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Identification of molecular markers associated with lycopene and carotenoid contents in tomato

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ABSTRACT

Tomato, one of the major fruit vegetables consumed all over the world, is an important source of micronutrients and, lycopene, an antioxidant that neutralizes the reactive oxygen species derived from free radicals. Twenty eight tomato varieties were grown following standard cultivation package. Lycopene content was high in variety Ruchi (105.41µg/g) and lowest was in Tomato Stone (10.53 µg/g). $OPC4_{950}$ and $OPC4_{300}$ markers showed significant correlation with lycopene by single marker analysis. In stepwise multiple regression analysis, three markers accounted for 45.96% relation with lycopene and $OPC4_{950}$ showed maximum association. Jaccard's coefficient analysis showed 46 to 92% genetic diversity among genotypes and correlation coefficient ranged from 66 to 99.98%. These results reveal that $OPC4_{950}$ can be used as potential marker in marker-assisted selection for the improvement of tomato with high lycopene and carotenoids contents.

Key words: Tomato, carotenoids, lycopene, RAPD markers.

INTRODUCTION

Tomato has become one of the most popular and widely grown vegetables in the world. Tomatoes contain significant amount of lycopene, b-carotene, magnesium, niacin, iron, phosphorus, potassium, riboflavin, sodium and thiamine (Jones, 6). Lycopene, is an antioxidant, neutralizes the reactive oxygen species derived from free radicals and the active compound is the carotenoids, lycopene (Ngyuen and Schwartz, 10). Hence, tomato based food products play a significant role in the protection of several forms of cancers (Garcia *et al*., 4; Giovanucci, 5) and vascular diseases (Su *et al*., 19). The antioxidant activity of carotenoids is probably dependent on: (i) number of conjugated double bonds, (ii) end groups (acyclic or cyclic), and (iii) functional groups (Stahl *et al*., 18). Based on these functional groups, the antioxidant potential can be rated as lycopene > a-carotene > b-carotene (Anguelova and Warthesen, 1).

Most of the modern elite tomato cultivars with high productivity are often low in lycopene content. Because of the nutritional importance and role in health care of the larger population world over, it becomes necessary to breed for vegetables with higher secondary metabolites and nutrients which help in the alleviation of the health disorders and diseases. Marker-assisted selection is art of the technology in plant breeding and crop improvement programs, helps in the study of genetic diversity, mapping, gene tagging, QTL analysis, fingerprinting and identification of suitable parents for breeding programs. RAPD markers are easy to use and have certain advantages compared to other molecular markers. DNA based markers are seldom influenced by the environment and are more in number unlike morphological markers, and hence, provide an excellent tool for marker-assisted crop improvement. The present study reports on the evaluation of the tomato genotypes for their carotenoids content, especially lycopene and the markers associated with high lycopene content in selected tomato varieties.

MATERIALS AND METHODS

Twenty eight tomato varieties were grown in the field following the package of practices, for cultivation. DNA was extracted from the young leaves. Fruits at four different stages (green, yellow, orange and red) were collected for estimation of lycopene and other carotenoids. Lycopene and carotenoids were estimated spectrophotometrically (Rodriguez, 13). Two grams of crushed tomato fruit pulp was weighed into 5 ml of hexane, agitated for 10 min. under dark. Centrifuged at 5,000 rpm for 10 min. (4°C) and supernatant was collected. The extraction protocol was repeated for three times, supernatants were pooled and volume made up to 20 ml. The absorbance for different carotenoids was measured at wavelength using a spectrophotometer at (Table 3). The concentration of carotenoids was calculated using their extinction coefficients.

$$Carotenoid = \frac{A \times V_1}{A^{1\%}} \times C^{1\%}$$

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where, A = Absorbance reading of the diluted sample; V_1 = Dilution factor (10x); $A^{1\%}$ = Absorbance of 1% solution; $C^{1\%}$ = Concentration of a 1% solution.

