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SHORT COMMUNICATION

A 54-kDa polypeptide identified by 2D-PAGE and bulked segregant analysis underlies differences for pH values in tomato fruit

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Abstract Fruit pH is an important quality attribute in tomato and it is defined during ripening. The aims of this work were to detect pericarp polypeptides associated with pH in an interspecific tomato BC_1 generation by 1D-PAGE and to identify those differentially expressed polypeptides by comparing 2D-PAGE protein profiles from bulked segregant analysis (BSA). Polypeptide patterns were resolved by 1D-PAGE in a BC_1 population obtained by crossing the cv. 'Caimanta' of *Solanum lycopersicum* (recurrent parental genotype) and the accession LA722 of *S. pimpinellifolium* (donor parental genotype). Putative QTL for fruit quality were detected by single point analysis. The presence of a 54-kDa band at the mature green stage (MG) carried by the wild genotype decreased the mean value of the pH trait. A BSA combined with 2D-PAGE was applied to the extreme phenotypes for pH in the BC_1 segregating population. Four differentially expressed spots were detected when the polypeptide patterns of the bulks were compared. The spots had the expected molecular mass (around 54-kDa), and they were present in the lower-pH bulk and absent in the higher-pH one. The spots were identified by MS MALDI-TOF and two of them showed homology with the ATP synthase CF_1 alpha subunit of *S. lycopersicum*. These results indicate that the association between the polypeptide marker and a fruit quantitative trait detected by 1D-PAGE not only would indicate genetic linkage but also could be directly related with the gene underlying the quantitative trait.

Keywords Fruit quality · Proteomics · SDS–PAGE · Solanum lycopersicum · Solanum pimpinellifolium

Abbreviations

1D-PAGE	One dimensional gel electrophoresis
2D-PAGE	Two dimensional gel electrophoresis
BSA	Bulked segregant analysis
kDa	Kilo Dalton
MG	Mature green stage
RR	Red ripe stage
SDS–PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Introduction

Tomato (*Solanum lycopersicum*) has emerged as the primary model for climacteric fruit ripening because this unique aspect of research cannot be studied in model plant species such as *Arabidopsis thaliana* or rice (Giovannoni 2004). Tomato fruit is a fleshy berry composed of an epidermis, a thick pericarp, and a placental tissue surrounding the seeds. Fruit ripening is the result of highly synchronized biochemical and physiological changes that occur during a relatively short period, and protein synthesis appears to be an essential component of this process

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(Grierson and Tucker 1983). The proteins expressed during fruit ripening can be used as molecular markers. In fact, the co-segregation of polypeptides in 1D-PAGE and fruit quantitative traits has been reported in segregating populations of tomato such as F_2, BC or RIL (Rodríguez et al. 2008, 2011; Gallo et al. 2011; Pereira da Costa et al. 2014). A polypeptide band with a specific mass weight is usually a mix of polypeptides; therefore, their quantification and further analysis by mass spectrometry became difficult. In peanut, it was demonstrated that 1D-PAGE was not useful to distinguish cultivars by comparing their protein storage profiles. However, four of 150 spots were differentially expressed among cultivars when the protein profiles were resolved by 2D-PAGE (Liang et al. 2006). These authors found that these proteins were isoforms of an allergen and proposed an association with resistance to aflatoxin contamination among peanut cultivars.

The 2D-PAGE method was useful for the analysis of hundreds to thousands of polypeptides differentially expressed in a few tomato cultivars (Rocco et al. 2006; Faurobert et al. 2007; Xu et al. 2013). Since this technique requires a large quantity of samples, its application to a large number of genotypes is limited. In consequence, the application of 2D-PAGE to analyze polypeptide profiles in segregating populations such as F_2, BC, or RILs is a very difficult task. This limitation can be overcome by combining bulked segregant analysis (BSA) and 2D-PAGE. The BSA method was developed to rapidly detect linkage between DNA markers and attributes with Mendelian inheritance (Michelmore et al. 1991), and recently it has been applied to detect QTL in tomato by using segregating populations (Rodríguez et al. 2013). Also, Chayut et al. (2015) have demonstrated the resolution power of bulk segregant RNA sequences in identifying genes related to commercially important traits of melon fruit.

