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QTL analysis of fruit antioxidants in tomato using *Lycopersicon pennellii* introgression lines

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Abstract Antioxidants present in fruits and vegetables may help prevent some chronic diseases such as cancer, arthritis, and heart disease. Tomatoes provide a major contribution to human dietary nutrition because of their widespread consumption in fresh and processed forms. A tomato introgression line population that combines single chromosomal segments introgressed from the wild, green fruited species *Lycopersicon pennellii* in the background of the domesticated tomato, *Lycopersicon esculentum*, was used to identify quantitative trait loci (QTL) for nutritional and antioxidant contents. The concentration of ascorbic acid, total phenolics, lycopene and β-carotene, and the total antioxidant capacity of the water-soluble fraction (TACW) were measured in the ripe fruits. A total of 20 QTL were identified, including five for TACW (*ao*), six for ascorbic acid (*aa*), and nine for total phenolics (*phe*). Some of these QTL (*ao6-2, ao6-3, ao7-2, ao10-1, aa12-4, phe6-2*, and *phe7-4*) increased levels as compared to the parental line *L. esculentum*. For lycopene content, we detected four QTL, but none increased levels relative to *L. esculentum*. The two QTL (*bc6-2* and *bc6-3*) detected for β-carotene increased its levels. The traits studied displayed a strong environmental interaction as only 35% of the water-soluble antioxidant QTL (including TACW, ascorbic, and phenolic contents) were consistent over at least two seasons. Also, only two QTL for phenolics were observed when plants were grown in the greenhouse and none was detected for ascorbic or TACW. The analysis demonstrates that the introgression of wild germplasm may improve the nutritional quality of tomatoes; however regulation appears to be complex with strong environmental effects.

Keywords Antioxidant · Ascorbic acid · Carotenoid · Phenolics · Tomato

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Introduction

Antioxidant compounds in plants are critical for protecting cellular processes from internal and environmental oxidative stresses. Lipid- and water-soluble compounds are involved in mediating damage to plant cells undergoing photosynthesis, respiration, or experiencing external stress. These same compounds, when consumed by animals and humans, are used in analogous ways for protection against oxidative stresses, which are associated with many human diseases (Demmig-Adams and Adams 2002). A substantial body of research exists that addresses the role these antioxidants play in the light harvesting, chlorophyll containing tissues of plants. However, with the exception of lycopene and β-carotene, relatively little is known about the genetic regulation of antioxidant compounds in the fruit tissues consumed by humans. Due to significant consumption levels of tomato products and well-developed genetic tools, tomato is an excellent system in which studies about the genetic controls of antioxidant accumulation in a major food crop can be undertaken.

Antioxidant compounds can be characterized as lipid-soluble or water-soluble. Most antioxidant capacity of fruits and vegetables is associated with ascorbic acid

(AsA), tocopherol, carotenoids, and phenolic compounds. Tomato fruit (*Lycopersicon esculentum* Mill. = *Solanum lycopersicum* L.) is an important source of lipid-soluble antioxidants in the human diet because of their relatively high content of carotenoids. Lycopene presence in plasma has been related to a reduction in the risk of prostate cancer (Giovannucci et al. 1995). Other carotenoids present in ripe tomato fruits include β-carotene and small amounts of phytoene, phytofluene, ζ-carotene, γ-carotene, neorosporene, and lutein (Khachik et al. 2002). Tomato fruit is also an important source of the water-soluble antioxidants, particularly AsA. Because tomatoes represent a major contribution to dietary nutrition worldwide (Willcox et al. 2003), there is a growing interest in the potential genetic improvement of tomato antioxidant levels either by traditional breeding methods (Ronen et al. 1999, 2000; Zhang and Stommel 2000) or by transgene incorporation (Giuliano et al. 2000; Romer et al. 2000). Improvement in tomato nutritional traits also offers the opportunity to determine basic information about the regulation of antioxidants in fruit crops.

In the United States, tomatoes rank first among the vegetables in per capita contribution of phenolic antioxidants (Vinson et al. 1996). However, in comparison to carotenoids relatively little is known about phenolic compounds in tomato. Martinez-Valverde et al. (2002) reported the presence of the flavonoids quercetin, kaempferol and naringenin, and the hydrocynamic acids caffeic chlorogenic, ferulic and p-coumaric acids. In addition to these, rutin (Minoggio et al. 2003), 5′-caffeoylquinic, and chalconaringenin (Proteggente et al. 2002) have been identified in tomato fruit. With the exception of tomatoes containing the *Aft* gene (Jones et al. 2003), tomato fruit does not contain significant levels of the phenolic anthocyanin pigments which contribute to antioxidant values in many other fruit crops.

Many cultivated plants have undergone a reduction in genetic variability from ancestral forms, and this is particularly pronounced in the tomato whose history of domestication and modern breeding have significantly depleted its natural variation (Miller and Tanksley 1990; Reto et al. 1993). Geneticists have recognized the value of the incorporation of variability from the germplasm of related wild species, but the problems of hybrid sterility and the incorporation of undesired characters have restricted the use of exotics (Rick and Tanskley 1983). In the last few years, the development of molecular marker assisted selection has enabled the development of a population of introgression lines (ILs) that combine relatively small sections of the wild, green fruited tomato species *Lycopersicon pennellii* Corr. (= *Solanum pennellii* (Corr) Darcy) in the background of *L. esculentum* (Eshed et al. 1992; Eshed and Zamir 1994a). The *L. pennellii* ILs represent the entire *L. pennellii* genome in 50 individual overlapping segments (Eshed et al. 1992; Eshed and Zamir 1994a, 1995). The average ratio of physical/genetic distance in the tomato genome is estimated to be approximately 750 kb/cM, in a genome of 950 Mbp and ~1250 cM (Tanksley et al. 1992). The average introgressed segment in the *L. pennellii* ILs is ~33 cM (Eshed and Zamir 1994b) or ~25 Mbp. Since the ILs are a homozygous and permanent resource, they provide a convenient means to map quantitative trait loci (QTL) with replication over multiple years and locations, which is less feasible with transient F_2 populations. QTL are established by comparing individual introgressions, which are independent of additional donor genome segments, unlike recombinant inbred or F_2 populations. The *L. pennellii* IL population has been successfully used to map QTL associated with a number of traits (Eshed and Zamir 1995; Eshed et al. 1996; Ku et al. 1999; Astua-Monge et al. 2000; Fridman et al. 2002). *Lycopersicon pennellii* has higher antioxidant enzyme activity than *L. esculentum* and up-regulated antioxidant activity in leaves and roots when exposed to stress inducing salt concentrations (Mittova et al. 2002a, b). The aim of this work is to identify genomic regions controlling the antioxidant capacity in tomato fruit by identifying QTL for ascorbic acid, lycopene, β-carotene, and phenolic compounds in the IL population.

Materials and methods

Plant material and experimental trials

Antioxidant traits were mapped in a library of ILs that contains a complete genome of *L. pennellii* (accession LA0716) in the background of *L. esculentum* (cv. M-82) (Eshed and Zamir 1995). LA0716 is a self-fertile, homozygous green fruited, indeterminate accession from Atico, Peru. M-82 is a red fruited, determinate, processing-type tomato. The genetic constitutions of the 50 ILs, as well as 26 sublines were described by Pan et al. (2000). The sublines were obtained as the product of backcrossing selected introgressed lines with M-82, creating smaller introgressed sections. Due to overlapping introgressions, effects observed in only one line of two overlapping segments can be ascribed to the unshared region. The *L. pennellii* ILs thus subdivide the tomato genome into 107 "bins" (Pan et al. 2000). Bin locations are referred to by chromosome number followed by letter identifying the bin. All lines contain a single homozygous introgression delineated by flanking RFLP markers. All seed were obtained from the C.M. Rick Tomato Genetics Resource Center at the University of California Davis.

