Theor Appl Genet (2013) 126:2163–2175
DOI 10.1007/s00122-013-2126-5

ORIGINAL PAPER

Validation and fine mapping of *lyc12.1*, a QTL for increased tomato fruit lycopene content

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Received: 1 January 2013 / Accepted: 8 May 2013 / Published online: 24 May 2013

Abstract Lycopene content is a key component of tomato (*Solanum lycopersicum* L.) fruit quality, and is a focus of many tomato-breeding programs. Two QTLs for increased fruit lycopene content, inherited from a high-lycopene *S. pimpinellifolium* accession, were previously detected on tomato chromosomes 7 and 12 using a *S. lycopersicum* × *S. pimpinellifolium* RIL population, and were identified as potential targets for marker-assisted selection and positional cloning. To validate the phenotypic effect of these two QTLs, a BC_2 population was developed from a cross between a select RIL and the *S. lycopersicum* recurrent parent. The BC_2 population was field-grown and evaluated for fruit lycopene content using HPLC. Statistical analyses revealed that while *lyc7.1* did not significantly increase lycopene content in the heterozygous condition, individuals harboring *lyc12.1* in the heterozygous condition contained 70.3 % higher lycopene than the recurrent parent. To eliminate the potential pleiotropic effect of fruit size and minimize the physical size of the *lyc12.1* introgression, a marker-assisted backcross program was undertaken and produced a BC_3S_1 NIL population ($n = 1{,}500$) segregating for *lyc12.1*. Lycopene contents from *lyc12.1* homozygous and heterozygous recombinants in this population were measured and *lyc12.1* was localized to a 1.5 cM region. Furthermore, we determined that *lyc12.1* was delimited to a ~1.5 Mb sequence of tomato chromosome 12, and provided some insight into potential candidate genes in the region. The derived sub-NILs will be useful for transferring of *lyc12.1* to other tomato genetic backgrounds and for further fine-mapping and cloning of the QTL.

Communicated by R. Visser.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-013-2126-5) contains supplementary material, which is available to authorized users.

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Introduction

A key goal of breeding for superior tomato (*Solanum lycopersicum* L.) fruit quality is to manipulate fruit color, which is directly related to the amount of carotenoids (primarily lycopene and β-carotene) that accumulate in ripe tomato fruit. Human plasma lycopene concentration has been shown to be inversely related with the incidence of certain types of cancer, and β-carotene, a precursor to vitamin A, is an essential part of the human diet (Rao and Rao 2007; Wu et al. 2004). Further, Stommel et al. (2005) demonstrate that deeper red color of fresh market tomatoes is positively associated with consumer preference. As lycopene is the overwhelmingly dominant carotenoid in ripe tomato fruit, one focus of genetic studies has been to identify genes or quantitative trait loci (QTLs) that control the accumulation of this compound. Thus, many researchers have focused on identifying and studying genes

involved in the biosynthesis of lycopene (and β-carotene) by conducting quantitative genetics studies using interspecific tomato populations segregating for fruit color (reviewed in Foolad 2007; Kinkade and Foolad 2013). These studies have uncovered deleterious alleles of carotenoid biosynthetic genes, as well as various alleles of light signaling genes, which modulate the intensity of carotenoid accumulation (Galpaz et al. 2008; Liu et al. 2003, 2004; Ronen et al. 1999, 2000; reviewed in Fraser and Bramley 2004). Unfortunately, while these mutants have greatly increased our understanding of carotenoid biosynthesis in tomato, their practical utility for tomato breeding is very limited (Sacks and Francis 2001). Simultaneously, studies have detected QTLs responsible for more transient portions of the phenotypic variance for fruit color in interspecific populations, rather than completely abolishing carotenoid biosynthesis or grossly altering photomorphogenesis. Several such studies have utilized red-fruited wild tomato species (Causse et al. 2002; Chen et al. 1999; Doganlar et al. 2002; Grandillo and Tanksley 1996; Saliba-Colombani et al. 2001; Tanksley et al. 1996), while others have used green-fruited wild tomato species (Bernacchi et al. 1998; Causse et al. 2004; Frary et al. 2004; Fulton et al. 1997, 2000; Liu et al. 2003; Rousseaux et al. 2005).

Despite the fact that many fruit color QTLs have been detected in tomato, few QTLs have been verified and isolated in subsequent populations, and even fewer are actively used for marker-assisted selection (MAS) in tomato-breeding programs (Foolad 2007). This discrepancy may be due to several reasons, including the low heritability associated with the detected QTLs, identification of QTLs in populations not useful for breeding purposes, population specificity of QTLs, and intellectual property restrictions. A few studies have assessed the stability of several fruit quality QTLs over the course of a marker-assisted backcross program (Chaïb et al. 2006; Lecomte et al. 2004). Although fruit color QTLs were not included in these studies, significant variation in the number and phenotypic effects of fruit quality QTLs were observed, which were reportedly originated from epistatic interactions between QTLs and the recipient background genotype or from the effect of other QTLs already present within the recipient genotype (Chaïb et al. 2006; Lecomte et al. 2004). These findings highlight the need for subsequent analyses of QTLs detected in experimental populations, as QTL number and effects may be altered in populations used for practical breeding purposes.

Previously, we reported the detection of two major QTLs, *lyc7.1* and *lyc12.1*, for increased fruit lycopene content in a *S. lycopersicum* (NCEBR-1) × *S. pimpinellifolium* (LA2093) RIL population, using colorimeter, spectrophotometer, and HPLC-based lycopene detection strategies (Ashrafi et al. 2009, 2012). Inherited from LA2093, each QTL increased fruit lycopene content by approximately 30 μg/g fresh weight based on HPLC assays conducted in multiple years, and *lyc12.1* alone explained >30 % of the phenotypic variation in visible fruit color (Ashrafi et al. 2012). The two QTLs were consistently detected in four successive RIL generations (F_7–F_{10}). These two QTLs do not correspond to the genomic positions of known carotenoid biosynthetic genes, and do not co-localize to QTL regions identified in previous QTL studies using other red-fruited wild species of tomato (Ashrafi et al. 2012; Kinkade and Foolad 2013). However, a *S. pennellii* LA716 (green-fruited) introgression line corresponding to the *lyc12.1* location results in yellowish fruit color as compared to the red color of the parental commercial cultivar M82 (Eshed and Zamir 1995; Liu et al. 2003). IL 12-4, which contains LA716 alleles in the *lyc12.1* genomic interval and *S. lycopersicum* M82 alleles at all other regions, accumulates 44.6 % of lycopene and 56.7 % of phytoene relative to the control M82 samples at the red ripe fruit stage (TFGD 2010).

