Quantitative Trait Loci Pyramiding Can Improve the Nutritional Potential of Tomato (*Solanum lycopersicum*) Fruits

Maria Manuela Rigano,[†,∥] Assunta Raiola,[†,∥] Gian Carlo Tenore,[‡] Daria Maria Monti,[#] Rita Del Giudice,[#] Luigi Frusciante,[†] and Amalia Barone*[,†]

[†]Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055 Portici (Naples), Italy

[‡]Department of Pharmacy, University of Naples Federico II, Via D. Montesano 49, 80131 Naples, Italy

[#]Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario M.S.Angelo, via Cinthia 4, 80126, Naples, Italy

ABSTRACT: *Solanum lycopersicum* represents an important source of antioxidants and other bioactive compounds. Previously two *Solanum pennellii* introgression lines (IL 7-3 and IL 12-4) were identified as carrying quantitative trait loci (QTL) increasing fruit ascorbic acid and phenolics content. Novel tomato lines were obtained by pyramiding these selected QTLs in the genetic background of the cultivated line M82. Pyramided lines revealed significant increases of total phenolics, phenolic acids, ascorbic acid, and total antioxidant activity compared to parental lines IL 7-3 and IL 12-4 and the cultivated line M82. In addition, tomato extracts obtained from the pyramided lines had no cytotoxic effect on normal human cells while exhibiting a selective cytotoxic effect on aggressive cancer cells. Therefore, the present study demonstrates that it is possible to incorporate favorable wild-species QTLs in the cultivated genetic background to obtain genotypes with higher nutritional value.

KEYWORDS: *Solanum lycopersicum*, antioxidant power, ascorbic acid, phenolics, phenolic acid, MTT test

INTRODUCTION

In recent years consumers are showing an increasing interest in functional foods, in particular in vegetable crops, encouraged by the evidence of the health effects of the Mediterranean diet. In this context tomato (*Solanum lycopersicum*) fruits are an important source of phytochemicals. Their consumption is associated with a reduced risk of cancer, inflammation, and chronic noncommunicable diseases (CNCD) including cardiovascular diseases (CVD), such as hypertension, coronary heart disease, diabetes, and obesity.[1] These effects are attributed mostly to the presence of hydrophilic (phenolics, folates, ascorbic acid) and lipophilic compounds (carotenoids and tocopherols).[2,3]

Among these, polyphenolic compounds are secondary metabolites implicated in plant defense and associated with therapeutic roles in inflammatory diseases including cardiovascular diseases, obesity, and type II diabetes, neurodegenerative diseases, cancer, and aging.[4] Flavonoids, which are located mainly in the skin, contribute to the aroma and color of the tomato fruit; they include quercetin, rutin, kampferol, naringenin, and catechin[5] and exert a protective action against rheumatoid arthritis[6] and intestinal inflammation.[7,8] Phenolic acids, equally distributed in the fruit, are responsible for the astringent taste of vegetables and include chlorogenic, ferulic, and gallic acids.[9] They act against DNA oxidation, have antitumor activity in humans, and control inflammation, cell differentiation, and proliferation.[10] Ascorbic acid (AsA) protects against oxidation of low-density lipoprotein (LDL) in vascular endothelial cells.[11] In tomato, AsA is highly bioavailable; thus, a regular consumption of tomatoes can increase cell protection from DNA damage induced by oxidant species.[12] Carotenoids contribute to photosynthesis in plants

by protecting them against photodamage.[13] They include non-provitamin A carotenoids, such as lutein and lycopene, and provitamin A carotenoids, such as α-carotene, β-carotene, and β-cryptoxanthin. Lycopene is responsible for the fruit's red color and is reported as the main carotenoid in tomato fruits for its strong antioxidative role associated with its ability to act as a free radical scavenger of reactive oxygen species (ROS). In any case, the relative contribution of lipid-soluble antioxidants to the total antioxidant activity in tomato fruits is much lower than the contribution from water-soluble antioxidants.[14]

The nutritional quality of the fruit constitutes one of the major objectives of tomato breeding. However, the synthesis of many of the compounds responsible for the nutritional quality of the tomato fruit is the result of coordinated activities that involve many of the primary and secondary metabolism pathways regulated by developmental, physiological and environmental signals. It is therefore necessary to identify key genes or quantitative trait loci (QTLs) that regulate processes important for the nutritional quality of the fruits. The use of a population of introgression lines (ILs) derived from wild tomato species is widely documented in tomato as a tool to dissect quantitative traits in their main genetic components, thus allowing those mostly influencing the traits under study to be identified.[15,16] A set of nearly isogenic lines was previously constructed that included single marker-defined introgressed genomic regions from the species *Solanum pennellii* into the genomic background of the cultivated *S. lycopersicum* variety

M82.[15] The *S. pennellii* ILs were used to map several QTLs associated with characters related to tomato fruit quality.[17,18] By using these lines, in our laboratory two QTLs increasing fruit phenolics and AsA content were detected on tomato chromosomes 7 and 12.[19−21] In a subsequent study, these QTLs were pyramided in the genetic background of the cultivated line M82 using marker-assisted selection. The efficacy of pyramiding to improve tomato traits has been previously reported. For example, Gur et al.[22] developed genotypes carrying three independent yield-promoting genomic regions introduced from *S. pennellii* that resulted in a hybrid with 50% higher yields than leading commercial varieties in multiple environments and irrigation regimens. Moreover, introgressions originating from *S. pennellii* were introduced into lines of processing tomato, and the resulting hybrid, AB2, is presently a leading variety in California.[23] In our case, IL 7-3 and IL 12-4 were crossed, and four genotypes of the F_3 progenies were selected that carry both introgressions 7-3 and 12-4 at the homozygous condition (DHO).[24] Because the combined effect of these QTLs is unpredictable, here we evaluated the nutritional quality of the four genotypes obtained by QTL pyramiding. We analyzed the amount of phytochemicals associated with antioxidant activity in the cultivated line M82, in the two introgression lines IL 7-3 and IL 12-4 and in the DHO genotypes. In particular, the hydrophilic and lipophilic antioxidant activity and contents of AsA, total phenolics, flavonoids, phenolic acids, total carotenoids, lycopene, and β-carotene were investigated. In addition, to assess the nutraceutical potential of the DHO lines, we tested the activity of tomato fruit extracts on the proliferation of human normal and cancer cell lines.