Field trial 2000

Six plants from each IL were transplanted on June 7, 2000 in the Vegetable Crops experimental field (University of California, Davis, CA, USA) in three replicated blocks, with two plants in each plot, in a randomized completely blocked design. Plants were

transplanted 3 feet apart in rows separated by 5 feet. The control line *L. esculentum* cv. M-82 was also present in the field trial as part of the main experiment. Because the wild parent, *L. pennellii* (LA0716), does not grow well under Davis field conditions, it was instead grown in the greenhouse, in three replicates.

At harvest, ripe fruit that had been fully exposed to sunlight and that represented typical fruit on plants in each block were selected from each plot. Fruits were refrigerated at 4°C (3–4 h) until they were processed on the morning of harvest. Approximately 500 g of whole fruits were macerated to uniformity with a hand-held blender in an ice bath. The complete trial was harvested within 2 weeks. Aliquots of the freshly macerated material were immediately taken to measure the total antioxidant capacity of the water-soluble fraction (TACW), the ascorbic acid, phenolics, lycopene and β-carotene concentrations, and the amount of total solids.

Field trials 2001 and 2002

During 2001 and 2002 plants were transplanted in the experimental field of Campbell Research and Development, Davis, CA, at the beginning of May. We used a complete randomized block design with three blocks. Each plot consisted of 25 plants spaced 1 foot apart in rows separated by 5 feet. In 2001 all of the ILs were planted and in 2002 a subset of selected ILs (50%) and sublines (31%) were planted. In both trials, the control *L. esculentum* cv. M-82 was represented five times per block to increase the statistical power for detecting differences. The *L. pennellii* (LA0716) parental line was planted in the field with one plot per block.

In 2001, a separate trial included sublines and their corresponding control ILs. We planted three blocks in plots of three plants per plot, 1 foot apart in rows separated by 5 feet. The control *L. esculentum* and the parental line *L. pennellii* were planted following a similar design.

Two ILs (IL6-2 and IL6-3) contained the *L. pennellii* *sp*+ allele for indeterminate growth habit. To test whether the results were influenced by plant habit we used determinate/indeterminate isolines in the background of VFNT Cherry (LA1221 *sp*+ and LA2705 *sp*) and the fresh market variety Gardener (LA3030 *sp*+ and LA3133 *sp*).

Each plot was harvested when 85% of the fruits were red ripe. We harvested 10 kg of ripe fruits per plot (i.e. three bulked fruit samples per accession per year except for M-82 for which we had 15 bulked fruit samples per year). The fruits were blended in a garbage disposal. Seeds and skin were extracted with a commercial seed extractor and kept refrigerated in 100 ml cups in an N-rich atmosphere until analysis, within 6 h. Aliquots were taken for measuring TACW, ascorbic acid, phenolics, lycopene and β-carotene concentration, and total solids. The parental line *L. pennellii* and F_1 hybrid (*L. pennellii* × *L. esculentum* M-82) were sampled to provide an estimate of *L. pennellii* homozygote and heterozygote values. The *L. pennellii* homozygote and heterozygote genotypes produce small yellowish-green fruit, which does not ripen in the same fashion as fruit of cultivated tomatoes. Therefore, fruit from these lines were handled somewhat differently at harvest. About 200 g of mature fruit were harvested and blended for 2 min in an ice bath. For *L. pennellii*, the fruit was judged mature when they were full size and contained ripe seeds. For F_1 hybrid, the fruits were harvested when yellow and soft.

Greenhouse trial 2002

For the 2002 trial, the same ILs and sublines that were analyzed in the field were also analyzed in the greenhouse. High levels of variability in the field lead us to test whether the greenhouse might provide us with a decreased level of environmental variability. We used a complete block design with three blocks. Plants were in plots of three plants, 1 foot apart in rows distant 2 feet. Each plant was planted in individual 500-ml pots and provided with a complete growing solution through an irrigation system with individual drips lines. The harvest protocol was similar to the one used for the field trials.

Quantification of antioxidants

The TACW of the tomato homogenates was determined by the total radical-trapping potential (TRAP) method; Lissi et al. (1995). Briefly, 1.5 ml of sodium phosphate buffer (pH 7.4 and 0.1 M) was added to 100 mg of fresh homogenate. Samples were centrifuged at 4500 g for 15 min at 4°C. For the assay, 10 µl of the sample supernatant was added to 200 µl of a 24 mM solution of 2,2-azo-bis (2amidinopropane) dihydrochloride (ABAP; Wako Chemicals USA Inc., Richmond, VA, USA) in phosphate buffer and 25 µl of 0.156 mM luminol (A4685, Sigma, St. Louis, MO, USA) in 0.1 N NaOH. The light emission was measured every 40 s for 2 h in a 96 well microplate luminescence counter (TopCount NXT, Packard Instruments, Meriden, CT, USA). We plotted light emission of the 200 time points. The time from the beginning of the measurement to the start of exponential light emission phase was considered the lag time. Final results are expressed in terms of the TROLOX standard equivalents (µM TROLOX per gram dry mass fruit). Trolox standards were included in each sample run. We took two independent aliquots from each sample and assayed duplicate samples for each aliquot. Values were corrected by the dry mass of solids in the aliquot.

Ascorbic acid

Ascorbic acid was measured using the enzymatic method described by Luwe et al. (1993). The method is based

on the complete oxidation of the ascorbic acid to dehydroascorbic by the exogenous ascorbate oxidase (EC 1.10.3.3, from *Cucurbita* sp.). One ml of 2% metaphosphoric acid in 2×10^{-4} M EDTA was added to 150 mg of fresh homogenate. Samples were centrifuged at 4000 *g* for 10 min at 4°C. Reaction assays contained 100 µl of samples and 700 µl of 0.1 M buffer sodium phosphate (pH 6.8), and were performed at room temperature. Ascorbic acid oxidation was started by the addition of 10 µl of ascorbate oxidase (0.1 unit μl^{-1}). An extinction coefficient for ascorbic acid of 14.3 mM^{-1} at 265 nm was used. We measured two independent aliquots from each sample. Values were corrected by the dry mass of solids in the aliquot.

L-ascorbic and dehydroascorbic acids were measured by HPLC to corroborate that the ascorbate present as dehydroascorbic acid was similar between ILs and did not exceed 10% (Zapata and Dufour 1992). Briefly, 2 g of frozen tomato homogenate that was kept at −80°C for 3 months were homogenized for 2 min with 18 ml of extraction buffer (0.1 M citric acid, 0.05% EDTA, 5% methanol) in an ice bath. Samples were filtered by four layers of cheesecloth, and centrifuged at 11,950 *g* at 2°C using a Sorvall RC-5C with a SS34 rotor (Global Medical Instrumentation Inc., Albertville, MN, USA). The pH was adjusted to 2.35–2.4 with HCl and supernatant was passed through a Sep-Pack C18 cartridge (Waters Associates, Milford, MA, USA). Three milliliters of the eluant were mixed with 1 ml of a 14.8-mM solution of 1,2-phenylenediamine dihydrochloride (Sigma) in 5% methanol. Samples were kept at room temperature in the dark for 37 min to allow the derivitization of the dehydroascorbate. Samples were injected in a C18 reverse phase HPLC column and detected using a variable wavelength UV detector (HP 1050 Series, Hewlett Packard). L-ascorbic was detected at 261 nm and dehydroascorbic at 348 nm.