To determine the practical potential of *lyc7.1* and *lyc12.1* for increasing the lycopene content of the cultivated tomato, we carried out a marker-assisted backcross program to simultaneously verify and isolate each QTL in an NCEBR-1 genetic background. NCEBR-1 is a multiple disease resistant, horticulturally superior, advanced fresh market breeding line developed by RG Gardner at the North Carolina State University (Gardner 1988). The availability of an NCEBR-1 × LA2093 RIL population (Ashrafi et al. 2009) as well as the graphical genotypes of the RI lines (Ashrafi et al. 2012) facilitated development of near-isogenic lines (NILs) and characterization of the two QTLs. Here we report fine mapping and genetic characterization of *lyc12.1*, a major QTL that significantly increases tomato fruit lycopene content. We also report co-dominant, flanking PCR-based markers that could be used in breeding programs to easily transfer this lycopene QTL to other tomato genetic backgrounds without having to return to the original wild accession or RI lines. Further, we discuss the implications of this research and suggest possible candidate genes underlying *lyc12.1* based on a preliminary genome annotation.

Materials and methods

Development and characterization of BC_2 population

Based on the *S. lycopersicum* (NCEBR-1) × *S. pimpinellifolium* (LA2093) RIL population and its genetic map reported by Ashrafi et al. (2009) and lycopene QTL analyses reported by Ashrafi et al. (2012), a superior F_{10} RI line (RIL37) was selected for marker-assisted backcrossing and

NIL development. This selection was based on the following criteria: high-fruit lycopene content as measured by HPLC (85 µg/g fresh FW in RIL37), presence of both *lyc7.1* and *lyc12.1* regions (inherited from LA2093), good line stability in lycopene content (over years and generations), and low LA2093 background genome content (~30 % in RIL37). The graphical genotype of RIL37 is displayed in Fig. 1, and the subsequent marker-assisted backcrossing scheme is described in Fig. 2. RIL37 was utilized as the pollen donor for hybridization with NCEBR-1 to produce a non-segregating BC_1 population, heterozygous at all LA2093-derived genomic intervals harbored by RIL37 and homozygous for NCEBR-1 alleles at all other loci. A single BC_1 plant was then hybridized with NCEBR-1 to produce a segregating BC_2 population. During June–October 2008, 189 random BC_2 individuals were grown to maturity under field conditions at the Pennsylvania State University Horticulture Research Farm, Russell E. Larson Agricultural Research and Education Center, Rock Springs, PA, phenotyped for fruit lycopene content using HPLC, and genotyped with molecular markers for foreground and background genomic regions. From these analyses it was determined that individuals harboring only *lyc7.1* (in heterozygous condition) were not producing significantly higher fruit lycopene content than the recurrent parent (described below in "Results"), and therefore in subsequent analyses the focus was placed on *lyc12.1*. From among the 189 BC_2 plants, 10 *lyc12.1* heterozygotes containing the least amount of LA2093 genomic background within the population were identified for further refining of the QTL.

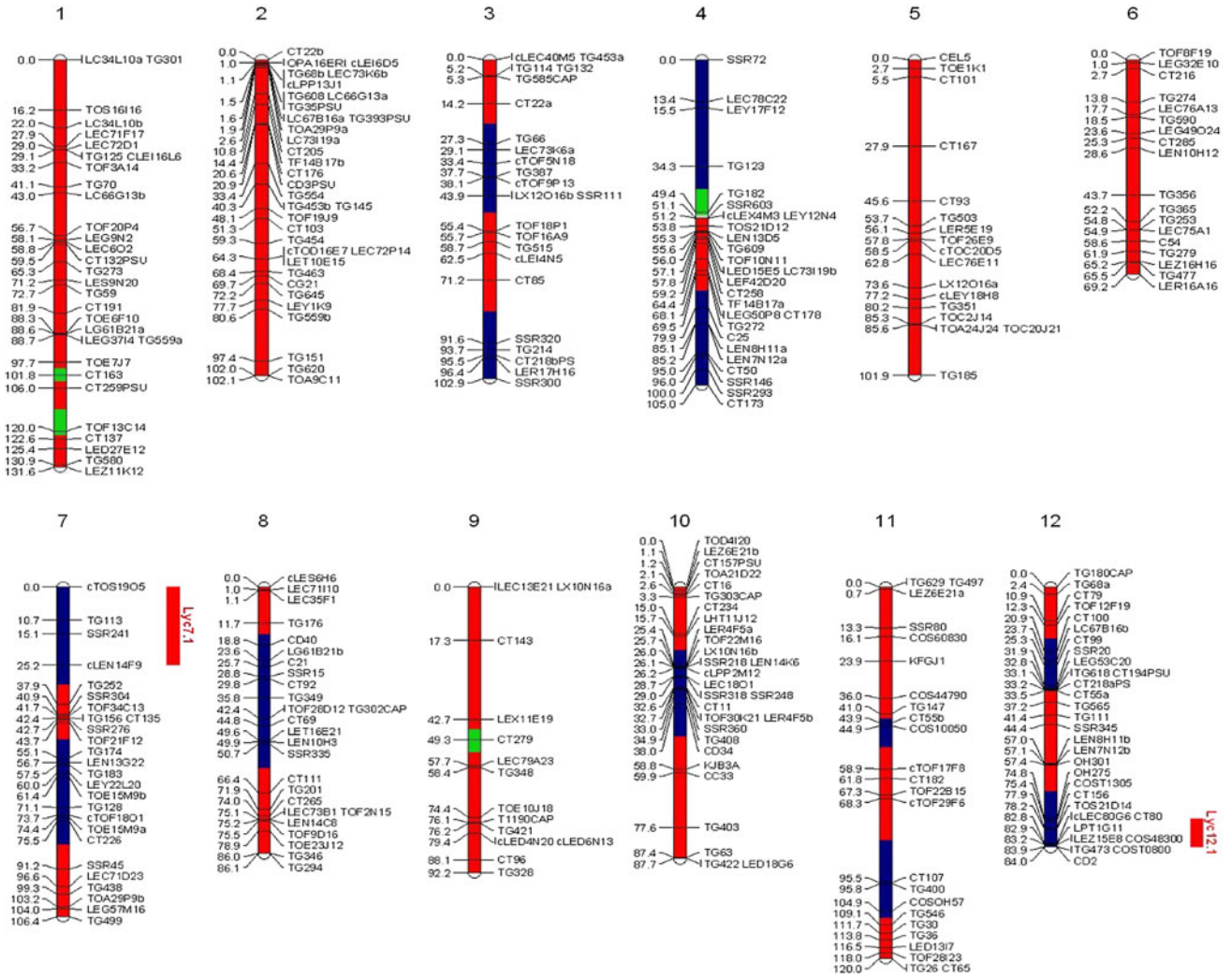


Fig. 1 Graphical genotype of recombinant inbred line #37 (RIL37) developed from crosses between *S. lycopersicum* NCEBR-1 and *S. pimpinellifolium* accession LA2093. *Blue* regions indicate genomic segments from LA2093, *red* regions indicate genomic segments from NCEBR-1 alleles, and *green* intervals are regions of heterozygosity. The locations of two lycopene QTLs (*lyc7.1* and *lyc12.1*) are denoted by *red bars* to the right of the chromosomes 7 and 12, respectively. RIL37 was used as the parental line for development of near-isogenic lines (NILs) and sub-NILs and fine mapping of *lyc12.1* via a marker-assisted backcross program using NCEBR-1 as the recurrent parent (color figure online)

