Associations among fruit firmness, morphological traits and RAPD markers in the 'firm' tomato mutant

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ABSTRACT

The Santa Clara Cultivar is widely known in the tomato-producing region of Viçosa (MG). Recently, tomato plants from the same cultivar with changes in morphological traits and post-harvest fruit characteristics have been identified. The inheritance study and the allelism test carried out by Schuelter et al. (2002) determined that a recessive gene with pleiotropic effects modified the expression of morphological traits, such as the color of stigma and fruits and the early leaf senescence. This gene, present in the 'firm' mutant (*frm*), was mapped on the tomato chromosome 10 region - the same region of the *lutescent-2* (*l-2*) gene. However, the identification and location of the genes that increase the firmness of 'firm' mutant fruits remain unknown. Therefore, the objective of this study was to analyze the inheritance of morphological and firmness characteristics of fruits which were modified by the mutation using RAPD markers. Results demonstrated that the genomic region comprising the *l-2* gene increases the fruit firmness, explaining from 6.27 to 25.09% the phenotypic variation for this trait along the 15 day-period of storage. However, the $AQ16_{747}$ and $AS8_{622}$ markers, mapped at 11.67 and 21.67 cM from the mutation *frm,* indicated that the further they were located from this region, the smaller proportion of the phenotypic variation they had. Thus, we can conclude that that the genomic region flanking the *l-2* gene also increases fruit firmness in the 'firm' mutant, a trait that has never been associated with this gene.

KEY WORDS: *Lycopersicon esculentum*, 'firm' Mutant, *lutescent-2* Gene, RAPD.

INTRODUCTION

Among the several problems of tomato crops, fruit perishability has been intensely focused due to the magnitude of post-harvest losses. From a total of 2.6 million t/year of the tomatoes produced in Brazil (FAO, 1998), the losses of the commercialized volume vary from 5 to 25% (Lana et al., 1999), without considering those losses occurred during crop production and transport, as well as the ones occurring after consumer purchasing. In order to solve some problems encountered during post-harvest fruit handling, different strategies have been used, such as the harvesting of mature-green fruits and a subsequent ethylene application; the production of transgenic varieties containing some ripening genes in anti-sense orientation, and finally the use of natural mutations in the heterozygote condition producing hybrids with an increased shelf life.

A great number of genes that modify tomato ripening have been previously identified and described (Tomes et al., 1953; Gray et al., 1994; Grierson and Fray, 1994). Plants containing *non-ripening* (*nor*), *ripening inhibitor* (*rin*) and *alcobaça* (*alc*) genes produce less softening fruits, less ethylene production and less intense color alteration during ripening (Tigchelaar et al., 1978; Mutschler et al., 1992). The *nor* (Tigchelaar et al., 1973) and *alc* (Mutschler, 1984) genes were mapped on chromosome 10, separated by 10 map-units (Mutschler, 1984). The *ripening inhibitor* gene is located in chromosome 5, which is strongly linked to the *macrocalix* (*mc*) gene (Tigchelaar et al., 1978).

Plants of cv. Santa Clara which presented pale yellow fruits in immature stages and red ones when overripe, and also slow maturation and firm-ripeness fruits have recently been identified in the producing region of Viçosa (MG). These plants present flowers with yellow stigmas and leaves with early senescence when

compared to wild plants (Schuelter et al., 1997), and were denominated 'firm' mutant (*frm*). These plants have similar characteristics to the effects caused by the *lutescent-1* (*l-1*) and *lutescent-2* (*l-2*) genes, which are located in chromosomes 8 and 10, respectively (Rick, 1980). Schuelter et al. (2002), based on the allelism test, reported a lack of gene complementation to morphological traits between the 'firm' mutant and *lutescent-2*, indicating that the mutation identified in cv. Santa Clara is also located in chromosome 10. However, the identification and location of the genes promoting an increase in firmness in the 'firm' mutant fruits are still unknown. Thus, the purpose of this study was to analyze the inheritance of some morphological and post-harvest fruit characteristics that were modified in the 'firm' mutant by using RAPD markers.

MATERIAL AND METHODS

Genetic material and growth conditions

The experimental material was a backcross population of 124 plants derived from a cross between 'firm' mutant and the accession BGH-6907 of the wild species *Lycopersicon cheesmanii*. In this case, interespecific crossings are preferred for genetic mapping due to the low level of polymorphism within cultivated tomatoes (Helentjaris et al., 1985; Miller and Tanksley, 1990).

