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ORIGINAL PAPER

Fine mapping a QTL for carbon isotope composition in tomato

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Abstract Carbon isotope composition ($\delta^{13}C$) and leaf water-use efficiency vary in concert in C3 plants, making $\delta^{13}C$ useful as a proxy for plant water-use efficiency. A QTL for $\delta^{13}C$ was detected in the *Solanum pennellii* chromosome fragment of IL5-4, an introgression line with *S. lycopersicum* cv. M82 background. M82 and IL 5-4 were crossed, and RFLP markers in the target region converted to PCR-based markers. Forty-one recombinants with an introgression fragment ranging in length from 1.1 to 11.4 cM were identified by marker assisted selection (MAS) among approximately 2000 F2 plants. A total of 29 markers were mapped within the introgression fragment unique to IL5-4. These markers divided the about 9 cM target region into nine intervals. A dominant QTL for $\delta^{13}C$, designated QWUE5.1 that explained 25.6% of the total phenotypic variance was mapped to an interval about 2.2 cM long. Twenty-one plants with a *S. pennellii* chromosome fragment shortened to a length of 2.0–9.1 cM by a second recombination event were generated by MAS of 1,125 F4 plants. Two near isogenic lines with high $\delta^{13}C$ (small negative value) and carrying QWUE5.1 on the shortest introgression fragments (about 7.0 cM) were identified. The markers and genetic stocks developed are valuable for cloning the gene underlying QWUE5.1, MAS of QWUE5.1, and fine-mapping genes/QTL located in this region.

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Introduction

Plant water-use efficiency (WUE), defined as the ratio of carbon gained to water transpired, determines yield in water-limited environments. Therefore, in principle, the natural genetic variation in WUE may be harnessed to increase crop yield. Interspecific variation for WUE was documented almost 100 years ago (Briggs and Shantz 1913, 1914). In more recent years, intraspecific variation has been also reported in a variety of crops, such as peanut (Hubick et al. 1988; Wright et al. 1994), barley (Hubick and Farquhar 1989), cotton (Quisenberry and McMichael 1991), wheat (Ehdaie and Waines 1993; Van den Boogard et al. 1997), cowpea (Ismail and Hall 1993; Ashok et al. 1999), and soybean (Mian et al. 1996, 1998). Yet, with few exceptions (e.g., Rebetzke et al. 2002; Richards 2004) limited improvement of WUE has been made through genetic selection. One difficulty is the lack of suitable methods to assess plant WUE since the measurements should be based

on water actually used by the plants rather than the total amount of water applied to the field or to pots in a greenhouse (Martin and Thorstenson 1988).

Carbon isotope composition ($\delta^{13}C$) in C3 plants is a measure of isotopic fractionation ($^{13}CO_2$ and $^{12}CO_2$) that occurs in the photosynthetic CO_2 fixation process, mainly at the initial carboxylation step catalyzed by Rubisco. The process favors assimilation of the light $^{12}CO_2$ form over the heavy $^{13}CO_2$. At a fixed ambient CO_2 concentration, $\delta^{13}C$ is negatively associated with the intercellular CO_2 concentration (C_i). At any moment in time, the C_i is also negatively correlated with leaf transpiration efficiency, where leaf transpiration efficiency is defined as the ratio of the photosynthesis rate to the transpiration rate (Farquhar and Richards 1984; Hall et al. 1996). Under water limitation, leaf transpiration efficiency is a major determinant of long term plant WUE. Because transpiration efficiency is positively linked to $\delta^{13}C$, it is not surprising that $\delta^{13}C$ has also been generally found to be positively associated with WUE (Farquhar and Richards 1984). Substantial genetic variance, small genotype-by-environment interaction (G × E), and high heritability for $\delta^{13}C$ have been reported for several species (Martin and Thorstenson 1988; Martin et al. 1989; Rebetzke et al. 2002; Richards et al. 2002; Comstock et al. 2005; Impa et al. 2005). These features, and the ability to measure $\delta^{13}C$ with minimal tissue destruction, make $\delta^{13}C$ an attractive surrogate for WUE in research and breeding programs (Farquhar and Richards 1984; Rebetzke et al. 2002; Richards et al. 2002).