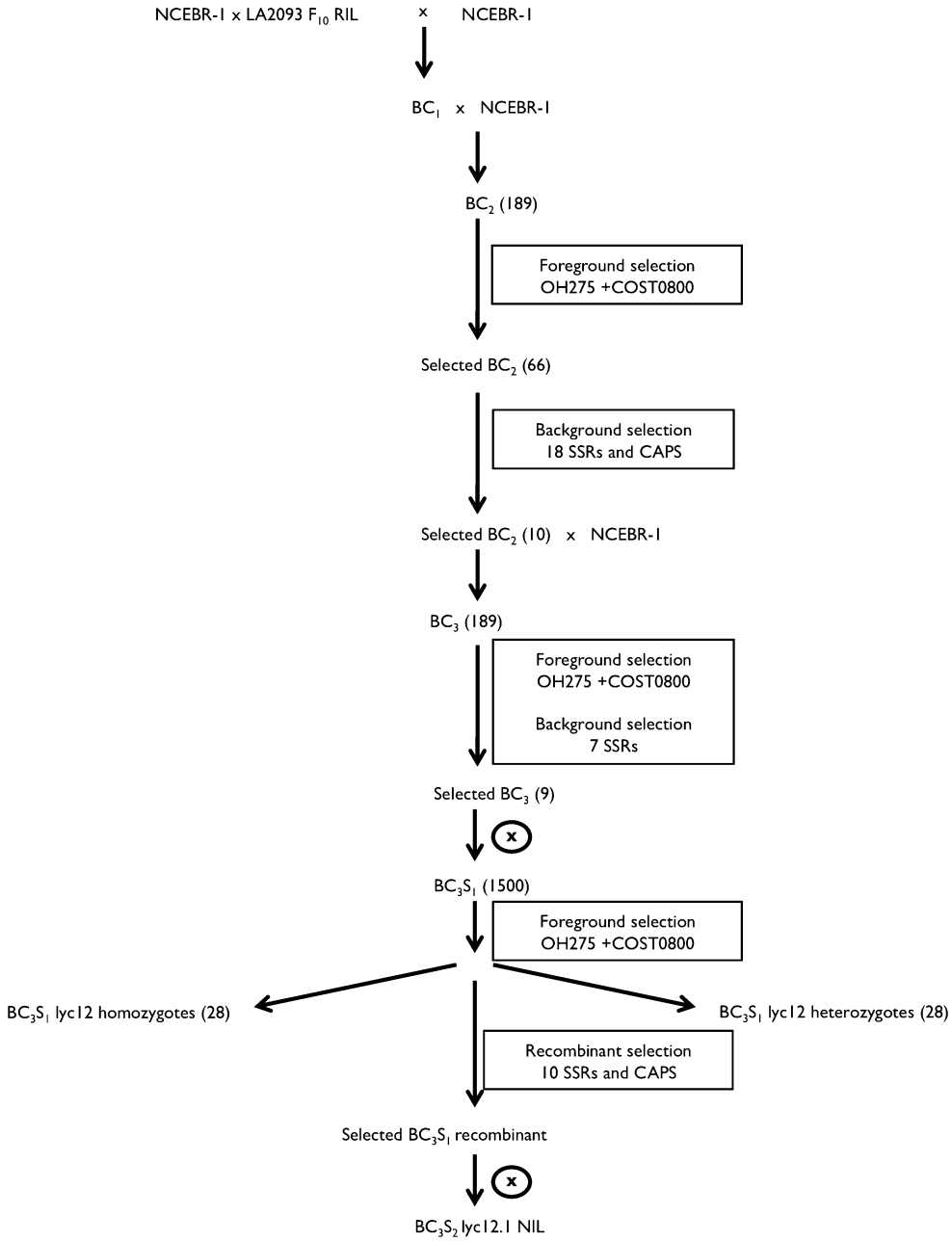


Fig. 2 Schematic representation of the marker-assisted backcross program to verify and fine map *lyc12.1*

Generation of new simple-sequence repeat (SSR) markers in the BC_2 population

To identify additional markers within the *lyc12.1* interval for fine-mapping purposes, sequenced BACs released by the Tomato Genome Sequencing Consortium (Mueller et al. 2005b) were BLASTed (Altschul et al. 1990) with sequences of markers already mapped within the *lyc12.1* region (Ashrafi et al. 2009; Mueller et al. 2005a). Four BACs with significant resultant *e* values (<0.0001) were scanned for SSR motifs. Flanking primer sequences to these motifs were generated with Primer3 (Rozen and Skaletsky 2000). Primer pairs were used to amplify NCEBR-1 and a BC_2 *lyc12.1* heterozygote with minimal LA2093 background genomic content, and resultant amplicons were analyzed for polymorphism. Two polymorphic markers from each BAC (a total of 8 markers) were identified and used to genotype the entire BC_2 population ($n = 189$). The markers were named according to the BACs from which they were derived (SSRG22.20, SSRG22.7, SSRM19.22, SSRM19.14, SSRP 12.3, SSRP12.18, SSRN06.9, SSRN06.22).

Development and characterization of BC_3S_1 population

The 10 selected BC_2 individuals harboring *lyc12.1* were propagated by vegetative cuttings, grown in a greenhouse

and hybridized with the recurrent parent (NCEBR-1) and produced 10 BC_3 progeny families (Fig. 2). From among these 10 BC_3 families, three families with the lowest number of detectable LA2093-derived background genomic intervals were selected for further analysis. Combined individuals from these three BC_3 families (total of 189 BC_3 plants) were screened with genetic markers for the presence of *lyc12.1* and the absence of any detectable background LA2093-derived intervals. This screening resulted in the identification of 9 BC_3 plants that harbored *lyc12.1* (in heterozygous condition) and did not have any detectable background LA2093-derived intervals. Suitable PCR-based markers for small segments of chromosomes 3 and 7 derived from LA2093 that may have been segregating in this population could not be mapped in these locations; however, these intervals did not contain any QTLs affecting the lycopene trait (Ashrafi et al. 2012). The 9 selected isogenic BC_3 plants (hereafter referred to as BC_3-NILs) were subsequently self-fertilized to produce a BC_3S_1 population ($n = 1{,}500$; hereafter referred to as "NIL population"), which was segregating for *lyc12.1* but homozygous for NCEBR-1 alleles at all other detectable loci.