■ MATERIALS AND METHODS

Chemicals and Reagents. Ascorbic acid, Trolox, gallic acid, ferulic acid, quercetin, and kampferol were purchased from Sigma (St. Louis, MO, USA). All solvents used (water, methanol, chloroform) were obtained from Fluka (Swizerland), whereas HPLC grade formic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Folin−Ciocalteu's phenol was purchased by AppliChem (Darmstadt, Germany). $FeCl_3$, trichloroacetic acid (TCA), Na_2CO_3, NaH_2PO_4, Na_2HPO_4, 2,2'-dipyridil, CH_3COOH, 2,4,6-tripyridyl-*s*-triazine (TPTZ), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and $K_2S_2O_8$ were obtained from Sigma-Aldrich, Na_2HPO_4 and H_3PO_4 were obtained from J. T. Baker (Germany), and HCl was obtained from Riedel-deHaën (Germany). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., USA) ultrasonic bath.

Plant Material and Growth Conditions. Seeds from IL 12-4 (LA4102), IL 7-3 (LA4066), and their parental line M82 (LA3475) were kindly provided by the Tomato Genetics Resource Centre (TGRC) (http://tgrc.ucdavis.edu/).[25] Genotypes carrying introgressions IL 7-3 and IL 12-4 at the homozygous condition (DHO) were obtained using marker-assisted selection as described in Sacco et al.[24] During the year 2013, the double-homozygous plants of the F_4 progenies and their parents were grown according to a completely randomized design with three replicates (10 plants/replicate), in an experimental field located in Acerra (Naples, Italy). Samples of about 20 full mature red fruits per plot were collected.

Tomato fruits were chopped, ground in liquid nitrogen by a blender (FRI150, Fimar) to a fine powder, and kept at −80 °C until the analyses.

Chemical Extractions. For the biochemical analyses, each tomato fruit sample consisted of 20 pooled fruits per plot. The extraction of hydrophilic fraction was carried out according to the procedure reported by Choi et al.[26] with minor changes. Briefly, frozen powder (2

g) was weighed, placed into a 50 mL Falcon tube, and extracted with 25 mL of 80% methanol into an ultrasonic bath (Branson 5200 Ultrasonic Corp.) for 60 min at 30 °C. The mixture was centrifuged at 14000 rpm for 10 min at 4 °C, and the supernatant was stored at −20 °C until analysis of total phenolic compounds and hydrophilic antioxidant activity (HAA). The pellet was extracted with 10 mL of chloroform (100%) with the aid of a pestle and was centrifuged at 4000 rpm for 5 min at 4 °C according to the method reported by Wellburn[27] with some modifications. This procedure was repeated five consecutive times, and supernatants were collected and stored at −20 °C until analysis of carotenoids and lipophilic antioxidant activity (LAA).

Carotenoid Determination. The evaluation of total carotenoids was carried out according to the method reported by Wellburn,[27] whereas lycopene and β-carotene were determined according the method reported by Zouari et al.[28] with slight modifications. All biological replicates of samples were analyzed in triplicate. Results were expressed as milligrams per 100 g fresh weight (FW).

Ascorbic Acid Determination. AsA quantification was carried out according to the method reported by Stevens et al.[18] with slight modifications. Briefly, frozen powder (500 mg) was placed in a 2 mL eppendorf tube, and 300 μL of ice-cold 6% TCA was added. The mixture was vortexed for 10 s, incubated for 15 min on ice, and centrifuged at 14000 rpm for 20 min at 4 °C. Then, 20 μL of supernatant for each assay was transferred in a 1.5 mL eppendorf tube with 20 μL of 0.4 M phosphate buffer (pH 7.4) and 10 μL of double-distilled (dd) H_2O. Then, 80 μL of color reagent solution was prepared immediately before assay by mixing solution A (31% H_3PO_4, 4.6% (w/v) TCA, and 0.6% (w/v) $FeCl_3$) with solution B (4% 2,2'-dipyridil (w/v) made up in 70% ethanol) at a proportion of 2.75:1 v/v. The mixture was vortexed and incubated at 37 °C for 40 min prior to measurement at 525 nm by a NanoPhotometer (Implen) using 6% TCA as reference. Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in nanomoles of AsA according to the standard curve, designed over a range of 0−70 nmol; then the values were converted into milligrams per 100 g FW.

Total Phenolic Compounds Determination. Total polyphenolic amount was evaluated by using Folin−Ciocalteu's assay as reported by Singleton et al.[29] with some modifications. In an eppendorf tube (1.5 mL) 62.5 μL of methanolic extract, 62.5 μL of Folin−Ciocalteu's phenol reagent, and 250 μL of dd H_2O were added and shaken. After 6 min, 625 μL of 7% Na_2CO_3 solution was added to the mixture. The solution was diluted with 500 μL of dd H_2O and mixed. After incubation for 90 min at room temperature, the absorbance against prepared reagent blank was determined at 760 nm by a NanoPhotometer (Implen). All biological replicates of samples were analyzed in triplicate. Total phenolic content of tomato fruits was expressed as milligrams of gallic acid equivalents (GAE) per 100 g FW.

