins Biotechnologies Pvt. Ltd. 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 (1) 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

Considerable variability for levels of total carotenoids, yellow and red carotenoids among accessions was recorded (Table 1). For total carotenoids in the fruits ranged from 0.004% in (LCA1074 : pale yellow colored fruit) to 0.47% (Byadagi Dabbi dark red fruited line), red carotenoids ranged from 0% in all yellow accessions to 0.27% in Byadagi Dabbi, whereas yellow carotenoids (includes β-carotene and β-criptoxanthin) ranged from 0.004% in LCA1074 to 0.2% in Byadagi Dabbi. In fruits of red *Capsicum,* during ripening process the levels of total carotenoids increases due to synthesis of carototenoids such as, capsanthin, capsorubin, β-cryptoxanyhin and zeaxanthin, in contrast, the total carotenoids levels do not increase and remain low during ripening in yellow peppers [11]. A similar trend was

Table 1. Mean carotenoids concentrations in pericarp of mature fully ripe *Capsicum* fruits

Accession	*Capsicum* species	Ripe fruit color	Red carotenoids (%)	Yellow carotenoids (%)	Total carotenoids(%)
Byadagi Dabbi	*annuum*	Dark red	0.27	0.2	0.47
EC631781	*annuum*	Dark red	0.2	0.082	0.282
Kashi Anmol	*annuum*	Dark red	0.16	0.11	0.27
EC631793	*annuum*	Red	0.11	0.09	0.2
Arka Abhir	*annuum*	Red	0.11	0.1	0.21
EC631778	*annuum*	Dark red	0.16	0.095	0.255
EC631783	*annuum*	Dark red	0.16	0.12	0.28
LCA206	*annuum*	Red	0.14	0.077	0.217
PMR14	*annuum*	Red	0.11	0.07	0.18
LCA 1071	*annuum*	Yellow	0	0.017	0.017
EC631786	*annuum*	Red	0.1	0.074	0.174
Sringeri Local	*annuum*	Red	0.05	0.07	0.12
EC631803	*chacoense*	Light red	0.03	0.032	0.062
Pant C1	*annuum*	Light red	0.07	0.046	0.116
EC631785	*annuum*	Orange red	0.01	0.044	0.054
PBC 80	*baccatum*	Orange	0	0.114	0.114
Ornamental	unknown	Orange	0.01	0.05	0.06
EC631815	*chinense*	Very light red	0.02	0.013	0.033
LCA1083	*annuum*	Yellow	0	0.013	0.013
LCA 1074	*annuum*	Pale yellow	0	0.004	0.004
LCA 1068	*annuum*	Yellow	0	0.033	0.033
LCA 1069	*annuum*	Yellow	0	0.045	0.045
LCA 1070	*annuum*	Yellow	0	0.045	0.045
JCA 283	*annuum*	Red	0.07	0.08	0.15
LCA 1081	*annuum*	Light yellow	0	0.077	0.077
EC631778	*annuum*	Dark red	0.16	0.095	0.255
Mean			0.0746	0.0691	0.1437
S.Em	-	-	0.00754	0.005	0.00817
C.D@1%	-	-	0.0286	0.0192	0.031

noticed in the present study showing that the total carotenoid content was highest in dark red lines (Byadagi Dabbi), followed by light red (EC 631815) and all non-red/ yellow peppers were found to have lower total carotenoids content (LCA1081). All the accessions with yellow coloured fruits did not contain red carotenoids content. Variations in respect of carotenoids have been earlier observed in *Capsicum baccatum* pubescens [12].

The 5'-3' end primers amplified a single, specific band of 1473 bp size in all red fruited lines and not in orange and yellow fruited lines except in LCA 1068 (Aparna) which share a yellow ripe fruit colour. PCR amplified products of three genotypes *viz.*, Byadagi Dabbi (Dark red), EC 631815 (light red) and LCA 1068 (Aparna; yellow) were cloned and sequenced and available nucleotide sequences for *Ccs* gene in public domains were downloaded by searching NCBI database. GenBank contained the Ccs mRNA sequence 499 amino acids long amplified from Yolo Wonder red fruited pepper (X77289) and other sequences are of