So far, the BSA method has not been applied to protein markers. The combination of BSA and polypeptide profiles would be a useful method to identify polypeptides that underlie the phenotypic differences displayed in a segregating population.

Fruit pH and titratable acidity are two important quality attributes in tomato and they are defined during fruit ripening. In fresh tomatoes, sugar, organic acid levels and aromatic volatiles define fruit flavor (Jones and Scott 1983). These traits are also related to the fruit shelf life attribute (Pinheiro and Almeida 2008; Centeno et al. 2011). For tomato-processing industry, fruit pH is important and it should be lower than 4.4 to assure the safety of the processed product (Rice and Pederson 1954; Fulton et al. 2002).

The aims of this work were to: (1) detect pericarp polypeptides associated with pH in an interspecific tomato BC_1 generation by 1D-PAGE; (2) identify those differentially expressed polypeptide by comparing 2D-PAGE protein profiles obtained with BSA. Afterwards, we tried to infer the biological relationship between the polymorphic polypeptides and the phenotypic trait evaluated.

Materials and methods

Plant material

An interspecific tomato BC_1 generation was derived from a cross between the Argentinean cultivar 'Caimanta' of *S. lycopersicum* (as recurrent parent) and the accession LA722 of *S. pimpinellifolium* (as donor parent). 'Caimanta' plants are compact, with a determinate growth habit, and large flat fruits, whereas the wild accession LA722 has an indeterminate growth habit and bears small and round fruits (Rodríguez et al. 2006). 'Caimanta' was obtained from INTA Cerrillos (Salta, Argentina) and the accession LA722 from the Tomato Genetic Resource Center, Department of Vegetable Crops, University of California (Davis, USA). The F_1 and BC_1 generations were obtained by manual crossing. Five plants per parental genotype and 50 BC_1 plants were transplanted in a greenhouse following a completely randomized design.

Fruit pH evaluation

Tomato juice pH was evaluated in three independent samples per plant obtained from three different fruits at the red ripe stage (RR) as defined by Giovannoni (2004). The normal pH distribution for both the parental genotypes and the BC_1 generation was tested according to Shapiro and Wilk (1965). A *t* test (Snedecor 1964) was used to compare mean values between parental genotypes. The distribution of frequencies for pH in the BC_1 generation was also analyzed and the genetic component of the total variance for the trait was estimated by ANOVA.

Pericarp total protein extraction and 1D-PAGE resolution

Three independent fruits from the parental genotypes and one from each backcross plant were harvested at the mature green (MG) and red ripe (RR) stages. Total proteins were extracted from the pericarp and resolved by 1D-PAGE according to Rodríguez et al. (2008). Equal amounts of polypeptides (20 µg) were loaded in a 12% polyacrylamide gel and run in a *Mini Protean II* electrophoresis cell (Bio-Rad, Hercules, CA, USA) for 1.5 h at 35 mA constant. Gels were stained overnight with 0.1% Coomassie Brilliant Blue R-250 solution. Then, they were destained in boiling

water, scanned and analyzed using GelPro Analyzer 3.0 software. This experiment was repeated twice. Total polypeptides and percentage of polymorphism between parental genotypes were calculated. Mendelian segregation (1:1, presence: absence) of polypeptides in BC_1 generation was probed by χ^2 test (Snedecor 1964).

Genetic association between polypeptide and pH phenotype segregation

The association between polypeptides and fruit pH was determined by single point analysis (Tanksley 1993). For this procedure, firstly a one-way ANOVA was performed, with polypeptide–genotype groups used as class variable. The R^2 values were used to estimate the percentage of total phenotypic variation explained by the polymorphic polypeptide (Liu 1998). Then, the interaction between polypeptides significantly associated with pH was analyzed by a two-way ANOVA.