DNA extraction was carried from air-dried tomato leaves as per the method described by Porebski (Porebski *et al*., 11). Hundred mg of leaf powder in 2.0 ml pre-warmed extraction buffer (100 mM Tris pH 8.0 containing 20 mM EDTA, 1.4 M NaCl, 1% β-mercapto ethanol, 3% CTAB) was incubated in water bath at 65°C for 30 min. with periodic shaking. Equal volume of chloroform: iso-amylalcohol (24:1 v/v) was added, vortexed gently and centrifuged at 12,000 rpm for 20 min. at 4°C. Aqueous phase was repeatedly washed with equal volume of chloroform: iso-amylalcohol (24:1 v/v). To the aqueous extract, 1/10th volume of 5M NaCl and equal volume of chilled iso-propanol was added, mixed gently, kept at -40°C for overnight to accentuate DNA precipitation. Centrifuged at 12,000 rpm for 20 min. at 4°C to recover DNA pellet. Pellet was washed with 70% aqueous ethyl alcohol and air-dried. Pellet was dissolved in 100 µl of TE buffer and incubated with 3 µl (10 mg/ml) of RNase for 2 h at 37°C. Washed with equal volume of phenol: chloroform: isoamylalcohol (25:24:1 v/v) and chloroform: iso-amylalcohol (24:1 v/v). DNA was precipitated by adding equal volume of chilled iso-propanol at -40°C for 2 h, centrifuged at 12,000 rpm for 20 min. DNA pellet was dissolved in 200 µl of TE buffer and stored at -40°C. DNA quantification was done at OD_{260} nm and diluted to a final concentration of 12.5 µg μl^{-1} and 2 µl of this DNA was used in PCR. DNA samples were amplified in 20 µl reaction mixture with a final concentration 1X PCR buffer, 200 mM dNTPs, 1.25 pmol primers, 1.6U *Taq* DNA polymerase and 25 ng of DNA template. Amplification reaction was carried out in PTC100 thermal cycler (MJ Research Inc. USA). Initial denaturation of template DNA was carried out at 94°C for 5 min. followed by 40 cycles of denaturation at 94°C for 1 min., primer annealing at 36°C for 1 min. and primer extension at 72°C for 2 min. The final extension was at 72°C for 10 min. Two hundred random primers of arbitrary sequence (Operon Technologies Inc. USA) were screened by PCR analysis. Of the 200 primers screened 10 primers, which produced strong, intense and unambiguous bands, were selected (Table 1). The PCR products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml).

Table 1. List of the selected RAPD primers with their sequences used in the amplication.

Primer No.	Sequence (5'-3')
OPA 4	5'-AATCGGGCTG-3'
OPA 7	5'-GAAACGGGTG-3'
OPA 8	5'-GTGACGTAGG-3'
OPB 3	5'-CATCCCCCTG-3'
OPB 4	5'-GGACTGGAGT-3'
OPC 4	5'-CCGCATCTAC-3'
OPC 8	5'-TGGACCGGTG-3'
OPC 11	5'-AAAGCTGCGG-3'
OPC 20	5'-ACTTCGCCAC-3'
OPE 7	5'-AGATGCAGCC3'

NTSYS-pc (Rohlf, 14) was used to analyze genetic similarity of genotypes. The data was analyzed using SAS v6.12 (SAS Institute, 15) and ANOVA was performed by Fisher's method using the General Linear Model (GLM). In SAS, the regression values (R^2) were calculated by SMA to evaluate correlation between each trait and the marker. The regression values (R^2) were also calculated by SMRA using SAS software to evaluate correlation between each trait and all markers.

RESULTS AND DISCUSSION

Carotenoids are efficient antioxidants capable of scavenging reactive oxygen species generated under conditions of photo-oxidative stress. Supplementation of β-carotene doses was found to protect skin against uv-induced erythema (Stahl *et al*., 18). Tomato is one of the rich sources of carotenoids and lycopene (3.88 to 8.78 mg/100g) and content varies significantly between cultivars (Lower and Thompson, 8; Sharma and Le, 16). High lycopene and carotenoid content was present in 'Ruchi' followed by 'Arka Keshav' and 'Vybhav' (Table 2). Whereas the cultivar, Tomato Stone had the lowest lycopene (11.19 µg/g) and carotenoids (β-cryptoxanthin- 11.79, xeaxanthin- 11.69 and β-carotene- 10.09 µg/g) contents.

