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Genetic control of modified genomic region in a *firm* ripening tomato (*Lycopersicon esculentum* Mill.) mutant

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ABSTRACT – *Studies involving the firm tomato (*Lycopersicon esculentum*) mutant have shown that pleiotropy or genetic linkage are responsible for modifications in morphological and postharvest traits. The objective of this report was to evaluate the hypothesis of pleiotropy or genetic linkage linked to morphologic traits and to verify the effect of QTL on fruit firmness. Plants of mutant firm and L. cheesmani were intercrossed; the F_2 and F_3 generations were analyzed for segregation of morphological traits and firmness, and the RAPD technique was used for the F_2 population. Results showed that the recessive pleiotropic gene is responsible for the morphological traits, but environmental and/or genetic factors affect the penetrance and expressivity of the mutation. By the RAPD analysis, a QTL was detected in the group represented by the markers AS-08_{622}, AQ-16_{747} and l-2 that explains 29.77% of the variation to fruit firmness.*

Key words: pleitropic mutant, penetrance, expressivity, RAPD markers.

INTRODUCTION

Tomato is economically the second most important vegetable, produced worldwide at around 115.95 million tons per year according to Camargo et al. (2006). In spite of the large volume, many factors are responsible for field losses, due to the susceptibility of the tomato plant to pathogens and pests, as well as during the posharvest stage, in particular due to inadequate fruit handling. From a total of 3.49 million tons of tomato produced in Brazil (Camargo et al. (2006), 14.92% fresh tomato is lost during postharvest due to physical damage and 60% due to inadequate packing (Vilela and Luengo 2002).

Postharvest practices have been developed to make storage easier, extend the shelf-life and minimize physical damages through handling. One of the most meaningful advances has however been achieved by using natural mutants as source of genes and techniques of genetic engineering for the development of commercial long shelf life and transgenic varieties with fruits with longer postharvest conservation (Gray et al. 1994).

Several genes have been identified in the *Lycopersicon esculentum* species, among them those that modify different aspects of fruit ripening. The pleiotropic mutants *never-ripe* (*Nr*), *non-ripening* (*nor*),

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ripening inhibitor (*rin*) and *alcobaca* (*alc*) produce fruits with reduced softening, lower ethylene production and less intense color alterations during ripening (Mutschler et al. 1992). These mutants have been used as potential gene donors for improvement programs that aim to obtain fruits with longer storage periods.

Recently, Schuelter et al. (2002) identified a new tomato mutant, the variety Santa Clara, denominated 'firm' (*frm*), with modifications in morphological traits, including early leaf senescence, yellow stigma color and cream-colored fruit, which is controlled by a recessive gene mapped in chromosome 10 in the region of gene *lutescent-2* (*l-2*). Furthermore, generation analysis showed that this mutant promotes modification in the postharvest life. The F_1 hybrids had intermediary shelf life compared to the parents and the additive genetic effects were more important to determine this trait (Schuelter et al. 2001). Later, Schuelter et al. (2003) investigated the genomic region that promoted the modifications of morphological and fruit firmness traits by using RAPD marker analysis, and it was verified that fruits of plants in the homozygotic recessive stage for the mutation presented less firmness losses. Besides, the morphological traits contributed most to the reduction of the firmness, indicating that the recessive pleiotropic gene also modifies this specific postharvest attribute. Still, the identification of two plants with pale unripe colored fruits, yellowish stigma and normal leaf senescence gave rise to the hypothesis that, contrary to the pleiotropy, these traits were controlled by linked genes.

The present study tested the hypotheses of pleiotropy or of genetic linkage for the control of leaf senescence, stigma and fruit color by analyzing the segregation of F_2 and F_3 populations, and by verifying the QTL effect associated to the reduced firmness loss, using RAPD marker and morphological analysis.

MATERIAL AND METHODS

Plant material

Plants of the *firm* mutant (*L. esculentum*) and *L. cheesmanii* (BGH-6908) were grown and intercrossed under greenhouse conditions to develop the segregating and non-segregating populations used in the present study. The use of interspecific crosses is justified in mapping studies involving tomato due to the low polymorphism level found in commercial varieties (Paterson et al. 1991).