Identification and Quantification of Individual Phenolic Compounds. An aliquot (1 mL) of the methanolic extract was passed through a 0.45 μm Millipore nylon filter (Bedford, MA, USA) before HPLC analysis. Free phenolic acids and flavonoids were separated and quantified by using a HPLC (Spectra System SCM 1000) fitted with an UV−visible detector. Separation of compounds was carried out on a reversed phase C18 Prodigy column (Phenomenex, 250 × 4.6 mm; particle size = 5 μm), preceded by a guard column (Phenomenex, 4 × 3.0 mm) of the same stationary phase. A gradient of water acidified with 5% formic acid (A) and methanol (B) at a flow rate of 1 mL/min was applied with the following modes: A = 60% (0 min), 40% (3−18 min), 0% (23−28 min), and 60% (30 min) according to the method reported by Ferracane et al.[30] with minor modifications. To determine total flavonoids and total phenolic acids concentration, the sum of all peaks present in the chromatogram was considered at two characteristic wavelengths of 365 and 287 nm, respectively. Each sample was analyzed by HPLC in triplicate. The quantification was carried out using the calibration curves of quercetin, gallic acid and ferulic acid in the ranges of 1−100, 0.5−10, and 0.01−1 μg/mL, respectively. The amount of phenolic compounds was expressed as milligrams of

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dx.doi.org/10.1021/jf502573n | *J. Agric. Food Chem.* 2014, 62, 11519−11527

quercetin equivalents (QE) per kilogram FW for quercetin and total flavonoids, as milligrams of GAE per kilogram FW for gallic acid and total phenolic acids, and as milligrams of ferulic acid (FA) per kilogram FW for ferulic acid.

Antioxidant Activity Determination. HAA was evaluated in the water-soluble fraction using two different methods, the ferric reducing/antioxidant power (FRAP) method[31] and the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method[32] with slight modifications.

The FRAP assay was carried out by adding in a vial (3 mL) 2.5 mL of acetate buffer, pH 3.6, 0.25 mL of TPTZ solution (10 mM) in 40 mM HCl, 0.25 mL of $FeCl_3 \cdot 6H_2O$ solution (12 mM), and 150 μL of methanolic extract. The mixture was incubated for 30 min in the dark, and then readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm using the spectrophotometer against a blank constituted by a mixture of reaction and 150 μL of extracting solution (CH_3OH/H_2O 80:20 v/v). The standard curve was linear between 20 and 800 μM Trolox. Results were expressed as micromoles of Trolox equivalents (TE) per 100 g FW.

The ABTS assay was based on the reduction of the ABTS$^{\bullet+}$ radical action by the antioxidants present in the sample. A solution constituted by 7.4 mM ABTS$^{\bullet+}$ (5 mL) mixed with 140 mM $K_2S_2O_8$ (88 μL) was prepared and stabilized for 12 h at 4 °C in the dark. This mixture was then diluted by mixing ABTS$^{\bullet+}$ solution with ethanol (1:88) to obtain an absorbance of 0.70 ± 0.10 unit at 734 nm using a spectrophotometer. Methanolic extracts (100 μL) were allowed to react with 1 mL of diluted ABTS$^{\bullet+}$ solution for 2.5 min, and then the absorbance was taken at 734 nm using a spectrophotometer against a blank constituted by ABTS$^{\bullet+}$ solution added with 100 μL of ethanol. The standard curve was linear between 0 and 20 μM Trolox.

LAA determination was carried out according to the ABTS assay, using the extract in chloroform. All biological replicates of samples were analyzed in triplicate. Results were expressed as micromoles of TE per 100 g FW.

The percentage of the variations of quantitative parameters among genotypes was calculated by using the following formula: % increase and/or decrease = (value in genotype 1 − value in genotype 2)/value in genotype 2) × 100. Total antioxidant activity (TAA) was calculated by adding LAA to HAA obtained by using the ABTS test. HAA contribution to TAA was evaluated by dividing HAA value obtained by using the ABTS test by TAA.

MTT Test Procedure. Human renal cortical epithelial cells HRCE (Innoprot, Biscay, Spain) were cultured in basal medium, supplemented with 2% fetal bovine serum, epithelial cell growth supplement, and antibiotics, all from Innoprot. Human HeLa adenocarcinoma cells, human renal epithelial cells (Hek 293), and murine BALB/3T3 and SV-T2 fibroblasts (ATCC) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 2 mM L-glutamine, and antibiotics. Cells were grown in a 5% CO_2 humidified atmosphere at 37 °C. Cells were seeded in 96-well plates (100 μL/well) at a density of 5 × 10³/well. Methanolic tomato extracts, obtained as reported above, were dried by rotovapor (R-210, Buchi), redissolved in dimethyl sulfoxide (DMSO) 5% in PBS, and then added to the cells 24 h after seeding for dose-dependent cytotoxicity assays. At the end of incubation, cell viability was assessed by the MTT assay. The MTT reagent, dissolved in DMEM in the absence of phenol red (Sigma-Aldrich), was added to the cells (100 μL/well) to a final concentration of 0.5 mg/mL. Following an incubation of 4 h at 37 °C, the culture medium was removed, and the resulting formazan salts were dissolved by adding isopropanol containing 0.1 N HCl (100 μL/well). Absorbance values of blue formazan were determined at 570 nm using an automatic plate reader (Microbeta Wallac 1420, PerkinElmer). The decrease in absorbance in the assay measures the extent of decrease in the number of viable cells following exposure to the test substances calculated by using the following formula: % inhibition of cells = ($A_{control}$ − $A_{test\ substance}$)/$A_{control}$ × 100. Three separate analyses were carried out with each extract. Control experiments were performed either by growing cells in the absence of the extract and

by supplementing the cell cultures with identical volumes of extract buffer (5% DMSO in PBS). The method used avoids any possibility of a DMSO effect on the results.