Production, identification and analysis of *lyc12.1* recombinants

The marker-assisted backcross strategy that was executed to produce the *lyc12.1* NIL population is shown in Fig. 2. Seedlings of 1,500 NIL individuals (BC_3S_1) were produced in the greenhouse, leaf tissue was collected from each individual, and genomic DNA was harvested for marker genotyping. All individuals were genotyped with the markers flanking the *lyc12.1* region (OH275 and T0800) to identify *lyc12.1* homozygotes, *lyc12.1* heterozygotes, *lyc12.1* recombinants, and NCEBR-1 homozygotes. Recombinants within the *lyc12.1* interval were identified when the two flanking markers showed differing genotypes in an individual. All such individuals ($n = 265$) were subsequently genotyped for 10 additional markers [SSRG22.20, SSRG22.7, T1305 (CAPS), SSRM19.22, SSRM19.14, SSRP12.3, SSRP12.18, 48,300 (CAPS), SSRN06.9, SSRN06.22] within the *lyc12.1* interval. The entire NIL population ($n = 1{,}500$) was grown to maturity under field conditions during the summer of 2009 at the Pennsylvania State University Horticulture Research Farm. Seeds resulting from self-hybridization (i.e., BC_3S_2 generation) were collected from all recombinants.

Marker genotyping

In the various populations subjected to PCR-based marker analysis, genomic DNA was extracted from tomato leaflets using a modified NaOH-based quick prep from Wang et al. (2003). In the BC_2 and BC_3 generations, DNA was harvested on a single-sample basis using manual tissue disruption. For the BC_3S_1 population, DNA was harvested in 96-well plate format using a QIAGEN Tissue Lyser II for tissue disruption. Simple-sequence repeat (SSR) and cleaved amplified polymorphic sequence (CAPS) markers residing in flanking locations of each LA2093-derived background genomic interval from RIL37 and those flanking *lyc12.1* (foreground markers OH275 and T0800) were used to genotype the populations (Fig. 2). The PCR reaction consisted of 3.4 μL H_2O, 0.8 μL F primer, 0.8 μL R primer, 5 μL DNA, and 10 μL 2× GoTaq Green Master Mix (Promega). For SSR and CAPS markers, reactions were amplified according to Ashrafi et al. (2009). SSR PCR products were electrophoresed on non-denaturing 6 % polyacrylamide gels using the Mega-Gel apparatus [(Wang et al. 2003); CBS Scientific]. CAPS PCR products were subjected to restriction digestion (10 μL PCR product, 7.8 μL H_2O, 1 μL 10× enzyme buffer, 0.2 μL BSA, and 2.2 U restriction enzyme) for at least 3 h, and electrophoresed on 2 % agarose gels.

Fruit collection, carotenoid extraction, and HPLC analysis

Red ripe fruit from the BC_2 population ($n = 189$) were harvested from the field-grown plants on September 18, 2008. For the NIL population (BC_3S_1), red ripe fruit from 227 *lyc12.1* recombinants, 28 *lyc12.1* heterozygotes, 28 *lyc12.1* homozygotes, and 28 NCEBR-1 homozygotes were collected from the field-grown plants on October 6, 2009. Healthy, fully ripe, non-diseased fruits were washed and an average of 5 fruits from each individual was weighed to obtain fresh fruit weight data. Further sample processing and carotenoid extraction was performed according to Ashrafi et al. (2012), omitting the aqueous extraction step. Although each BC_2 fruit purée sample was extracted twice, each NIL fruit purée sample was extracted once. Five μL of each fruit extract was injected into a Shimadzu Prominence HPLC system with inline degasser, autosampler, column oven (30 °C), and UV/Vis dual wavelength detector. All other HPLC parameters were the same as those reported in Ashrafi et al. (2012).

Statistical analyses

Descriptive statistics and Student's *t* tests were obtained/conducted using Minitab v10 and Microsoft Excel v2008. Single marker analyses were performed using Windows QTL Cartographer v2.5 (Zeng 1994). Graphical genotypes were visualized using GGT v2.0 software (Wageningen University). The genetic map of the *lyc12.1* region was constructed using JoinMap (Kyazma; http://www.kyazma.nl/index.php/mc.JoinMap).

Results

BC_2 lycopene analysis

To verify the effects of the two lycopene QTLs (*lyc7.1* and *lyc12.1*) detected in our previous study (Ashrafi et al. 2012), the RIL37-derived BC_2 population, segregating for both QTLs and 10 background genomic intervals, was analyzed for lycopene content. A histogram displaying the population distribution is shown in Fig. 3. The BC_2 population mean was 46.6 μg lycopene/g fresh fruit weight (μg/g FW), whereas the mean lycopene content for NCEBR-1 (the recurrent parent) was 34.5 μg/g FW. The distribution failed the Anderson–Darling test for normality, and a somewhat bimodal shape was evident. The distribution of fruit lycopene content in the population coincided strikingly with the presence of *lyc12.1* in the different BC_2 individuals (Fig. 3). In this population, "*lyc12.1*" was defined as the genomic region inherited from LA2093 between markers OH275 and T0800 (approximately 10 cM; Fig. 1). In the group of individuals with lycopene content ranging from 60 to 85 μg/g FW (Fig. 3 red bars), *lyc12.1* was present in 95 % of the individuals, of which only 10.5 % were recombinant within the *lyc12.1* region. In the group of individuals with lycopene content ranging from 45 to 55 μg/g FW (Fig. 3 orange bars), 56.7 % of individuals had a *lyc12.1* allele, of which 32.3 % were recombinants. Finally, in the group of individuals with lycopene content ranging from 20 to 40 μg/g FW (Fig. 3 green bars), only 17.4 % of individuals could have had the *lyc12.1* allele, of which 72.7 % were recombinant. Clearly, BC_2 individuals with higher fruit lycopene content were enriched for *lyc12.1*, suggesting significant involvement of *lyc12.1* in fruit lycopene content.

