Journal of Plant Molecular Breeding (2016) 4(2): 26- 32
DOI: 10.22058/jpmb.2016.25532

RESEARCH ARTICLE

# Identification of Linked Markers for Delayed Fruit Ripening in Tomato Using Simple Sequence Repeat (SSR) Markers

Pavan Kumar Velpula[1, 2*], Dwarkesh Singh Parihar[2], Rajasekhar Pinnamaneni[3]

[1] Jawaharlal Nehru Technological University, Hyderabad, India
[2] Bioseed Research India, Agribusiness Innovation Park, International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India
[3] Deptartment. of Biotechnology, Sreenidhi Institute of Science and Technology, Hyderabad, India

**Abstract:** Tomato (*Solanum lycopersicum* L.) is an important vegetable crop and acts as model plant for fruit development studies. Besides that, post-harvest damage is a devastating phenomenon often associated with ripening process in tomato which in turn leads to greater yield loss. Understanding the genetics, molecular and biochemical pathways is the key to overcome the existing situation. In the present study, we have identified a delayed ripening mutant and used in identification of linked marker for delayed fruit ripening. Initially, BML-03 (delayed ripening mutant line) was crossed with BIL-29 (normal ripening inbred line) to produce $F_2$ population. Bulked segregate analysis was carried out using 245 SSR markers. Out of which, five SSRs were found to be polymorphic between parental lines and respective bulks along with a segregating genotype of mapping population. A population of 227 $F_2$ plants was screened with five polymorphic SSR markers and the data were used in linkage analysis. Three SSR markers were found to be co-segregating with the delayed ripening phenotype and resulted in a linkage map which covered the map distance of 3.4 cM. Out of 3 markers TGS0070 was found to be closely linked to the fruit ripening locus and was successfully validated using other ripening specific $F_2$ population BML-28 x BIL-3.

**Keywords**: Marker validation, MapDisto 1.7.7.0.1.1 (XL2007), Molecular mapping, SSR, Tomato fruit ripening

## INTRODUCTION

Studies on fruit related aspects have immense importance as it is a major source for human dietary consumption and provides good amount of nutrition (Motamedzadegan and Tabarestani 2011). Among all other fruit related traits, fruit ripening is one of the most important trait as it is directly related to the post-harvest damage (Passam et al. 2007). Fruit ripening is a change in form, pigmentation, aroma, texture, flavor, and nutrient composition. Fruits of many species undergo several modifications like change in cell wall structure and texture, starch to sugar conversion, increased susceptibility to post-harvest pathogens, modifications in pigment biosynthesis, and heightened levels of flavor and aromatic volatiles during the maturation and ripening processes (Klee and Giovannoni 2011). Furthermore, most of the ripening characteristics have a negative impact on the quality attributes like decrease in shelf life, changes in firmness, prone to microbial infection, shipping and storage. In most of the cases fresh market tomatoes are harvested and allowed to ripen artificially to overcome the post-harvest damage due to mechanical, biotic or abiotic factors. This process doesn't meet the consumer's expectation like

*Corresponding author (✉): pavank.velpula@gmail.com
Received: 24 October 2016 / Revised: 12 March 2017
Accepted: 15 March 2017

naturally ripened tomatoes and adversely affect the quality attributes (Foolad 2007).

In the recent past, considerable amount of research has been carried out at molecular level to understand the biology of fruit development, especially in the process of fruit ripening; this resulted in establishing the basic metabolic pathways and the effect of environmental changes on fruit development and ripening (Barry et al. 2005; Zhang et al. 2009; Martel et al. 2011). In addition, Tomato is an excellent model plant to explore the fruit development and ripening attributes as it is rich in well characterized ripening mutants (Moore et al. 2002). Studies on ripening inhibitor (rin), non- ripening (nor) and colorless non ripening (Cnr) have provided novel insights in ripening behavior of tomato fruit (Klee and Giovannoni 2011). Lot of knowledge has been obtained on molecular regulation of each ripening parameter, especially cell wall metabolism and ethylene biosynthesis, which is facilitated by the advances in the technology over the past two decades (Giovannoni 2001). Understanding the key control points of ripening process and its manipulation will prevent the post-harvest damage while rescuing quality attributes.