Total phenolics

The amount of total phenolics in extracts was determined according to Folin-Ciocalteu's procedure Singleton and Rossi (1965). One milliliter of 99:1 of methanol:HCl (1 N) was added to 100 mg of fresh homogenate. Extraction was at room temperature for 24 h on a shaker in the dark. The extraction was centrifuged at 9000 *g* for 1 min at room temperature. Four hundred microliters of the supernatant, 450 µl of Folin-Ciocalteu's reagent (diluted 1:6 reagent/distilled water), and 250 µl of a 6% solution of sodium bicarbonate were incubated for 90 min at 20°C. Absorbance was measured at 725 nm. The total phenolic concentration was expressed in terms of gallic acid equivalents (µmol g^{-1} dry mass fruit), based on a gallic acid standard curve for each experiment. Because ascorbic can also react with the Folin-Ciocalteu's reagent, we subtracted the corresponding absorbance from the measurement at 725 nm. Values were corrected by the dry mass of solids in the aliquot.

Carotenoids

To measure lycopene and β-carotene 50 mg of frozen (−80°C) homogenate was extracted in 3.5 ml of 4:3 (v/v) ethanol/hexane overnight at room temperature in a shaker at 150 rpm, keeping the samples out of bright light. To separate the hexane phase, 0.5 ml of water was added, the samples were vortexed and left for 10 min. Absorbance of the hexane fraction was measured at 510 nm for lycopene and 452 nm for β-carotene (Zscheille and Porter 1947). For δ-carotene, we measured the absorbance of the hexane fraction at 446 nm (Taungbodhitham et al. 1998). During the first season we determined which tomato lines accumulate δ-carotene by assessing the spectral absorption of the hexane fraction. Values were corrected by the dry mass of solids in the aliquot. We used spectrophotometric determinations for lycopene, β-carotene and δ-carotene because this is a relatively fast and accurate method of determining levels of these compounds in tomato fruit. We therefore do not present data on other minor carotenoids, which may be measured by HPLC.

Dry mass

The dry mass was measured using two methodologies. For the 2000, we took 2 g aliquots for each sample and measured the dry mass after drying it for 3 h at low drying rate on an automate environmental speed-vac (AES1010-120, Savant Instruments Inc., Farmingdale, NY, USA) and then for 24 h in an oven at 70°C. For 2001 and 2002, we used a microwave moisture balance analyzer (CEM Labwave 9000, Amphotech Ltd., Beverly, MA, USA) in which we dried 2 g of tomato paste until mass change over 10 s was less than 0.2 mg (AOAC Official method 42.1.09; *JAOAC* 68, 1081, 1985).

Statistical analysis

Significance of QTL was determined by comparing mean values of individual ILs to the control M-82 genotype. We considered that there was a QTL when the chromosomal segment had an effect 30% or larger or when it had and effect of 25% or larger but it also was consistent over at least 2 years. For those lines used both in 2001 and 2002, we perform a factorial ANOVA to assess year and IL effects. Because of the significant interaction between IL and year ($P < 0.05$), analysis of variance and the Dunnett's test (Dunnett 1955) for a complete block design ($n = 3$) were used to study the differences between ILs and control M-82 ($n = 15$) for TACW, ascorbic acid, total phenolics, lycopene, and β-carotene concentration in the field and greenhouse experiments for each year separately (Figs. 1, 2, 3). In spite of the significant IL × year interaction, we presented a 3 years average graph in the

Figs. 1, 2, 3 to show the interannual variability (represented by the SE) and the relation between the values for the IL and the *L. esculentum* M-82 control. Differences between the responses of the ILs for a particular chromosome in the fine mapping analysis were assessed by a Duncan test (SAS Institute 1999).

The difference between determinate and indeterminate habit (*sp* genotypes) in different genetic backgrounds were assessed in a split plot ANOVA. Regression analysis was used to determine the percentage of variability in the TACW explained by ascorbic acid and/or total phenolics concentration.

Results

Antioxidant capacity

The TACW of fruit from the parental line *L. pennellii* was always higher than the TACW of fruit of *L. esculentum* cv M-82 ($P < 0.01$; Fig. 1). *Lycopersicon pennellii* had an average TACW almost two times higher than *L. esculentum*. The F_1 did not differ from the *L. esculentum* parent (Fig. 1, 2002). We identified 5 QTL for TACW in the replicates over 3 years. Three of these

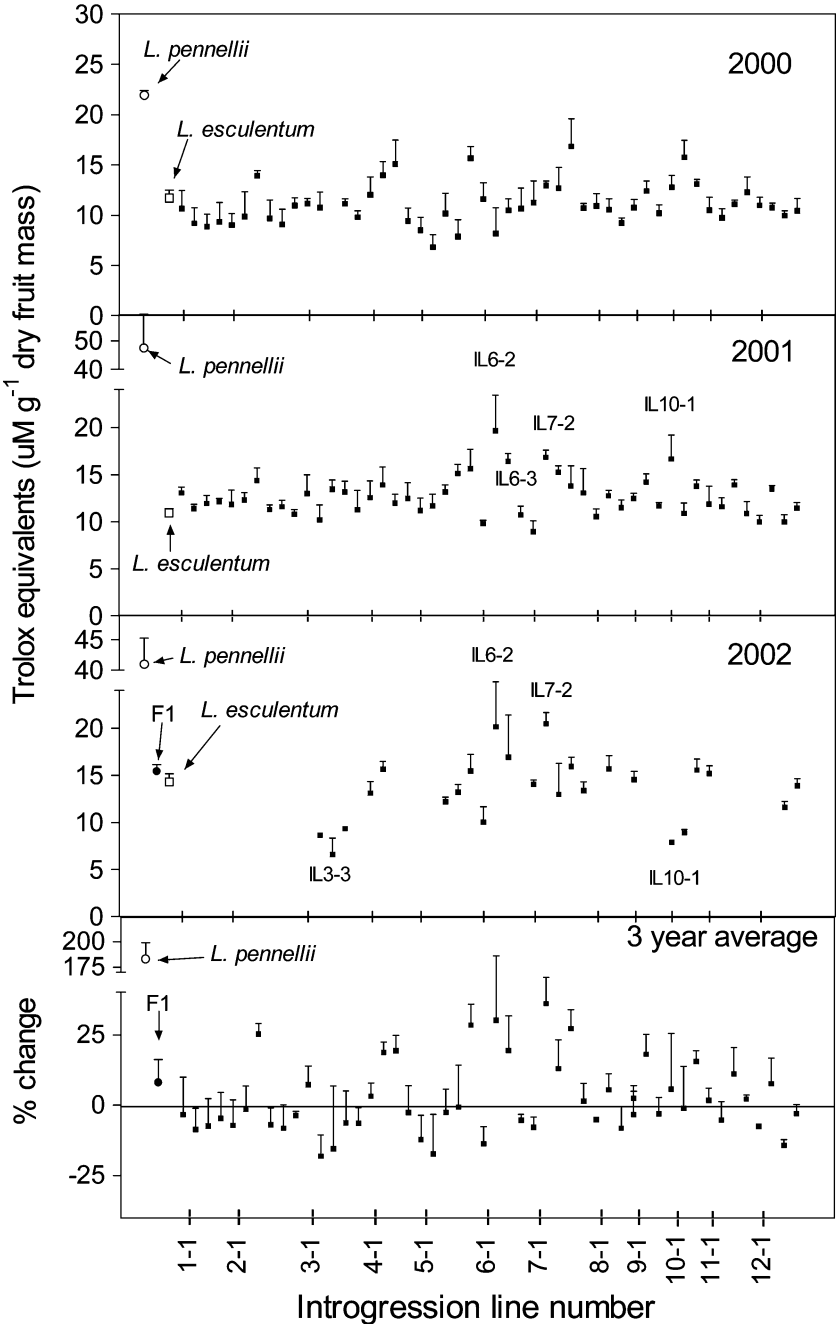

Fig. 1 Total water-soluble antioxidant capacity in mature tomatoes from *L. pennellii* ILs. The TACW is expressed in trolox equivalents (μM g^{-1} of dry fruit mass) using the TRAP assay (total radical-trapping potential). The mean ± SE ($n = 3$) for each IL and year are presented. In the bottom panel, the 3-year mean percentage change relative to *L. esculentum* for each IL is presented. The ILs that was different from *L. esculentum* ($P < 0.05$, Dunnett's test) is labeled. The first introgression for each chromosome is indicated on the axis (i.e. 1-1). Missing points indicate that the introgression was not included in the trial