Statistical Analyses. Quantitative results were expressed as the mean value ± SD for three replicates. Differences among genotypes for each evaluated parameter were determined by using SPSS (Statistical Package for Social Sciences) Package 6, version 15.0. Significance was determined by comparing mean values of DHOs to the common controls M82, IL 7-3, and IL 12-4 through a factorial analysis of variance (one-way ANOVA) with LSD post hoc test at a significance level of 0.05. Principal component analysis (PCA) was carried out using the same version of SPSS. For the MTT test all of the data are representative of at least three independent experiments (with comparable results). Statistical significance of the data was evaluated by t test analyses.

■ RESULTS AND DISCUSSION

Tomato plants represent one of the major sources of antioxidants and other bioactive compounds that benefit nutrition and human health. It is well-known that carotenoids, phenolics, and AsA alone are not responsible for tomato functional effect, but they act synergistically.[12] In this study we report the characterization of novel tomato lines obtained by QTL pyramiding to combine different wild chromosomal regions and the evaluation of their phytochemical parameters in comparison with parental lines.

Carotenoids. In Table 1 the levels of total carotenoids, lycopene, and β-carotene in M82, the ILs, and DHO lines are

Table 1. Lycopene, β-Carotene, and Total Carotenoids Contents in the Tomato Lines Analyzed[a]

genotype	lycopene (mg/100 g FW)	β-carotene (mg/100 g FW)	total carotenoids (mg/100 g FW)
M82	10.09 ± 0.38a	0.67 ± 0.03a	11.10 ± 0.41a
IL 12-4	6.66 ± 0.14b	0.41 ± 0.01b	7.15 ± 0.09b
IL 7-3	12.70 ± 0.20c	0.58 ± 0.01c	13.56 ± 0.22c
DHO1	6.15 ± 0.241b	0.40 ± 0.02b	6.78 ± 0.261b
DHO2	5.73 ± 0.64bd	0.39 ± 0.05b	6.40 ± 0.72bd
DHO3	5.75 ± 0.23bd	0.33 ± 0.03bd	6.29 ± 0.18bd
DHO4	6.85 ± 0.20b	0.42 ± 0.01b	7.61 ± 0.21b

[a]Values are means ± SD ($n = 9$). Values with different letters are significantly different ($p < 0.05$).

reported. The cultivated line M82 showed a mean value of carotenoids of 11.10 ± 0.41 mg/100 g FW. The genotype IL 7-3 exhibited a significant ($p < 0.05$) increase (22.19%) of total carotenoids content compared to the cultivated line M82. On the contrary, IL 12-4 showed a significant decrease of total carotenoids content (35.6%) compared to M82. The DHO genotypes were characterized by an amount of carotenoids significantly lower than in M82, with a decrease ranging between 31.42 and 43.30% and comparable to the amount calculated in IL 12-4. In all of the genotypes lycopene constituted about 90−94% of total carotenoids, whereas β-carotene represented about 4−6% of total carotenoids. The reduction of carotenoids in the DHO lines and in IL 12-4 compared to the control M82 could be explained by taking into consideration the detection and fine-mapping of one major QTL (*lyc12.1*) for lycopene content in the chromosomal region 12-4.[33,34] In fact, wild alleles of the region 12-4 influenced negatively the levels of these metabolites in the tomato fruits of the DHO lines, even contrasting and overcoming the positive influence of the wild alleles located on the region 7-3 that may contribute increasing carotenoids levels. Therefore, we can

speculate that a negative interaction exists between genes and/or transcription factors located in the *S. pennellii* regions 12-4 and 7-3. However, it is noteworthy that the amount of these metabolites calculated in the DHO lines and in IL 12-4 are comparable to those found in some cultivated tomato lines.[2,35−37]

Ascorbic Acid. The average AsA concentration in red ripe fruit of M82 was 14.27 ± 1.30 mg/100 g FW. Genotypes IL 12-4 and IL 7-3 showed significant increases (40 and 45%, respectively) compared to the cultivated genotype (Figure 1).
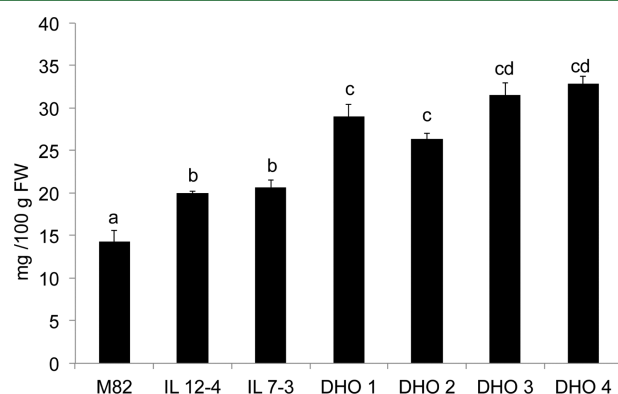

Figure 1. Ascorbic acid content (mg/100 g FW) in tomato lines analyzed. Values are means ± SD (*n* = 9). Values with different letters are significantly different (*p* < 0.05).

The amount of AsA calculated in M82 and in the IL lines is in agreement with the presence of QTLs for high AsA content previously identified in the 12-4 and 7-3 introgressed regions.[19,24] The mean value of ascorbic acid calculated in the DHO lines was between 28.99 and 32.84 mg/100 g FW. The DHO genotypes showed a significant increase of AsA content ranging between 84 and 130% compared to M82. Interestingly, the DHO genotypes also showed a significant mean increase of AsA content of 50% compared with IL 12-4 and of 45% compared to IL 7-3, indicating that in this case QTL pyramiding is effective in increasing AsA content. In addition, the DHO lines analyzed showed a higher AsA level compared to common commercial varieties,[2] for which a value of 20 mg/100 g FW was established as normal.[38] The high levels of AsA in DHOs may not only increase the nutritional value of these genotypes but might also have an effect on plant health, because AsA biosynthesis has been related to resistance to biotic and abiotic stresses.[39] Finally, the increase of AsA levels in the DHO lines compared to M82, but mostly compared to the two ILs, demonstrated that it is possible to have a positive interaction of the two wild chromosomal regions 7-3 and 12-4 on the level of this metabolite in tomato fruit.