ANOVA was carried out to asses the variation across the cultivars and the stages of harvest. The results indicate that carotenoids level show variation across the cultivars and the stages of fruit development (Table 3). Biochemical studies of 28 tomato varieties were conducted to study the difference between diverse tomato genotypes and significant difference among genotypes with these traits was identified (Table 3). The genetic diversity analysis based on the carotenoids and lycopene contents gave rise to two major clusters containing 17 and 11 cultivars each (Fig. 3). Cultivar Utpan was singled out from the cultivars in a minor cluster, otherwise each of the sub- clusters of the two main clusters, contain two to four cultivars with closer similarity coefficient. Significant difference among genotypes for the lycopene was observed (F = 1.67) at 5% probability level. Cultivar 22 (Ruchi) was found to has highest mean value for lycopene followed by Arka Keshav, Vybhav and Sankranti (Table 2), while Tomato Stone had the lowest lycopene. Such genotypic variations have been observed in peach flesh masses

Table 2. Carotenoid and lycopene contents of tomato cultivars grown under Bangalore conditions.

Genotype	Lycopene (µg/g)	β-carotene (µg/g)	β-cryptoxanthin (µg/g)	Zeaxanthin (µg/g)	α-carotene (µg/g)
Indam-2105	34.78	34.47	36.31	36.02	31.37
Sankranti	47.15	44.88	47.29	46.91	43.05
PKM-1	30.82	31.64	33.33	33.06	29.27
Indam-2108	43.29	41.28	43.50	43.15	36.16
Indam-2	38.16	37.55	39.57	39.25	35.06
Arka Abha	15.94	13.25	13.96	13.84	12.92
Arka Meghali	11.11	11.83	12.47	12.37	10.70
Vybhav	52.85	48.10	50.68	50.27	46.74
Tomato Stone	10.53	11.19	11.79	11.69	10.09
Nandi	23.38	22.12	23.31	23.12	25.65
Vaishali	15.36	14.02	14.77	14.65	10.33
Rakshita	28.02	24.31	25.61	25.40	20.85
Tomato Rohini	41.35	39.61	41.73	41.40	38.38
Rohini-2	36.14	35.11	36.99	36.69	28.78
Rashmi Improved	35.65	37.55	39.57	39.25	34.50
Ranjani	32.17	27.97	29.47	29.23	30.81
Ramya	41.84	41.67	43.90	43.55	38.87
D-4	28.50	26.49	27.91	27.69	25.46
Utpan	31.01	27.78	29.27	29.03	25.83
Indam-88-2	12.37	14.15	14.91	14.78	14.51
Indam-13	24.54	27.26	28.73	28.49	25.46
Ruchi	105.41	103.78	109.35	108.47	98.40
Arka Keshav	62.22	60.70	63.96	63.44	58.18
Arka Vikas	28.89	23.15	24.39	24.19	28.17
Yalandur Local	12.75	13.50	14.23	14.11	12.92
Kashmiri-1	14.59	15.56	16.40	16.26	14.51
Kashmiri-2	20.39	17.10	18.02	17.88	17.34
Cherry tomato	22.71	25.98	27.37	27.15	23.12
Mean	13.79	13.95	14.69	14. 58	13. 33
Critical Difference (CD)	14.234	14.025	14.778	14.660	13.413
Coefficient of Variation (%)	72.999	71.111	71.113	71.118	71.136

Note: Coefficient of variation was calculated at the 5% level of significance.

(Quilot *et al*., 12). Fluctuation in the concentration of template DNA had an effect on the PCR amplification product. Low concentrations (10-15 ng) of template DNA resulted in poor amplification of small fragments and at higher concentration of template DNA (40-50 ng) smear was produced. The 25 ng of template and 200 µM dNTP was found adequate for generating reproducible RAPDs. Among 200 random primers screened, ten primers OPA4, OPA7, OPA8, OPB3, OPB4, OPC4, OPC8, OPC11, OPC20 and OPE7 (representative pictures are shown in Fig. 1) that produced intense and reproducible bands were selected for PCR amplification. Determination of diversity between elite germplasm and adapted cultivars will provide an estimate of genetic variation among segregating progenies for developing new pure-lines (Manjarrez *et al*., 9), and degree of heterosis in progenies of parental combinations (Cox and Murphy, 3; Barbosa *et al*., 2). Clustering of pattern of tomato genotypes was carried out using Jaccard's coefficient for RAPD marker data and correlation coefficient for morphological and biochemical data (Figs. 2 & 3). The similarity index ranges from 46 to 92% among genotypes based on RAPD analysis and from 49 to 99.98% based on carotenoids and lycopene contents. Genotypes were clustered into two main groups at

Table 3. ANOVA for carotenoids content in tomato cultivars and stages of fruit maturation.