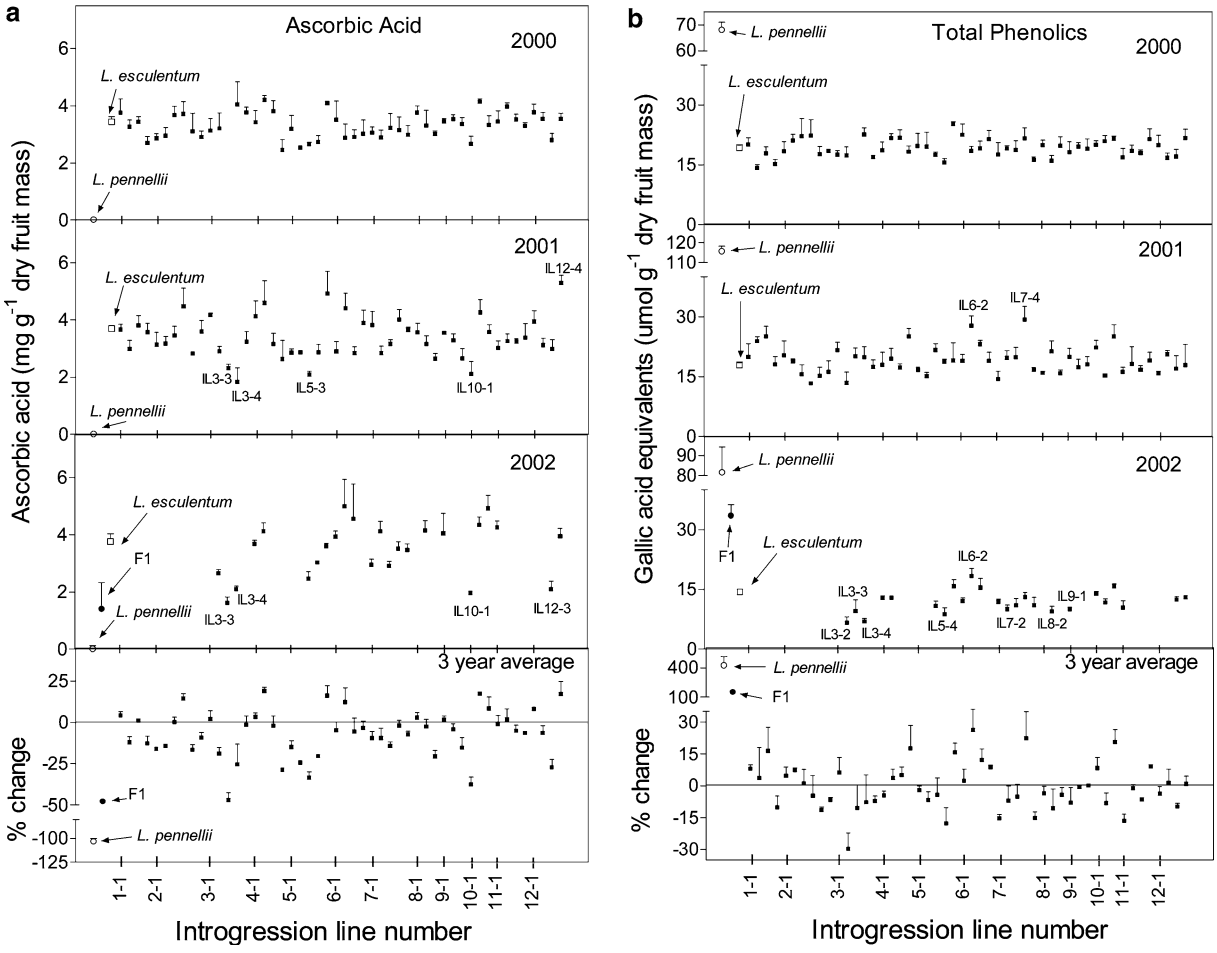

Fig. 2 Ascorbic acid (**a**) and phenolic (**b**) contents of mature tomatoes from *L. pennellii* ILs. Ascorbic acid concentration was measured using the ascorbate oxidase enzymatic method and total phenolics using Folin-Ciocalteu method and the data are expressed in gallic acid equivalents (μmol g^{-1} of dry fruit mass). The mean ± SE ($n = 3$) for each IL and year are presented. In the bottom panel, the 3-year mean percentage change relative to *L. esculentum* for each IL is presented. The ILs that was different from *L. esculentum* ($P < 0.05$, Dunnett's test is labeled). The first introgression for each chromosome is indicated on the axis (i.e. 1-1). Missing points indicate that the introgression was not included in the trial

QTL (*ao6-2*, *ao7-2*, and *ao10-1*) were observed during two out of three seasons, 2001 and 2002, while the rest were detected in only one season (Fig. 1, *ao3-3* and *ao6-3*). The TACW of *ao3-3* in 2002 was 54% lower than M-82, while the TACW of *ao6-3* in 2001 was 50% higher than in the M-82 control. The QTL *ao6-2* and *ao7-2* were 80% and 54% higher than M-82 in 2001 and 47% and 43% higher than M-82 in 2002, respectively. On the other hand, *ao10-1* was 55% higher in 2001 but 45% lower than *L. esculentum* M-82 in 2002.

Ascorbic acid content

The average ascorbic acid concentration was about 3.6 mg g^{-1} dry mass in ripe fruit from *L. esculentum*. The concentration of ascorbic acid in *L. pennellii* fruit was low or undetectable, and was intermediate (1.4 mg g^{-1} dry fruit mass) in the F_1 hybrid ($P < 0.01$, Fig. 2a). We identified six QTL in 3 years of field experiments (Fig. 2a) that affect ascorbic acid concentration in the IL as compared to the control. Three of these QTL (*aa3-3, aa3-4*, and *aa10-1*) were observed during 2001 and 2002, while the other QTL (*aa5-3, aa12-3*, and *aa12-4*) were only detected in one season. The ascorbic acid concentration in the fruit of the only high ascorbic QTL (*aa12-4*) was 44% higher than the *L. esculentum* M-82 control in 2001. The other QTL were always lower than *L. esculentum* M-82. The *aa3-3* was 37% and 56% lower than *L. esculentum* M-82, *aa3-4* was 51% and 44% lower, and *aa10-1* was 42% and 47% lower than *L. esculentum* M-82 for 2001 and 2002, respectively. The *aa5-3* was 44% lower than *L. esculentum* M-82 in 2001 and *aa12-3* was 44% lower than *L. esculentum* M-82 in 2002.