BC_1 plants and progenitors were grown in pots under greenhouse conditions. Seeds were sowed in polystyrene boxes containing commercial substrates, and twenty-five days after seed sowing the plantlets were transferred to 10-L pots containing a 5:3:2 (v/v) mixture of soil, organic compound and sand. This mixture presented medium fertility and pH around 6.0.

Evaluation of phenotypic traits

Leaf early senescence, yellow-pale fruit color and yellowish stigmas were evaluated in the whole backcross population. Five fruits were harvested from each plant of the population and taken to the laboratory, sprinkled with a solution of Ethrel (1.000 ppm) for a uniform ripening and stored in a humid chamber at 25°C and 95% relative humidity afterwards. Fruit softening was verified every three days, using the Calbo and Nery method (1995).

DNA extraction and RAPD assay

DNA from parental and segregating plants was extracted following the method described by Fulton et al. (1995). Amplifications were performed in 25µL of the reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.8 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of a 10 base primer (Operon Technologies Inc., Boulevard, CA, USA), 5-10 ng of genomic DNA and 1 U of DNA Taq polymerase. Amplifications were carried out in a polystyrene tray with 96 wells in a PT-100 thermal cycler (MJ Research, Waltham, MA, USA).

The DNA amplification program consisted of 42 cycles of 1 minute at 92°C, 1 min at 35°C, and 2 min at 72°C. Amplification products were resolved in a 1.4% agarose gel, containing 0.2 mM ethidium bromide in a 0.5% TAE buffer. Images were stored in an Eagle Eye II photo-documentation system (Stratagene, La Jolla, CA, USA).

Molecular data analysis

The identification of RAPD markers linked to morphological traits that were modified by the mutation was done by using the Bulked Segregant Analysis (BSA) proposed by Michelmore et al. (1991). Each of the contrasting DNA bulks consisted of eight individuals from the backcross population showing mutant and normal phenotypes, respectively. For the initial screening, 865 RAPD primers were evaluated between the bulks, and later the polymorphism of interest was analyzed in the whole population, together with the morphological traits for the presence (1) or absence (0) of the bands or the phenotype, respectively.

All markers were assayed for the Mendelian segregation using the chi-squared test at 5%, and genetic distances between markers were determined by the software MAPMAKER v 3.0 (Lander et al., 1987), with a minimum LOD of 3.0 and a maximum recombination frequency of 0.4. The map distance was calculated by using the Kosambi function.

The consistency of the fruit firmness characteristic, measured every three days during the storage period of 15 days, was verified in the 124 BC_1 individuals using the repeatability coefficients, as described by Cruz and Regazzi (1994). The associations between the markers and phenotypic data were evaluated by single and multiple regression analyses using JUMP software (SAS Institute Inc., 1994).

RESULTS AND DISCUSSION

Segregation of Morphological Characters

The segregation analysis of the morphological characters for the interespecific cross (Table 1) indicated that the 'firm' mutant (*frm*) is regulated by a recessive gene with pleiotropic effects for early leaf senescence, yellow-pale color in immature fruits and yellowish stigmas. This complies with the results of Schuelter et al. (2002) in an intra-specific cross. However, only two individuals of the backcross population presented yellow-pale immature fruits, yellowish stigmas and senescent leaves at the end of the crop cycle. Therefore, the results obtained in this study indicate the presence of modifying genes linked to the genomic region of the mutation that may act on the reduction of leaf senescence severity. In addition, it can also be inferred that epistatic gene interactions could be responsible for reducing the effects promoted by the mutation, such as leaf senescence. Nevertheless, further experiments should be done to prove the hypothesis of genetic linkage and/or pleiotropy through the analysis of segregation of progenies derived from self-fertilization of individuals with normal leaf senescence and yellowish fruits and stigmas.

Association of RAPD markers with fruit firmness

Two polymorphisms, present in non-mutant BC_1 individuals, were identified among the 826 RAPD primers amplified among the progenitors and contrasting bulks. The markers $AQ16_{747}$ and $AS08_{622}$ (Figure 1) presented an expected Mendelian segregation (c^2 = 0.524 and 0.669), which was mapped at 11.67 and 21.67 cM from the 'firm' mutant, respectively (Figure 2). The 'firm' mutation was mapped using morphological traits, once it was determined by a single recessive gene (Table 1). According to Schuelter et al. (2002), this genomic region can be considered in the tomato chromosome 10, where the *lutescent-2* gene was mapped (Rick, 1980).