orange cultivars, wildtype Ccs sequences deposited (4). Multiple alignments of the nucleotide sequences from the coding regions were performed and the presence of structural changes in the sequences was found (Table 2). Yellow fruited line, LCA 1068 (Aparna) coding region sequence showed 99% similarity to the Byadagi Dabbi (dark red fruited line) except for a single nucleotide change at 935bp with adenine (A) substituting guanine (G). And several SNPs were observed in EC 631815 (light red fruited line which is *Capsicum chinense* accession) spanning throughout the coding region of the gene. A SNP was found in Byadagi Dabbi *i.e.* guanine (G) substituting adenine (A) at 1273bp when the sequences were compared with Yolo Wonder (red fruited) sequence. Multiple alignment of the deduced amino acid sequences were also performed (Fig. 1) and the presence of amino acid changes was observed in the sequences (Table 3). In yellow fruited line LCA 1068 (Aparna), R312K (Arginine 312 Lysine) was present, which might have resulted in change in phenotypic expression from red to yellow color. In an exotic accession EC 631815 (light red fruited line) belonging to *C. chinense* many specific non-silent changes and two amino acid changes i.e., G79R (Glycine 79 Arginine) and K229M (Lysine 229 Methionine) were recorded, whereas in Byadagi Dabbi, K425 E (Lysine 425 Glutamic acid) was present.

The relationship between the presence of structural genes for carotenoid biosynthesis and phenotypic variability of fruit colors at ripe stage has been studied using genetic approaches in diverse *Capsicum* spp. [13-14] and these studies have reported that *Ccs* gene either deleted or absent in yellow and

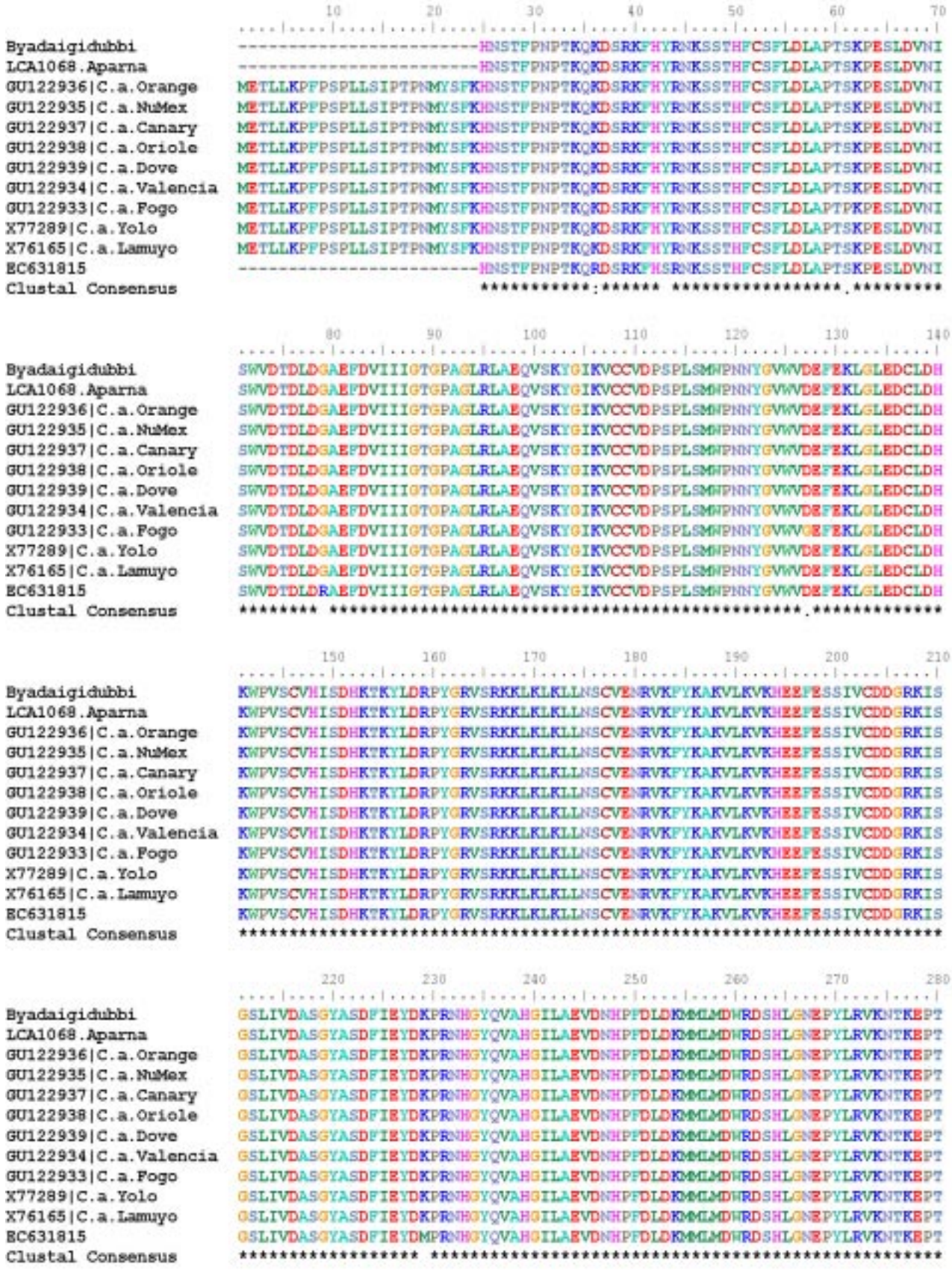
Table 2. Single Nucleotide Polymorphisms identified in coding region sequence of Capsanthin capsorubin synthase gene