The concentration of dehydroascorbic acid (oxidized ascorbic acid) did not exceed 10% of the total ascorbate concentration when the samples were measured by HPLC and its content was similar between lines (data not shown). For this reason, we only used

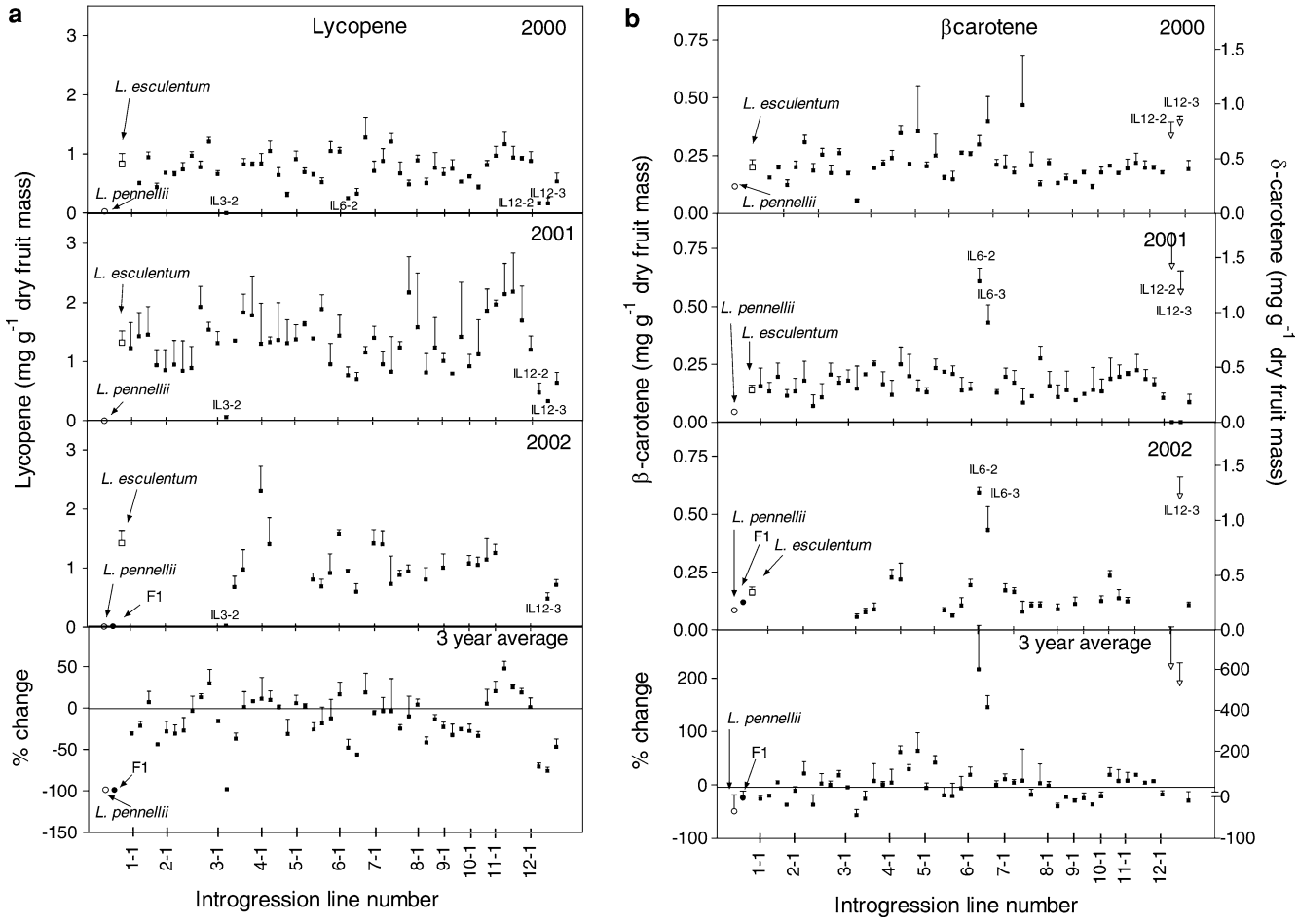

Fig. 3 Lycopene (**a**) and β-carotene (**b**) concentrations present in mature tomatoes from selected ILs. The lycopene and the β-carotene concentrations are expressed in mg g^{-1} of fruit DW. The mean ± SE ($n = 3$) for each IL and year are presented. In the bottom panel, the 3-year mean percentage change relative to *L. esculentum* for each IL is presented. The ILs that was different from *L. esculentum* ($P < 0.05$, Dunnett's test) is labeled. The first introgression for each chromosome is indicated on the axis (i.e. 1-1). Missing points indicate that the introgression was not included in the trial. The levels of δ-carotene corresponding to the *right axis* are shown for IL12-2 and 12-3, which are represented by an *open triangle*

the data of the amount of ascorbic acid measured by the enzymatic method to compare IL. In the *L. pennellii* fruits the amount of dehydroascorbate measured changed dramatically depending on the homogenization method. Samples that were treated similarly to the rest of the ILs (blended at 4°C and than frozen) had undetectable levels of ascorbic acid. In these samples the ascorbate was only present in the dehydroascorbic acid form. On the other hand, samples that were ground with liquid nitrogen and preserved frozen until measurement, had dehydroascorbate content that did not exceed 10% of the total ascorbate and the content of ascorbic acid was about half that found in *L. esculentum* fruits (1.9 mg g^{-1} dry fruit mass). Similarly, when we ground F_1 fruit with liquid nitrogen, the content of ascorbic acid was 3.8 mg g^{-1} dry fruit mass, as was the case for *L. esculentum*. In contrast there was no change in the content of dehydroascorbate in the *L. esculentum* fruit when the fresh fruit was ground with liquid nitrogen.

Phenolic content

The total phenolic concentration in fruit from *L. pennellii* (85 μmol gallic acid g^{-1} dry fruit mass) was more than 4× higher than it was in fruit of *L. esculentum* cv M-82 (18 μmol gallic acid g^{-1} dry fruit mass) ($P < 0.01$, Fig. 2b). As observed for the concentration of ascorbic acid, the total phenolic concentration in fruit of the interspecific F_1 was intermediate between the two parental lines (34 μmol gallic acid per gram dry fruit mass). We detected nine QTL controlling the accumulation of total phenolics, but only one of them, *phe6-2*, was observed during two field seasons with 60% and 27% higher content than *L. esculentum* M-82 for 2001 and 2002, respectively. The other QTL (*phe3-2, phe3-3, phe3-4, phe5-4, phe7-2, phe7-4, phe8-2*, and *phe9-1*) were only observed in one field season. *Phe7-4* had a total phenolic concentration 66% higher than *L. esculentum* M-82 in 2001. Surprisingly, most of the QTL were negative (i.e. the *L. pennellii* alleles were associated with

lower phenolic concentrations) and were only detected during the 2002 field season. These QTL had phenolic concentrations 30–50% lower than the *L. esculentum* M-82 control in 2002 (Fig. 2b).

Lycopene and β-carotene

Lycopersicon pennellii fruits had undetectable levels of lycopene as expected of this green-fruited species. Similarly fruits of the F_1 hybrid, which ripens to a greenish-yellow color, did not contain detectable lycopene ($P < 0.01$, Fig. 3a). The QTL *ly3-2*, which includes the segment containing the *r* gene (phytoene synthase) from *L. pennellii* and the *ly12-2* and *ly12-3*, which includes the *Del* (lycopene-ϵ-cyclase), had undetectable or very low levels of lycopene. During the 2000 season, another QTL associated with reduced lycopene concentration in the fruits was detected. The QTL *ly6-2* had 50% lower concentrations of lycopene than *L. esculentum* M-82.

The concentration of β-carotene was lower in *L. pennellii* than in *L. esculentum* fruit in all three seasons and intermediate in the F_1 ($P < 0.05$, Fig. 3b). We detected two QTL with higher (*bc6-2* and *bc6-3*) β-carotene concentrations than *L. esculentum*. The QTL *bc6-2* and *bc6-3*, which correspond to the *B* gene were observed for at least two seasons. We also observed that the *bc12-2* and *bc12-3* had high levels of δ-carotene.