Molecular analysis of tomato has advantage of having relatively small genome size and simple diploid genetics with wide variety of molecular markers (0.9 pg/haploid genome; Arumuganathan and Earle, 1991) thousands which have been mapped on to different genetic maps (Tanksley et al. 1992). Most of the tomato fruit characters like fruit development, size, shape, color, ripening, organoleptic quality and yield have been identified as QTLs (Van Der Knaap and Tanksley 2001; Causse et al. 2004; Semel et al. 2006; Chapman et al. 2012; Kinkade and Foolad 2013). High-density genetic maps for the regions spanning the ripening inhibitor (rin) and (non-ripening) nor loci on chromosomes 5 and 10 spanning was constructed by using combination of bulked segregation analysis as well as classical restriction fragment length polymorphism (RFLP) (Giovannoni et al. 1995).

Molecular mapping of ripening and ethylene response loci on the tomato RFLP map will allow us both the identification and characterization of putative gene sequences related to identified single gene and quantitative trait loci influencing fruit development and ethylene response (Giovannoni et al. 1999). Two quantitative trait loci (QTLs) associated with early ripening were identified and mapped using $F_2$ population derived from a cross between 'E6203'(normal ripening) and 'Early Cherry'(early ripening) (Doganlar et al. 2000). Quantitative trait mapping revealed a single major gene on chromosome 7 (named sun) to be responsible for the varietal development of fruit (Van Der Knaap and Tanksley 2001).

## MATERIALS AND METHODS

### Plant material

Parental lines BML-3 and BIL-29, which were showing contrasting phenotype with respect to fruit ripening, were collected from the inhouse germplasm lines at Bioseed Research India, AIP, ICRISAT, Hyderabad, India. These lines were crossed to produce $F_1$ seed and $F_2$ seed was derived by selfing $F_1$ plants in the glass house. Total 227 $F_2$ plants were used to evaluate phenotypic segregation of fruit ripening. Another $F_2$ population was produced by crossing BML-3 with BIL-28 for validation of the co-segregating marker. DNA extraction has been done using modified CTAB method described by Doyle & Doyle (1987). Samples were purified and concentrations were optimized to 20ng/ul using agarose gel electrophoresis. Phenotyping was carried out by synchronizing flowering time across the population and the number days were calculated from the date flowering to the fruit ripening.

### Polymerase chain reaction (PCR)

PCR was performed in a volume of 10µl in Kbio-Science Hydrocycler. The reaction mixture contained 1µl of 10x buffer, 0.75 µl of 2 mM dNTPs, 0.1µl of 10µM each primer, 0.1µl of *Taq* polymerase (NEB), 1 µl of 20ng/ µl DNA template. PCR reaction was carried out with initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min. Final extension was carried out at 72°C for 5 min. 3% agarose gel was used to perform electrophoresis in 0.5x TBE buffer for 2 h at 5 V/cm, constant voltage. The gels were visualized on a 302 nm UV trans-illuminator and image was captured with a Bio-Rad gel documentation system.

### Bulked segregate analysis

Bulked segregate analysis (BSA) was carried out using bulks of each parent along with extreme phenotypes in the population and a segregating bulk. Bulks were prepared by pooling 10 samples of each type and total 245 SSRs were screened to check the polymorphism (Giovannoni et al. 1995).

### Statistical analysis

### Chi-square test

Phenotypic and genotypic frequencies were subjected to chi-square test to predict the goodness of fit for the segregation of $F_2$ population.

$$x^2 = \sum_I \frac{(O_i - E_i)^2}{E_I}$$

Where, $O_i$ is the observed number of cases in category $i$, and $E_i$ is the expected number of cases in category $i$ (Bentler and Bonnett 1980).

### Linkage analysis

Linkage analysis was carried out using mapmaker EXP 3.0 function embedded in MapDisto 1.7.7.0.1.1 (XL2007) (Lorieux 2012). Markers were grouped based on recombination fraction and maximum likelihood estimates. Kosambi mapping function was used to calculate centimorgan (cM) distance.