Genotype	Nucleotide position (bp)														
	107	128	181	235	312	380	630	686	714	935	1089	1265	1291	1273	1293
Byadagi Dabbi	A	A	T	G	T	A	C	A	T	G	T	C	A	**G**	T
EC631815	G	C	T	C	A	A	T	T	T	G	C	C	A	A	C
APARNA	A	A	T	G	T	A	C	A	T	**A**	T	C	A	A	T
GU122937	A	A	T	G	T	A	C	A	T	G	T	C	G	A	T
GU122936	A	A	T	G	T	A	C	A	T	G	T	C	G	A	T
GU122939	A	A	T	G	T	A	C	A	T	G	T	C	G	A	T
GU122938	A	A	T	G	T	A	C	A	T	G	T	C	G	A	T
GU122934	A	A	T	G	T	A	C	A	T	G	T	C	G	A	T
GU122935	A	A	T	G	T	A	C	A	T	G	T	C	G	A	T
GU122933	A	A	C	G	T	G	C	A	C	G	T	-	A	A	T
X77289	A	A	T	G	T	A	C	A	T	G	T	C	A	A	T
X76165	A	A	T	G	T	A	C	A	T	G	T	C	A	A	T

Table 3: Identified amino acid substitutions in the Capsanthin capsorubin synthase gene coding region

Genotype	Amino acid position (bp)						
	61	79	127	229	312	425	431
Byadagi Dabbi	S	G	D	K	R	**E**	T
EC631815	S	R	D	M	R	K	T
APARNA	S	G	D	K	**K**	K	T
GU122937	S	G	D	K	R	K	A
GU122936	S	G	D	K	R	K	A
GU122939	S	G	D	K	R	K	A
GU122938	S	G	D	K	R	K	A
GU122934	S	G	D	K	R	K	A
GU122935	S	G	D	K	R	K	A
GU122933	P	G	G	K	R	K	A
X77289	S	G	D	K	R	K	T
X76165	S	G	D	K	R	K	T

GU 122937- Canary orange, GU122936-orange Grande, GU122939-Dove, GU122938- Oriole, GU122934 Valencia, GU122935- NuMex Sunset, GU122933-Fogo, X77289-Yolo Wonder and X76165-Lamuyo


Byadaigidubbi
LCA1068.Aparna
GU122936|C.a.Orange
GU122935|C.a.NuMex
GU122937|C.a.Canary
GU122938|C.a.Oriole
GU122939|C.a.Dove
GU122934|C.a.Valencia
GU122933|C.a.Fogo
X77289|C.a.Yolo
X76165|C.a.Lamuyo
EC631815
Clustal Consensus