The lack of polymorphisms closely linked to the *l-2* gene on the tomato chromosome 10 may be overcome by using a higher number of AFLP markers in a cross with more divergent progenitors such as *L. penelli* and *L. chmielewskii*, frequently used in mapping studies (Azanza et al., 1994; Chetelat et. al., 1995; Giovannoni et al., 1999). Another strategy might be converting the $AQ16_{747}$ and $AS08_{622}$ markers in SCARs in order to locate them in a better-saturated tomato map. Since genetic factors related with fruit ripening have been identified in different positions along chromosome 10 (Kinzer et al., 1990; Giovanonni et al., 1999), confirming the segregation among these markers and other fruit ripening genes would be very useful.

The regression analyses (Figure 3) using fruits derived from the backcross population detected that 'firm' mutant fruits presented a higher shelf life along the 15-day storage period than the heterozygous fruits. Although the differences in fruit firmness have decreased with storage time, the average values of the homozygous fruits for *frm* mutation were statistically higher than for the heterozygous determined by the *t*-test (data not shown). The repeatability coefficients for fruit firmness differed significantly along the storage period, with high values observed at 12 and 15 days of storage (Table 2). As the highest performance estimates were found at 15 days of storage in both genotypes, this data was used in the association analyses of the fruit firmness with the RAPD markers and the 'firm' mutant.

The regression analysis identified a genetic factor which caused a delay in fruit softening (Table 3) in the genomic region containing the *frm* mutation. This fact

Table 1. Segregation of morphological characters modified by the mutation identified in the 'firm' mutant of the cv. Santa Clara (*frm*), derived from the cross between *frm* and the accession BGH-6907 from the wild species *Lycopersicon cheesmanii* - *lc*.

Generation	Genotype or cross	Number of plants		Expected ratio	χ^2	Probability
		Wild Type	Mutant			
P_1	lc	12	0	1:0	-	-
P_2	frm	0	12	0:1	-	-
F_1	lc × frm	12	0	1:0	-	-
BC_1	(lc x frm) × frm	62	60	1:1	0.033	0.85

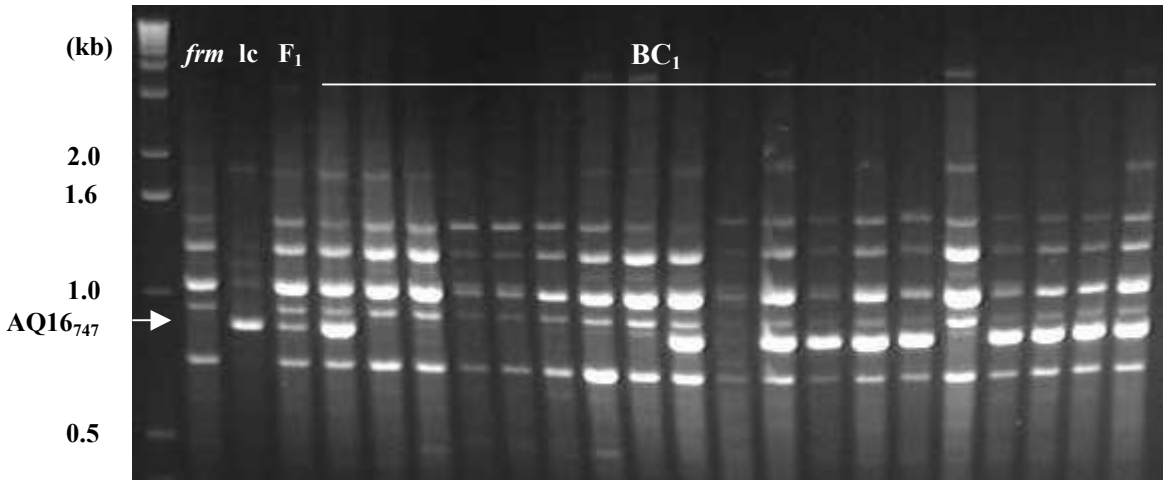

Figure 1. Amplification profile generated by primer OPAQ16 identifying the $AQ16_{746}$ marker associated to the 'firm' mutant (*frm*). Lane 1: molecular weight marker (1kb ladder); Lane 2: 'firm' mutant (*frm*); Lane 3: *L. cheesmani* (*lc*); Lane 4: progeny of cross *frm* x *lc*. F1; Lane 5: progenies of backcross (F1 x *frm*). BC_1.

explained 12.94% of the total phenotypic variation after 15 days. Additionally, the $AQ16_{747}$ and $AS8_{622}$ markers indicated that the further they were mapped from the *frm* mutation, the smaller proportion of fruit firmness occurred. These results confirm the allelism test reported by Schuelter et al. (2002), suggesting the existence of a genetic factor that promotes reduction in fruit softening in the 'firm' mutant, and is located in the genomic region where *lutescent-2* gene is mapped.

