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Near-isogenic lines enhancing ascorbic acid, anthocyanin and carotenoid content in tomato (*Solanum lycopersicum* L. cv Micro-Tom) as a tool to produce nutrient-rich fruits

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ABSTRACT

A great emphasis of plant research is being placed on developing crops with increased nutritional value. Generating plant materials suitable for controlled studies as potential tools for pre-breeding is still the main hurdle. Improvements are needed in generating different allele combinations to stack various nutrients into a single genotype, without losses in fruit yield or quality, and in testing the specific effects of nutrients in their original matrix, avoiding the noise caused by the characteristic mix of compounds. An elegant approach in both pre-breeding and diet supplementation tests is the use of near-isogenic lines (NILs). Here, we tap on the large pool of monogenic mutants and natural genetic variation available in tomato to create a series of NILs in the genetic background of the cultivar Micro-Tom (MT). We describe the introgression of the mutations *Anthocyanin fruit* (*Aft*), *atroviolacium* (*atv*), *Aubergine* (*Abg*), *Beta-carotene* (*B*), *old-gold crimson* (*og*) and *high pigment 1* and *2* (*hp1, hp2*) and characterize their fruit metabolic profiles in single, double and triple mutant combinations. We show that Brix can be raised without yield penalty, along with increases in lycopene, β-carotene and ascorbic acid, and a concomitant enhancement of anti-oxidant capacity. As proof-of-concept of the suitability of stacking alleles for breeding nutrient-rich tomatoes, we introduce three mutations leading to uniformly purple fruits and enhanced nutrient contents from MT into a commercial cherry tomato cultivar.

1. Introduction

There is considerable evidence associating healthy eating habits with a reduced risk of chronic disease and obesity (WHO, 2003). High consumption of fruits and vegetables, in particular, is strongly correlated with significant reductions in cardiovascular disease and cancer (Hung et al., 2004). As public awareness of the importance of switching to plant-based diets grows, a greater emphasis of plant research will be placed on developing crops with increased nutritional value (De Sa and Lock, 2008). The development of nutrient-rich vegetables and fruits requires improvement in both (i) the capacity to test different combinations of alleles (pre-breeding) in order to stack different kinds of nutrients into a single genotype, ideally, with no loss in fruit yield or quality, and (ii) the capacity to test the effectiveness of the genotypes produced. The latter is usually achieved through diet experiments in either animal models or humans, which have the downside that identifying and characterizing the contributions of any single compound in a mixture of nutrients is difficult. The alternative approach of

Abbreviations: AA, ascorbic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GAE, gallic acid equivalents; GM, genetically modified; HPLC, high-performance liquid chromatography; MT, Micro-Tom; NILs, near-isogenic lines; SSC, soluble solids content.

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Table 1
Mutations introgressed into the cv. Micro-Tom background.

Mutant	Gene function	Origin	Reference
Beta carotene (*B*)	Chromoplast-specific lycopene β-cyclase (Cyc-B), which converts most fruit lycopene into β-carotene	LA1401 (*S. galapagense*)	Lincoln and Porter (1950) and Pecker et al. (1996)
crimson (*og*)	Defective for a chromoplast-specific lycopene β-cyclase (Cyc-B), which abolishes the conversion of lycopene into β-carotene; a null allele of *B*	cv. Ourovelho	Mustilli et al. (1999) and Mohr (1979)
atroviolacium (*atv*)	Natural variation from *S. cheesmaniae*, probably a non-functional allele of a negative regulator of photomorphogenesis	LA0797 Hybrid	Kendrick et al. (1994)
high pigment1 (*hp1*)	Defective for a gene homologous to DDB1A of *Arabidopsis*, which codes for a protein interacting with DET1 (HP2), a repressor of photomorphogenesis	LA3004 cv. Rutgers	Lieberman et al. (2004) and Peters et al. (1989)
high pigment 2 (*hp2*)	Defective for a gene homologous to *DET*1 of *Arabidopsis*, a negative repressor of photomorphogenesis	LA2451 cv. Manapal	Mustilli et al. (1999)
Anthocyanin fruit (*Aft*)	Natural variation from *S. chilense*, probably coding for an R2R3 MYB transcription factor	LA1996	Jones et al. (2003) and Schreiber et al. (2012)
Aubergine (*Abg*)	Natural variation from *S. lycopersicoides*, probably allelic to *Aft*	LA3668	Kerckhoffs et al. (1997) and Rick et al. (1994)

testing purified compounds in diets, on the other hand, hampers the study of molecules in the particular chemical forms in which they are most commonly consumed, and in their original biochemical matrix, which has a large impact on bioavailability (Martin et al., 2011). An elegant alternative to overcome these hurdles is the use of near-isogenic lines (NILs) (Martin et al., 2011). NILs are genetically identical lines, except for one or a few loci, and represent a powerful tool to carry out detailed analyses of the physiological and molecular basis of key traits within a fixed genetic background. NILs have been used extensively as a pre-breeding tool for various crops (Edmeades et al., 2004).

