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Research Article

QTL MAPPING OF FRUIT FIRMNESS RELATED LOCI IN TOMATO USING SNP MARKERS SYSTEM

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ABSTRACT

Fruits such as tomato are important source for nutrition and acts as essential component for balanced diet. Ripening of tomato involves in softening of cell wall structure and remodeling, which results in reduced firmness and shortening of shelf life. This is one of the main factors in post-harvest damage and leads to greater yield loss. Understanding the key molecular mechanisms underlying fruit ripening will be helpful to overcome this problem. It is also evident from the previous studies that, ripening process is the consequence of decrease in the fruit firmness. In our study we assessed significant variation for fruit firmness in a population derived by crossing firmness specific parent BML-3 and a normal parent BIL-29 along with some secondary traits. The observed phenotypic variation in fruit firmness, fruit breadth, fruit length and total soluble solid (TSS) indicated the potential use of these traits in selection indices. Genotyping of F2 mapping population was carried out using48 polymorphic SNPs with respective KAS Parassays. A genetic linkage map was constructed using 48 SNPs which consists of 6 linkage groups and covered the map distance of 817cM. Identification of QTLs for the adjusted means of each trait using the genetic linkage map resulted in identification of 2 QTLs for firmness on linkage group 5 (LG5),which together explained 62.2% phenotypic variation. A pair of flanking markers has been developed and validated to facilitate rapid selection fruit firmness in marker assisted introgression programs.

INTRODUCTION

Fruits such as tomato are important source for nutrition and acts as essential component of balanced diet (Passam *et al*. 2007). However, tomato fruit quality is determined by three major components such as physical, chemical and sensory. Physical components include fruit weight, firmness and color; chemical components include dry matter weight, sugar content, titratable acidity, pH and also the contents of several aroma volatiles and pigments, whereas sensory components include taste, aroma and texture attributes(Giovannoni *et al*. 1995).

Wild germplasm is a potential source for genetic variability, disease resistance and agronomically important traits (Menda *et al*. 2014). Gene introgressions from wild species often associated with the linkage drag which intern has negative effects and hinders the crop improvement (Haggard *et al*. 2013). It can be overcome by locating the trait of interest and selecting only for those regions in introgression programs. Usually, the process of locating genomic regions responsible for the trait of interest involves molecular markers and some statistical methods. If the resulted trait is determined by more than one genomic regions and called quantitative trait (QTL) (Foolad 2007). Never the less, QTL mapping for fruit related traits like fruit weight, soluble solid content, pH, fruit color, and fruit firmness has already been done and identified the regions controlling them using different populations derived from inter-specific crosses (Ashrafi *et al*. 2012; Zhang *et al*. 2012). Among all, fruit firmness is considered to be the highly complex trait as it is determined by several factors such as cell wall structure, turgor and cuticle properties which intern controlled by different genes and pathways (Chapman *et al*. 2012). So far, most of the research was centered on molecular aspects of fruit ripening using tomato as a model system. Tomato ripening involves softening of cell wall structure by degradation and remodeling(Eriksson *et al*. 2004).

Several single gene mutants have been identified and established their underlying genes which have a global effect on ripening (Moore *et al*. 2002). However, molecular mechanisms regulating the firmness during ripening process are poorly understood. Development of inter specific

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introgression lines along with advances in quantitative genetics enables us to identify complex traits. Furthermore, traits like fruit size, parthenocarpy and transition from allogamous flowers to autogamous flowers have been identified using ILs of wild tomato species(Frary *et al*. 2004).

The lower number of molecular markers in tomato makes it difficult in studying genetic relations and linkage analysis. None the less, their genomic coverage and throughput makes them inefficient. These limitations are being overcome Single Nucleotide Polymorphisms (SNPs) and next generation sequencing projects. SNPs are becoming more important due to their bi-allelic nature and locus specificity (Sim *et al*. 2012). Utilization of SNPs is being increased with their wide acceptance in conjunction with novel genotype platforms(Kim *et al*. 2014). In the present study, we have identified QTLs determining fruit firmness with 1089 putative SNPs, which were evenly distributed throughout the genome, using Kbio sciences genotyping platform.