Five F_1 plants derived from the interspecific cross were self pollinated to obtain the F_2 population. One hundred and fifteen F_2 plants were grown together with the parents in 10 L pots in a greenhouse. One hundred and fifteen F_3 families, with an average of 21 plants, were grown under field conditions to verify the occurrence of morphological trait segregation, from which 92 were analyzed for fruit firmness. The parent generations F_1 and F_2 were grown in a greenhouse and in the Biotechnology Laboratory of the Universidade Paranaense, and the F_3 generation was planted at the Research Station of the Pioneer seed company, both in the county of Toledo (PR).

Evaluation of morphological traits

Plants of the F_2 and F_3 populations were evaluated for the traits leaf senescence and fruit and stigma color upon formation of the first fruits. In the F_3 families, the fruits were harvested at the mature-green stage and stored at 25 ºC and 70 % relative air humidity. The fruit firmness was assessed by the flattening method described by Calbo and Nery (1995).

DNA purification, bulk establishment and RAPD analysis

Leaf tissue DNA was extracted from plants of the parents and F_1 and F_2 generations by the method described by Fulton et al. (1995), and the BSA strategy (Michelmore et al. 1991) was used for the RAPD marker analysis. Four DNA bulks of the F_2 population were obtained. Each bulk was assembled by the DNA mixture of four F_2 plants; two bulks consisted of plants with early leaf senescence, yellow stigma and cream-colored fruits and the other contained plants of normal senescence, greenish stigma and green-colored immature fruits. Three hundred and seventy-six primers were used and those that generated polymorphic amplification products in the bulks, to test the entire F_2 population for a confirmation of the linkage analysis of the marker to gene *l-2* and determine the existing genetic distance between them.

The amplification reactions by the RAPD technique were performed as described by Williams et al. (1990), in a volume of 25 mL, containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.8 mM $MgCl_2$; 0.2 mM of each one of the deoxinucleotides (dATP, dTTP, dGTP, dCTP), 0.2 mM decamer primers, 5-10 ng genomic DNA and one unit of Taq DNA polymerase per reaction. The DNA was amplified in PCR tubes (0.2 mL) in a Thermo Hybrid PX2 (Thermo) thermal cycler. The DNA amplification program consisted of one denaturation step at 92 ºC for one minute, pairing with the DNA template at 35 ºC for one minute and an extension step at 72 ºC for two minutes. The products of 42 amplification cycles were separated electrophoretically in agarose gel (1%), containing 0.2 mM ethidium bromide, in 0.5% TBE buffer. The amplified fragments were visualized under UV light and the images recorded by a Vilber Lourmat (model Doc Print) gel documentation system.

Data analysis

The segregation of the RAPD markers and morphological traits was tested by the χ^2 test. In the analysis of co-segregation of the RAPD markers and morphological traits the criterion of minimum LOD score of 3.0 and frequency of maximal recombination of 0.35 was used to infer on the linkage between two loci. The recombination frequencies were converted to centiMorgans (cM) through the Kosambi function (1944). The linkage analysis and establishment of the linkage group was performed using software GQMOL (Universidade Federal de Viçosa, Viçosa, MG – Brazil). The confidence interval for the estimate of recombination frequency was obtained based on the approximation with normal distribution at 99% probability (Schuster and Cruz 2004).

The analysis of association between the RAPD markers and morphological data with the fruit firmness was realized by simple and multiple regression analysis, and a QTL was analyzed by simple interval mapping. The stepwise procedure (Draper and Smith 1981) was used for the selection of variables in the multiple regression analysis. The cutting point for the QTL significance determination in the interval mapping was obtained by the permutation test of Churchill and Doerge (1994), comprising 1.000 permutations. The intervals were mapped using software QTL Cartographer (Basten et al. 1998).

RESULTS AND DISCUSSION

Evaluation of the morphological traits

The evaluation of F_1 plants derived from the cross involving the mutant 'firm' and the species *L. cheesmanii* (Table 1) detected the green fruit coloration, flowers with greenish stigmas and normal leaf senescence.