Field versus greenhouse trials

To study the stability of the detected QTL in different environments, we compared results from a greenhouse trial (2002) with those of the field trial (2002). In the greenhouse none of the ILs differed from the *L. esculentum* control with respect to TACW or the ascorbic acid concentration (data not shown). Only the fruit of IL3-2 and IL12-4 differed from the M-82 control, and these lines had lower concentrations of phenolics. In general, the variability between ILs was much lower in the greenhouse than in the field.

Fine mapping with sublines

Sublines containing smaller *L. pennellii* introgressions than the original ILs was used to provide higher resolution for detected QTL. The high TACW QTL *ao7-2* ($P < 0.05$) could be localized to bin 7-G of chromosome 7 between markers TG199 and CD48 because IL7-1 and IL7-3 had TACW different than IL7-2 ($P < 0.05$; Fig. 4a). Also on chromosome 7, a QTL associated with high phenolic concentrations, *phe7-4* ($P < 0.05$), was not shared by other overlapping introgressions (IL7-2, IL7-3, IL7-5, IL 7-5-5, and $P < 0.05$) and only marginally shared by the subline IL7-4-1 ($P < 0.10$), which localized the QTL to bin 7-D, between markers CT158 and CD54 or to bin 7A, around marker CT52. In chromosome 6, the high phenolic *phe6-2* and high TACW *a06-2* QTL was restricted to the bin 6-E between markers CT146 and TG279, in the overlapping section between IL6-2 and IL6-3 (data not shown).

On chromosome 10 we detected a QTL for low ascorbic acid concentration. The QTL (*aa10-1*) was localized to bin 10-B between the markers TG540 and TG280 as the overlapping ILs 10-1-1 and 10-2 had values marginally higher (IL10-1-1 and $P < 0.10$) or higher (IL10-2 and $P < 0.05$) than that observed in IL10-1 (Fig. 4b). Finally, the high ascorbic acid QTL *aa12-4* was not present in the adjacent ILs thereby delimiting it to bin 12-H, the region around marker CT276.

Effect of indeterminate habit

An independent trial was performed to determine whether the QTL associated with high TACW and high phenolic concentration observed in IL6-2 was related to its indeterminate growth habit conferred by the *L. pennellii* allele of the *sp* gene (*sp*+). In this trial, we tested the same fruit traits in *sp*/*sp*+ isolines in two different genetic backgrounds (VFNT Cherry and Gardener). We found that VFNT Cherry had higher concentrations of ascorbic acid and phenolics than the Gardener fresh market variety but there were no differences associated with *sp* genotype (Table 1). In the VFNT Cherry background, the TACW was higher in the determinate than in the indeterminate plants. These results suggest that the QTL *ao6-2* and *phe6-2* are not related to the indeterminate growth habit of the IL6-2 line.

The percentage of total solids was higher in the indeterminate plants than in the determinate plants in both genetic backgrounds (Table 1). As expected, the average fruit mass was higher for Gardener than for VFNT Cherry and in the former genetic background, fruits from *sp*+ plants were heavier than those of *sp*. For VFNT Cherry, yield was higher on the determinate plants (Table 1). It should be noted however that yield was measured at a single point in time which tends to bias yield estimates in favor of the determinate line.

Correlation between antioxidant capacity and phenolics or ascorbate

Variation in phenolic concentration explained 22–29% of the variability in TACW (Fig. 5). Surprisingly, the percentage of variability in TACW explained by the ascorbic acid concentration changed dramatically between years. In 2001 less than 1% while in 2002, 51% of the variability in TACW was explained by ascorbic acid (Fig. 5). As expected there was no relation between the concentrations of lipid-soluble lycopene or β-carotene and the TACW measured on the water-soluble fraction (data not shown).

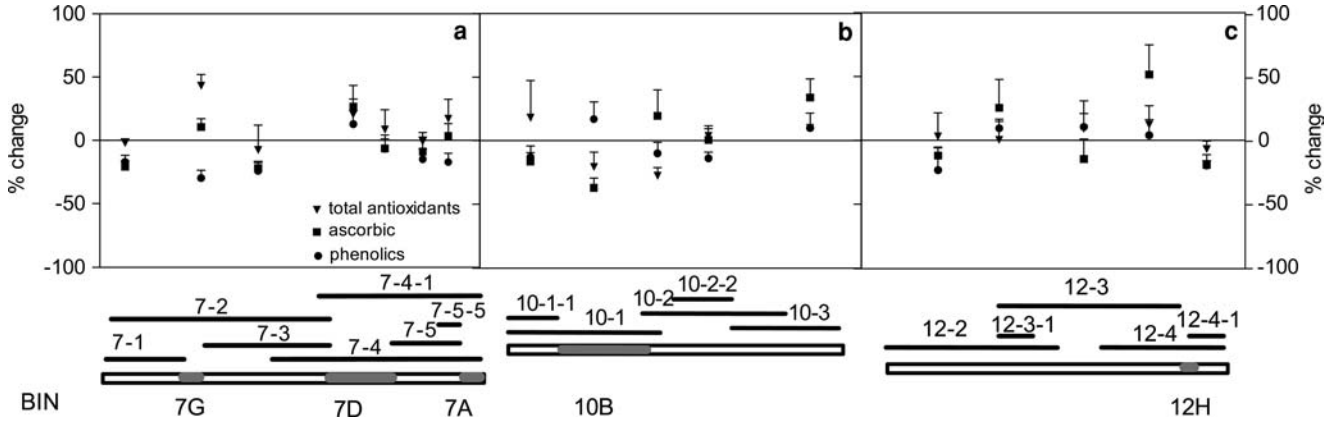

Fig. 4 Water-soluble antioxidants in mature tomatoes from the ILs and sublines in chromosomes 7 (**a**), 10 (**b**), and 12 (**c**). The TACW (*inverted filled triangle*), ascorbic acid (*filled square*) and total phenolics (*filled circle*) concentrations are expressed in terms of the percent change with respect to *L. esculentum* control for the corresponding year and block. *Values* represent the mean ± SE ($n = 6$) for data from the 2001 subline trial and the 2002 field trial. The open rectangles under the *graph* represent the complete chromosome and the *shaded areas* represent the fine mapped QTL. The *solid rectangles* over the *chromosome* represent different IL or sublines. The corresponding label is over each represented section

Table 1 Comparison of the ascorbic acid concentration, total phenolics, and TACW in tomato samples from two different genetic backgrounds (VFNT Cherry tomato and the fresh market Gardener) and the two allelic forms for the indeterminate character

Genetic background	Growth Habit (*sp*)	Ascorbic acid (mg g^{-1} dry weight)	Total phenolics (μmol gallic acid g^{-1} dry weight)	TACW (μM trolox g^{-1} dry weight)	Percentage of solids	Fruit mass (g)	Yield (kg per plant)
Cherry	*sp*	$4.91 \pm 0.26^{*}$	$19.8 \pm 1.51^{*}$	$21.5 \pm 2.22^{*}$	$4.11 \pm 0.04^{*}$	$13.3 \pm 0.26^{*}$	$25.7 \pm 1.67^{*}$
	+	$5.41 \pm 0.12^{*}$	$16.3 \pm 0.35^{*}$	$15.2 \pm 1.68^{**}$	$6.10 \pm 0.13^{**}$	$13.2 \pm 0.40^{*}$	$15.2 \pm 0.50^{**}$
Gardener	*sp*	$3.27 \pm 0.12^{**}$	$13.9 \pm 2.52^{**}$	$15.6 \pm 1.36^{**}$	$4.33 \pm 0.01^{*}$	$65.4 \pm 5.22^{**}$	$17.25 \pm 1.65^{**}$
	+	$3.19 \pm 0.18^{**}$	$11.3 \pm 0.58^{**}$	$13.6 \pm 1.38^{**}$	$5.59 \pm 0.17^{**}$	$102.8 \pm 4.33^{***}$	$17.16 \pm 1.91^{**}$

