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ARTICLE

Tomato second cycle hybrids as a source of genetic variability for fruit quality traits

Pereira da Costa JH[1,2*], Rodríguez GR[1,2], Liberatti DR[2], Mahuad SL[2], Marchionni Basté E[2], Picardi LA[2,3], Zorzoli R[2,3] and Pratta GR[1,2]

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Abstract: *The objective of this study was to investigate the phenotypic and molecular variability in a F_2 generation derived from a SCH (Second Cycle Hybrid) in order to detect QTLs for some fruit traits of tomato. Genome coverage at different levels was achieved by three types of molecular markers (polypeptides, sequence-related amplified polymorphism-SRAP and amplified restriction fragment polymorphism - AFLP). Different degrees of polymorphism were detected by SRAP and AFLP at the DNA structure level and also by polypeptides at the DNA expression level. The first two markers, associated with phenotypic variation, detected QTLs involved in important agronomic traits such as fruit shelf life, soluble solids content, pH, and titratable acidity. New gene blocks originated by recombination during the first cycle of crossing were detected. This study confirmed that the observed phenotypic differences represent a new gene rearrangement and that these new gene blocks are responsible for the presence of the genetic variability detected for these traits.*

Keywords: *Recombinant inbred lines, Solanum lycopersicum, genetic recombination, gene blocks.*

INTRODUCTION

In the last 30 years, significant progress has been made in tomato breeding by replacing open-pollinated varieties by hybrids (Figueiredo et al. 2015). A physiological *plateau* was reached when both yield and fruit quality had been improved (Grandillo et al. 1999). However, the advent of genomics has transformed breeding strategies. As a result, the improvement of tomato cultivars is expected to continue in the future. Recombinant Inbred Lines (RILs) allow increase the genetic variability by recombination and chromosome rearrangements, introducing new gene blocks. One way to profit from these new rearrangements is to develop Second Cycle Hybrids (SCH). These genotypes are generated by hybridization among RILs (Hills et al. 2003, Ipsilandis et al. 2006). According to Pratta et al. (2003) and Pereira da Costa et al. (2009), *Solanum pimpinellifolium L.* could be an appropriate parental genotype to improve fruit quality traits. These authors found that its hybrids with cultivated tomato had longer fruit shelf life compared to commercial cultivars and also better fruit quality in terms of color, texture and flavor. Though *S. lycopersicum L.* and S. *pimpinellifolium* have significant phenotypic differences, only minor variation was found among them at the genomic level (about 0.6 %) (Tomato Genome Consortium 2012). It can be postulated that these differences are due to protein

***Corresponding author:**
E-mail: jpereira@unr.edu.ar

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[1] IICAR-CONICET (Instituto de Investigaciones en Ciencias Agrarias de Rosario - Consejo Nacional de Investigaciones Científicas y Técnicas), Universidad Nacional de Rosario, Facultad de Ciencias Agrarias, Campo Experimental JF Villarino CC 14 (S2125ZAA), Zavalla, Santa Fe, Argentina2 Universidad Nacional de Rosario
3 IICAR-CONICET, CIUNR (Consejo de Investigaciones de la Universidad Nacional de Rosario), Universidad Nacional de Rosario

functions (Michael and Alba 2012) and consequently to transcriptome regulation. As Giovannoni (2004) pointed out, the fruit quality could be conditioned by the developmental stage of the fruits at harvest time.

Different techniques have generated large amounts of data of gene expression during the development of climacteric and non-climacteric tomato fruits (Aharoni and O'Connell 2002, Henniget al. 2004, Grimpletet al. 2005, Moyle et al. 2005, Terrier et al. 2005). Carotenoid pigment accumulation and fruit softening distinguish at least two ripening stages: mature green and red ripe (Rick 1978, Giovannoni 2004). Although the polymorphism of polypeptide profiles is relatively low, they have been successfully used as molecular markers in various species to characterize genotypes or biological processes (Castro et al. 2006, Rodríguez et al. 2011).