## RESULTS

Total 227 $F_2$ plants were successfully grown and growth conditions were monitored regularly. All the plants were recorded for time of fruit ripening by synchronizing the flowering time and there was a clear differentiation in the expression of the phenotype. Phenotype for fruit ripening was segregated in 3:1 ratio and given goodness of fit (Table 1). BSA using bulks of each parent along with extreme phenotypes in the population and a segregating bulk resulted in 5 polymorphic markers out of 245 SSR markers tested (Figure 1).

These markers were used to screen the mapping population to find out the allelic frequencies. All the markers were segregating in accordance Mendelian ratio and the marker segregation was confirmed using chi square test (*P<0.05*) (Table 2). The polymorphic markers data was used in linkage analysis, which resulted in a single linkage group consisting 3 markers TGS0070, TGS504 & TGS2519, while, the other two markers were found to be unlinked. Total distance of the map was 3.39cM (Figure 2, left). Among the three markers, TGS0070 found to be tightly linked to the delayed ripening trait (Figure 2, left). Marker validation using other $F_2$ mapping population (BML-3 x BIL-28) suggested that co-segregation of the TGS0070 was consistent with ripening specific locus and given chi-square value of 0.049 ($p<0.05$) with the slow ripening phenotype (Figure 3 and Table 3).

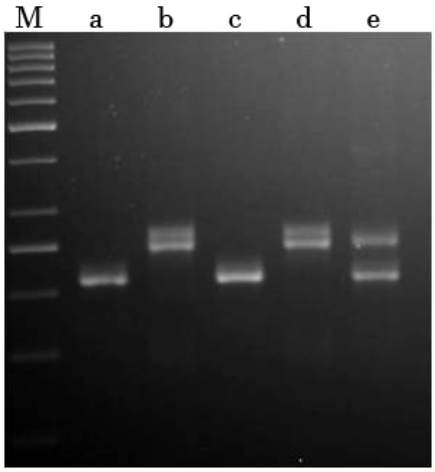


**Figure 1:** Bulked segregate analysis using the bulks of each parent along with bulks of extreme phenotypes and a segregating bulk (TGS0070).

**M)** DNA marker,
**a)** BML-3,
**b)** BIL-29,
**c)** Slow ripening bulk,
**d)** Normal ripening bulk,
**e)** Segregating bulk.

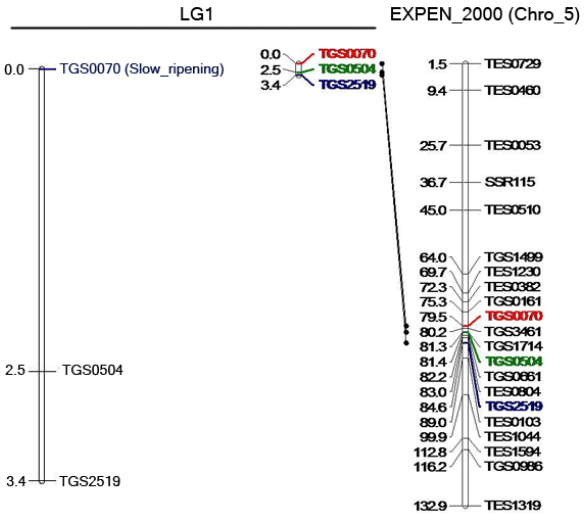


**Figure 2.** Linkage map constructed using three co-segregating SSR markers (left), Comparative analysis using the tomato EXPEN_2000 genetic linkage map (right).

**Table 1:** Chi-square test for segregation of ripening phenotype in the F2 mapping population.

| No. | Marker | *x2* 3:1 | *p*-value | Normal ripening | Slow ripening | No. of Individuals |
|---|---|---|---|---|---|---|
| 1 | Ripening | 0.618 | 0.05 | 167 | 60 | 227 |