Trait	Source of variation	SS	df	MS	F	P-value	F crit.
Lycopene	Between stages	14534.65	3	4844.88	47.82	6.88E-18	2.72
	Between genotypes	4585.94	27	169.85	1.68*	0.04	1.62
	Error	8205.70	81	101.30			
	Total	27326.29	111				
β-carotene	Between stages	12842.94	3	4280.98	43.53	7.43E-17	2.72
	Between genotypes	3995.12	27	147.97	1.50	0.08	1.62
	Error	7966.98	81	98.36			
	Total	24805.04	111				
β-cryptoxanthin	Between stages	14257.91	3	4752.64	43.52	7.44E-17	2.72
	Between genotypes	4435.40	27	164.27	1.50	0.08	1.62
	Error	8845.03	81	109.20			
	Total	27538.35	111				
Zeaxanthin	Between stages	14027.23	3	4675.74	43.51	7.49E-17	2.72
	Between genotypes	4365.29	27	161.68	1.50	0.08	1.62
	Error	8704.25	81	107.46			
	Total	27096.77	111				
α-carotene	Between stages	11542.02	3	3847.34	42.77	1.15E-16	2.72
	Between genotypes	3783.65	27	140.13	1.56	0.06	1.62
	Error	7286.88	81	89.96			
	Total	22612.55	111				

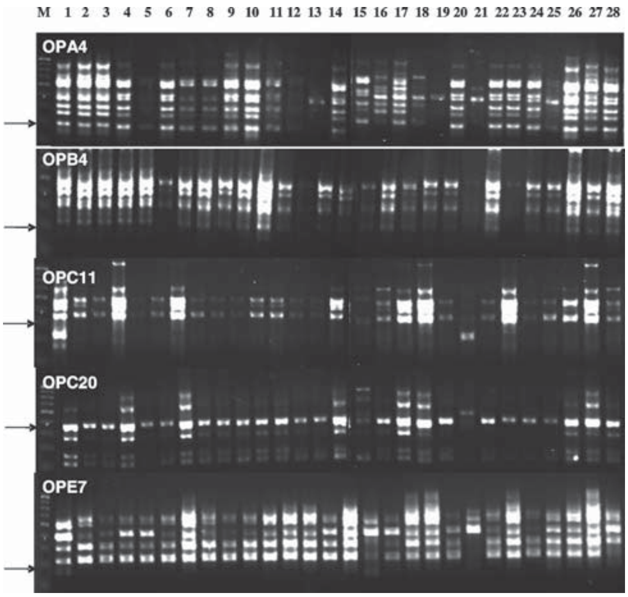

Fig. 1. PCR amplicons of tomato genotypes using selected RAPD primers (OPA4, OPB4, OPC11, OPC20 and OPE7). M. 100 bp DNA markers, 1-28 tomato genotypes as listed in Table 1.

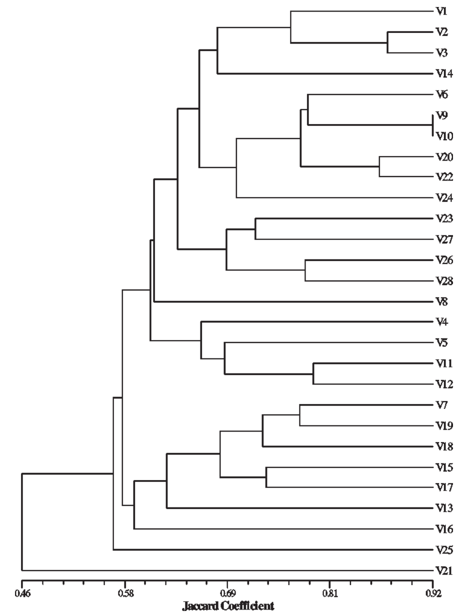

Fig. 2. Genetic diversity of 28 tomato genotypes (V1-V28, list as given in Table.1) based on RAPD data by using Jaccard's coefficient.