Sequence-related amplified polymorphism markers (SRAPs) are DNA markers that preferentially amplify the expressed genomic regions (Li and Quiros 2001). These markers have been previously used in different species (Ruiz et al. 2005, Cravero et al. 2007, Mahuad et al. 2013). Other commonly used DNA markers are AFLP (Amplified Fragment Length Polymorphism), uniformly distributed in the tomato genome (Saliba-Colombaniet al. 2001), with a high chromosome coverage. Therefore, they were used to generate various inter- and intraspecific maps as well as to assess the genetic diversity of tomato cultivars, for genotyping (fingerprinting) and to detect QTLs of different fruit traits in interspecific crosses (Zhang and Stommel 2001, Lecomte et al. 2004, Pratta et al. 2011). Due to the different nature of genome coverage (expressed regions vs. random regions) of these three types of molecular markers (polypeptides and SRAP vs. AFLP), they could be interesting molecular tools to detect QTLs for fruit quality traits. As pointed out, the genetic recombination that occurs during the generation of the SCHs would produce genetic variability for these traits. Consequently, the aim of this study was to assess the amount of genetic variability at the phenotypic and molecular levels in the F_2 generation derived from a SCH and also to detect QTLs for some fruit quality traits. In addition, we demonstrated that new gene blocks were originated by recombination during the first cycle of crossing and that they were preserved even in the F_2 derived from the selfed SCH.

MATERIAL AND METHODS

Plant material

By antagonistic-divergent selection for weight and fruit shelf life, Rodríguez et al. (2006) obtained 17 RILs. These were derived from the F_2 generation of an interspecific cross between LA722 of *S. pimpinellifolium* and the Argentinean cultivar Caimanta of *S. lycopersicum* (first-cycle parental genotypes). The RILs ToUNR1, ToUNR8, ToUNR9, ToUNR15, and ToUNR18 were selected based on their combining ability. Then the five RILs were crossed according to a diallel cross (Model II without reciprocal crosses) to select the best SCH among the 10 possible F_1s to initiate a new breeding process. Due to the high values of specific combining ability for fruit weight and shelf life and the significant values of general combining ability of its parents, the hybrid ToUNR9 x ToUNR15 was selected to obtain the segregating F_2 population by selfing (Marchionni Basté et al. 2010). Fifteen plants of each parental genotype and the F_1 and 180 F_2 plants were assessed in a greenhouse in a completely randomized design.

Phenotypic analysis

The following fruit traits were evaluated: weight (W; g), diameter (D; cm), height (H; cm), shape (Sh; height/diameter), and shelf life (SL; in days from harvest to fruit softening or excessive wrinkling), following the methodology proposed by Garg et al. (2008). In addition, the soluble solids content (SS, °Brix), measured with a hand refractometer, pH, titratable acidity of the homogenized juice (TA, g), pericarp thickness (PT, mm), locule number (LN), and fruit firmness (F), on the equatorial plane in two opposite areas of the fruit measured with a durometer type Shore A (Durofel DFT100, 0.10 cm^2) were assessed.

Molecular analysis

Pericarp polypeptide profiles

Total pericarp proteins were extracted from fruits harvested at two ripening stages: Mature Green (MG, at least 10% of the fruit surface red) and Red Ripe (RR, 90% of the fruit surface red). Three independent pericarp samples (fruits from

three different plants) per parental genotype and F_1, and one sample per F_2 plant were extracted from fruit harvested at each ripening stage. Proteins were extracted and resolved on SDS-PAGE following the protocol proposed by Rodríguez et al. (2008). Equal amounts of polypeptides (20 ug) were run for 1.5 h at a constant current of 35 mA on denaturing polyacrylamide gels (12 % v/v). Gels were stained with a 0.1% solution of Coomassie Brilliant Blue R-250 and destained with boiling water, scanned and analyzed with software Gelpro Analyzer 3.0.

DNA Markers

Young leaves were collected from the parents, F_1 and each F_2 plant and stored at -80 °C. The DNA was extracted with a commercial kit (Wizard ® Genomic DNA Purification Kit of Promega ®). PCR amplifications were performed in duplicate for parental genotypes and F_I.

Sequence-Related Amplified Polymorphism (SRAP)

Four of the primer combinations previously selected for their high level of polymorphisms (Mahuad et al. 2013) were used to characterize the parental genotypes, and F_1 and F_2 plants. The amplification protocol proposed by Li and Quiros (2001) was used with some modifications, as described by Mahuad et al. (2013). The bands were codified with a number (SI, SII, SIII, or SIV), according to the primer combination, followed by another number indicating the order of the band on the gels (e.g. SIV.26).