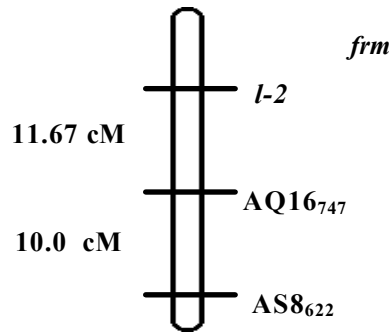

Figure 2. Mapping of RAPD markers and 'firm' mutation (*frm*) in the BC_1 population derived from the cross between the cv. Santa Clara 'firm' mutant (*L. esculentum* Mill) and BGH-6907 (*L. cheesmanii* Riley).

CONCLUSIONS

The chromosome region where the *lutescent-2* gene is mapped allows for early leaf senescence, as well as yellowish color of fruits and stigmas in the interespecific cross between the 'firm' mutant and *Lycopersicon cheesmanii*. Morphological characters were either controlled by a recessive gene with pleiotropic effects or by two recessive genes with epistatic gene interactions for fruit and plant color. The RAPD markers $AQ16_{747}$ and $AS8_{622}$ were mapped at 11.67 and 21.67 cM from the *frm* mutation, which indicates that the further they were located from this genomic region, the smaller proportion of the phenotypic variation for fruit firmness they had.

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RESUMO

Associação entre firmeza de frutos, caracteres morfológicos e marcadores RAPD no mutante de tomate 'firme'

A cultivar Santa Clara é bastante difundida entre os produtores de tomate da região de Viçosa (MG). Recentemente, foram identificadas plantas de tomate

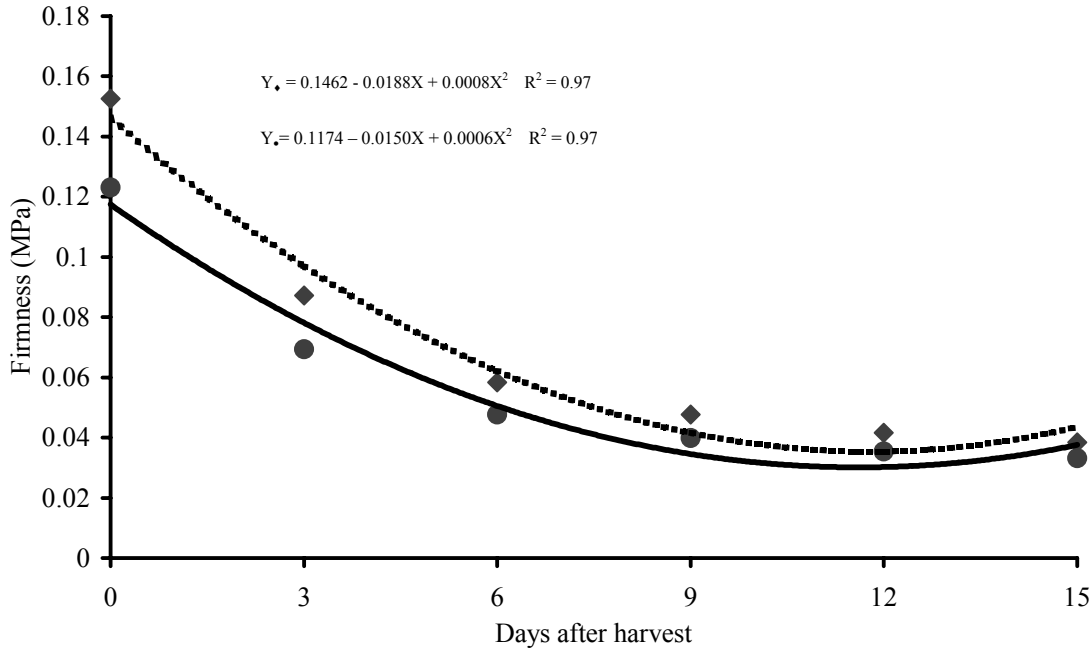

Figure 3. Effect of storage time on the firmness of BC_1 tomato fruits, expressed in Mega Pascal (MPa), for 14 days.