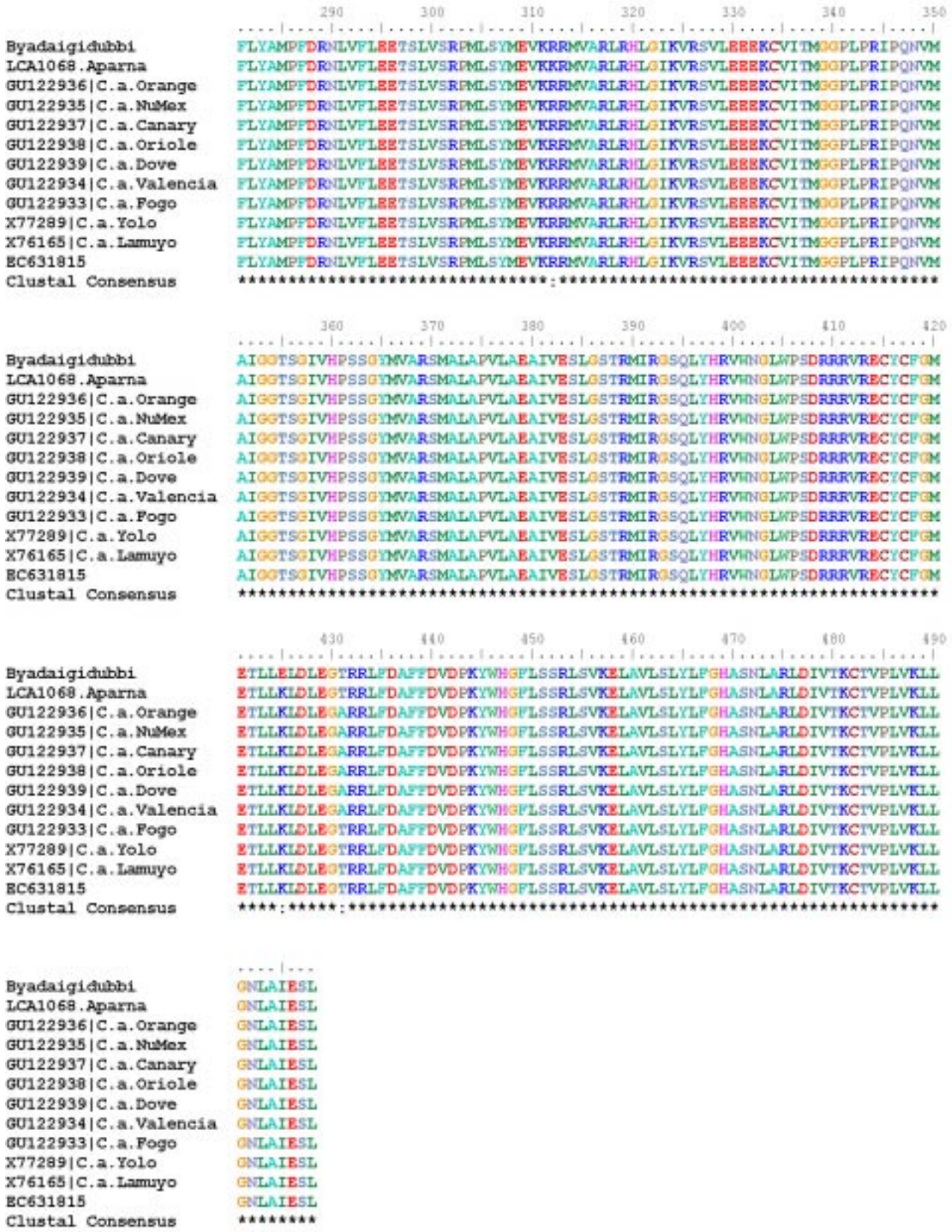


Fig.1. Multiple alignment of deduced amino acid sequences of *Ccs* coding region with corresponding published sequences

orange colored pepper fruits. Absence of *Ccs* gene in two yellow cultivars *i.e* Jaune de Pignerolle and Golden Summer [7] and *Ccs* gene deletion in cultivars with yellow fruits at 211bp and 220bp from 3' end were reported. However, in the present study it was found that *Ccs* gene coding region was also present in yellow fruit coloured line, LCA 1068 (Aparna; selection released from Horticulture Research Station, LAM farm, Guntur). The findings of the present study are in accordance with those of Ha *et al*. [11] who reported for the first time that coding and promoter regions are present in yellow peppers, but not in white peppers. Presence of *Ccs* gene coding region was reported in the seven orange cultivars studied and a novel allele *Ccs-3* variant (contained a deletion of cytosine at nucleotide position 1283 of gene) from Fogo cultivar (orange fruited) showing higher â-carotene content was recovered recently [4]. Based on the present study, it can be concluded that *Ccs* gene coding sequences were present in yellow and orange peppers. A novel allele was recovered from Byadagi Dabbi (dark red fruited with high total carotenoids, C^R and C^Y content) a popular land race being cultivated in Karnataka, India which is valued in domestic and international market for its unique traits like high color value and low pungency. These *Ccs* variants could provide candidate molecular markers for selection of pepper lines with high dark red color and yellow carotenids. These novel allelic variants can be effectively used in breeding for cultivars with higher pro-vitamin A bioactive compounds that could help in alleviating vitamin A deficiency and providing nutritional security in developing countries.

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