Although both dry matter production and agronomic yield have been found to be correlated with $\delta^{13}C$, whether these correlations are positive or negative appears to depend on the availability of water in the growth environment (Farquhar and Richards 1984; Condon et al. 1987; Martin and Thorstenson 1988; Martin et al. 1999; Rebetzke et al. 2002). Recently the two wheat cultivars Rees and Drysdale were bred by selecting for $\delta^{13}C$ (Richards 2004). They were developed for dryland wheat production in Australia and have proven to be successful there.

QTL for $\delta^{13}C$ have been identified in barley (Handley et al. 1994; Teulat et al. 2002), soybean (Mian et al. 1996), *Stylosanthes scabra* (Thumma et al. 2001), *Brassica oleracea* (Hall et al. 2005), rice (Price et al. 2002) and *Arabidopsis thaliana* (Juenger et al. 2005). Some QTL for $\delta^{13}C$ were coincident with QTL for transpiration efficiency (Thumma et al. 2001; Hall et al. 2005), supporting the expectation that transpiration efficiency and $\delta^{13}C$ are under the same genetic control. Masle et al. (2005) mapped a QTL for $\delta^{13}C$ and transpiration efficiency on chromosome 2 of *A. thaliana*. They concluded that ERECTA, a gene with manifold effects in plant morphogenesis, underlies the QTL. The way by which ERECTA controls transpiration efficiency and $\delta^{13}C$ may be through its known effects on stomatal density, epidermal cell expansion, and mesophyll cell proliferation.

Considerable progress has been made to understand WUE in tomato. Martin and colleagues (Martin and Thorstenson 1988; Martin et al. 1999) found that the drought tolerant *Solanum pennellii* (formerly *Lycopersicon pennellii*) had greater WUE and less negative $\delta^{13}C$ than the cultivated species *Solanum lycopersicum* (formerly *Lycopersicon esculentum*). They also noted that $\delta^{13}C$ better reflected season-long WUE than instantaneous transpiration efficiency measured by gas exchange on individual leaves. Importantly, season-long WUE was strongly negatively correlated with plant dry weight in an F2 cross. Thus, although WUE could be improved by selecting high $\delta^{13}C$ values, selecting high $\delta^{13}C$ alone would also reduce plant size. Given the limited amount of recombination in the F2, it is not clear whether this correlation is due to pleiotropy or linkage between different loci underlying the two traits, and whether there exist *S. pennellii* alleles that would confer high WUE to *S. lycopersicum* without reducing plant dry weight.

Identification and marker-assisted selection of QTL for WUE by means of $\delta^{13}C$ is a particularly promising way to break negative pleiotropy between WUE and yield in C3 species, since individual QTL should vary with respect to their pleiotropic effects.

Martin et al. (1989) identified three RFLP markers that explained much of the genetic variance for $\delta^{13}C$ in F3 and BC1S1 tomato populations. Using differential display and cDNA library screening, Zhu et al. (1998) isolated and sequenced a gene that appeared to contribute to the difference in WUE between *S. lycopersicum* and *S. pennellii*. This gene encodes Rubisco activase, a critical enzyme that enables Rubisco to catalyze CO_2 fixation more efficiently in photosynthesis.

Recently, Comstock et al. (2005) evaluated the stability of $\delta^{13}C$ in greenhouse-grown *S. lycopersicum* and *S. pennellii* with respect to variable growth conditions. They found that the main environmental effects were generally small, and that the G × E interaction across the divergent treatments was much smaller than for other associated measures, such as relative growth rate, photosynthesis rate, biomass allocation pattern, and specific leaf area. Thus, the genetic difference in WUE between these two species is sufficiently stable to be of potential agronomic importance.