Isogenic lines with increased nutrient contents have already been produced in tomato by expressing the snapdragon transcription factors *Delila* (*Del*) and *Rosea1* (*Ros1*), which led to the accumulation of anthocyanins in the fruit (Butelli et al., 2008a). Supplementing the diet of *Trp53*$^{-}$/$^{-}$ mice with anthocyanin-rich tomatoes increased their lifespan (Butelli et al., 2008a) when compared to the isogenic control. In that work the authors profited from the short life cycle, reduced adult size and genetic transformation capability of the tomato cultivar Micro-Tom (MT) (Meissner et al., 1997). The genetic resources available in tomato, such as a large collection of monogenic mutants (http://tgrc.ucdavis.edu/) and a wide pool of natural genetic variation (Stevens and Rick, 1986), have also been exploited in MT through conventional breeding (Campos et al., 2010). In our laboratory, we have previously used NILs in MT, differing only in one or a few alleles, to address diverse questions of plant physiology and development (Campos et al., 2009; Carvalho et al., 2011; Lombardi-Crestana et al., 2012).

Here, we aimed at creating nutrient-rich tomato NILs carrying the mutations *Anthocyanin fruit* (*Aft*), *atroviolacium* (*atv*), *Aubergine* (*Abg*), *Beta-carotene* (*B*), *old-gold crimsom* (*og*) and *high pigment 1* and *2* (*hp1, hp2*) from their original genetic backgrounds into MT. We characterized the metabolic profiles of the fruits in the resulting NILs in single, double and triple mutant combinations, and compared their yield and total soluble solids content (Brix). We suggest that such a collection in a single genetic background could be useful for diet supplementation studies, and also as a pre-breeding tool to test allele combinations with the aim of producing nutrient-rich commercial varieties. As proof-of-concept of the latter, we combined three mutations to produce a genotype with nutritionally valuable traits, such as increased levels of anthocyanins, β-carotene, lycopene and vitamin C. Lastly, we prove the feasibility of producing a commercial crop with added health benefits by introducing all three mutations into a commercial cherry tomato cultivar (VFNT).

2. Material and methods

2.1. Plant material and growth conditions

The near-isogenic lines (NILs) in *Solanum lycopersicum* L. cv. Micro-Tom (MT) carrying the alleles of interest (Table 1) were produced as described in Fig. 1. Briefly, *S. lycopersicum* cv Micro-Tom (MT) and the cultivar carrying the mutation (in Fig. 1 exemplified by *atv*) were crossed, using the former as the female parent. The F_1 was backcrossed (BC) using MT as a recurrent female parent and the resulting BC_1 seedlings were screened for characteristic dwarfism of MT (Martí et al., 2006). Selected plants were selfed, producing BC_1F_2 seeds. The process was repeated until the theoretical proportion of MT genome was >99% (Stam and Zeven, 1981). All experiments were performed in BC_6F_4 homozygous plants.

To generate the double mutants *Aft/hp2*, *Aft/hp1* and *Abg/hp2*, homozygous single mutants were crossed and screened for phenotype in the F_2 generation. The triple mutant *Aft/atv/hp2* was generated by crossing the double mutant *Aft/hp2* with the homozygous mutant *atv*. The double and triple mutants, once identified, were self-fertilized to obtain the respective populations. Plants were grown in 8 L rectangular plastic pots containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, São Paulo, Brazil) and expanded vermiculite, supplemented with 1 g L^{-1} of NPK 10:10:10 and 4 g L^{-1} of dolomite limestone ($MgCO_3 + CaCO_3$). Plants were kept in a greenhouse at an average mean temperature of 28 °C; 11.5/13 h (winter/summer) photoperiod, and exposed to 250–350 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) by natural radiation reduced with a reflecting mesh (Aluminet-Polysack Industrias Ltda, Leme, SP, Brazil). At flowering time (~35 days after sowing), plants were sprayed twice at 14-day intervals with 1 g L^{-1} Peters 20-20-20 fertilizer. Yield (total fruit weight per plant) was calculated using 12 plants. Uniformly ripe fruits, at the corresponding ripening stage, were harvested and part of a sample was immediately used to determine agronomic parameters and carotenoids content. The remainder of the sample was used to determine phenolics, flavonoids, ascorbic acid and antioxidant activity in different fractions of the fruit (flesh and peel). Flesh and peel were handled separately, thoroughly homogenized by powdering in liquid N_2, and stored at −70 °C until analysis.

2.2. Soluble solids content (SSC)

SSC was measured in the juice of 10 ripe fruits of each genotype using a digital refractometer with automatic temperature compensation (Atago PR-101, Bellevue, WA).