MATERIALS AND METHODS

A mapping population of 188 F2 plants was developed by crossing fruit firmness specific parent BML3 and normal parent BIL-29. The parental lines were provided by Bioseed Research India, ICRISAT, Hyderabad, from the available germplasm pool and were sowing differential expression in the fruit phenotype. Plants were grown in the field and their growth condition was monitored periodically. Leaf tissue was collected at true leaf stage and DNA extraction was carried out using modified CTAB method. Quality and Quantity of the DNA was determined using 0.8 % agarose gel. Final concentration of the DNA was adjusted to 5ng/ ul and stored for further usage.

Total 10 fruits per each plant were collected at fully ripened stage and their firmness was measured using penetrometer on the scale of 1-10. Similarly, Breadth, Length were measured with measuring scale in mm and TSS was measured with refractometer. Average of 10 measures for each plant was calculated and used for analysis.

Statistical data analysis

Estimation of significance of variation

The mean value of the trait data was subjected to analysis of variance (ANOVA) using the statistical analysis package (SPSS) software. The phenotypic and genotypic variances were also estimated according to the method suggested by (Burton and DeVane 1953) using the formula:

$$\sigma^2{}_g = \frac{(MS_g - MS_e)}{r}$$
$$\sigma^2{}_p = \sigma^2 g + \sigma^2 e$$
$$\sigma^2 = MS_e$$

Where, $\sigma^2{}_g$=genotypic variance, $\sigma^2{}_p$ = phenotypic variance, $\sigma^2{}_e$ =environmental variance, MS_g= Mean square due to genotypes, MS_e = Error mean square, and r= number of replications.

The coefficient of variations for phenotype and genotypewere estimated using the formula adopted by (Sabesan *et al*. 2009)as:

$$PCV = \frac{\sigma^2{}_P}{\bar{x}}$$
$$GCV = \frac{\sigma^2{}_g}{\bar{x}}$$
$$ECV = \frac{\sigma^2{}_e}{\bar{x}}$$

Where, $\sigma^2{}_p$ = phenotypic variance, $\sigma^2{}_g$ = genotypic variance, $\sigma^2{}_e$ = Environmental variance, and $\bar{x}$ = Grand mean for the trait x; PCV, GCV, and ECV = phenotypic, genotypic and environmental coefficient of variation respectively.

Estimate of heritability and expected genetic advance Heritability (h^2) in broad sense for all characters was computed using the formula adopted by (Allard 1960)

$$h^2 = \frac{\sigma^2{}_p}{\sigma^2{}_g} \times 100$$

Where, $\sigma^2{}_g$=genotypic variance, $\sigma^2{}_p$ = phenotypic variance, h^2=heritability.

Genetic advance as part of the mean (GA) for each trait was calculated using the formula by (Allard 1960).

$$GA = k\sqrt{\sigma^2{}_p h^2}$$
$$GAM\ (as\ \%\ of\ the\ mean) = \frac{GA}{\bar{x}}$$

Where, k = selection differential (at 5% selection intensity), $\sigma^2{}_p$ = phenotypic variance, h^2= heritability and $\bar{x}$ = grand mean.

Linkage analysis and QTL mapping

Parental lines were genotyped with a set of 1089 SNPs, for which KASP assays were designed at LGC genomics (formerly Kbiosciences) facility in London, UK. Genotyping of the 188 F2 mapping population was carried out with 56 polymorphic SNPs using the SNP line platform. Genetic linkage map was constructed using Mapmaker Exp 3.0 software using two point analysis and informativeness criteria LOD=3.0. Recombination frequencies between linked loci were transformed into centimorgan (cM) distances using Haldane mapping function. Identification of QTLs for the adjusted means of each trait was carried out using Mapmaker/QTL.

RESULTS

Continuous variation in phenotypic data of fruit firmness, breadth, length and TSS suggested that traits were segregating quantitatively (Table 1& Figure 1).

Table 1 Range, mean, standard error, standard deviation and variance of different traits

Trait	Minimum	Maximum	Mean	Mean Std. Error	Std. Deviation	Variance
Firmness	3.50	10.00	5.194	0.069	0.946	0.896
Width	3.00	6.00	4.233	0.040	0.551	0.304
Length	3.00	6.50	4.859	0.053	0.686	0.471
TSS	2.00	6.20	4.247	0.048	0.651	0.424

Analysis of the variance the traits suggested the potential use of these phenotypes for mapping of QTLs and studying the responsible loci (Table 2). Among the four traits estimated for phenotypic and genotypic variance fruit firmness showed high variance.