In order to use the genetic variation from *S. pennellii* to improve WUE in *S. lycopersicum*, a more detailed molecular and phenotypic characterization of the QTL in these two species is needed. We chose to address this matter by using an introgression line (IL) mapping population, which consists of 50 lines that each contains a unique introgressed segment of the *S. pennellii* genome in an otherwise pure *S. lycopersicum* genetic background (Eshed and Zamir 1994). Collectively, the IL cover the whole genome, and they have been previously genotyped. This population has a number of advantages: QTL are unambiguously mapped to well-delimited

intervals, QTL are expressed against the *S. lycopersicum* background, and the clean background permits rapid isolation of near isogenic lines (NIL) differing only in a small region surrounding the QTL (Eshed and Zamir 1995). Our objectives were to identify QTL for δ^{13}C in the IL mapping population, fine-map the QTL with the greatest influence on δ^{13}C, develop markers to facilitate MAS of WUE into commercial cultivars, and develop NIL to further isolate the QTL for detailed genetic and physiological characterization.

Materials and methods

Whole-genome scan

Eshed and Zamir (1994) developed a set of 50 *Solanum pennellii* chromosome segmental introgression lines (IL) from a cross between *S. pennellii* accession LA716 and *S. lycopersicum* cv. M82, a processing tomato cultivar that has been widely used in tomato breeding and genetics research. Each IL contains a single unique homozygous *S. pennellii* chromosome fragment estimated to span an average of 33 cM. The fragments overlap and collectively cover the tomato genome (Eshed and Zamir 1994).

Six replicate sets of the 50 IL were grown in randomized blocks in a greenhouse at Boyce Thompson Research Institute, Ithaca, NY, and each plant was individually phenotyped for δ^{13}C. All plants were grown in a potting mixture composed of 3:1:1 screened and pasteurized fritted clay:sand:topsoil with dolomitic lime, gypsum, superphosphate, and a micronutrient supplement (Micromax Plus, Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) added as amendments at rates of 2.7, 4.5,1.1, and 0.85 kg m^{-3}, respectively. Starting one week after germination, the plants were fertilized once every other day with Peters Excel 15:5:15 (Scotts-Sierra Horticultural Products Co.) providing 100 μg N g^{-1} potting mixture. The plants were exposed to a 14-h photoperiod, 50–60% relative humidity, and the irradiance was augmented by artificial light resulting in more than 500 μmol m^{-2} s^{-1} PAR in mornings and evenings and above 1,000 μmol m^{-2} s^{-1} PAR during midday. The day/night temperature was 28/22°C. A more detailed description of the growth conditions is given by Comstock et al. (2005).

Several leaflets from the youngest fully expanded leaves of about 3-week-old plants were sampled for isotope analysis. Carbon isotope measurements were conducted on dry leaf powder at the Cornell Stable Isotope Laboratory (Cornell University, Ithaca, NY, USA) using a Finnigan Matt Delta Plus ratio mass spectrometer as reported by Comstock et al. (2005). The International Atomic Energy Agency standard Pee Dee limestone was used to calculate the carbon isotope composition, δ^{13}C, of plant materials. In this paper we consistently use the δ^{13}C (carbon isotope composition) denotation even in those cases where cited publications use the Δ (carbon isotope discrimination) denotation. Discrimination values are normally calculated from sample δ^{13}C values as described by Comstock et al. (2005) using a δ^{13}C value of CO_2 in the air equal to −8‰.

Analysis of variance (ANOVA) was used to assess heterogeneity in δ^{13}C among the lines, and post-hoc contrasts were performed to compare individual lines against M82. Based on the results of the whole-genome scan, line IL5-4 was chosen for further fine-mapping and NIL development.

Molecular markers

The markers used in fine-mapping were developed as described below. Genomic DNA was extracted from fresh leaf tissue of *S. pennellii* accession LA716, *S. lycopersicum* cv. M82 and IL 5-4 using the DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA, USA).