Amplified Fragment Length Polymorphism (AFLP)

Six AFLP primer combinations selected for their high level of polymorphism among parental genotypes (Liberatti et al. 2013) were used. The AFLP profiles were obtained following the amplification protocol proposed by Liberatti et al. (2013). The bands were codified with a capital letter indicating the primer combination followed by a number indicating the position of the band on the gels (e.g. X4)

Statistical Analysis

Normal distribution of each trait was tested by the Shapiro-Wilk test (Shapiro and Wilk 1965). The mean values of parental genotypes and F_1were compared with a *t*-Student test (Snedecor 1964). Broad-sense heritability (H^2, $H^2 = \sigma^2_g / \sigma^2_p$), where σ^2_g is genetic variance and σ^2_p is phenotypic variance) was calculated using variance components from ANOVA for a completely randomized design, according to Falconer and Mackay (1996). All statistical analyses were performed with software InfoStat Version 1.0 (Di Rienzo et al. 2001). With a view to the dominant nature of all molecular markers used in this study (polypeptide profiles, SRAP and AFLP), a χ^2 test (Snedecor 1964) was used to verify the expected segregation of 3:1 (presence: absence) in the F_2 generation. Only bands with Mendelian inheritance in parents and F_2 were taken into account for the QTL detection. The *de novo* bands (present in the first-cycle but not second-cycle parental genotypes and *vice versa*) were evaluated according to Liberatti et al. (2013). The association between molecular markers and fruit quality traits was analyzed by the single point method (single point analysis) (Tanksley1993). One-way ANOVA was performed with markers as classification variables. All markers with Mendelian inheritance were used to determine the existence of gene blocks. The LOD threshold for accepting a linkage group (LG) was 3.0, estimated by software JoinMap4.0, with the following settings: Rec = 0.40, LOD = 3.0 and Jump = 5. Recombination values were converted to genetic distances using the Kosambi (1943) mapping functions.

RESULTS AND DICUSSION

All phenotypic traits had normal distribution. The second cycle parental genotypes (ToUNR15 and ToUNR9) were significantly different for all traits except for fruit shelf life and firmness (Table 1). The results for weight and shelf life were consistent with those reported by Rodríguez et al. (2006), who characterized ToUNR9 as a genotype with short shelf life and low fruit weight and ToUNR15 as one with short shelf life and high fruit weight (Table 1). However, the frequency distribution in the F_2 (ToUNR15 x ToUNR9) generation for fruit shelf life (Figure 1e) shows wide phenotypic variation despite the similarity of the parental genotypes values. Even though H^2 was 0.38 ± 0.02 for shelf life, transgressive segregation was observed in the F_2 generation (Figure 1e), indicating that QTLs for shelf life, and other attributes with very high H^2 values (SS, pH and TA), could be detected in this population (see Table 1, shown in gray). When the polypeptide

Table 1. Mean values, standard error for each evaluated traits in second cycle parental genotypes (ToUNR15 and ToUNR9), F_1 and F_2 generation and broad-sense heritability (H^2)

Fruit traits	ToUNR15	ToUNR9	F_1 (15x9)	F_2 (15x9)	H^2
D (cm)	3.42 ± 0.09c	1.95 ± 0.06a	2.85 ± 0.04b	2.66 ± 0.03	0.63 ± 0.02***
H (cm)	2.90 ± 0.07c	1.75 ± 0.04a	2.53 ± 0.03b	2.39 ± 0.02	0.62 ± 0.06***
Sh	0.85 ± 0.02a	0.90 ± 0.01b	0.90 ± 0.01b	0.91 ± 0.01	0.24 ± 0.01***
W (g)	21.59 ± 1.04c	4.49 ± 0.32a	12.95 ± 0.32b	10.84 ± 0.36	0.63 ± 0.02***
SL (days)	15.13 ± 1.49a	15.39 ± 0.43a	16.37 ± 0.84a	16.22 ± 0.28	0.38 ± 0.02***
SS (°Brix)	6.84 ± 0.11b	5.07 ± 0.06a	7.20 ± 0.07b	8.38 ± 0.07	0.85 ± 0.02***
pH	4.84 ± 0.05b	4.40 ± 0.02a	4.74 ± 0.04ab	4.68 ± 0.01	0.73 ± 0.02***
TA (g)	0.31 ± 0.01a	0.43 ± 0.02b	0.31 ± 0.02a	0.41 ± 0.01	0.78 ± 0.06***
F	46.70 ± 0.96a	50.50 ± 0.61ab	51.76 ± 1.50b	51.80 ± 0.28	0.52 ± 0.03***
LN	4.42 ± 0.22c	2.00 ± 0.00a	2.95 ± 0.11b	2.60 ± 0.06	0.48 ± 0.02***
PT	0.39 ± 0.02c	0.19 ± 0.01a	0.29 ± 0.02b	0.25 ± 0.01	0.67 ± 0.02***