**Table 2:** Chi-square test for segregating SSR markers in the $F_2$ mapping population (BIL-29 x BML-3).

| No. | Marker | *x2* 1:2:1 | *p*-value | AA | Aa | aa | No. of Individuals |
|---|---|---|---|---|---|---|---|
| 1 | TGS0070 | 5.67 | 0.05 | 41 | 123 | 62 | 226 |
| 2 | TGS0504 | 2.86 | 0.23 | 46 | 123 | 58 | 227 |
| 3 | TGS2519 | 3.32 | 0.19 | 45 | 123 | 59 | 227 |
| 4 | TGS0871 | 0.92 | 0.63 | 63 | 109 | 55 | 227 |
| 4 | TES0312 | 3.10 | 0.21 | 67 | 109 | 49 | 225 |

**Table 3:** Chi-square test for co-segregating marker TGS0070 in the F2 mapping population (BIL-28 x BML-3).

| No. | Marker | *x2* 1:2:1 | *p*-value | AA | Aa | aa | No. of Individuals |
|---|---|---|---|---|---|---|---|
| 4 | TGS0070 | 1.391 | 0.49 | 27 | 47 | 19 | 93 |

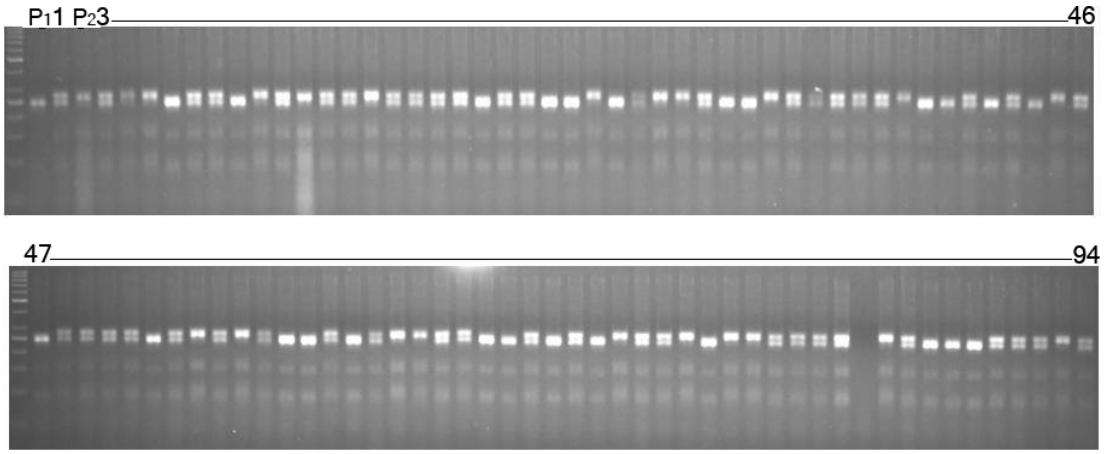


**Figure 4:** Validation of co-segregating marker TGS0070 using $F_2$ mapping population derived by crossing BIL-28 x BML-3. P1: BML-03, P2: BML-28, 1-94: $F_2$ population.

## DISCUSSION

Mapping population derived by crossing ripening specific parental lines (BML-3 x BIL-29) exhibited clear differentiation with respect to ripening time. This supports the statement that tomato is an excellent model plant to explore the fruit development and ripening attributes as it is rich in well characterized ripening mutants (Moore et al. 2002). Furthermore, phenotype for fruit ripening was segregated in (normal) 3: 1 (slow) ratio, which is a common characteristic of most of the dominant phenotypes, and followed the Mendal' law (Table 1). When it comes to the genotyping, the markers used in this study were evenly distributed across all chromosomes to make sure the coverage of entire genome, which was helpful in scrutinizing the ripening determining locus. The drastic reduction of marker number, i.e. 5 out of 245, after BSA could be due to the level of polymorphism, which is relatively less in tomato (Areshchenkova and Ganal 2002). These five polymorphic SSRs were considered to be highly informative based on the previous studies conducted by Shirasawa et al. 2010. Screening of these 5 markers using the mapping population derived by crossing the two ripening specific parents revealed the segregation distortion of the 4 markers (80%) and similar results were reported by Liu et al. 2011. The polymorphic