STS and CAPS markers

Several RFLP markers previously mapped to the introgression fragment of IL5-4 (Fig. 1) were converted into PCR-based markers. This was done by designing primers based on sequences of genes and the RFLP probes TG69, TG351, CT80, TG60, CHS3, T1541 and T1584 published at the Solanaceous Genomic Network database (http://sgn.cornell.edu/). Primer sequences and annealing temperatures are given in Table 1. PCR amplification was performed in a 25-μl reaction mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 0.4 μM of the forward and reverse primers, 0.04 U/μl of Taq DNA polymerase (New England Biolabs, Inc., Ipswich, MA, USA) and 0.4 ng/μl template DNA. PCR amplification was carried out for 36 cycles (denaturation at 94°C for 1 min, the primer-specific annealing temperature listed in Table 1 for 1 min, extension at 72°C for 1 min, plus denaturation at 94°C for two additional minutes at the beginning and extension at 72°C for four additional minutes at the end). PCR products were electrophoresed in 2% agarose and stained with ethidium bromide. PCR products with a scorable size difference between IL 5-4/LA716 and M82 were classified as sequence tagged site (STS) markers.

The remainder of the PCR products, which were invariant in size, were purified using Qiagen PCR purification columns (Qiagen, Inc.). Direct sequencing was carried out using BigDye terminator chemistry on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Restriction site polymorphisms were identified within the assembled and aligned sequences. Those which could successfully be scored after digestion with a restriction enzyme were classified as cleaved amplified polymorphic

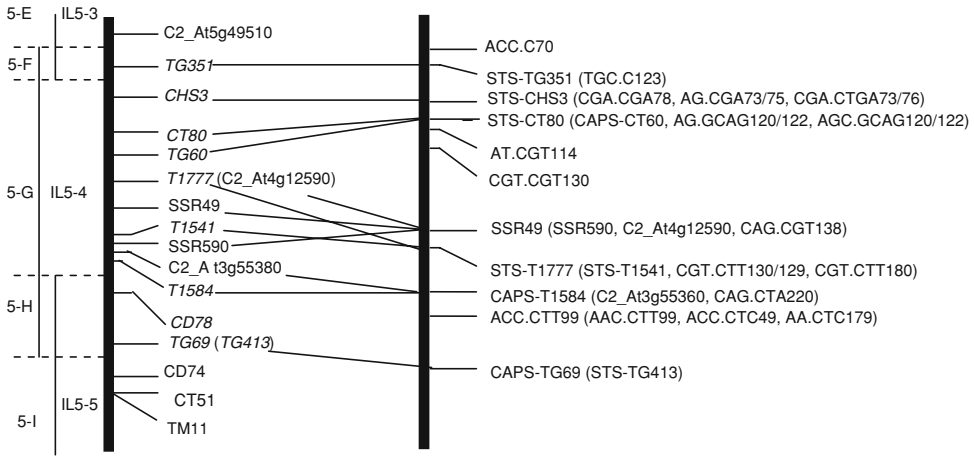

Fig. 1 Positions of introgressed chromosome fragments in IL lines IL 5-3, IL 5-4 and IL 5-5 (*thin solid lines on the left*), and comparison of positions of CAPS and STS markers developed in this study with those of corresponding RFLP markers. The introgressed chromosome fragment in IL 5-4 spans segments 5-F, 5-G, and 5-H (Pan et al. 2000). Nine out of ten RFLP markers (in *italics*) mapped in this region were converted into PCR-based markers. The map on the left is based on an F2 population derived from *S. lycopersicum* cv. LA925 × *S. pennellii* accession LA716 (http://soldb.cit.cornell.edu/cview/map.pl?map_id=9). The map on the right is from the present study. Additional markers mapped to the same loci as the leading markers are shown inside parentheses

Table 1 Primer sequences, annealing temperatures, and polymorphic restriction enzymes for STS and CAPS markers