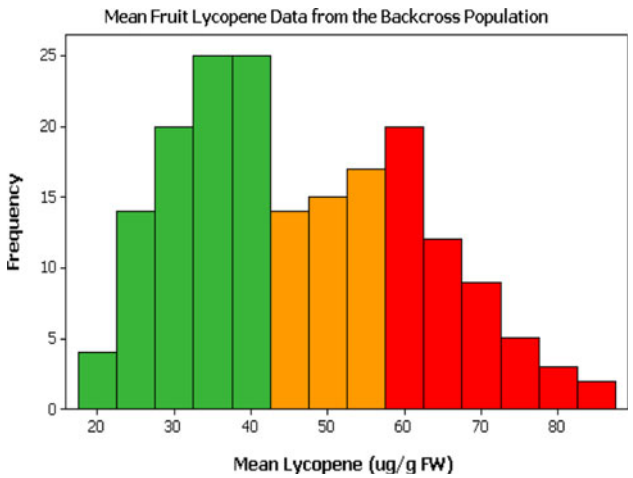


Fig. 3 Histogram distribution of the lycopene content of the BC_2 population. In the *red* region, 95 % of the individuals may harbor a *lyc12.1* allele (10.5 % of these individuals recombined within the *lyc12.1* interval). In the *orange* region, 56.7 % of the individuals may possess a *lyc12.1* allele (32.3 % of these individuals were recombinants). In the *green* region, 17.4 % of the individuals may contain *lyc12.1* (72.7 % of these were recombinants) (color figure online)

To explore whether the foreground and background intervals from LA2093 were exerting a phenotypic effect on lycopene content, a single marker analysis was performed after measuring lycopene content and genotyping the BC_2 population with foreground and background markers. Table 1 displays the results for foreground regions. *lyc12.1* markers (OH275 and T0800) were significantly ($p < 0.001$) associated with higher fruit lycopene in each of the two extraction replicates as well as in the mean dataset, exerting an average effect of 22.1 μg lycopene/g FW. In comparison, *lyc7.1* markers (SSR241 and cLEN14F9) were significantly ($p \leq 0.01$) associated with higher fruit lycopene content only in Rep II of the BC_2 population (Table 1). Further, the difference between the means of individuals with and without *lyc7.1* was only 4.1 μg lycopene/g FW, and not statistically significant. Among all the background markers examined, the only marker that exhibited association with lycopene content was SSR20 on chromosome 12, which is located outside of the *lyc12.1* QTL interval (data not shown). This association was most likely due to a loose linkage with *lyc12.1*, since no QTL was detected in this location based on the mapping analysis in the NCEBR-1 × LA2093 RIL population (Ashrafi et al. 2012). Boxplots of lycopene concentration from the various genotypic groups (subpopulations) within the BC_2 population and the results of *t* tests comparing the mean lycopene content of these subpopulations to NCEBR-1 are displayed in Fig. 4. On an average, individuals harboring *lyc12.1* had 24.3 μg/g FW more lycopene than NCEBR-1 (a 70.3 % increase). The mean lycopene concentration of any subpopulation containing individuals harboring *lyc12.1* was significantly ($p < 0.05$) greater than the NCEBR-1 level (indicated by the horizontal line). No genotypic class containing *lyc7.1* without *lyc12.1* was significantly different in fruit lycopene content from the mean of the BC_2 population. However, the "Y *lyc7*" class (i.e., individuals having *lyc7.1* QTL) was significantly higher in fruit lycopene content than NCEBR-1 ($p = 0.037$), but this class also contained some individuals harboring *lyc12.1*.

Finally, although the average fruit weight of the BC_2 population (115.6 g) was significantly lower than that of NCEBR-1 (144 g), fruit weight was not significantly correlated with lycopene content in this population ($r = -0.108$, $p = 0.147$). Overall, these results support *lyc12.1* as a major QTL that significantly increases lycopene content in this population with strong additive or dominant effects independent of fruit size.

BC_3S_1 population construction and fine mapping

The objective of this marker-assisted backcross program was to introgress *lyc12.1* into the genetic background of

Table 1 Single marker analysis of foreground markers used for selection for lyc7.1 and lyc12.1, based on the lycopene content data from two extraction replicates (Rep I and Rep II), and the mean of the two replicates, in the BC2 population

Marker	Chromosome	Map position	Rep I		Rep II	
			$\mu_M - \mu$	p	$\mu_M - \mu$	p
OH275	12	74.78	23.823	**<0.001**	14.907	**<0.001**
T0800	12	83.92	26.877	**<0.001**	17.269	**<0.001**
SSR241	7	15.10	4.695	0.124	5.629	**0.001**
cLEN14F9	7	25.20	3.019	0.322	4.542	**0.010**

μ_M mean lycopene content of all individuals at marker M, μ mean lycopene content of all individuals at all loci, p significant level for testing the null hypothesis (H0): $\mu_M - \mu = 0$

Bold values denote significant differences

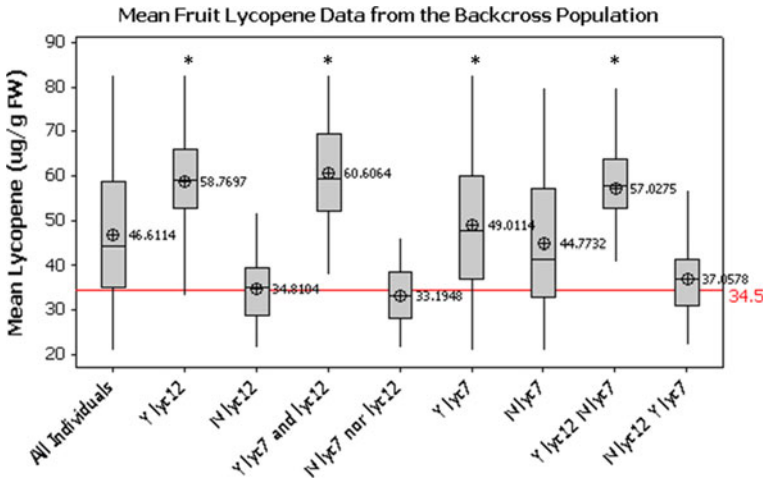


Fig. 4 *Boxplot* displaying lycopene data from the entire BC_2 population and from different subpopulations of individuals with or without *lyc7.1* and *lyc12.1*. Each subpopulation that contained individuals with *lyc12.1* had a significantly higher mean lycopene concentration than the recurrent parent, NCEBR-1 (indicated by the *red horizontal line*). *Y* yes, *N* no (color figure online)

breeding line NCEBR-1 as rapidly as possible using a foreground and background MAS strategy. Several polymorphic SSR markers within the foreground and background intervals, identified via syntenous relationships between the NCEBR-1 × LA2093 F_7 RIL map (Ashrafi et al. 2009) and the EXPEN-2000 high-density map (Mueller et al. 2005a), were mapped in this study. Using these markers, BC_2 individuals harboring *lyc12.1* region and minimal LA2093 genomic background were identified. In these individuals, at least 8 of the 10 detectable background intervals were eliminated, indicating an 80 % rate of return. Three such BC_2 individuals with identical graphical genotypes were hybridized with NCEBR-1 and generated BC_3 progeny ($n = 189$), which were subsequently genotyped to identify 9 individuals heterozygous at the *lyc12.1* location but homozygous for NCEBR-1 alleles at all other detectable locations. The nine selected BC_3 individuals were grown to maturity and self-pollinated to produce a BC_3S_1 population (hereafter the "NIL" population). Thus, only two marker-assisted backcross generations were required to isolate *lyc12.1* in an NCEBR-1 genetic background starting from the original recombinant inbred line, RIL37.