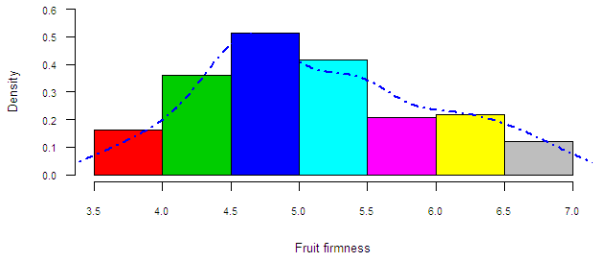

Figure 1 Variation of fruit firmness in F2 mapping population (BIL 29 x BML-3)

Table 2 Mean squares from analysis of variance of different traits

Statistic	Firmness	Width	Length	TSS
Mean square	2.203*	0.450**	0.974**	0.565
Std. error	0.069	0.040	0.053	0.048

** Highly Significant at 0.01% level * Significant at 0.05% level.

Phenotypic coefficient of variability (PCV) values ranged between 15.13% for fruit width and 16.93% for firmness. Genotypic coefficient of variability (GCV) values ranged between 15.05 for fruit width and 16.70 for fruit firmness. Heritability estimates for the four traits were between 0.97 and 0.98. Genetic gain as a mean for each trait was 15.01, 14.88, 15.84 and 14.83 respectively (Table 3).

Identification of QTLs for fruit firmness, width, length and TSS using the genetic linkage map resulted in identification of 2 QTLs for fruit firmness, whereas QTLs for other 3 traits were not found at given threshold (LOD=2.5). The identified QTLs Frm_1.1 and Frm_1.2 were located on LG5 in the marker interval BTS_4829 and BTS_5942. Phenotypic contribution of each QTL was 32.2% and 30% at a LOD score of 6.23 and 2.80 respectively (Figure 3).

DISCUSSION

Fruit ripening is a complex process which involves in deterioration of cell wall structure and softening of the fruit via remodeling. This process results in decreased firmness which leads to shortening shelf life. In the light of previous studies, ripening starts with ethylene signaling pathway as the key, which intern triggers changes in different cellular networks (Cara and Giovannoni 2008). Furthermore, MADS box transcription factors plays an important role in activating ethylene signaling pathway (Martel *et al*. 2011). Several single gene mutants like non-ripening (nor), ripening inhibitor (rin), never ripen (nr) and green ripe have been identified and characterized. These mutations have a global effect on ripening process (Eriksson *et al*. 2004; Barry and Giovannoni 2006; Zhong *et al*. 2008; Martel *et al*. 2011).

Table 3 Estimates of phenotypic, genetic variance, phenotypic (PCV) and genotypic (GCV) coefficients of variation, heritability (h2), genetic advance (GA) and GA as percentage of mean (GAM) of 4 traits

Trait	G. Variance	GCV %	P. Variance	PCV %	E Variance	ECV%	(H^2)	GA	GA as % age of Mean
Firmness	0.145	16.70	0.149	16.93	0.004	2.77	0.97	0.78	15.01
Width	0.096	15.05	0.097	15.13	0.001	1.53	0.98	0.63	14.88
Length	0.143	17.15	0.145	17.27	0.002	2.02	0.98	0.77	15.84
TSS	0.097	15.11	0.099	15.26	0.002	2.17	0.97	0.63	14.83

Among 1089 SNPs used to screen the parental lines of mapping population56 SNPs were found to be polymorphic, out of which 48 assays were found to be segregating and found significant on chi-square test. A genetic linkage map constructed using 48 SNP markers resulted in total 6 linkage groups with given LOD score threshold value(LOD=3.0). Total distance of the genetic linkage map was covered about 817 cM with an average distance between each marker 17.64 cM. Comparison of genetic linkage map with respective physical map suggested the integrity of the marker loci in their segregation with respect to their position (Figure 2).

In the present study, we have studied fruit firmness, breadth, length and TSS using F2 mapping population (BIL-29 x BML-3) to find out the loci responsible for these traits. Analysis of variance of the traits suggested the potential use of these phenotypes for mapping of QTLs and studying the responsible loci (Table 2). In addition, PCV values were higher than respective GCV values for all the traits examined as expected. According to (Mesele *et al*. 2015) PCV and GCV values generally more than 20% are considered as high, whereas values less than 10% are considered to be low and values between 10 and 20% to be medium.