Phenolic Compounds. The levels of total phenolic compounds were calculated in M82, ILs, and DHO lines. Results obtained from Folin−Ciocalteu's assay are reported in Figure 2. The cultivated line M82 showed a mean value of 18.12 ± 0.91 mg GAE/100 g FW. IL 12-4 did not exhibit a significant variation compared to M82, whereas IL 7-3 showed a significant increase (35.43%) compared to the cultivated genotype. The data obtained in the present study are in agreement with the levels reported for M82, IL 12-4, and IL 7-3 by Sacco et al.[24] Therefore, we could confirm the presence of a major QTL for fruit phenolic accumulation mapping to the introgressed region 3 of chromosome 7. DHO lines showed a
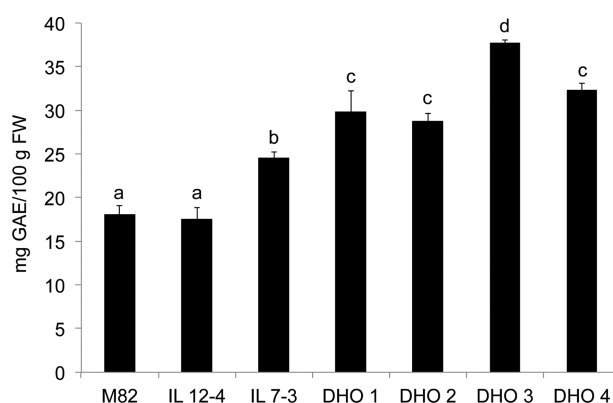

Figure 2. Total phenolics content (mg GAE/100 g FW) in tomato lines analyzed. Values are means ± SD (*n* = 9). Values with different letters are significantly different (*p* < 0.05).

significant increase of phenolics compared to the control M82 (ranging between 59.09 and 107.92%) and to IL 12-4 (ranging between 64.24 and 114.65%), but, interestingly, also compared to IL 7-3 (ranging between 17.46 and 53.52%). In the DHO lines the calculated amount of phenolic compounds ranged between 30 and 38 mg/GAE 100 g FW. These values are comparable with those observed by García-Valverde et al.[36] and by Martínez-Valverde et al.[40] in some tomato cultivated lines and higher than those reported by George et al.[35] and Hernández et al.[41]

Phenolic acids and flavonoids were also detected by HPLC (Table 2). Quercetin and gallic acid represent the majority of detected phenolics, whereas kampferol was not detected in any analyzed lines. These results are not in line with those reported by Slimestad et al.,[42] who observed that chlorogenic acid was the major phenolic acid and rutin the major flavonoid together with naringenin. This variability can be attributed mainly to the diverse genetic sources adopted in the different studies.[43] In particular, in our case quercetin was present in M82 at the mean level of 25.70 ± 4.26 mg QE/kg FW, and in IL lines it did not show significant variations compared to M82. By contrast, in DHOs a significant decrease ranging between 67.00 and 76.00% was found. Despite this decrease, values found for quercetin in the pyramided lines were higher than those reported by Vallverdú-Queralt et al.[44] in cultivated tomatoes. The same trend observed for quercetin in tested lines was observed for total flavonoids. The amount of total flavonoids in pyramided lines is comparable to that reported by Slimestad et al.[42] in different cultivars of tomato.

Total phenolic acids were significantly higher in IL 7-3 and DHO lines compared to M82 and IL 12-4. In particular, DHO genotypes showed an increase ranging between 94.11 and 154.16% compared to M82 and between 77.39 and 132.27% compared to IL 12-4. Interestingly, the DHO genotypes also showed a significant mean increase of total phenolic acid content up to 50.64% compared to IL 7-3. Frusciante et al.[2] established an optimum concentration of these compounds in tomato of 53.6 mg/kg FW. Therefore, the amount of total phenolic acid calculated in M82 in this study exceeded the recommended concentration of 53%, whereas in DHOs it exceeded this value of 197−288%.

There was also an increase in the level of gallic acid in the DHO genotypes compared to M82 and IL 12-4. These results are noteworthy because it has been demonstrated that gallic acid exerts a protective effect on diabetes due to its

Table 2. Flavonoids, Ferulic Acid, Gallic Acid, and Total Phenolic Acids Evaluated by HPLC[a]

genotype	quercetin (mg QE/kg FW)	kampferol (mg QE/kg FW)	total flavonoids (mg QE/kg FW)	ferulic acid (mg FA/kg FW)	gallic acid (mg GAE/kg FW)	total phenolic acids (mg GAE/kg FW)
M82	25.70 ± 4.26a	nd	27.53 ± 4.22a	0.34 ± 0.05a	78.48 ± 8.88a	81.91 ± 9.86a
IL 12-4	23.82 ± 3.02a	nd	26.52 ± 2.14a	0.35 ± 0.03a	86.02 ± 15.99a	89.63 ± 15.48a
IL 7-3	21.57 ± 5.27a	nd	28.00 ± 5.60a	0.58 ± 0.05b	129.99 ± 3.55b	138.20 ± 4.51b
DHO1	8.13 ± 0.74b	nd	12.87 ± 0.06b	0.51 ± 0.09b	147.58 ± 10.66b	208.19 ± 18.79c
DHO2	8.43 ± 0.72b	nd	12.81 ± 0.04b	0.56 ± 0.15b	98.58 ± 12.44a	159.00 ± 3.76d
DHO3	6.19 ± 0.28b	nd	9.73 ± 0.61b	0.62 ± 0.07b	131.25 ± 1.78b	187.02 ± 15.58c
DHO4	6.55 ± 0.07b	nd	11.29 ± 0.66b	0.53 ± 0.01b	147.58 ± 17.77b	184.63 ± 19.63cd

[a]Values are means ± SD ($n = 9$). Values with different letters are significantly different ($p < 0.05$).