In a previous study (Ashrafi et al. 2012), *lyc12.1* was delimited to an ~10 cM segment. However, for fine mapping and identifying candidate genes underlying *lyc12.1*, additional genetic mapping within the *lyc12.1* interval was required. We generated an NIL population ($n = 1{,}500$) segregating only for *lyc12.1*, genotyped the whole population with PCR-based markers flanking the QTL location, and identified 265 recombinants. Two PCR-based markers reported by Ashrafi et al. (2009) along with eight additional SSR markers developed in this study were then genotyped in the recombinant subpopulation ($n = 265$). The accurate genetic map of the *lyc12.1* region was determined (Fig. 5). Meanwhile, red ripe fruits from 227 *lyc12.1* recombinants and individuals from each of the following genotypic classes were harvested and analyzed for lycopene content: *lyc12.1* homozygotes ($n = 28$), *lyc12.1* heterozygotes ($n = 28$), and NCEBR-1 homozygotes (i.e., no *lyc12.1*; $n = 28$). The *lyc12.1* homozygotes had a mean lycopene content of 49.9 µg/g FW, heterozygotes a mean of 52.4 µg/g FW, and NCEBR-1 homozygotes a mean of 38.2 µg/g FW. The breeding line NCEBR-1 displayed a mean of 35.0 µg lycopene/g FW. In the NIL population, individuals homozygous or heterozygous for *lyc12.1* were statistically indistinguishable from each other ($p = 0.292$), indicating that *lyc12.1* had dominance effects on fruit lycopene content. Furthermore, both of these

classes were significantly higher in lycopene content than NCEBR-1 ($p < 10^{-6}$). Enhanced fruit color was also evident in NILs containing *lyc12.1* upon visual comparison between ripe fruit from these genotypes and NCEBR-1 (Fig. 6). Further, no significant difference in fruit weight was observed between the *lyc12.1* NILs (204 g) and the recurrent parent NCEBR-1 (205 g), indicating that any observed differences in fruit weight in the BC_2 population were due to other genes residing in the segregating background intervals. Although the phenotypic effect of *lyc12.1* on fruit lycopene content in the NIL population was not as strong as it was in the BC_2 population (most likely due to the unfavorable environmental conditions in 2009), the increased lycopene content in *lyc12.1* NILs was statistically significant and visibly apparent (Fig. 6).

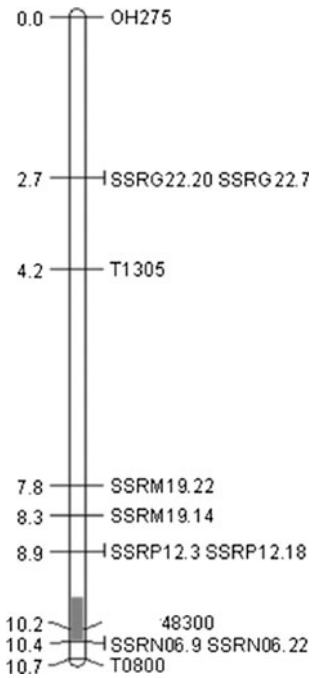
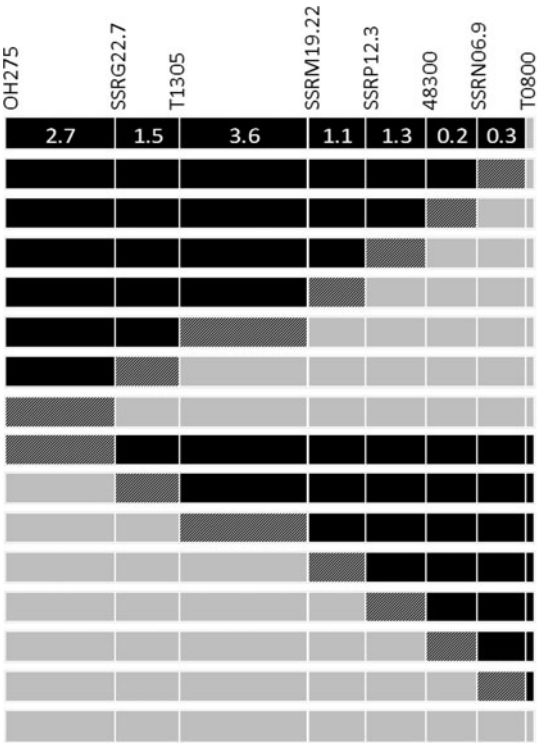


Fig. 5 Genetic map of the full *lyc12.1* interval (~10 cM) based on the BC_3S_1 mapping ($N = 1{,}500$). Genetic distance from marker OH275 is indicated on the *left side* of the sub-chromosome. The *shaded region* is the possible location of *lyc12.1* as determined by fine mapping

Because *lyc12.1* appeared to be completely dominant, recombinants that were homozygous for LA2093 alleles at one *lyc12.1* flanking marker and heterozygous at the other flanking marker were unusable for fine-mapping purposes. Only individuals heterozygous at one flanking marker and homozygous for NCEBR-1 alleles at the other were informative. Figure 7 displays graphical genotypes of various sub-NILs (*lyc12.1* recombinants) and their fruit lycopene contents. A comparative analysis of the different sub-NILs indicates that *lyc12.1* is most likely delimited to the 1.5 cM region between SSRP12.3 and SSRN06.9 (Fig. 7). A close inspection of the different sub-NILs indicates that *lyc12.1* is possibly located between markers 48,300 and SSRN06.9, which are approximately 0.2 cM apart, due to the fact that the mean of the group of individuals that recombined between SSRP12.3 and 48,300 was not significantly higher than NCEBR-1. A mean comparison of all the recombinants having the 48,300 + SSRN06.9 segment ($n = 52$; mean = 54.3 µg lycopene/g FW) with those lacking it ($n = 56$; mean = 36.0 µg lycopene/g FW) indicated a significant difference ($p < 10^{-9}$). Further, the mean lycopene content of the sub-NILs having the segment between markers 48,300 and SSRN06.9 was also significantly higher than the recurrent parent, NCEBR-1 (mean = 36.0 µg lycopene/g FW). Owing to the close proximity of markers 48,300, SSRN06.9 and T0800, we were not able to recover a large number of sub-NILs that had only the 48,300 + SSRN06.9

Fig. 6 Ripe fruit from a *lyc12.1*-containing near-isogenic line (*bottom*) and NCEBR-1 (*top*)



Mean [lycopene]	% Diff	N
55.0	57.1	30
65.4	86.9	2
60.9	74.0	1
36.3	3.7	12
no data	n/a	n/a
36.2	3.4	16
35.0	0.0	11
37.6	7.4	13
45.8	30.9	12
51.2	46.3	11
49.6	41.7	17
49.3	40.9	6
57.8	65.1	3
35.6	1.7	3
36.4	4.0	1
34.8	0.0	30