D: diameter. **H**: height. **Sh**: shape. **W**: weight. **SL**: shelf life. **SS**: soluble solids content. **TA**: titratable acidity. **F**: firmness. **LN**: locule number. **PT**: pericarp thickness. Different letters indicate significant differences ($p < 0.05$)
***$p < 0.001$

profiles of the second cycle parental genotypes ToUNR15 and ToUNR9 were compared, 27% and 8% of polymorphism were found at the mature-green and red-ripe stages, respectively. A high number of monomorphic bands were found at both ripening stages. Perhaps the selection process to obtain these RILs fixed the same alleles in some loci in the parental genotypes ToUNR15 and ToUNR9. No bands with Mendelian inheritance were found at the mature-green stage, but only one polypeptide of 75.6 kDa with Mendelian inheritance was found at the red-ripe stage, though without any association with a phenotypic trait. These results indicated that the phenotypic differences between parents have no correlation with the polymorphism in polypeptide profiles. These kinds of molecular markers were useless to detect associations among polypeptide profiles and phenotypic traits in this genetic background generated by crossing ToUNR15 with ToUNR9. Other authors (Rodríguez et al. 2011, Pereira da Costa et al. 2014) detected QTLs by polypeptide profiles in other genetic backgrounds. Probably the parental genotypes for those studies were genetically more divergent than the RILs studied here.

A different situation was observed when SRAP markers were analyzed. A total of 214 SRAP bands were detected between the second cycle parental genotypes and the F_2 generation. A mean of 53 bands was detected by primer combinations. The combinations 2 and 4 were the most polymorphic (percentages ranged from 29.7% to 22.4%). The mean number of bands detected by SRAP primer combinations was consistent with those reported by Mahuad et al. (2013), Pereira da Costa et al. (2014) and Ruiz et al. (2005). Nine bands with Mendelian inheritance were used for the association analysis. A total of 9 QTLs ($p < 0.01$) were found (Table 2A). The proportion of phenotypic variance explained by each QTL ranged between 5 and 10%. QTLs for soluble solids content, pH, pericarp thinness and fruit height were detected (Table 2A). These results support the hypothesis of Mahuad et al. (2013), suggesting that it would be possible to find SRAP markers associated with tomato fruit quality traits because a high consensus was observed between molecular and phenotypic diversity when a diallel design of 5 RILs and their hybrids was evaluated. The presence of fragment SIV.7 ($p <0.002$), originated from ToUNR15, produced a decrease in SS and pH. A high soluble solids content is a desirable trait in breeding programs for being associated with a better fruit flavor (Stevens et al. 1979). In all cases, the SRAP bands of ToUNR9 were associated with an increase in sugar content, while the SRAP bands inherited from ToUNR15 had the opposite effect (Table 2A). These results were expected according to the parental values for the traits. For fruit height, the effect of band SI.55 was consistent with the phenotype observed in second-cycle parental genotypes. Accordingly, ToUNR9 had fruits with smaller height and size (Table1) and contributed to one SRAP band that diminished the mean values of these traits. These results demonstrate that the selection by RILs development fixed alleles in ToUNR15 andToUNR9 with opposite effects on fruit size and soluble solids content.

A total of 735 AFLP bands were detected of which 496 (67%) were polymorphic. Mendelian inheritance was confirmed for 19 bands (4%) and 13 QTLs were detected by AFLP markers. One QTL (X4, p=0.001) for fruit shelf life decreased the mean value in the plants with this band. This QTL was contributed by To UNR15 (Table 2B).