Marker	Forward primer sequence (5′ > 3′)	Reverse primer sequence (5′ > 3′)	Temperature (°C)	Restriction enzymes
STS-CT80-1	GAGGTTGCTGACAAATGTGG	TCACAATATTCAAAGGGATTCG	57	*Bst*71I, *Dde*I, *Sna*BI, *Fok*I, and *Hsp*92II
STS-CT80-2	AGTCGAGGA(T,A)ATGTGTCACC	AGAAATCTGACCTCATTCCA	55	
CAPS-TG60	GGCTGAAGTGAAGAAAAGTAAGGAT	GGAATCCAGAAGAGAAAAGTTCTAC	52	*Alu*I and *Mbo*II
CAPS-CHS3	GTGAATACATGGCTCCTTCTTTG	ACTATGGAGCACAACAGTCTCAAC	60	*Alu*I, Sau96I, *Ban*II, *Bsp*1286I, *Bsr*SI, *Hae*III, *Dde*I, *Fok*I, *Hin*dIII, *Hin*fI, *Tru*9I, and *Sac*I
STS-CHS3	TGGCACTTCAACCACCACTA	GCTAAAATGGTCACCGTTGC	50	
CAPS-T1584	CCTTCCTTTCCATCAAAGAGC	GGTTGGGCTTCAACATTGC	51	*Bsa*MI, *Hpa*I, and *Tru*9I
CAPS-TG69	TGCCATAACCCAGTTGAACA	TTGGAGTATGATTCCTTCAATGAG	50	*Bsa*HI and *Eco0*109I
STS-T1777	TTAGATGGTGTGGTATTGACTTATGAAC	AACCAAGAACAACTATGTGAATAGACTG	50	
STS-TG351-1	ACAACATCACCAGCCACAAG	TGAAACTGCCGCAGAAGAAT	50	
STS-TG351-2	GGCCTTGAAATTTGACCTGC	CAGGAACGGAATCAAGAAGC	50	
STS-TG413	CAGCAAATGGGTTTTCTTCC	TGTGCATTTTCTTAAACCACAAA	50	
STS-T1541-1	AGCCTAAAGAGCCCGAAAAG	GTTGTGGTTGTGGGGGAAG	50	
STS-T1541-2	CCCTCAAATTCGAGACCAAG	GCTTCTCTGCAACCACCACT	50	

sequence (CAPS) markers. The restriction enzymes for each CAPS marker are given in Table 1. In addition to the markers described above, we used one STS (C2-At3g55360) and one CAPS (C2-At4g12590) marker previously mapped on chromosome 5 (http://soldb.cit.cornell.edu).

SSR markers

We also used SSR49 and SSR590 that had been previously developed (http://sgn.cornell.edu) using an IR-4200 DNA Analyzer (Li-Cor, Inc., Lincoln, NE, USA) following the

method described by Xu et al. (2006). In brief, a tailed PCR primer was used for SSR analysis by adding a 19-base M13 oligonucleotide sequence (M13 tail) to the 5′-end of each forward SSR primer. Thus, each SSR reaction used three primers: two unlabeled SSR primers, one of which had an attached M13 sequence tail, and one infrared fluorescence dye-labeled M13 primer (Li-Cor, Inc.).

AFLP markers

Amplified fragment length polymorphism (AFLP) markers (Keygene, Inc., Rockville, MD, USA) were developed to supplement the STS, CAPS and SSR markers. Genomic DNA was digested with *Pst*I and *Mse*I restriction enzymes. Twenty *Pst*I primers and 35 *Mse*I primers with one to four selective nucleotides were screened to obtain informative primer pairs detecting polymorphic bands between the two parents in the target region. Table 2 lists primer sequences used in pre-amplification and selective amplifications. AFLP bands were scored using an IR-4200 DNA Analyzer (Li-Cor Inc.) following the protocol of Xu et al. (2006). AFLP markers are designated in this paper according to the selective nucleotide sequences of the *Pst*I and *Mse*I primers and the molecular size of each marker. For example, the selective nucleotide sequences of the *Pst*I and *Mse*I primers of AFLP marker CAG.CGT138 are CAG and CGT, respectively, and the molecular size of this marker is 138 bp. For co-dominant AFLP markers, the molecular sizes of both the M82 and IL5-4 alleles are given.