Among the 115 plants evaluated in the F_2 generation (Table 1), 93 had green stigma (normal type) and 22 yellow stigma (mutant type), in agreement with the segregation hypothesis of 3:1 ($\chi^2 = 2.13$, P=14.60%). For the joint evaluation of the morphological traits modified by mutation in F_2 generation, we observed plants with greenish stigma, normal leaf senescence and green unripe fruits or those with yellow stigma, early leaf senescence and pale colored unripe fruits, with exception of two plants. This result is similar to the one obtained by Schuelter et al. (2003) in a backcross population that also involved the mutant 'firm' and the wild tomato species of *Lycopersicon cheesmanii*.

Of the two plants that did not present co-segregation for the three traits, one plant had yellow stigma, pale unripe colored fruits and normal leaf senescence (PS1) and the other greenish stigma, green unripe fruits and early leaf senescence (PS2). In the evaluation of the F_3 families we observed that in the progeny of plant PS1 all 22 plants presented the mutant phenotype, indicating that the F_2 plant PS1 was homozygous for mutation in spite of presenting normal leaf senescence. Likewise, in the progeny of plant PS2, all 22 plants presented the normal phenotype, indicating that the F_2 plant PS2 was homozygous as well, without mutant alleles, in spite of its early leaf senescence (Table 2). Furthermore, other results in F_3 did not agree with the phenotyping in F_2. The families 48, 134 and 145, whose F_2 plants had mutant phenotype (yellow stigma, pale unripe fruit and early leaf senescence) segregated, indicating that the F_2 plants that originated from these families were heterozygous for mutation.

The results obtained in the F_2 and F_3 generations indicate that the lack of co-segregation for the traits stigma color, color of the unripe and senescence in some plants is due to incomplete penetrance of a pleiotropic gene. Considering the 115 plants evaluated in the F_2

Table 1. Segregation of morphological traits modified by the mutation identified in the Santa Clara tomato cultivar (*L. esculentum* - *frm*) derived from the cross of the firm mutant (gene *l-2*) with the wild species (*L. cheesmanii* – lc)

Generation	Genotype or cross	Number of plants		Expected Proportion	χ^2	Probability (%)
		Normal Type[1]	Mutant Type[2]			
P_1	lc	5	0	1:0	-	-
P_2	*l-2*	0	8	0:1	-	-
F_1	lc × *l-2*	5	0	1:0	-	-
F_2	(lc × *l-2*)⊗	93	22	3:1	2.13	14.60

[1] green fruits in the immature phase, green stigma or normal leaf senescence
[2] cream-colored fruits in the immature phase, yellow stigma or early leaf senescence

Table 2. F_3 families derived from the interspecific cross involving *L . cheesmanii* and *L. esculentum* with phenotypic standard modified to the mutation identified in the firm mutant

Families F_3	Phenotype F_2	Plants observed in F_3 (N:M)	Expected proportion in F_3 (N:M)
48	Mutant Type	14:8	0:1
94	PS1	0:22	-
130	PS2	22:0	-
134	Mutant Type	12:9	0:1
145	Mutant Type	4:17	0:1

N:M – Normal type: Mutant type; PS1 – cream-colored fruits, yellow stigma and normal senescence; PS2 - green fruits, green stigma and early senescence

generation, the confidence interval, which contains 99% of the recombination frequency estimate, is 0±0.002 cM. If the 92 F_3 families are considered, the confidence interval for the recombination frequency estimates would be $0\pm5.31x10^{-8}$ cM. At this point, the hypothesis of gene linkage suggested by Schuelter et al. (2003) could be discarded, confirming the previous conclusion of Schuelter et al. (2002) that a recessive pleiotropic gene determines the traits modified by mutation.

By definition, incomplete penetrance is the presence of a particular genotype expressing the corresponding phenotype in a given percentage of a specific population (Griffiths et al. 2002). The phenomenon has been described in plant and animal species as a result of genetic interactions, of environmental fluctuations and of epigenetic variations in the genetic expression (Rakyan et al. 2002). In our study, the occurrence of incomplete penetrance can be justified by the interspecific cross, which has many segregating genetic loci in the first selfing generation and is subjected to the influence of modifying genes and the environment. In this context, the appearance of different degrees of green pigmentation in the leaves, fruits and stigmas was verified, which can characterize the gene *lutescent-2* in its variable expressions.