Table 2. Estimates of repeatability (r) and determination (R^2) coefficients for firmness of heterozygous (wild type) and homozygous (mutant - *frm*) tomato fruits for the mutation of the backcross population derived from the cross between the 'firm' mutant and the accession BGH-6907 (*L. cheesmannii*).

Genotype	Method[1/]	Day 0		Day 3		Day 6		Day 9		Day 12		Day 15	
		r	R^2	R	R^2	r	R^2	r	R^2	r	R^2	r	R^2
Wild Type - Heterozygous	1	0.53	85.01	0.50	83.22	0.63	89.39	0.64	89.71	0.69	91.86	0.70	92.08
	2	0.53	84.99	0.49	82.90	0.62	88.99	0.62	88.91	0.67	90.98	0.67	91.21
	3	0.53	85.11	0.52	84.27	0.64	89.78	0.65	90.18	0.72	92.69	0.72	93.02
	4	0.53	85.25	0.51	83.82	0.63	89.47	0.64	89.81	0.70	92.12	0.70	92.22
	5	0.53	85.17	0.50	83.36	0.63	89.41	0.64	89.75	0.70	92.04	0.70	92.17
Mutant - Homozygous	1	0.32	70.59	0.43	78.87	0.43	79.38	0.38	75.32	0.46	81.18	0.68	87.55
	2	0.33	70.94	0.43	79.17	0.44	79.50	0.38	74.90	0.45	80.64	0.68	87.48
	3	0.39	76.33	0.43	79.27	0.44	79.68	0.38	75.76	0.47	81.43	0.69	87.86
	4	0.36	73.66	0.43	79.12	0.44	79.54	0.39	76.33	0.47	81.86	0.69	87.82
	5	0.35	72.63	0.43	78.95	0.44	79.48	0.39	75.95	0.47	81.69	0.69	87.72

[1/] Methods: 1. ANOVA: Model with one variation factor; 2. ANOVA: Model with two variation factors; 3. Principal components: covariance matrix; 4. Principal components: correlation matrix; 5. Structural analysis: correlation (medium r).

Table 3. Estimates of determination coefficients (R^2) and the respective significance levels (P) obtained by the regression analysis between genetic markers and firmness of tomato fruits in six ripening stages.

Genetic Markers	Day 0		Day 3		Day 6		Day 9		Day 12		Day 15	
	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
$AS8_{622}$	7.84	0.003	2.78	0.849	2.89	0.079	0.09	0.757	1.93	0.151	3.74	0.045
$AQ16_{747}$	15.48	0.000	7.17	0.005	4.31	0.031	0.22	0.627	2.25	0.121	3.92	0.040
frm	25.09	0.000	21.10	0.000	18.56	0.000	6.27	0.009	15.74	0.000	12.94	0.0001

da mesma cultivar com modificações em caracteres morfológicos e de pós-colheita de frutos. Pelo estudo de herança e teste de alelismo realizados por Schuelter et al. (2002), determinou-se que um gene recessivo com efeitos pleiotrópicos modifica a expressão dos caracteres morfológicos, como a cor de estigma e de frutos, bem como a ocorrência de senescência foliar precoce. O gene, presente no mutante 'firme foi mapeado na região do cromossomo 10 onde se encontra o gene *lutescent-2* (*l-2*). No entanto, os questionamentos quanto a identificação e localização de genes que promovem o aumento da firmeza de frutos do mutante 'firme' permanecem sem uma elucidação adequada. Assim, o presente trabalho teve como objetivo estudar a herança dos caracteres morfológicos e da firmeza de frutos modificados pela mutação, empregando-se a técnica de marcadores moleculares RAPD. Os resultados demonstraram que a região genômica do gene *l-2* promove aumento da firmeza de frutos, sendo explicados de 6,27 a 25,09 % da variação fenotípica nos diferentes períodos de avaliação da firmeza. Já os marcadores $AQ16_{747}$ e $AS8_{622}$, mapeados a 11,67 e 21,67 cM da mutação *firme*, explicaram tanto menor proporção da variação fenotípica quanto mais distantes estavam localizados dessa região. Assim, conclui-se que a região flanqueando o gene *l-2* promove também o aumento na firmeza de frutos no mutante 'firme', uma característica que nunca havia sido associada a esse gene.

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