Fig. 7 Graphical genotypes and fruit lycopene content phenotypes (μg/g fruit FW) of sub-NILs developed from the introgression of *lyc12.1* from *S. pimpinellifolium* accession LA2093 into *S. lycopersicum* breeding line NCEBR-1. *Solid black bars* represent homozygous LA2093 segments, *dark gray bars* indicate areas of recombination, and *light gray bars* represent homozygous NCEBR-1 segments. Approximate genetic map distances between the associated markers are indicated in the *top bar*. % Diff" is the percent difference between the mean of the recombinant class and the mean value for NCEBR-1, 35.0 μg lycopene/g FW

or the SSRN06.9 + COST0800 segment. Therefore, based on this experiment, *lyc12.1* is most likely located either between markers 48,300 and SSRN06.9 or between markers SSRP12.3 and 48,300 (but very tightly linked to 48,300). Further analysis, including additional markers, is needed to determine the exact location of *lyc12.1*. BLAST searches using sequences from the corresponding BACs revealed that segment SSRP12.3 + SSRN06.9 resides on a contiguous genome sequence scaffold of 1.5 Mb.

Discussion

Unsatisfactory tomato fruit quality attributes, including lack of dark red color, are major consumer complaints and potential limitations to supermarket tomato sales, especially in the northeastern US Stommel et al. (2005) reported that consumers associated deep red color of tomato fruit with improved quality and taste, and that upon limiting the ability to discern fruit color, this association was abolished. Thus, in addition to the supposed health benefits of lycopene, it is economically imperative to develop commercial tomato varieties with improved fruit color. Although many fruit color QTL mapping experiments were previously conducted using various interspecific populations of tomato (Chen et al. 1999; Fulton et al. 2000; Liu et al. 2003; Rousseaux et al. 2005; Saliba-Colombani et al. 2001), no major QTLs (i.e., QTLs explaining more than 10 % of the phenotypic variation) directly useful for increasing fruit lycopene content of commercial tomatoes were identified [except for the *old-gold crimson* (og^c) mutant gene (Ronen et al. 2000); reviewed in Foolad (2007)]. In fact, of the ~126 fruit color QTLs/genes identified in different mapping studies, only 4 genes/QTLs affecting tomato fruit color have been fine-mapped or cloned and most of these decrease fruit lycopene content, as they represent deleterious mutations of key carotenoid biosynthetic enzymes [reviewed in (Foolad 2007)].

The overall goals of this research were to verify the phenotypic effects of two lycopene QTLs (*lyc7.1* and *lyc12.1*), which we had identified in a previous study (Ashrafi et al. 2012), and to develop NILs and sub-NILs for genetic characterization of these two QTLs. In the RIL-derived BC_2 population, where the two QTLs were examined individually and in combination, *lyc7.1* did not show statistically significant effects on fruit lycopene content, although the BC_2 heterozygotes harboring this QTL alone had somewhat higher fruit lycopene content than the recurrent parent NCEBR-1 (Fig. 5). This lack of significant effect contrasted with the detection of *lyc7.1* in the RIL population in multiple years and generations (Ashrafi et al. 2012). The disparity could be due to the fact that in the BC_2 generation these QTLs were only present in the heterozygous condition. It is possible that either the effect of *lyc7.1* is recessive, its additive effect is small and thus negligible in the heterozygous condition, or there were unfavorable QTL × environment interactions that negated the phenotypic effect of this QTL. Further, in a

tomato-breeding program with the goal of developing commercial hybrid cultivars with high fruit lycopene content, it is highly desirable to use genes or QTLs with dominance effects. The use of dominantly inherited traits eliminates the need to incorporate the desired genes/QTLs in both parents of the hybrid. For these reasons, we decided to focus on *lyc12.1* in further experiments.

Unlike *lyc7.1*, *lyc12.1* significantly increased fruit lycopene content in the heterozygous condition in the BC_2 population (Fig. 5). This observation suggested that *lyc12.1* either had strong additive effects or the contributing allele from the high-lycopene *S. pimpinellifolium* parent (LA2093) had dominance effects. In the BC_3S_1 NIL population, however, it was determined that there were no significant differences in fruit lycopene content between heterozygous and homozygous *lyc12.1* NILs, suggesting that *lyc12.1* had dominance effects. This and the finding that *lyc12.1* had large effects heighten the utility of this QTL for production of commercial tomato hybrids with high fruit lycopene content.

Under favorable field growing conditions in 2008, on an average, *lyc12.1* contributed to ~70 % increase in fruit lycopene content in BC_2 individuals, as compared to NCEBR-1, and in a rather poor growing climate in 2009, the increase was ~52 % in BC_3S_1 individuals. As a comparison, the most commonly used gene source for high fruit lycopene content is the old-gold crimson (og^c) allele of the *Beta* gene, which increases fruit lycopene content by approximately 25 % in homozygous status [(Ronen et al. 2000); J Scott, pers. commun.]. As a part of the current research, we have fine-mapped *lyc12.1* and developed NILs that contain a 1.5 cM introgression from LA2093 containing *lyc12.1* and otherwise are homozygous for NCEBR-1 alleles at all other detectable loci. The presence of *lyc12.1* in a cultivated tomato genetic background and the availability of tightly linked, co-dominant PCR-based markers flanking *lyc12.1* will facilitate incorporation of this QTL to other tomato genetic backgrounds via simple MAS and without any obvious linkage drag issue.

Linkage drag is a major concern when transferring a desirable trait from a wild to a cultivated genetic background. In tomato, several introgressions from wild species have failed to be implemented in commercial breeding programs, at least initially, mainly due to this predicament (Brouwer et al. 2004; Brouwer and St. Clair 2004). However, in several such situations, introgressions were attempted using a wild accession or early segregating populations as the genetic source to be disseminated to the relevant research community. The current study, however, might be the first reported research to utilize an RI line with the specific aim of transferring well-defined fruit quality QTLs to elite tomato germplasm, while also minimizing the donor segment and testing QTL effects under practical field conditions. Lecomte et al. (2004) reported the transfer of several QTLs for fruit firmness, soluble solids content, locule number, and titratable acidity into commonly grown tomato varieties, but the sizes of donor introgressions in the various resultant lines were not entirely known and varied widely. Although significant introgression × genetic background effects were detected, it is unknown whether or not the effects were due to QTLs or negative linkage drag (Lecomte et al. 2004). However, minimizing the amount of background and linked foreground introgressions is imperative for reducing the effect of linkage drag, precisely assessing QTL effects in unrelated genetic backgrounds, and for increasing the immediate utility of detected QTLs to other breeding programs. This was clearly demonstrated by Neeraja et al. (2007), who described the introgression of an important submergence tolerance QTL into the *Swarna* variety of rice. In the present study, the development of NILs and sub-NILs containing *lyc12.1* in the background of a cultivated tomato and identification of tightly linked molecular markers facilitate clean transfer of this QTL to other tomato genetic backgrounds.