Pericarp total protein pooling using the extreme phenotypes for pH

From the BC_1 segregating generation, ten individual plants with the lowest pH mean value and presence of 54-kDa at the MG band were selected, hereafter referred to as low-pH bulk. Other ten individual plants were selected which exhibited the highest mean pH value and absence of 54-kDa at MG, hereafter called high-pH bulk. Low-pH and high-pH protein bulks were added separately to seven volumes of IEF buffer (8 M urea, 2% w/v CHAPS, 20 mM DTT and 2% w/v carrier ampholytes pH 3–10NL) (Bio-Rad, Hercules, CA, USA). This procedure was done in order to remove the anionic detergent SDS from the proteins and to replace it with a zwitterionic detergent as CHAPS that is compatible with IEF (Görg et al. 2004). IPG strips (13 cm, pH 3–10NL Bio-Rad Ready Strip) were rehydrated overnight with 250 mL of IEF buffer containing 200 mg of protein.

2D-PAGE resolution, differential spot identification and MS–MALDI–TOF analysis

Proteins were focused using an *EttanIPGphor 3 Isoelectric Focusing Unit* (GE Healthcare, England, UK) at 20 °C, applying 100 V (60 min), 500 V (60 min), 1000 V (60 min) and 8000 V for a total of 72 KVh. After focusing, proteins were reduced by incubating the IPG strips with 1% w/v DTT for 10 min and alkylated with 2.5% w/v iodoacetamide in 10 mL of 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS and 0.001% Bromophenol Blue for 10 min at 25 °C. Second dimension electrophoresis (2D-PAGE) was carried out using a *SE 600 Ruby Standard Dual Cooled Vertical Unit* (GE Healthcare, England, UK). A 10% polyacrylamide gel (18 cm × 24 cm × 1.5 mm) in 25 mM Tris–HCl (pH 8.3), 1.92 M glycine and 1% w/v SDS, with 3 W/gel was applied for 5 h. 2D-PAGE gels were stained with colloidal Coomassie G-250 and scanned using a flatbed Scanner. Spot detection and matching were performed by visual comparison with molecular weight markers loaded on the same gel. Differential spots were manually removed from the gel and processed for MS–MALDI–TOF analysis at CEQUIBIEM, Buenos Aires, Argentina. The results of the sequences obtained were contrasted with database at NCBI (http://www.ncbi.nlm.nih.gov/).

Results and discussion

Phenotypic and genetic analysis for pH attribute in the BC_1 population

The mean pH values were not significantly different between the parental genotypes ($p > 0.05$). However, there was a broader phenotypic variation in the BC_1 generation (Fig. 1a). The genetic component of the total variance was 96%, indicating that a strong genetic component explains the phenotypic variation in this segregating population. Similar results for this attribute have been found in other tomato wide crosses (Saliba-Colombani et al. 2001; Rodríguez et al. 2011). Various studies have reported QTL for pH distributed throughout the tomato genome. Paterson et al. (1988) found five QTL for the pH attribute at chromosomes 3, 6, 7, 8 and 10, which account for 48% of the phenotypic variance. Saliba-Colombani et al. (2001) also found two QTL for pH (at chromosomes 11 and 12) when RILs derived from an intraspecific cross were evaluated. Particularly, the BC_1 population of this study was also used for QTL detection by SSR molecular markers (Pereira da Costa et al. 2013). Six QTL for pH were detected at chromosomes 1 (two QTL), 5, 8, 9, and 12. This result confirms that this trait is genetically controlled.