Most associations detected by AFLP markers were related to the traits with greatest genetic variability in F_2, i.e. SS,

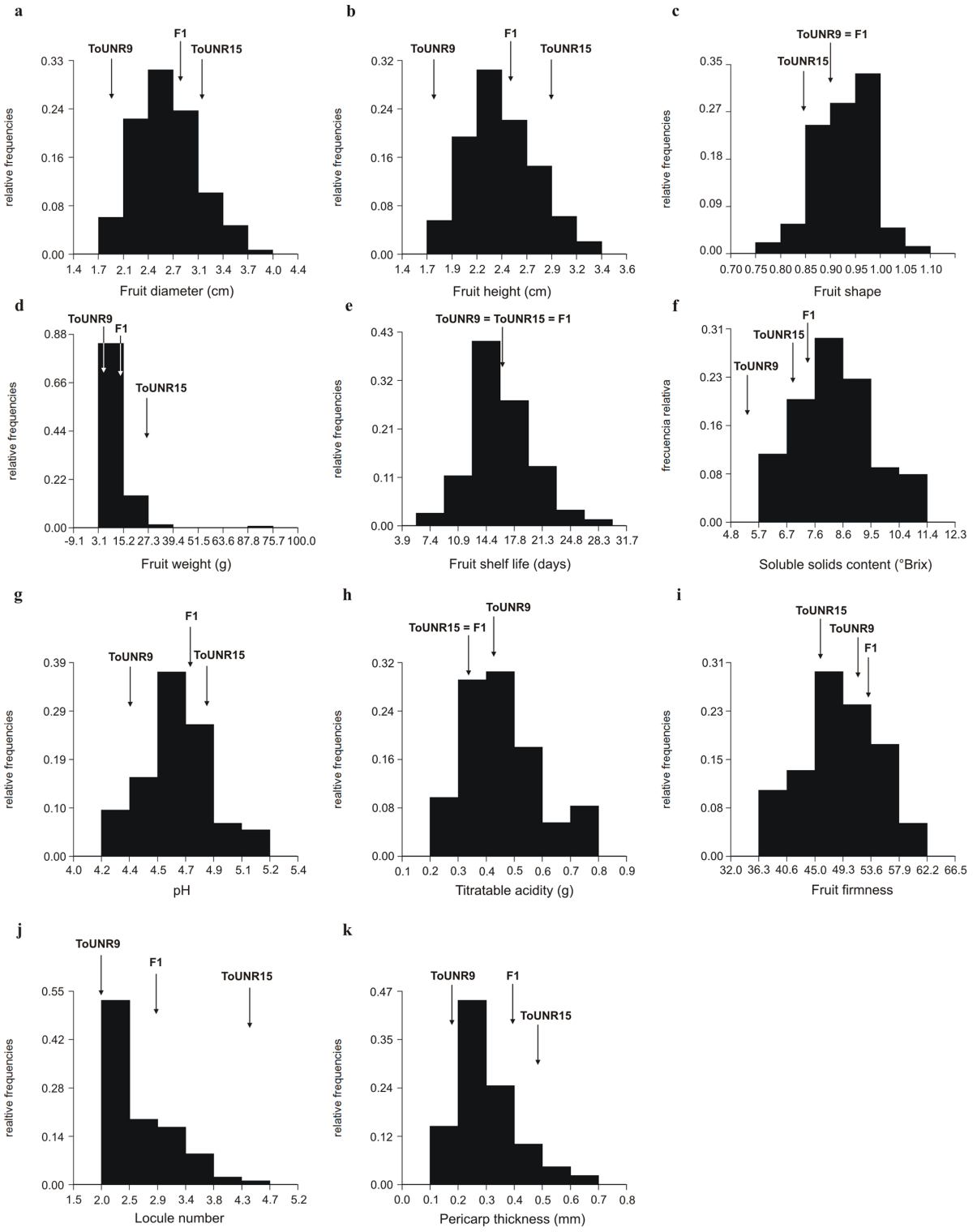

Figure 1. Frequency distributions for each phenotypic trait evaluated in F_2 (ToUNR15 x ToUNR9) generation. Arrows indicates mean values of each trait in parents (ToUNR15 and ToUNR9) and the F_1.