To further examine the importance of *lyc12.1* in producing high fruit lycopene content and the use of MAS strategy for transferring this QTL, in the BC_2 population we inspected the marker genotypes of the "high lycopene" individuals, i.e., those individuals with a lycopene content one standard deviation (SD) greater than the BC_2 population mean. Of the 28 "high lycopene" individuals, 23 had both *lyc12.1* flanking markers and the remaining 5 had only one of the flanking markers. Further, the mean lycopene content of individuals with both *lyc12.1* flanking markers was 1.6 SD greater than the mean of the BC_2 population, and no "high lycopene" individual was identified with homozygous genotypes for NCEBR-1 alleles at both flanking markers (i.e. with no *lyc12.1* QTL). These analyses indicated that most or all of the "high lycopene" individuals contained *lyc12.1*, and thus MAS alone for *lyc12.1* would be highly effective in significantly increasing the lycopene content of the selected population. At the same time, however, since a few BC_2 individuals were identified that contained both *lyc12.1* flanking markers, but yet did not attain the "high lycopene" characteristic, MAS alone may be <100 % efficient in selecting for individuals with "high lycopene" content. This is most likely due to the influence of environmental factors on production of lycopene in the fruit. Further, as the majority or all of the phenotypically selected "high lycopene" individuals had *lyc12.1* QTL, it is expected that phenotypic selection for this trait will also be effective. This is consistent with our previous estimate of a moderate-to-high heritability for this trait (Ashrafi et al. 2012). Moreover, in the *Tomato Genetics and Breeding Program* at Penn State, we have successfully incorporated

this high-lycopene trait to numerous fresh market and processing tomato-breeding lines, mostly via phenotypic selection (MR Foolad, unpublished information). Several improved lines are currently available to tomato breeders via signing a material transfer agreement.

It would be of great scientific interest to uncover the molecular basis of *lyc12.1*. Because no carotenoid biosynthetic enzymes are currently known to map to the *lyc12.1* region, identifying the gene and functional polymorphism underlying *lyc12.1* would provide further understanding of the carotenoid biosynthetic process. We are also unaware of the prevalence of this allele in the tomato cultigen, or whether *lyc12.1* is specific to LA2093. In some tomato genotypes, such as NCEBR-1 as well as breeding lines and cultivars containing og^c, there is more lycopene accumulation near the fruit pericarp and less accumulation in the interior of the fruit (Torres and Andrews 2006). Our general observation has been that *lyc12.1* seems to also enhance fruit color in the interior of ripe fruits so that there is no discernible decrease in redness within the fruit flesh (Fig. 6).

Because the sequences of the markers flanking the 1.5 cM *lyc12.1* region are known, we obtained the tomato genomic sequence between them, as well as the tomato EST, unigene, and protein sequence datasets, from SGN (Mueller et al. 2005a). We then performed a preliminary unigene alignment, using the bioinformatics program MAKER (Cantarel et al. 2008) and the Apollo genome annotation and curation editor (Lewis et al. 2002), to identify putative tomato genes in the *lyc12.1* region. This resulted in the preliminary alignment of 144 known unigenes to the ~1.5 Mb *lyc12.1* region (Supp. Table 1). To leverage known annotations and previous gene identification research in *Arabidopsis thaliana*, each unigene was then BLASTed against the latest TAIR (The Arabidopsis Information Resource) gene build. Based on these preliminary annotations, many potential genes encoding different types of proteins were identified, some of which are known to be involved in the regulation and production of primary and secondary metabolites (Supp. Table 2). The most interesting classes include biosynthetic/metabolic enzymes, bHLH and MYB transcription factors, and SnRK1-related signaling proteins.

One of the most interesting candidates is SGN-U577978. The top *A. thaliana* BLAST match for this unigene is 15-*cis*-*zeta*-carotene isomerase (*Z-ISO*; At1g10830.2; *e* value $= 1 \times 10^{-94}$), a carotenoid biosynthetic enzyme recently isolated by Chen et al. (2010) in maize and *A. thaliana.* Z-ISO is localized in the chloroplast and catalyzes the conversion of 9-15-9′-tri-*cis*-*zeta*-carotene to 9-9′-di-*cis*-*zeta*-carotene in *A. thaliana* and *Zea mays* (Chen et al. 2010). This step in carotenogenesis (upstream of lycopene) also occurs autocatalytically in the presence of light; thus, Z-ISO is responsible for the production of the isomer required by CRTISO for further conversion to lycopene in tissues where light is not present [such as maize endosperm or the interior of a tomato fruit (Chen et al. 2010)]. According to the Tomato Functional Genomics Database (TFGD), SGN-U577978 localizes to the plastid and is expressed in tomato fruit (Mueller et al. 2005b; TFGD 2010). Spatial differences in *Z-ISO* expression or Z-ISO localization within tomato fruit could explain spatial differences in lycopene accumulation within tomato fruit. However, whether or not the observed spatial difference in carotenoid accumulation between NCEBR-1 and *lyc12.1* NILs can be linked to Z-ISO remains unknown. Further studies, utilizing the NILs and tightly linked markers developed in this research, are required to elucidate the underlying molecular mechanism of *lyc12.1*.

In conclusion, the identification and analysis of genes that increase fruit lycopene content in tomato would be beneficial from a scientific point of view as well as for practical reasons, including tomato breeding and consumption. As a result of our research, significant progress toward this goal has been achieved, including the identification and verification of two major QTLs (*lyc7.1*, *lyc12.1*) for increased fruit lycopene content. The individual effect of *lyc12.1* has been verified in inbred backcross and NIL populations and demonstrated that *lyc12.1* increased the lycopene content of ripe tomato fruits by 52–70 %. Flanking PCR-based markers tightly linked to *lyc12.1* have been developed and marker-assisted breeding experiments demonstrated that selection for this QTL significantly increased tomato fruit lycopene content. The marker information provided here can readily be employed to transfer the high-lycopene trait to other tomato-breeding lines commonly used for production of commercial hybrid cultivars. The marker information can also be used to further discern the basis of the gene(s) underlying the high fruit lycopene QTL, *lyc12.1*.

Acknowledgments This research was supported in part by Agricultural Research Funds administered by the Pennsylvania Department of Agriculture, the Pennsylvania Vegetable Marketing and Research Program, and the College of Agricultural Sciences at the Pennsylvania State University. The authors graciously thank Dr. Randolph Gardner for providing seed of breeding line NCEBR-1, Dr. Hamid Ashrafi for assistance with constructing genetic maps, and all Penn State staff and undergraduates who helped with field experiments and data collection.

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