Table 3. Total Antioxidant Activity (TAA) and Its Lipophilic (LAA) and Hydrophilic (HAA) Fractions Evaluated by ABTS and FRAP Analyses[a]

genotype	ABTS LAA (μmol TE/100 g FW)	ABTS HAA (μmol TE/100 g FW)	FRAP HAA (μmol TE/100 g FW)	TAA (μmol TE/100 g FW)
M82	104.79 ± 3.38a	131.61 ± 13.34a	115.36 ± 11.89a	236.40 ± 16.72a
IL 12-4	83.27 ± 2.74b	153.21 ± 5.17b	154.52 ± 15.72b	236.48 ± 7.91a
IL 7-3	112.05 ± 3.27a	145.43 ± 3.10ab	143.93 ± 4.57b	257.48 ± 6.37a
DHO1	84.01 ± 3.88b	183.86 ± 6.42c	179.64 ± 2.63c	267.87 ± 10.30a
DHO2	85.13 ± 5.80b	253.98 ± 3.87d	229.14 ± 3.18c	339.11 ± 9.67b
DHO3	79.76 ± 4.76b	246.07 ± 4.40d	207.86 ± 4.02c	325.83 ± 9.16b
DHO4	88.75 ± 0.50bc	262.73 ± 3.38d	289.71 ± 9.66d	351.48 ± 3.88bc

[a]Values are means ± SD ($n = 9$). Values with different letters are significantly different ($p < 0.05$).

antihyperglycemic and antilipid peroxidative capacity as well as its strong antioxidant power.[45] In addition, a significant increase in the level of ferulic acid in the DHO lines compared to the cultivated line and IL 12-4 was detected. Also, the increase of ferulic acid in the DHO lines is significant from a nutritional point of view, because a positive effect derived from the combination of ferulic acid with AsA, in particular associated with the protection of the myocardium, has been demonstrated.[46]

Taken together, the data obtained from two independent assay procedures (Folin−Ciocalteau and phenolics estimation by HPLC-UV procedure) clearly indicated that the amount of phenolic compounds, in particular of phenolic acids, was increased in DHOs compared to ILs and M82, and above all compared to IL 7-3. This suggested that on the region 12-4 there are wild alleles that synergistically interact with those mapping to the 7-3 region, resulting in increased phenolic levels. On the contrary, the decrease of total flavonoid in the DHO lines must be due to negative interactions between the two wild regions. These findings highlight that detailed information regarding the interaction effects between detected QTLs and between QTLs and the recipient background genotype are necessary to design an efficient pyramiding strategy for increasing tomato nutritional quality.

Antioxidant Activity. Results regarding lipophilic (LAA), hydrophilic (HAA), and total antioxidant activity (TAA) of genotypes analyzed are reported in Table 3. In the cultivated M82 line, the LAA antioxidant activity showed a mean level of 104.79 ± 3.38 μmol TE/100 g FW. A significant 20% lower value of LAA was calculated in genotype IL 12-4 compared to the cultivated line, whereas in IL 7-3 the mean value of LAA (112.05 ± 3.27 μmol TE/100 g FW) was comparable to the value obtained in M82. In DHO lines, the detected values were not significantly different from those of IL 12-4 and showed a decrease compared to M82 ranging between 15.30 and 23.88%. Our LAA results confirmed the data obtained for carotenoids that constitute the major fraction of lipophilic compounds in

tomato fruit. Nevertheless, the LAA decrease in the DHO lines and in IL 12-4 (estimated by ABTS) was lower than the decrease calculated for total carotenoids and lycopene. This is probably due to the presence of other lipophilic compounds such as tocopherol and lipophilic phenolics, as well as to the synergic effect of compounds contributing to LAA.[47]

The HAA determined by FRAP and ABTS tests in M82 showed values of 115.36 ± 11.89 and 131.61 ± 13.34 μmol TE/100 g FW, respectively. The parental lines IL 7-3 and IL 12-4 showed increased values of HAA compared to the cultivated line. Values calculated in DHO lines were significantly different from M82, with a mean increase of 96% by FRAP test and 80% by ABTS test. Compared to IL 12-4, DHO genotypes revealed significant mean increases of 46.63 and 54.46% according to FRAP and ABTS tests, respectively, and compared to IL 7-3 mean increases of 57.43 and 62.72% in the two assays. The mean contribution of HAA to TAA was estimated to be 55.67% in M82, 64.78% in IL 12-4, and 56.48% in IL 7-3, whereas in DHOs these percentages were between 68.63 and 75.52%. Overall, the TAA in DHOs revealed significant increased values in comparison to the cultivated M82 and IL lines, and this increase was attributed mostly to a higher contribution of HAA to TAA. Our data on HAA and TAA were comparable to those reported by Cano et al.,[48] and the contribution of HAA to TAA was comparable to that found by Kotíková et al[3] and Arnao et al.[49]

Comprehensively, our data demonstrated that there is a positive interaction between the two wild regions 7-3 and 12-4 that caused an increment of HAA. On the contrary, there is a negative influence of the wild alleles of the region 12-4 on LAA. Therefore, the improvement of the nutritional quality in DHO lines can be attributed mostly to the increment of the hydrophilic fraction that includes the majority of tomato phytochemicals, despite a general decrease of the lipophilic fraction.