pH, and TA (Tables1 and 2). However, associations for pericarp thickness, firmness and locule number, with intermediate values of genetic variability, were also found. A similar result was found for fruit shelf life in spite of being a trait with low H^2. The occurrence of a band associated with more than one trait could be explained by pleiotropic effects or by a strong linkage between two or more QTLs underlying these different traits (Kearsey and Pooni 1996). Co-localized associations are frequent. In fact, co-localized association for soluble solid content and sugar content, fruit weight and locule number were also found by Xu et al. (2013) in an association mapping study for fruit quality traits of cultivated tomato and related species. Results from Tables 3 and 4 suggest that both types of DNA markers (SRAP and AFLP) allowed QTL detection. Polypeptide profiles were not useful to mark genetic regions that could be involved in the quantitative variation in this segregating generation derived from a SCH. They would probably be more valuable if the parental genotypes were more divergent. DNA markers are more effective because they cover a higher proportion of the genome and can distinguish a larger amount of molecular polymorphism. For same traits QTLs were found using both SRAP and AFLP. From an operational point of view, the SRAP markers were simpler, less expensive and quicker. They can detect the same extent of associations but with a considerably smaller number of total bands compared to AFLP markers (214 against 735, respectively). Gene blocks due to recombination events together with a tight linkage were found by genetic linkage analysis (Figure 2). The genomic regions belonging to the same linkage group (LG) were produced by fragments inherited from first cycle parental genotypes. Nevertheless, some bands assessed in ToUNR9 and ToUNR15 and conserved in generation F_1 (Mendelian inheritance in F_2), were defined as *de novo* bands (Liberatti et al. 2013). Even if they were absent or monomorphic in Caimanta and LA722 (first cycle parental genotypes), inheritance was stabilized in the second cycle parental genotypes as well as in the segregating generation (see examples in LG1, LG2, LG3, and LG4 in Figure 2).

Table 2. Detected associations in F_2 (ToUNR15 x ToUNR9) with SRAP (Sequence-related Amplified Polymorphism) markers (**A**) and AFLP (Amplified Fragment Length Polymorphism) markers (**B**)

A

Bands	Trait	Mean P	nP	Mean A	nA	R^2	p-value	Origin[1]
SI.55	H (cm)	2.36	110	2.55	30	0.05	0.0062	ToUNR9
	SS (°Brix)	8.57	66	7.72	22	0.08	0.0009	
	PT (mm)	0.24	67	0.29	21	0.06	0.0026	
SI.60	LN	2.54	73	2.91	15	0.05	0.0063	ToUNR15
SIV.7	SS (°Brix)	8.13	73	9.19	16	0.10	0.0002	ToUNR15
	pH	4.64	61	4.82	13	0.10	0.0001	
	PT (mm)	0.26	73	0.21	16	0.07	0.0020	
SIV.26	SS (°Brix)	8.48	68	7.80	21	0.05	0.0074	ToUNR9
SIV.41	pH	4.63	58	4.79	16	0.09	0.0002	ToUNR15

B

Band	Traits	Mean P	nP	Mean A	nA	R^2	p-value	Origin[1]
X4	SL (days)	15.36	69	18.64	21	0.12	0.0010	ToUNR15
X14	TA (g)	0.41	54	0.50	13	0.07	0.0020	ToUNR15
X67	SS (°Brix)	8.07	51	8.66	32	0.05	0.0100	ToUNR15
X91	SS (°Brix)	8.10	65	8.99	18	0.08	0.0009	ToUNR15
	pH	4.61	53	4.79	17	0.13	0.0001	
	TA (g)	0.45	51	0.37	16	0.06	0.0042	
	PT (mm)	0.26	66	0.22	20	0.05	0.0080	
X137	F	47.58	60	50.94	31	0.07	0.0015	ToUNR15
	PT (mm)	0.26	63	0.21	23	0.07	0.0016	
R79	SS (°Brix)	8.04	67	9.15	23	0.14	0.0001	ToUNR15
P7	SS (°Brix)	8.68	24	6.89	6	0.24	0.0007	ToUNR9
	LN	2.42	26	3.05	6	0.19	0.0030	
	PT (mm)	0.22	26	0.34	6	0.27	0.0003	

[1] Origin (parental genotype) of DNA amplification fragments are indicated. **Mean P**: mean value for F_2 plants with presence of band, **nP**: number of plant with presence of band, **Mean A**: mean value for F_2 plants with absence of band, **nA**: number of plants with absence of band, **R^2**: fraction of phenotypic variation, **p-value**: probability associated, **H**: fruit height, **SS**: soluble solids content, **PT**: pericarp thickness, **LN**: locule number, **SL**: fruit shelf life, **TA**: titratable acidity.