Physiologically, the recessive allele of gene *lutescent-2* modifies the balance of chlorophyll and carotenoid. Chloroplasts were partially converted into chromoplasts (Fornasiero and Bonatti 1985), which culminated in the reduction of the photosynthetic rate and the early yellowing of plant structures (Fornasiero and Bonatti 1985, Schuelter et al. 2002). As the photosynthetic process is highly influenced by the environmental conditions and the mutation substantially contributes to this physiological process, the occurrence of incomplete penetrance and variable expressivity is reinforced. Nevertheless, it must be highlighted that the occurrence of incomplete penetrance and variable expressivity was not detected in intraspecific crosses (Schuelter et al. 2002).

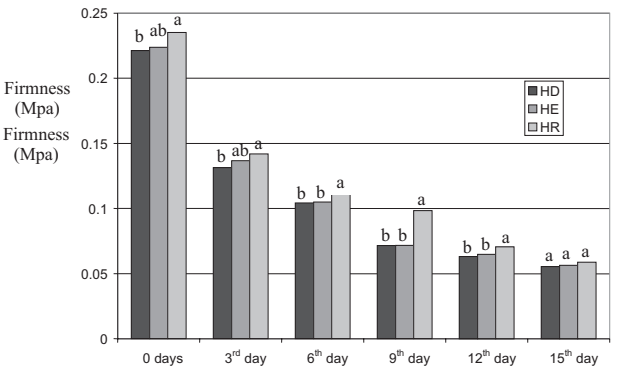

Figure 1. Loss of fruit firmness, expressed in Mega Pascal (MPa), in homozygous dominant (HD), heterozygous (HE) and recessive plants (HR) for the gene *l-2*, by the method described by Calbo & Nery (1995) in F_3 families. Equal letters, in the same evaluation period, indicate that the means did not differ from each other by Tukey´s test at 5% probability

Analysis of fruit firmness and repeatability of the data

The fruit firmness of the 92 F_3 families (Figure 1), analyzed by Tukey´s test at 5% probability evidenced that the homozygous recessive plants (HR) for mutation were firmer than the homozygous dominant (HD) throughout the storage period, except on the last day of evaluation. The greatest difference in fruit firmness was however observed during the first nine days of storage and more uniform losses thereafter. The fruit firmness of the dominant homozygous plants, on the other hand, did not differ statistically from the heterozygous for mutation. Physiologically, Moura et al. (2005) verified that the fruits of the mutant 'firm' in different maturation stages presented lower ethylene and CO_2 levels than the cultivar Santa Clara. Besides, the fruits of the mutant 'firm' presented a different activity pattern of ACC oxidase with a delayed increase of polygalacturonase activity. Since ethylene and the polygalacturonase are directly involved in the modification of the fruit firmness of tomato, the results obtained in the present study corroborate the ones obtained by Moura et al. (2005) indicating the occurrence of differential expression of the mutant allele.

By the estimation of repeatability coefficients (data not shown) of the F_3 families, which allow a verification of the quality of the firmness data, variations in the estimate magnitudes were observed according to the periods of firmness evaluation. The highest values were attained after 6, 9, 12 and 15 days of fruit storage, with repeatability and determination coefficients of over 0.6 and 0.8, respectively. These results allow the affirmation that the replication of the genotypes from one evaluation to the other was regular. Data of fruit firmness can therefore be used for analyses of association with markers (molecular or morphological).

Analysis of RAPD markers and loss of fruit firmness

By the DNA data analysis of the F_2 plants, using the BSA method (Michelmore et al. 1991), three polymorphic patterns were identified in the bulks, in the markers A-08_{725}, AS-08_{622} and AQ-16_{747}. In all polymorphisms, the presence of bands was verified in the parent and in the bulk presenting normal phenotype, and absence of mutant phenotype in the parent and in the bulk, in other words, the molecular markers were arranged in *cis* with the morphological markers.