Accordingly, by the PCA we observed that the separation of DHOs from M82 and from the two ILs is mainly due to the

hydrophilic fraction (Figure 3). Indeed, PC1 explained 71.92% of the variation in the data and was associated positively with
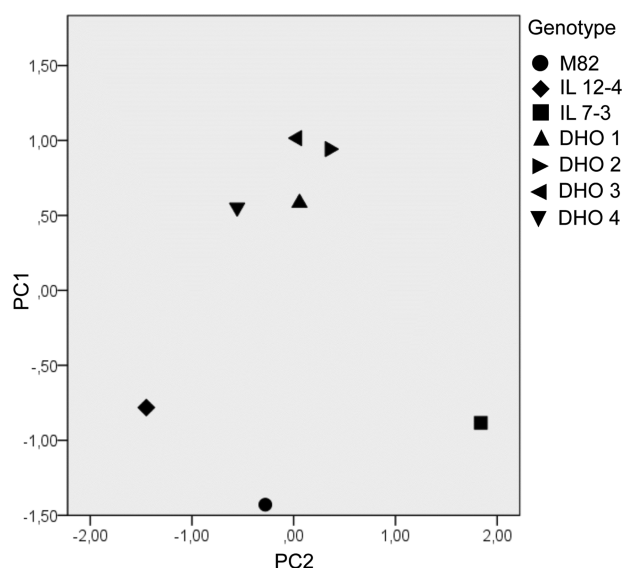

Figure 3. Principal component analysis (PCA) of metabolic profiles of analyzed genotypes.

AsA, phenolic acids, gallic acid, ferulic acid, total phenolics, total flavonoids, quercetin, hydrophilic ABTS, and FRAP (loading values > 0.60). PC2 explained 18.09% of the variation in the data and was associated positively with total carotenoids, lycopene, β-carotene, and lipophilic ABTS (loading values > 0.60). The PCA output shows an evident separation of both IL 12-4 and IL 7-3 from M82, mainly attributable to PC2 that included the lipophilic compounds and their contribution to the antioxidant activity. DHOs were well separated from the parental lines for both PC1 and PC2 and from M82 mainly for PC1, which represented the hydrophilic fraction of the analyzed metabolites and their relative antioxidant activity.

Effects of Tomato Extracts on Cell Viability. Finally, the biological activity of extracts from M82, IL 7-3, IL 12-4, and DHO has been tested in our study by using two normal cell lines (Figure 4): a primary human cell line derived from kidney (HRCE) and an immortalized murine fibroblast cell line (BALB/3T3). The viability of cells treated for 48 h with increasing amounts of tomato extracts was tested by the MTT

reduction assay, as an indicator of metabolically active cells. In Figure 4 the results of dose−response experiments are shown. The values are the average of three independent experiments, each carried out with triplicate determinations. We observed that M82, IL 7-3, and IL 12-4 extracts had a cytotoxic effect on the HRCE cells and on the BALB/3T3 cells, which was very evident at the highest concentration tested. On the contrary, DHO extracts had no effect, independent of the concentration used on primary cells (HRCE cells), and small effects only at the highest concentration used (24 mg/mL) on immortalized normal cells (BALB/3T3 cells). The cytotoxic effect of M82, IL 7-3, and IL 12-4 extracts could be due to the presence in these genotypes of higher levels of compounds with reported antiproliferative activity such as flavonoids[50] compared with the lower levels observed in the DHO lines (Table 2). In fact, it has been demonstrated that exposure to high levels of flavonoids may potentially damage cells.[51] To test the anticarcinogenic potential of the tomato genotypes, we performed the same experiments using cancer cell lines of the same anatomical origin of normal cells (Figure 5). We examined the inhibition of the rapidly proliferating human cancer cell lines (HeLa cells), of BALB/3T3 murine fibroblasts transformed with SV40 virus, and of human renal cancer cell lines (Hek293). As shown in Figure 5, we observed a strong and selective cytotoxic effect of DHO extracts, particularly evident at the highest concentration used on all of the cancer cell lines tested. Generally, a lower cytotoxic effect was observed with the M82, IL 7-3, and IL 12-4 extracts. The effects of DHO extracts on cancer cells could be also dependent on the content of total phenolic acids[54] and on the presence of gallic acid, a compound known to have a selective cytotoxic effect on cancer cells.[55] The mechanism for the known selective effects of plant phenolics on cancer cell growth is still under debate and may also involve differences in gene expression between normal and tumor cell lines.[50] At low concentrations all of the tomato extracts caused an initial increase in HeLa and SV-T2 cell growth followed by inhibition of growth at higher concentration. This result was not observed on Hek293 cells. The proliferative effect here observed has been reported for several plant extracts used at low concentration on different cancer cells.[26,52,53] This could be due to the fact that cancer cells are actively proliferating; thus, the presence of nutrients in the plant extracts in combination with low levels and/or low
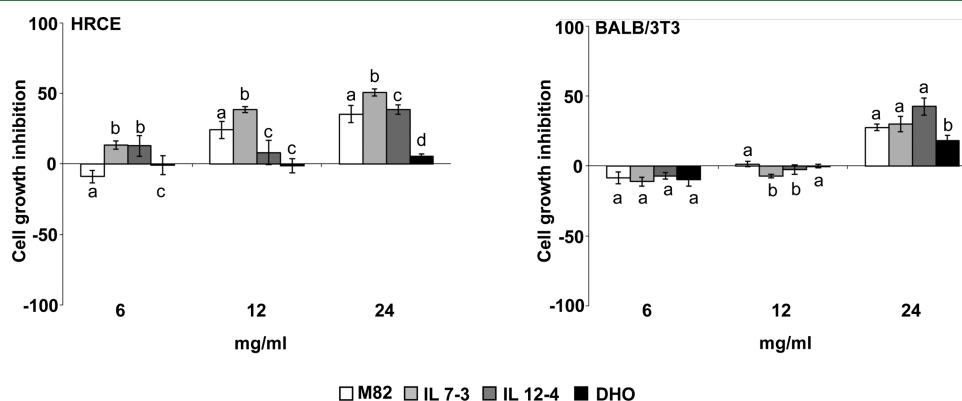