Polypeptides associated to pH attribute in 1D-PAGE

1D-PAGE polypeptide profiles in the parental genotypes showed 27 bands at the MG and 28 at the RR stage, with a similar level of polymorphism for each stage, 70 and 64%, respectively. At MG, three bands (54, 45 and 38-kDa) showed Mendelian inheritance. The 54-kDa band was associated with pH ($p = 0$ 0.01). Figure 1b shows the 1D-PAGE polypeptide profiles at MG for several BC_1 plants; the 54-kDa polypeptide is indicated by an arrow. Two bands at the RR (66 and 54-kDa) showed Mendelian

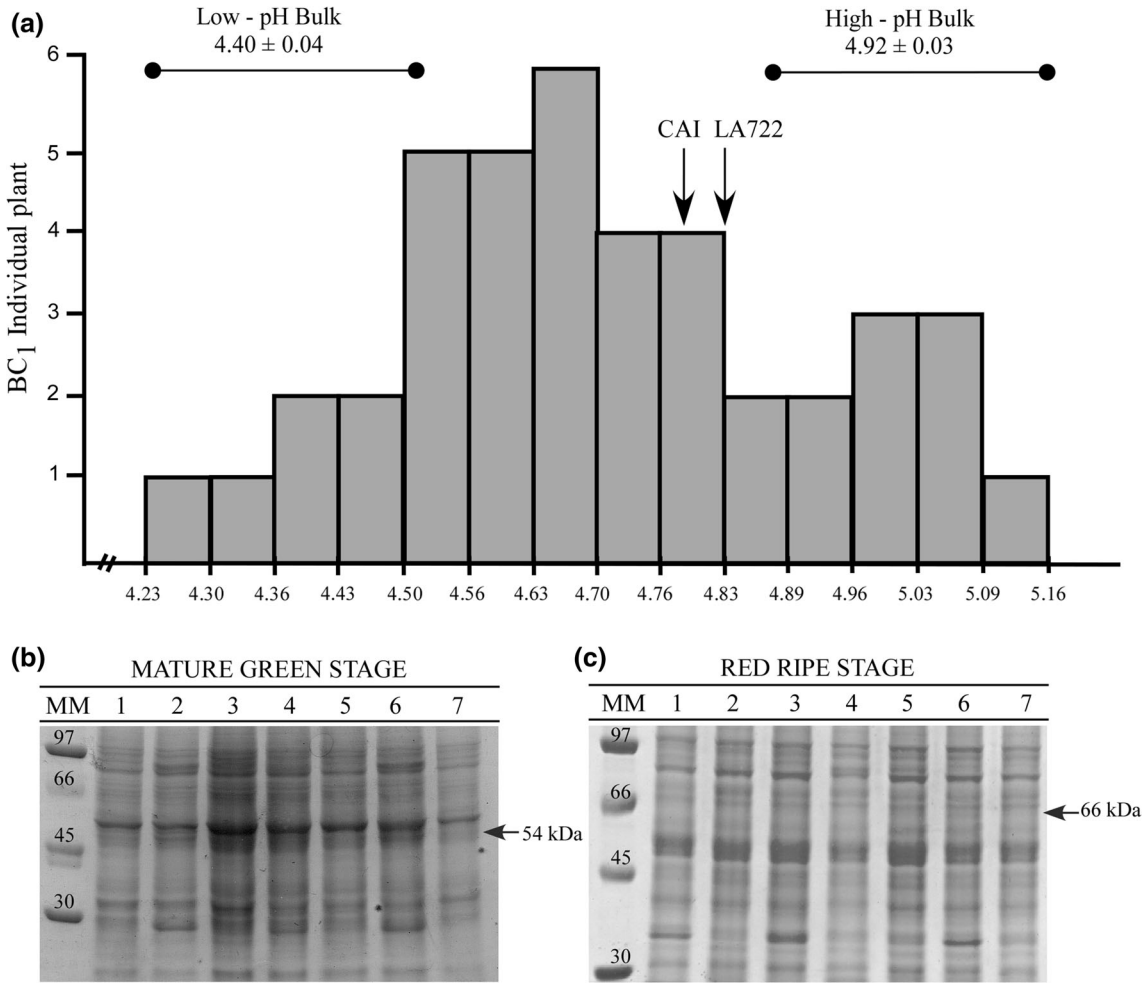

Fig. 1 **a** Distribution of frequencies for pH values at RR ripening fruit stage in the BC_1 generation (cross between cv. Caimanta of *Solanum lycopersicum* (CAI), as current parental genotype and LA722 of *S. pimpinellifolium*, as donor parental genotype). The *arrows* indicate the mean values for pH in the parental genotypes. The *intervals* at the *top* of the *distribution* show the extreme phenotypic individuals used for BSA. **b** Polypeptide bands detected by 1D-PAGE at mature green ripening fruit stage. **c** Polypeptide bands detected by 1D-PAGE at red ripe ripening fruit stage. *Lane 1* size-marker protein (MM). *Lanes 1–7* correspond to pericarp polypeptide profiles of BC_1 individuals. *Arrows* indicate polymorphic polypeptides associated with fruit pH