The markers A-08_{725}, AS-08_{622} and AQ-16_{747} segregated in a proportion of 3:1 (presence:absence of band). Moreover, the evaluation of the F_2 population based on the segregation pattern in F_3 generation showed that the morphological marker (*l-2*) associated to stigma color, fruit color and leaf senescence segregated in the proportion 1:2:1 (Table 3), confirming the genetic control of one gene for mutation.

In the analysis of co-segregation for the molecular and morphological markers, it was verified that the markers AQ-16_{747} and AS-08_{622} are linked to chromosome 10, in the region of the *lutescent-2* gene, flanking it, where AQ-16_{747} is at 9.3cM from *l-2* (LOD 12.91) and AS-08_{622} is at 15.16cM from *l-2* (LOD 8.11) (Figure 2). Although the marker A-08_{725} had presented polymorphism in the bulks, the analysis of the F_2 population did not confirm the genetic linkage between this marker and gene *l-2*. The probability that a RAPD locus is polymorphic in an analysis of a bulk containing eight plants, without being linked to the gene of interest is 10%.

Data from the regression analysis involving the markers AS-08_{622}, A-08_{725}, AQ-16_{747}, and *l-2* with the data of fruit firmness of plants of the F_3 population (Table 4), showed that marker AQ-16_{747} was the one that contributed most to determine the firmness (R^2 = 23.46/9th day), followed by the morphological marker (*l-2*) (R^2 = 20.10/9th day) and by the marker AS-08_{622} (R^2 =

Table 3. Genetic-statistical analysis by the c^2 test for the modified morphological traits in the ´firm´ mutant in the gene locus *l-2* and polymorphic RAPD fragments (A-08_{725}, AS-08_{622} and AQ-16_{747}) in the F_2 generation

Marker	Classes					Hipotesis	χ^2	Probability
	P	A	HD	HE	HR			
AS-08_{622}	67	22	-	-	-	3:1	0.004	95.12
A-08_{725}	72	18	-	-	-	3:1	1.22	7.33
AQ-16_{747}	66	25	-	-	-	3:1	0.297	58.59
l-2	-	-	27	41	24	1:2:1	1.283	52.66

P: band presence; A: band absence; HD: dominant homozygous; HE: heterozygous; HR: recessive homozygous

Table 4. Estimates of determination coefficients (R^2) and the respective levels of significance (P) obtained by the analysis of simple linear regression between genetic markers and the fruit firmness in six different storage periods

	Day 0		Day 3		Day 6		Day 9		Day 12		Day 15	
Marker	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P	R^2	P
AS-08_{622}	1.01	0.65	0.30	0.61	5.84	0.02*	17.04	<0.01*	6.40	0.02*	4.01	0.06
A-08_{725}	0.07	0.80	0.10	0.76	0.06	0.81	0.44	0.54	0.09	0.77	0.04	0.83
AQ-16_{747}	2.23	0.15	2.07	0.17	3.45	0.07	23.47	<0.01**	2.99	0.10	3.17	0.09
l-2	1.76	0.24	1.25	0.29	2.26	0.21	20.10	<0.09*	1.49	0.27	0.41	0.57

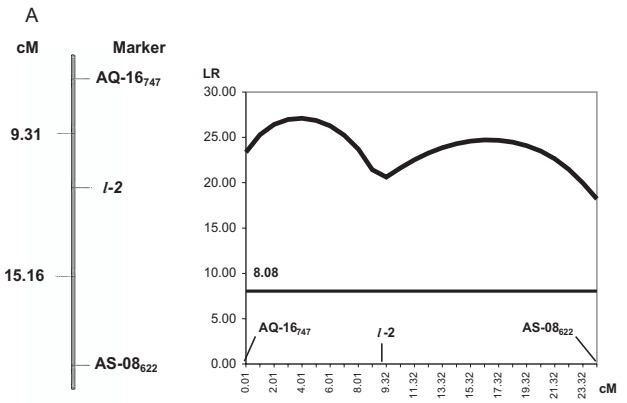

Figure 2. Genetic linkage between the RAPD and morphological markers (l-2) in the F2 population of the cross ´firm´ mutant x *L. cheesmanii* (A) and QTL for tomato fruit firmness (B), localized in the region of locus l-2, obtained by simple interval mapping analysis. The cutoff value for the likelihood ratio (LR=8.08) was obtained by the permutation test with 1.000 permutations, at a level of significance of 5%