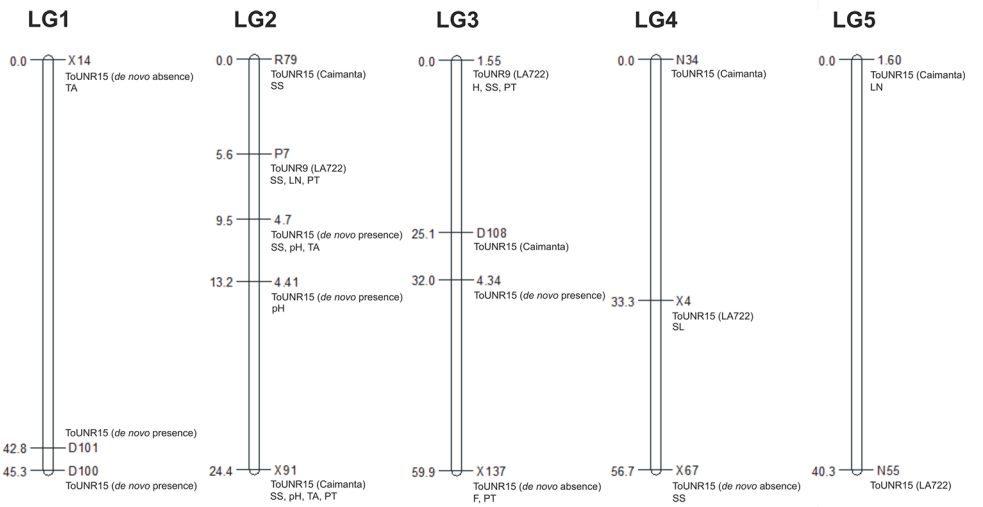

Figure 2. Linkage group (LG) constituted by molecular markers with Mendelian inheritance. The origin of molecular markers in second cycle parental genotypes (ToUNR15 and ToUNR9) and firstcycle parental genotypes (between brackets, Caimanta of *S. lycopersicum* and LA722 of *S. pimpinellifolium*) are indicated. The d*e novo* bands were defined according to Liberatti et al. (2013). The phenotypic traits associated with the molecular markers are also indicated. **TA**: titratable acidity, **SS**: soluble solids content, **LN**: locule number, **PT**: pericarp thickness, **H**: height, **F**: firmness, **SL**: shelf life.

The five linkage groups spanned 226.6 cM (about 15% of the whole tomato genome, based on previous maps reported by Khialparast et al. 2013 and Tanksley et al. 1992), with a mean interval length of 13.3 cM between markers. It was found that 17 of 26 (65%) markers with Mendelian segregation belong to some linkage groups. The number of markers per linkage group ranged from 2 (LG5) to 5 (LG2). On average, 3.4 markers per group were detected. The LG3 was the largest (59.9 cM) and LG2 had the smallest genetic linkage distances. Although all polymorphic polypeptides with Mendelian segregation were considered in the analysis they do not belong to any linkage group. The most interesting group was LG2, for being the shortest. During the first cycle of crosses, new gene blocks from chromosome regions of Caimanta and LA722 were created by recombination. They were conservatively inherited and located at LG2. Moreover, the markers belonging to this LG were associated with fruit quality traits (SS, pH, TA, LN and PT) in robust agreement with those observed in the parental RILs for these traits. This fact implies that ToUNR9 and ToUNR15 genotypes fixed alleles with opposite effects during the selection process. A similar result was found for pericarp thickness, a trait for which the QTL detected in LG3 had opposite effects on the linkage markers (X137 increases its mean value and SI.55 decreases it). These results are consistent with the breeding approach proposed by Bai and Lindhout (2007), who stated that early selection for fruit quality traits is rarely performed in crosses derived from wild germplasm, as many generations may be needed to remove the deleterious genes that accompany the introduced genes, due to linkage drag. When the parental lines reach a high level of homozygosity (F_4 to F_6), crosses are made to test hybrids.

Phenotypic characterization of the two tomato RILs derived from an interspecific cross, the F_1 generation (SCH) between them and the segregating F_2 generation allowed the identification of genetic variability for several fruit quality traits. Different polymorphism levels were detected by polypeptide profiles, SRAP and AFLP markers, showing that the observed phenotypic differences are associated with gene variation. Only SRAP and AFLP markers were able to detect QTLs for fruit quality traits of agronomic interest. This study demonstrated the presence of new gene blocks by chromosome rearrangement and recombination. These results suggest that it is possible to go on with a tomato breeding plan to generate phenotypes with higher performance for fruit quality in the studied F_2 generation.

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