The mean ± SE ($n = 3$) is presented
*,**,***Statistical significant difference ($P < 0.05$) between genetic backgrounds and allele forms

Discussion

Carotenoids

Quantitative trait loci associated with carotenoids and tomato fruit color using introgression populations of *L. pennellii* (e.g. Ronen et al. 1999; Liu et al. 2003), *L. peruvianum* (= *S. peruvianum*) (Fulton et al. 1997) and *L. hirsutum* (= *S. habrochaites*) have been previously described (Bernacchi et al. 1998). QTL found in this paper for lycopene and β-carotene concentrations agree with known carotenoid mutations present in tomato. Bin 3-C has previously been described as harboring a single gene mutation *r yellow-flesh* (Fray and Grierson 1993). The recessive allele of this gene results in the loss of function of the phytoene synthase gene which catalyzes the conversion of geranylgeranyl diphosphate to phytoene and as a result the fruits do not accumulate lycopene. Bin 6-E is also a known mutation of the carotenoid biosynthetic pathway. The high β-carotene concentration measured in *bc6-2* and *bc6-3* is related to the presence of the wild species allele of the lycopene-β-cyclase that results in accumulation of β-carotene (Ronen et al. 2000). Lastly, the *Delta* (*Del*) locus present in bin 12-E (see following discussion) results in the accumulation of δ-carotene due to the presence of the *L. pennellii* allele of the lycopene-ϵ-cyclase (Ronen et al. 1999).

Two other lycopene QTL (*ly4.1* and *ly11.1*) were reported by Saliba-Colombani et al. (2001) for a recombinant inbred line population derived from a cross with *L. esculentum* var. cerasiforme. We did not find a significant difference in the lycopene content (mg g^{-1}dry fruit mass) between IL11.1 and *L. esculentum* M-82 and only a marginal difference between IL4.1 and *L. esculentum* M-82 in 2002. Also, we did not find a significant difference for the lycopene content of IL4-4 and M-82. The chromosomal segment 4-H has been reported to have an impact on internal fruit color (Monforte et al. 2001; Liu et al. 2003) and lycopene content. The differences at these loci in our study compared to previous reports could be related to the fact that in our study we express the data as the dry mass of the fruit. This could be the case for IL4-4 that harbors a locus (allele) that results in severe cuticular cracks that appear prior to ripening (Eshed and Zamir 1995). Due to these cracks, fruits of this specific IL tend to lose a substantial amount of

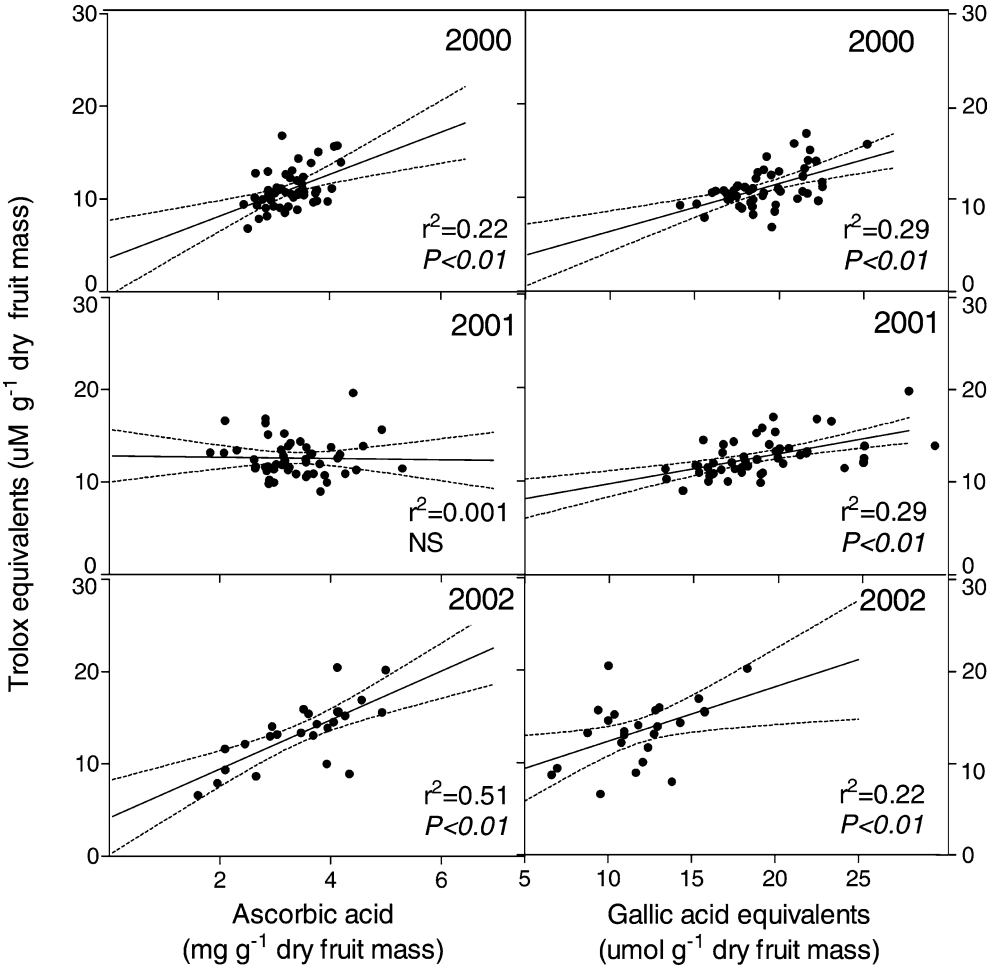

Fig. 5 Regression between ascorbic acid or total phenolic concentration and the TACW for each of the 3 years. *Data* represent the mean for each tested line (and subline during 2002 trial). The *solid lines* represent the linear regression and the *dashed line* the 95% confident intervals

moisture, resulting in higher metabolite concentrations making the measured lycopene content on the fresh mass basis high. When we compared the lycopene content on fresh basis, IL4-4 was 45% higher than M-82 in 2001 ($P < 0.05$; data not shown) compared to the 1% lower than M-82 on dry weight basis for the same year (Fig. 3).

Delta locus maps to IL 12-2 and 12-3

The high δ-carotene QTL that we mapped between markers CT79 and TG111 (Bins 12-D and 12-E) differed in location from that proposed by Ronen et al. (1999) (Bin 12-C). Ronen et al. restrict delta to IL12-2 and exclude it from 12-3 based on the mapped location in relation to CT79. One possible explanation for this discrepancy is a mislabeling of the IL12-3. Another possibility is a reversal in the order between delta and CT79 in the backcross F_2; map by Ronen et al. (1999). If this were the case, delta should lie close to CT79 on IL12-3 and also be present in the IL12-3-1 subline. IL12-3-1 does not contain high levels of δ-carotene. Consequently our data would suggest Bin 12-E as the location of delta. Additional evidence that both IL12-2 and 12-3 contain delta and that IL12-3-1 lacks this locus comes from carotenoid values similar to ours reported by other researchers (http://zamir.sgn.cornell.edu/Qtl/Html/home.htm). Delta codes for lycopene ϵ-cylase, which produces δ-carotene at the expense of lycopene and β-carotene. Due to the lack of a β-ring, δ-carotene has no vitamin A activity (Olson 1989).