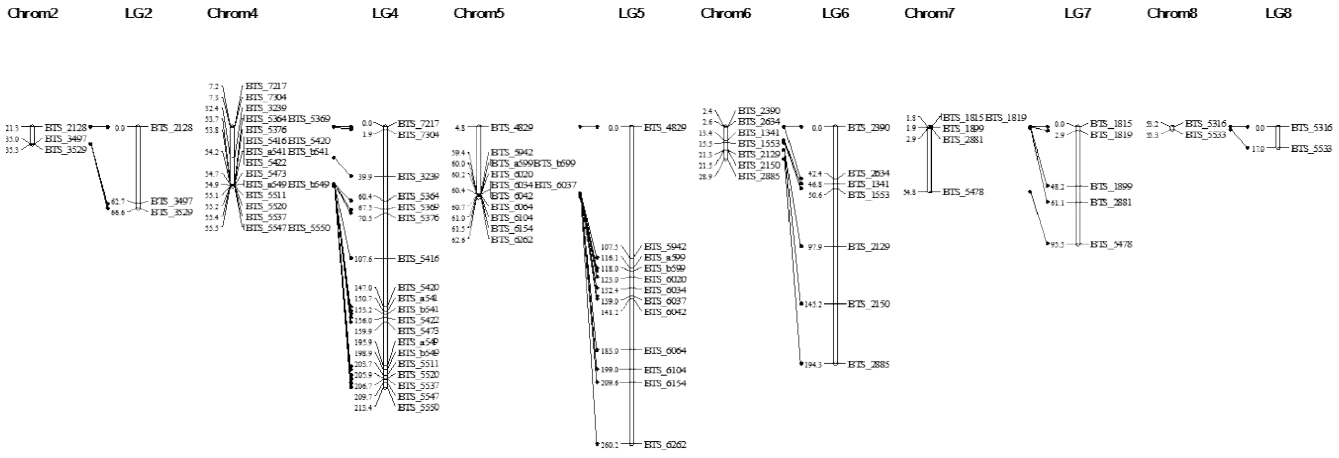

Figure 2 Genetic linkage map using 48 SNP markers using a segregating F2 population (BIL-29 x BML-3) and comparison with physical map.

The moderate GCV values of these traits indicated that the possibility of improving these trait through selection. Relatively high values for heritability of the four traits analyzed suggested the minimal interference of environmental factors in the expression of the trait. Similarly the moderate values for genetic advance indicated the possibility for prediction of the gain under selection pressure. According to (Sabesan *et al.* 2009), high heritability values along with the high genetic advance are generally more helpful in predicting gain under selection than heritability estimates alone. Therefore, genetic enhancement of these characters would be easier with estimated values (Table 3).

The diversity analysis between two parental lines with 1089 SNPs revealed the extent of polymorphism (14.78%) which is quite less compared to other crops species. It indicates that, even though, genetic diversity among cultivated tomato varieties is very low, the parental lines shown sufficient polymorphism to carry out the mapping experiment. Out of 48 polymorphic assays 16 assays were consistent with the segregation ($p<0.05$) while the other 32 assays found to be slightly distorted. Segregation distortion (SD) is defined as abnormal segregation ratio of hybrid offspring's at some genetic loci deviating from the mendelian ratio. Segregation distortion results from the incompatibility among genes from different parents, which could be due to loss-of-function or gain-of-function gene interactions (Xian-Liang *et al.* 2006). Segregation distortion is a frequently observed occurrence in mapping populations generated from crosses involving divergent genotypes (Reflinur *et al.* 2014).