inheritance patterns, but only the 66-kDa polypeptide was associated with pH ($p = 0.04$) (Fig. 1c). The presence of the 54-kDa polypeptide at MG and of 66-kDa at RR, both carried by the wild parental genotype, was associated to a decrease in the mean pH value. The 54 and 66-kDa explained 11 and 12% of phenotypic variance, respectively. The interaction between both polypeptides was not significant ($p = 0.27$). According to Etienne et al. (2013), pH is determined at the mature green stage during fruit ripening, when malate and citrate acids are converted into phosphoenolpyruvate. This conversion is linked to the activation of gluconeogenesis and occurs when sugars start to accumulate during fruit ripening. Also, Almeida and Huber (1999) found that the pH of pericarp showed little (si querés decir "poca variación", entonces "little" va solo, sin "a") variation during the transition from MG to RR. In our study, we found two polypeptides associated to fruit pH, one at MG and the other at RR. Although both polypeptides had significant effect on fruits pH, the 54-kDa polypeptide at the MG stage was the most significant and therefore we decided to study it further.

In a segregating generation such as F_2 or BC, the co-segregation of polymorphic polypeptides with fruit quality traits allows identifying genetic associations. The phenotypic differences among genotypes within these generations explained by the presence or absence of a specific polypeptide could make this polypeptide a functional marker of the trait under study.