Table 2 AFLP primer combinations

Pre-amplification primers:
*Pst*I:GACTGCGTACATGCAG
*Mse*I: GATGAGTCCTGAGTAA
Selective amplification primers:
*Pst*I: pAAC, pACA, pACT, pACC, pACG, pAGC, pAGG, pTGC, pCAG, pCGA,
pACTG pACGC, pTAGC, pGTCG, pCGT, pGCTG, pGTG, pAG, pAT, and pAA
*Mse*I: mC, mAGC, mTGC, mCAA, mCAT, mCAC, mCAG, mCTA, mCTT, mCTC,
mCTG, mCCT, mCGA, mCGT, mCGC, mCGG, mGAC, mGTG, mGCG, mTGC,
mATGA, mACAG, mACGT, mACTG, mAGAC, mAGCT, mAGGC, mAGTG,
mCACG, mCGCT, mACGC, mCAGT, mCTGA, mCGAC, mGCAG

Selective amplification primer sequences of *Pst*I and *Mse*I are prefixed by p and m, respectively

Fine mapping

Marker-assisted identification of recombinant F2 and F3 plants

A cross between M82 and IL 5-4 was made in a greenhouse at Oklahoma State University, Stillwater, OK, USA. The resultant F1 generation was then selfed to produce F2 plants. The F2 population, consisting of approximately 2,000 plants, was grown for 3–4 weeks in small plastic pots after which each F2 plant was genotyped with two PCR markers flanking the QTL region, STS-TG351 and CAPS-TG69. F2 recombinants with a heterozygous genotype at one marker locus and a homozygous genotype at the other were allowed to produce selfed F3 seed.

Unlike during marker development when fresh leaf tissue was used to extract DNA, a protocol based on Whatman FTA Classic (Whatman, Inc., Clifton, NJ, USA) filter paper cards was adapted to screen recombinants in F2 and F3 and to develop NIL. Leaf tissue was excised from 2-week-old plants. A rubber mallet was used to crush one leaflet onto a Whatman FTA card. After the FTA card was dried at room temperature for at least 1 h, or sometimes stored dry for several days, a 2-mm disc was collected with a hole punch and placed in a PCR tube. Genomic DNA was purified according to the manufacturer's instruction with modifications. In brief, 100 µl of FTA purification reagent was added to the PCR tube, and the disc was washed by pipetting the purification reagent 10–20 times. After the purification reagent was discarded, the wash was repeated with new purification reagent, followed by two additional washes with 0.1× TE buffer, and two final washes with distilled water. The disc was then dried at room temperature for 1 h or at 45°C for 20 min in a thermal controller before it was used for PCR analysis.

STS-TG351 and CAPS-TG69 were employed to screen recombinants in the segregating population using DNA deposited on Whatman FTA cards. STS-TG351 was scored as follows. For each 10 µl PCR reaction, one 2-mm diameter Whatman FTA DNA disc was added to a PCR mixture containing 1× PCR buffer, 200 µM dNTP, 2.5 mM $MgCl_2$, 0.9 unit Taq polymerase, 0.5 pmol of each primer (one of them purchased from Li-Cor, Inc., labeled with a fluorescent dye), 0.5 µl 10% PVP, and 0.5× BSA. PCR was performed with an initial step of 94°C for 5 min, followed by 36 cycles of 20 s at 94°C, 20 s at 50°C, and 30 s at 72°C with a final step of 7 min at 72°C. PCR products were separated in a 6.5% denaturing polyacrylamide gel on an IR-4200 DNA Analyzer (Li-Cor, Inc.). Electrophoresis conditions were set at 1,500 V, 40 W, 35 mA, and 50°C for 3 h in 1× TBE buffer. Gels were prepared using a 68-well comb and 0.8 µl of sample was loaded into each well.

For CAPS-TG69, one Whatman FTA card DNA disc was used for each PCR reaction. The 20 µl of PCR mixture contained 10 pmol of each primer and 12 µl of REDExtract-N-Amp PCR ReadyMix (Sigma, Saint Louis, MO, USA). The PCR profile consisted of an initial step of 94°C for 2 min followed by 36 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C with a final step of 7 min at 72°C. Following incubation at 37°C for 12 h, 15 µl of PCR product was digested with 15 µl of mixture containing 5 units of *Bsa*H1, 1× NEB buffer 4, and 1× BSA. The digested PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide.