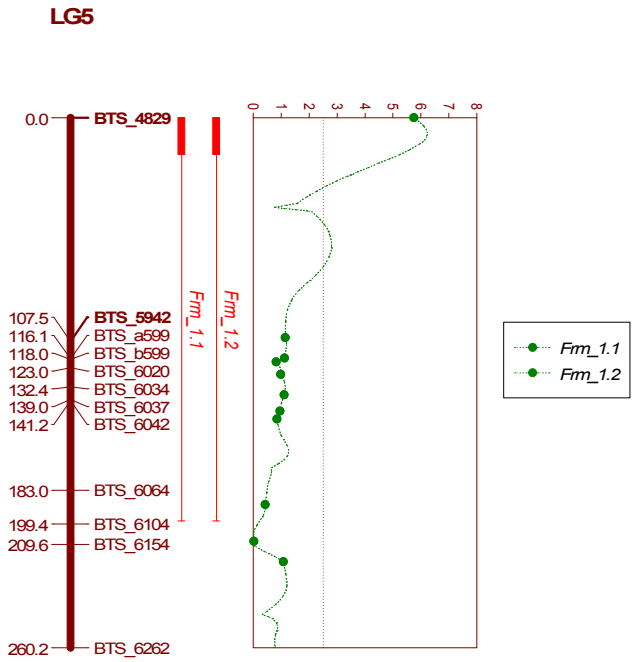

Figure 3 Location of QTLs Frm_1.1 and Frm_1.2 on linkage group 5.

A genetic linkage map was constructed using 48 markers formed 6 different linkage groups with a total distance of 817cM with an average distance between each marker was 17.64 cM, which is in accordance with the previously reported linkage maps (Bachlava *et al.* 2012). A comparative map was created with the genetic map and the available physical map to find out the location of the SNPs and confirmed their consistency in segregation. QTL mapping for fruit firmness, fruit breadth, fruit length and TSS detected two QTLs for firmness, while the other traits fall under minimum threshold value of LOD=2.5 (Figure3). Dominant nature of the QTL Frm_1.1 (Dominance= 1.157) indicated the origin of the firmness determining allele is form firmness specific parentBML-3. Whereas, negative sign for Frm_1.2 (Dominance= -0.746) indicated the origin of favorable allele from BML-29 (Table 4).

Table 4 QTLs identified for fruit firmness

QTL	Marker Interval	Linkage group	Position	LOD	Phenotypic contribution	Dominance
Frm_1.1	BTS_4829 - BTS_5942	LG5	8.0cM	6.23	32.2%	1.0065
Frm_1.2	BTS_4829 - BTS_5942	LG5	62.0	2.80	30.0%	-0.7466

Furthermore, sequence analysis located the flanking markers on distal portion of chromosome 5 of Tomato EXPEN_2000 genetic map. This suggests that genes involving in determination of fruit firmness intern regulate the ripening process (Fujisawa *et al.* 2011). Previous studies on QTL mapping of fruit firmness revealed Firs.p.QTL2.1 to Firs.p.QTL2 on chromosome 2. This could be due to degree of diversity of fruit firmness conferring loci at genomic level (Chapman *et al.* 2012). Consistent sources of firmness and critical information on number and effectiveness of different genomic regions conferring firmness will be useful in successful breeding.

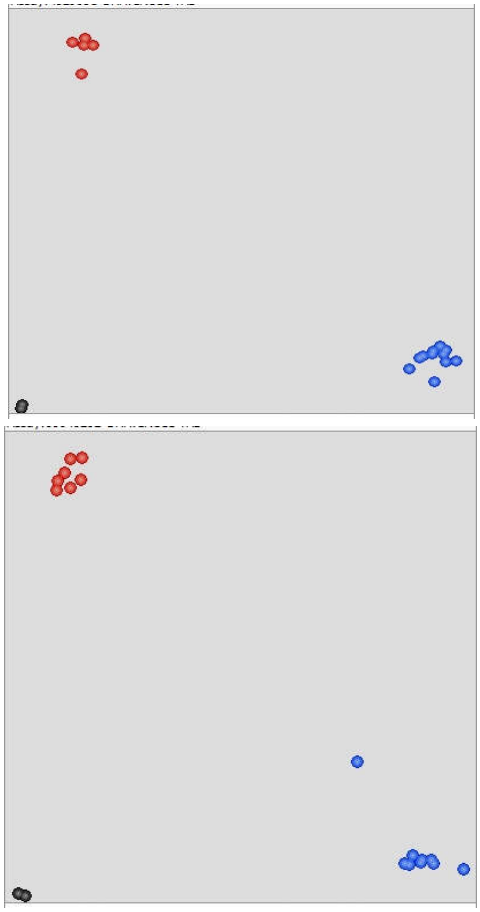

Figure 4&5 Validation of flanking marker BT_4829 and BTS_5942 using 16 ripening specific genotypes.

The flanking markers were further confirmed by investigating the fruit firmness specific germplasm (Figure 4&5). Markers

BTS_4829 (Frm_1.1) and BTS_5942 (Frm_1.2) were successfully differentiated all the genotypes into two groups with respect to the phenotype.

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