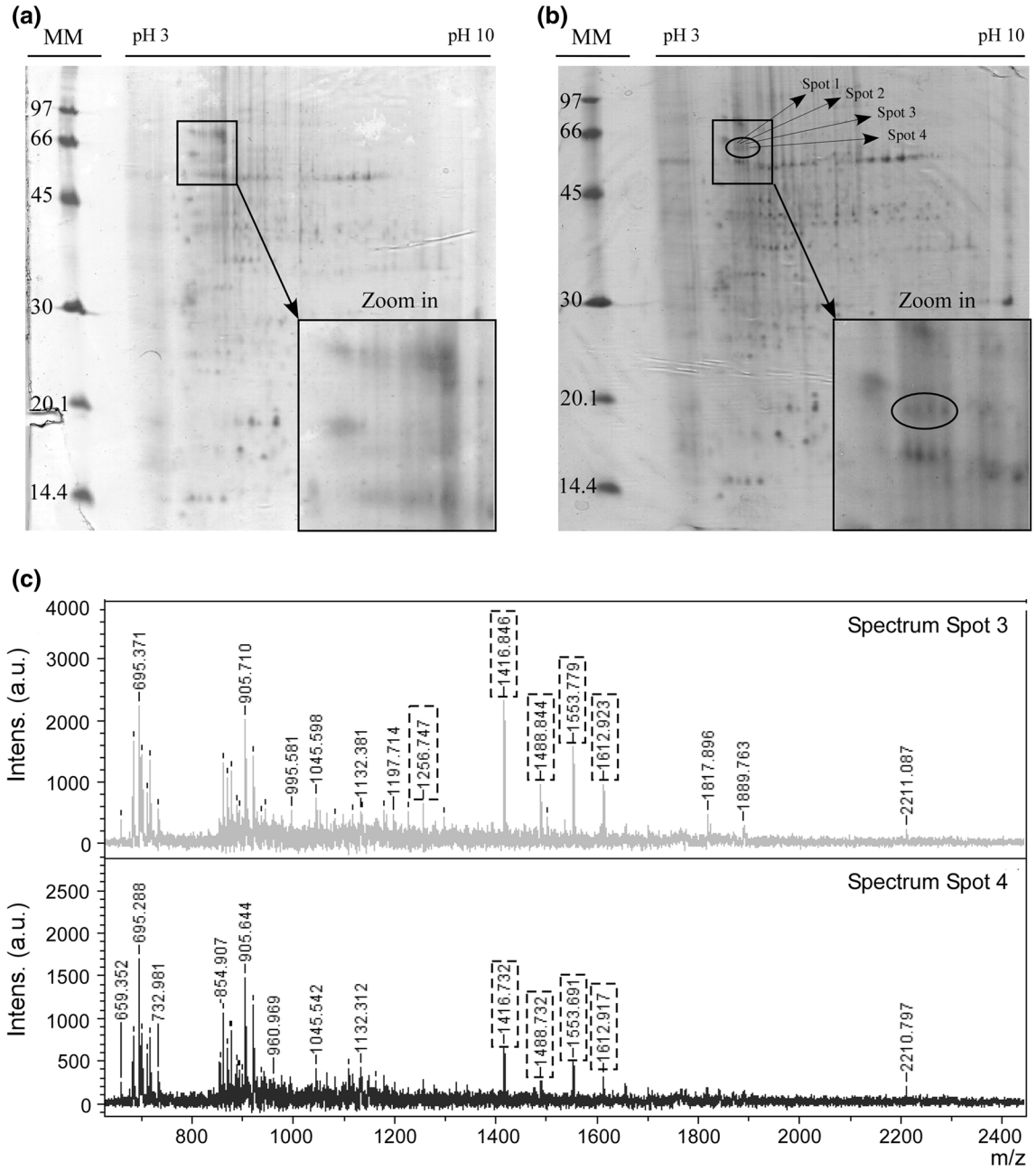

Fig. 2 a 2D-PAGE for the high-pH bulk samples, at the right corner is a zoom in the studied area of the gel. **b** 2D-PAGE for the low-pH bulk samples, at the *right corner* is a zoom in the studied area of the gel. *Arrows* indicate the presence of four differential expressed spots in this bulk. **c** MS-spectra corresponding to differential *spots 3* and *4* detected on low-pH bulk pericarp protein samples

BSA combined with 2D-PAGE to identify differentially expressed polypeptides

Previous studies have demonstrated the usefulness of pericarp polypeptide profiles in finding associations with fruit quality traits in different types of tomato segregating populations (Rodríguez et al. 2008; Gallo et al. 2011; Pereira da Costa et al. 2014). However, it has never been evaluated if the association between the polypeptide polymorphism and fruit attributes was due to a genetic linkage or if the polypeptide had a function directly related to the fruit attribute. To test this hypothesis, we followed a BSA approach, making protein bulks with those individuals that showed more extreme values for pH in the BC_1 population (Fig. 1a). The pH mean value for the low-pH bulk was 4.40 ± 0.04, and for the high-pH bulk it was