Transgressive segregation observed for QTL from *Lycopersicon pennellii*

In contrast to the relatively abundant information on QTL for carotenoids in tomato fruits, there is no previous work that focuses on QTL for water-soluble antioxidants, including ascorbic acid and phenolics. Tomatoes are an important source of water-soluble antioxidants, particularly AsA. Due to the high consumption levels of tomatoes (average US consumption is ~90 lbs per person per year) and the substantial ascorbic acid content (approximately 4 mg/100 g FW), tomatoes represent a major contribution to dietary nutrition worldwide (Willcox et al. 2003). In spite of the fact that *L. pennellii* fruit has very low levels of ascorbate, we detected a high ascorbate QTL (*aa12-4*) that could be a valuable tool to improve tomato nutritional value. In addition two regions IL10-2 and IL4-2 had ascorbate levels approximately 20% higher than M-82 in all years and these regions have also been

found to have high ascorbic acid at least in one of two seasons (http://tomet.bti.cornell.edu/). Although the levels for these two regions were not statistically significant, the stability of the phenotype indicates the likely presence of valuable loci.

There are many examples of transgressive segregation in tomato populations derived from interspecific crosses. For example, for traits associated with fruit color, eight QTL were identified that improved tomato color in a population derived from the green-fruited *L. hirsutum* (Bernacchi et al. 1998). Three QTL that improved fruit color were also detected in an *L. peruvianum* Miller (L.) (= *S. peruvianum* L.) cross (Fulton et al. 1997). Rick (1974) observed transgression for soluble solids from *L. chmielewskii* (= *S. chmielewskii*). Devicente and Tanksley (1993) observed transgressive segregation for 36% of the QTL observed in a *L. pennellii* × *L. esculentum* F_2 population. Moreover, in this paper we detected one QTL (*aa12-4*) that improved ascorbic acid content although the parental line has very low levels of ascorbic acid in the fruit.

Low ascorbate in *Lycopersicon pennellii*

The low ascorbate in *L. pennellii* fruit could be the result of reduced biosynthesis or differences in antioxidant metabolism. *Lycopersicon pennellii* has been shown to have higher constitutive activities of superoxide dismutase, ascorbate peroxidase, and dehydroascorbate reductase in leaves (Shalata and Tal 1998). Ascorbate peroxidase consumes ascorbate in the process of reducing H_2O_2. If a similar enzymatic activity is present in *L. pennellii* fruit, our measurements may be affected by rapid utilization of ascorbate. This possibility is partially supported by the fact that when we reduced the interaction with oxygen by grinding the fruits with liquid nitrogen instead of blending them, the ascorbate content measured increased from 0 mg g^{-1} to 1.9 mg g^{-1} dry weight for *L. pennellii* and from 1.4 mg g^{-1} to 3.8 mg g^{-1} in the F_1, bringing the levels in the F_1 similar to those observed in *L. esculentum*. Hence, something in the *L. pennellii* antioxidant metabolism consumes ascorbic acid during the homogenization process. The differences in the metabolism did not affect the pH, as the pH of blended and frozen mature *L. pennellii* (pH = 4.26) was similar to the pH of blended and frozen mature *L. esculentum* and higher than that of fruit ground in liquid nitrogen (pH = 3.87). These findings suggest that ascorbate levels in the fruit are regulated by mechanisms, which appear to be distinct between *L. pennellii* and *L. esculentum*.

Lycopersicon pennellii introgression lines as a tool to understand antioxidant regulation

The QTL identified in this study are a valuable tool for understanding the regulation and biosynthetic pathways of ascorbic acid and phenolics. For example, *L. pennellii* fruits have total phenolic levels about four times higher than *L. esculentum* fruits, while phenolic composition is similar and primarily composed of flavonols (data not shown). It has been recently shown that overexpressing chalcone isomerase from *Petunia hybrida* in tomato dramatically increased flavonol content (Muir et al. 2001). The authors suggested that a single gene encoding chalcone isomerase could represent the sole rate-limiting step in the flavonol synthesis in tomato. However, we found that no single IL could reproduce the high concentration of flavonols seen in *L. pennellii* fruits (Fig. 2), suggesting that the regulation of flavonoid biosynthesis is more complex than previously proposed. This argument is also supported by the observation that the levels of phenolics in the F_1 were intermediate between the two parental lines.

Studies of fruit size in tomato provide evidence supporting the concept that loci underlying complex traits may be uncovered using a single introgression approach. For example, a similar QTL analysis found at least 28 QTL associated with the small-size fruit of wild *Lycopersicon* sp. However, a further analysis of this complex regulation showed that one of these QTL, *fw2.2*, appears to be controlled by a single locus, which increases fruit mass up to 30% (Alpert and Tanksley 1996).

Environment: a strong factor in antioxidant regulation

The traits studied appear to be under strong environmental control. Most QTL were only observed during one out of the three seasons. This was more obvious for the water-soluble antioxidants (only 35% of the QTL were observed over more than one season) than for the water insoluble antioxidants (80% of the QTL were observed more than one season). Moreover, when the ILs were tested in a greenhouse trial, most QTL were not observed. Because of their roles for protecting against oxidative stress, many environmental factors (e.g. water stress, temperature, and light) play a key role in inducing antioxidants. Consequently the value of QTL analysis conducted in greenhouse or growth chamber conditions may be limited as phenotypes in these studies may differ from those observed in field grown conditions. Other researchers have demonstrated the induction of key enzymes in the flavonoid biosynthetic pathway (i.e. phenylalanine ammonium lyases and chalcone synthase) by light, particularly by ultraviolet radiation (Beggs and Wellmann 1985). Additionally, the L-galactose dehydrogenase, which oxidized the L-gal to L-galactono-1,4-lactone, is induced by photosynthetically active radiation (Gatzek et al. 2002). While many QTL observed in our field studies were not significant in every season, the direction of the effect was highly consistent. In combination with the quantitative nature of the antioxidant phenotype these results imply that the genetic effect of single introgressions may not be as strong as season-to-season

environmental differences. Some of the variation observed could be due to plant-to-plant variation within small number of plants (6) per accession analyzed in year 1. In years 2 and 3, 25 plants for each rep were utilized, but plant-to-plant variation cannot be assessed since bulked samples were employed.

Phenolic contribution to antioxidant capacity in tomato

It has been proposed that phenolics and ascorbic acid are the major contributors to the water-soluble total antioxidant capacity in fruits and vegetables (e.g. Wang et al. 1996; Kahkonen et al. 1999; Ou et al. 2002). For example, in tomato Minoggio et al. (2003) found that the total contents of phenolics accounted for 80% of the variability in total antioxidant capacity. On the other hand, Martínez-Valverde et al. (2002) found a poor relation between antioxidant capacity and the content of quercetin and chlorogenic acid (two of the most important contributors to the total phenolics in tomato). In our 3-year study, total phenolics never explained more than 29% of the variability in the TACW. Also, ascorbic acid content represented a relatively small contribution to the TACW, explaining on average less than 25% of its variability. Similarly, Lavelli et al. (2000) found high antioxidant activity in tomato samples that had low ascorbic acid concentration. A question that arises from these results is which other compounds are present in tomato fruits that confer antioxidant capacity in the water-soluble fraction.

Our results demonstrate the feasibility of improving tomato fruit quality through an increase in their antioxidant content. We have identified QTL on chromosomes 6, 7, 10, and 12 that potentially improve TACW, vitamin C, and phenolics concentrations. *Lycopersicon pennellii* introgressions IL6-2 and IL 7-2 could be used to improve the TACW in tomato fruit and IL6-2 could as well as be used to improve its total phenolic content. On the other hand, only IL12-4 could be used to improve tomato fruit ascorbic acid content but its effect show a strong environmental dependency. Also, novel QTL associated with the improvement of the nutritional value of fruits can be found in wild-species even when positive effects would not be expected from the phenotype of the parental (e.g. positive ascorbic acid QTL from *L. pennellii* in this study). However, QTL for traits that display an important environmental control may not be expected to be apparent under all environments.

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