Figure 4. Dose−response curves obtained upon treatment of HRCE and BALB/3T3 cells with extracts from M82, IL 7-3, IL 12-4, or DHO after 48 h. Cell growth inhibition was assessed by the MTT assay and expressed as described under Materials and Methods. All values are given as means ± SD ($n \geq 3$). Within each treatment, values with different letters are significantly different ($p < 0.05$).
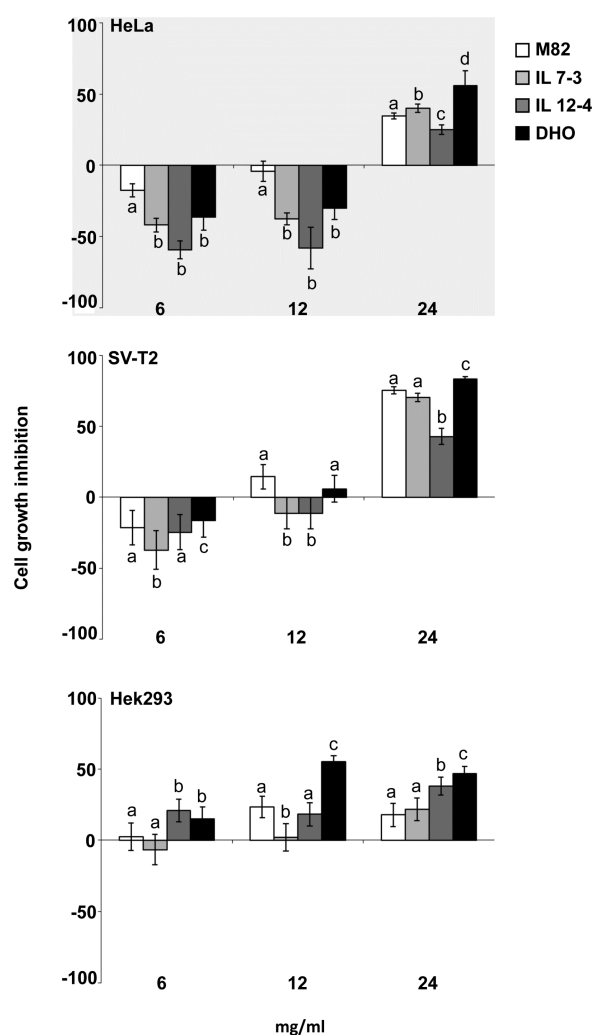
Figure 5. Dose−response curves obtained upon treatment of HeLa, SV-T2, and Hek 293 cells with extracts from M82, IL 7-3, IL 12-4, or DHO after 48 h. Cell growth inhibition was assessed by the MTT assay and expressed as described under Materials and Methods. All values are given as means ± SD ($n \geq 3$). Within each treatment values with different letters are significantly different ($p < 0.05$).

susceptibility to active compounds may have a positive effect on cell growth, as suggested by Choi et al.[26]

In conclusion, the combination of multiple QTLs, QTL pyramiding, offers a straightforward and useful way for improving target traits in crop plants.[56] Here we demonstrated that it is possible to incorporate favorable wild-species QTLs in the same genetic background to obtain genotypes with a reproducibly higher nutritional value. In particular, we have shown that QTL pyramiding resulted in genotypes with higher AsA and phenolics and increased the total antioxidant power. In addition, crude extracts from the pyramided genotypes showed antiproliferative activity only on human cancer cells, whereas they did not affect the growth of normal cells. On the contrary, extracts from the cultivated genotypes and the ILs did not show the same selective cytotoxic effect but inhibited proliferation of normal cells. The pyramided QTLs should now be transferred in cultivated genetic backgrounds different from M82 to verify that they might exert the same increase of the total antioxidant activity and the same selective cytotoxic effect on aggressive cancer cells. In particular, because compounds such as vitamin C (that are increased in DHO lines) are heat-labile, the

pyramiding strategy here used to increase tomato nutritional value could be applied to varieties that are used for fresh consumption.

Consequently, these novel genotypes could be used as genetic material for breeding schemes aimed at generating new hybrids or improved varieties with higher nutritional levels. However, to use these DHO lines as breeding materials, it will be important to understand if introgressed wild regions determine taste alterations and/or allergenic effects. Finally, further studies aimed at dissecting the introgressed region into sublines, as well as in-depth analyses of candidate genes, will be necessary to elucidate the underlying mechanisms of the selected QTLs. These studies will help also to disrupt the linkage between the genes that promote AsA content and those that contribute to low carotenoid levels in region 4 of chromosome 12, thus facilitating the use of IL 12-4 as a source of favorable alleles for higher AsA.

■ AUTHOR INFORMATION

Corresponding Author
*(A.B.) E-mail: ambarone@unina.it. Phone: +39-081-2539491. Fax: +39-081-2539486.

Author Contributions
[||]M.M.R. and A.R. contributed equally to this work.

Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); AsA, ascorbic acid; CNCD, chronic noncommunicable diseases; CVD, cardiovascular diseases; dd, double distilled; DHO, double homozygote; FRAP, ferric reducing/antioxidant power; FW, fresh weight; GAE, gallic acid equivalents; HAA, hydrophilic antioxidant activity; IL, introgression line; LAA, lipophilic antioxidant activity; LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCA, principal component analysis; QE, quercetin equivalents; QTL, quantitative trait locus; ROS, reactive oxygen species; TAA, total antioxidant activity; TCA, trichloroacetic acid; TE, Trolox equivalents; TGRC, Tomato Genetics Resource Centre; TPTZ, 2,4,6-tripyridyl-s-triazine

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