4.92 ± 0.03. These mean values were significantly different ($t = 10.9$; $p < 0.0001$). The MG ripening stage was chosen for this experiment because the 54-kDa polypeptide had the highest level of association with the pH trait and because this is when the fruit begins ripening. Protein samples from fruit pericarps at MG were analyzed by 2D-PAGE. After comparison between gels, four spots with expected molecular mass (~54-kDa) and differential expression between the bulks (Fig. 2a, b) were picked and processed for MS–MALDI–TOF analysis at CEQUIBIEM, Buenos Aires, Argentina (Fig. 2b; spots 1, 2, 3 and 4). The analysis of spot 1 had a good quality spectrum and several significant peaks; however, it was not possible to predict any protein by homology using BLAST at NCBI database (http://www.ncbi.nlm.nih.gov/). Spot 2 did not show any significant result because the quality of the sample was not good enough. A good spectrum quality and significant peaks were resolved from spots 3 and 4. These two spots were identified by mass spectrometry analysis (Fig. 2c) and showed high homology with the ATP synthase CF1 alpha chain of *S. lycopersicum* (YP_514837.1, GI: 89280620, http://www.ncbi.nlm.nih.gov/protein/YP_514837.1, last access September 22, 2016). The nominal mass of the protein is 55,434, the calculated pI is 5.14, and its total length is 507 residues. The matched peptides for spot 3 when compared to the original sequence were: IVNTGTVLQVGDGIAR (Mr 1612.923), IAQIPVSEAYLGR (Mr 1416.846), LIESAAPGIISR (Mr 1256.747), ASSVAQVVTTLQER (Mr 1488.844), and EAYPGDVFYLHSR (Mr 1553.779), which covered 13% of the total length (rectangles with dashed lines in Fig. 2c). For spot 4, the matched peptides were four, three being the same as those for spot 3 except for the peptide LIESAAPGIISR, which covered 11% of the total length of the protein.

The ATP synthase CF1 alpha chain subunit catalyzes the formation of ATP from ADP and inorganic phosphate (Senior et al. 2002) at expense of the transmembrane electrochemical proton potential difference. It is a large protein complex comprising a membrane domain that carries out proton transport and a cytoplasmic compartment domain (F1) that catalyzes ATP synthesis. In fruits, more than 90% of the cell volume is occupied by the vacuole; therefore, the measurement of this attribute in the juice is directly related to the pH in this organelle (Nanos and Kader 1993). A high concentration of hydrogen ions inside the vacuole is created and maintained by a variety of enzymes associated with the outer membrane of the cells (Müller et al. 1996). Although further experiments are needed to confirm this hypothesis, tomato wild species might have isoforms of this kind of enzymes that produce differences in the mechanism of hydrogen accumulation in the fruit cell. Some data in the Solanaceae family database (http://www.solgenomics.net) seem to support this hypothesis. When we performed BLAST (protein to protein) with the four spot sequences as queries and the tomato genome protein sequence (ITAG release 2.40) as subject, the ASSVAQVVTTLQER (Mr 1488.844) sequence was not found. Neither was it found when we performed BLAST (protein to translated sequence) with the tomato genome chromosome (build 2.50). On the other hand, this polypeptide was found when BLAST (protein to translated nucleotide) was carried out with the transcriptome sequence of the wild species *S. pimpinellifolium* and *S. peruvianum*, but it was not found in the transcriptome sequence of *S. pennellii*. These results support the hypothesis that the ATP synthase CF1 alpha chain has isoforms in tomato germplasm.

As a conclusion, pericarp protein profiles obtained with 1D-PAGE allowed detection of a polypeptide associated with pH of tomato fruit in a segregating generation. Although the association between the polypeptide marker and the fruit quantitative trait indicates linkage between the gene controlling this attribute and the gene codifying the polypeptide, in this study we demonstrated that the polymorphism in the polypeptide would be directly related with the gene underlying the quantitative trait. These results demonstrate that the 54-kDa polypeptide of fruit pericarp at MG stage may serve as a functional molecular marker in tomato. The combination of BSA and 2D-PAGE would make it possible to use the latter in breeding programs as tool for marker assisted selection.

Author contribution statement JHPdC, TAV, RZ and GRR designed the research; JHPdC, TAV and RZ conducted the research; JHPdC, TAV, GRP, LAP, RZ and GRR analyzed the data; JHPdC, TAV and GRR wrote the paper; GRR had primary responsibility for the final content. All authors have read and approved the final manuscript.

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