markers data was subjected for linkage analysis and LOD scores were calculated at 3.0 threshold value (Giovannoni et al. 1999). The linkage group formed was consisting 3 markers TGS0070, TGS504 & TGS2519 covering the total distance of 3.39cM. The average marker distance was 1.13 cM which is in relation to the previous studies conducted by (Zhang et al. 2002). Comparison of the marker positions with the previously available tomato genetic map EXPEN_2000 (Chromosome 5) submitted to sol genomics indicated their consistency in segregation (Figure 2, right). It is also evident that loci controlling the fruit ripening are present on Chromosome 5 (Giovannoni et al. 1995). Based on this study, we have successfully identified a SSR marker TGS0070 which is tightly linked to the slow fruit ripening trait. In addition, the marker was validated using the other $F_2$ mapping population (BML-3 × BIL-28) and can be used in marker assisted introgression of slow fruit ripening trait into elite cultivars of hybrid parental lines (Table 3).

## REFERENCES


Areshchenkova T, Ganal MW (2002) Comparative analysis of polymorphism and chromosomal location of tomato microsatellite markers isolated from different sources. Theor Appl Genet 104:229–235.

Barry CS, McQuinn RP, Thompson AJ (2005) Ethylene insensitivity conferred by the Green-ripe and Never-ripe 2 ripening mutants of tomato. Plant Physiol 138:267–275.

Bentler PM, Bonnett DG (1980) Significance tests and goodness of fit in the anlaysis of covariance structures. Psychol Bull 88:588–606.

Causse M, Duffe P, Gomez MC (2004) A genetic map of candidate genes and QTLs involved in tomato fruit size and composition. J Exp Bot 55:1671–1685.

Chapman NH, Bonnet J, Grivet L (2012) High-Resolution Mapping of a Fruit Firmness-Related Quantitative Trait Locus in Tomato Reveals Epistatic Interactions Associated with a Complex Combinatorial Locus. Plant Physiol 159:1644–1657.

Doganlar S, Tanksley SD, Mutschler MA (2000) Identification and molecular mapping of loci controlling fruit ripening time in tomato. TAG Theor. Appl. Genet. 100:249–255.

Foolad MR (2007) Genome mapping and molecular breeding of tomato. Int J Plant Genomics.

Giovannoni, J. (2001) Molecular biology of fruit maturation and ripening. Annual review of plant biology, 52(1), 725-749.

Giovannoni J, Yen H, Shelton B (1999) Genetic mapping of ripening and ethylene-related loci in tomato. Theor Appl Genet 98:1005–1013.

Giovannoni JJ, Noensie EN, Ruezinsky DM (1995) Molecular genetic analysis of the ripening-inhibitor and non-ripening loci of tomato: A first step in genetic map-based cloning of fruit ripening genes. Mol Gen Genet 248:195–206.

Kinkade MP, Foolad MR (2013) Validation and fine mapping of lyc12.1, a QTL for increased tomato fruit lycopene content. Theor Appl Genet 126:2163–2175.

Klee HJ, Giovannoni JJ (2011) Genetics and Control of Tomato Fruit Ripening and Quality Attributes. Annu. Rev. Genet. 45:41–59.

Liu X, You J, Guo L (2011) Genetic Analysis of Segregation Distortion of SSR Markers in F2 Population of Barley. J Agric Sci 3:172–177.

Lorieux M (2012) MapDisto: Fast and efficient computation of genetic linkage maps. Mol Breed 30:1231–1235.

Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ (2011) The Tomato MADS-Box Transcription Factor RIPENING INHIBITOR Interacts with Promoters Involved in Numerous Ripening Processes in a COLORLESS NONRIPENING-Dependent Manner. PLANT Physiol. 157:1568–1579.

Moore S, Vrebalov J, Payton P, Giovannoni J (2002) Use of genomics tools to isolate key ripening genes and analyse fruit maturation in tomato. J Exp Bot 53:2023–2030.

Motamedzadegan A, Tabarestani HS (2011) Tomato Processing, Quality, and Nutrition. In: Handbook of Vegetables and Vegetable Processing. pp 739–757

Passam HC, Karapanos IC, Bebeli PJ, Savvas D (2007) A review of recent research on tomato nutrition, breeding and post-harvest technology with reference to fruit quality. Eur J Plant Sci Biotechnol 1 (1):1–21.

Semel Y, Nissenbaum J, Menda N (2006) Overdominant quantitative trait loci for yield and fitness in tomato. Proc Natl Acad Sci U S A 103:12981–12986.