5.84/6th day, R^2 = 17.04/9th day, R^2 = 6.40/12th day). Marker A-08_{725}, on the other hand, was not linked to the genomic region with the modifications in the morphological traits, nor did it contribute to fruit firmness. In the joint regression analysis, where the stepwise method of variables was used, the markers AQ-16_{747} and AS-08_{622} were significant with R^2 of 26.41% (P=0.029).

By analyzing the fruit firmness data of the 9th day in the linkage group obtained by the molecular markers and gene *l-2* (Figure 2), a QTL associated to tomato fruit firmness was detected in the region of gene *l-2*. This QTL accounted for 29.77% of the variation in tomato fruit firmness and presented complete dominance (d/a=1.26), with negative values for the additive and dominance effects (a=-1.47 and d=-1.86), indicating that the dominant alleles diminish fruit firmness. The broad and narrow-sense heritability of the fruit firmness, based on the mean square expectations, were 60.56% and 58.73%, respectively. Thus, the QTL associated to locus *l-2* explained 49.16% of the genetic variance and 50.70% of the additive genetic variance for fruit firmness.

This QTL associated to gene *l-2* had already been identified by Schuelter et al. (2003) in a backcross population. In this study, the use of a F_2 population to obtain the molecular data and F_3 families for the

phenotypic data allowed an estimation of the additive and dominance effects of the QTL on loss of firmness, besides confirming the hypothesis of pleiotropy of gene *l-2* for the traits fruit firmness, early leaf senescence and stigma and fruit color. Gene *l-2* is localized in chromosome 10 of the tomato plant, where genetic factors related to ripening have also been mapped earlier, such as the polygalacturonase gene (Della Penna et al. 1986) and the mutants *non-ripening* (Tigchelaar 1978) and alcobaca (Mutschler 1984).

CONCLUSIONS

The results obtained based on the evaluation of the F_2 population and the F_3 families indicate that a pleiotropic recessive gene *(l-2)* determines the yellow stigma color, early leaf senescence and the pale color of unripe fruit. This gene presents incomplete penetrance and variable expressivity for the modified morphological traits in the firm mutant. The fruits derived from recessive homozygous plants for gene *l-2* are firmer than the homozygous and heterozygous dominant ones. A QTL was identified in the linkage group formed by the AS-08_{622}, AQ-16_{747} and *l-2* markers explaining 29.77% of the variation for the reduction of the fruit firmness during ripening studied in the F_3 generation.

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Controle genético de região genômica modificada no mutante de tomate firme (*Lycopersicon esculentum* Mill.)

RESUMO – *Estudos envolvendo o mutante de tomate 'firme' (*Lycopersicon esculentum*) tem indicado que pleiotropia ou ligação gênica são responsáveis pelas modificações em caracteres morfológicos e de pós-colheita de frutos. Assim, o presente trabalho teve como objetivos testar as hipóteses de pleiotropia ou de ligação gênica e verificar o efeito de QTL associado à firmeza de frutos. Plantas do mutante firme e da espécie L. cheesmanii foram intercruzadas, verificando-se nas gerações F_2 e F_3, o padrão de segregação para os caracteres morfológicos e de firmeza, enquanto que a técnica de RAPD foi analisada em indivíduos da população F_2. Os resultados indicaram que um gene recessivo pleiotrópico determina os caracteres morfológicos, porém fatores ambientais e/ou genéticos afetam a penetrância e expressividade. Pela análise de marcadores RAPD, detectou-se que um QTL no grupo de ligação formado pelos marcadores AS-08_{622}, AQ-16_{747} e l-2 explicam 29,77% da variação para a perda de firmeza de frutos.*

Palavras-chave: Lycopersicon esculentum*, mutante 'firme', penetrância, expressividade, marcadores RAPD.*

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