Jaccard's coefficient of 58%, and Indam-13 variety with minimum similarity (46%) and Yalandur Local (0.57) are distinct from others. Many sub-groups of two main groups were constructed with increasing of Jaccard's

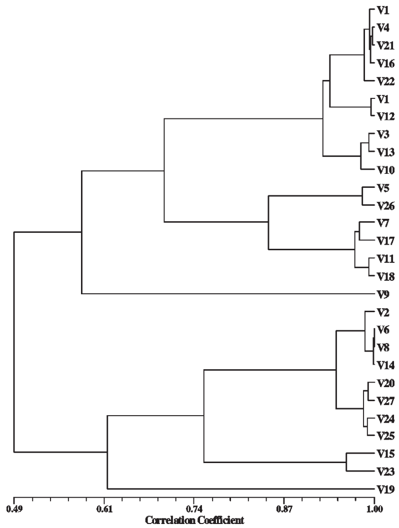

Fig. 3. Diversity of 28 tomato genotypes (V1-V28, list as given in Table 1) based on carotenoids and lycopene contents.

coefficient. Relation between Tomato Stone and Nandi was the nearest with Jaccard's coefficient (0.92). Genotypes were also clustered into two main groups based on biochemical analysis. Differences among genotypes with correlation coefficient based on carotenoids and lycopene content ranged from 49 to 99.9%. The largest group consists of many sub-groups with differences in correlation coefficient. In this group, cultivar (Utpan) was separate from others and relation between Arka Abha and Vybhav was the closest. Single marker analysis revealed that $OPC4_{950}$ and $OPC4_{300}$ are highly correlated with lycopene. For the α-carotene, $OPC4_{950}$ and $OPC4_{300,}$ contributed more than 47%. Similarity for β-carotene, $OPC4_{950}$ and $OPC4_{280}$ were also found contributing more than 47%. For the β-cryptoxanthin, $OPC4_{950}$ and $OPC4_{280}$, contributed at 47.21%. Furthermore markers $OPC4_{950}$ and $OPC4_{300}$ contributed more than 47% with zeaxanthin. All the markers related to individual carotenoids were positive with PE (Table 4).

Three markers accounted for 45.96% for lycopene content, $OPC4_{950}$ showed the maximum association. OPA 8_{270} and OPB 4_{320} contributed to 30% towards α-carotene. The results of the present study reveal primer OPC4 alone produces markers which had high correlation with carotenoids and lycopene content. Coupling of molecular markers (RAPDs) using multiple regression analysis, would allow the better use of biodiversity of crops for improvement in yield and qualitative traits (Kurata, 7).

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Table 4. Single marker analysis and stepwise multiple regression analysis of tomato cultivars in relation to RAPD markers and their correlation with lycopene and carotenoid contents.

Marker	Lycopene		α-carotene		β-carotene		β-cryptoxanthin		Zeaxanthin	
	SMA	SMRA	SMA	SMRA	SMA	SMRA	SMA	SMRA	SMA	SMRA
OPA 4_{250}		0.0308*								
OPA 7_{700}		0.1349**								
OPA 7_{950}		0.0687*								
OPA 8_{270}				0.1848*						
OPB 3_{350}						0.1124*		0.1123*		0.1123*
OPB 4_{320}				0.1165*						
OPC 11_{170}		0.0973*								
OPC 20_{300}			0.1582*	0.1582*						
OPC 4_{280}					0.1595**		0.1595**			
OPC 4_{300}	0.1349**		0.1526**						0.1595**	
OPC 4_{750}	0.1631*	0.1631*	0.1479*		0.1404*		0.1404*		0.1404*	
OPC 4_{950}	0.3139**	0.3139**	0.3175**		0.3126**		0.3126**		0.3126**	
OPC 8_{400}			0.1440*		0.1548*	0.1548*	0.1548*	0.1548*	0.1548*	0.1548*
OPC 8_{600}				0.1417*						
OPE 7_{700}		0.0484*								

*, ** significance at 5 and 1%, respectively.

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