Shirasawa K, Asamizu E, Fukuoka H (2010) An interspecific linkage map of SSR and intronic polymorphism markers in tomato. Theor Appl Genet 121:731–739.

Tanksley SD, Ganal MW, Prince JP (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141–1160.

Van Der Knaap E, Tanksley SD (2001) Identification and characterization of a novel locus controlling early fruit development in tomato. Theor Appl Genet 103:353–358.

Zhang LP, Khan A, Niño-Liu D, Foolad MR (2002) A molecular linkage map of tomato displaying chromosomal locations of resistance gene analogs based on a Lycopersicon esculentum x Lycopersicon hirsutum cross. Genome 45:133–46.

Zhang M, Yuan B, Leng P (2009) The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. J Exp Bot 60:1579–1588.

# شناسایی نشانگرهای مرتبط با تاخیر در رسیدگی گوجه فرنگی با استفاده از نشانگرهای توالی‌های تکراری ساده (SSR)

**پاوان کومار ولپولا[۱،۲*]، وارکش سینگ پاریهار[۲]، راجاسیخار پیناماننی [۳]**

[۱]دانشگاه تکنولوژی جواهرلعل نهرو، حیدرآباد، هند

[۲]مرکز تحقیقات بذر هند، پارک فناوری کشاورزی، موسسه تحقیقات بین‌المللی گیاهان زراعی نواحی نیمه خشک، پاتانچرو، هند

[۳]گروه بیوتکنولوژی، موسسه علوم و تکنولوژی سرینیدهی، حیدرآباد، هند

*نویسنده مسئول: pavank.velpula@gmail.com

## چکیده

گوجه فرنگی (*Solanum lycopersicum L.*) یک گیاه زراعی مهم بوده که در مطالعات مراحل نموی میوه بعنوان یک گیاه از اهمیت بسیاری برخوردار است. خسارت پس از برداشت غالباً با فرایند رسیدگی در گوجه‌فرنگی همراه بوده و منجر به کاهش بیش از پیش عملکرد می‌شود. حل این مشکل مستلزم درک مسیرهای ژنتیکی، مولکولی و بیوشیمیایی دخیل در فرایند رسیدگی میوه می‌باشد. در تحقیق حاضر، ضمن شناسایی لاین موتانت با خاصیت تاخیر در رسیدگی، نشانگرهای پیوسته به تاخیر در رسیدگی میوه نیز شناسایی و استفاده گردید. در ابتدا، برای تشکیل جمعیت *F2*، لاین *BML-03* (لاین موتانت تاخیر در رسیدگی) با *BIL-29* (لاین اینبرد رسیدگی نرمال) تلاقی داده شد. تجزیه و تحلیل تفرق توده‌ای (BSA) با استفاده از ۲۴۵ نشانگر *SSR* انجام شد. از میان این نشانگرها، ۵ نشانگر *SSR* بین لاین‌های والدینی و دو نمونه بالک‌شده از ژنوتیپ‌های جمعیت در حال تفرق، چندشکلی نشان دادند. غربال جمعیت *F2* شامل ۲۲۷ گیاه با ۵ نشانگر چندشکل *SSR* صورت گرفت و داده‌های ژنوتایپینگ در تجزیه و تحلیل لینکاژ استفاده شد. سه نشانگر *SSR* با فنوتیپ تاخیر در رسیدگی پیوستگی نشان دادند و منجر به یک نقشه لینکاژی با فاصله ۳/۴ سانتی مورگان شد. از میان این سه نشانگر، نشانگر *TGS0070* از پیوستگی بالایی با جایگاه رسیدگی میوه برخوردار بوده بطوریکه کارایی آن در جمعیت *F2* دیگر حاصل از تلاقی *BML-28 × BIL-3* ( جمعیت اختصاصی رسیدگی میوه) نیز تایید شد.

**کلمات کلیدی:** اعتبارسنجی نشانگر، *MapDisto 1.7.7.0.1.1 (XL2007)*، نقشه‌یابی مولکولی، *SSR*، رسیدگی میوه گوجه‌فرنگی