Two homozygous recombinant plants from each F3 family were randomly selected and analyzed with the AFLP, SSR, CAPS and STS markers described above. The break point of each recombination event was determined from the marker genotypes in homozygous recombinants. All remaining F3 plants were then scored with an internal marker and, based on the three-marker genotype and the known break point of each recombination event, the genotype of each remaining F3 plant was inferred. Homozygous recombinants were used for fine-mapping of δ^{13}C in the F3 generation, while a select set of heterozygous recombinants were used for NIL development after selfing and screening the progeny for individuals with a second recombination breakpoint as described below. Those plants with a second breakpoint are here denoted double recombinants.

Phenotyping the F3 plants

Fifteen F3 seed from each selected recombinant F2 were planted in 14-cm diameter (2.5 l) plastic pots in a greenhouse at Cornell University, Ithaca, NY, USA for determination of δ^{13}C at three to four weeks post germination. Potting media was 2:1:1:1 vermiculite:peat:sand:perlite with dolomitic lime and micronutrient supplement (Unimix III, The Scotts Company, Marysville, OH, USA) added as amendments at 2.7 and 0.64 kg m^{-3}, respectively. Fertilization was as described above for the whole genome scan. Leaf tissue sampling and the subsequent carbon isotope analysis were also conducted as for the whole genome scan.

QTL analysis

The additive (*a*) and dominance (*d*) effects of individual DNA marker loci within the introgressed chromosomal segment were estimated among M82 × IL5-4 F2:3 progeny using mixed model analysis of variance, where DNA marker locus effects were fixed and other effects were random (Littell et al. 1996). The experimental design was hierarchal with F2 progeny nested in a DNA marker locus and F3 progeny nested in F2 progeny. Statistical analyses were performed using SAS PROC MIXED (http://www.sas.com; SAS, Cary, NC, USA) for unbalanced data. The values of *a* and *d* were estimated using orthogonal contrasts among least square means, and the degree of dominance was calculated as |*d*/*a*| (Falconer and MacKay 1996; Littell et al. 1996; Lynch and Walsh 1998). The reduction in sums of squares associated with a DNA marker locus and the proportion of the phenotypic variance explained by a DNA marker (R^2) were estimated using SAS PROC GLM.

Development of near isogenic lines

Heterozygous F3 individuals from two families (5-4-5 and 5-4-28) were transferred to larger pots and allowed to self. A total of 1,125 F4 progeny were screened for the presence of two recombination events within the IL5-4-5 interval using markers STS-CT80 and CAPS-TG69, and the IL5-4-28 interval using STS-TG351 and STS-T1777, following the same procedures used to score markers in previous generations (see above). Double recombinants were transferred to larger pots and selfed.

The selfed progeny of the double recombinants (the F5 generation) were screened with the same family-specific markers as used for the F4 generation in order to identify homozygous double recombinants. The homozygous double recombinants carrying the QTL for δ^{13}C with the shortest introgressed fragment were considered to be the final NIL. The NIL were then screened using remaining markers to delineate the recombination breakpoints as finely as possible. The NIL were grown in small pots and sampled for determination of the δ^{13}C phenotype as described above. The NIL were transferred to larger pots and selfed for bulk seed amplification. Seed from the NIL with the smallest *S. pennellii* chromosomal insert has been deposited in the C.M. Rick Tomato Genetic Resource Center at the University of California, Davis, CA, USA.

Results

Whole genome scan of the IL population

Several segments of the *S. pennellii* LA716 genome, when introgressed into the *S. lycopersicum* cv. M82 background, resulted in significantly altered δ^{13}C relative to M82 (Fig. 2). The line with the least negative δ^{13}C (greatest WUE) was IL5-4, which was more extreme than either parent, and differed from M82 by about 0.9‰. Line IL6-1 had about 0.5‰ less negative δ^{13}C than M82. A number of lines had significantly more negative δ^{13}C than M82, including IL2-3, IL2-4, IL12-2, IL3-5, IL7-2 and IL9-2.

Because of its potential agronomic value, and because it appears to be in a recombinogenic region of the genome (i.e., not close to a centromere or telomeres), the QTL

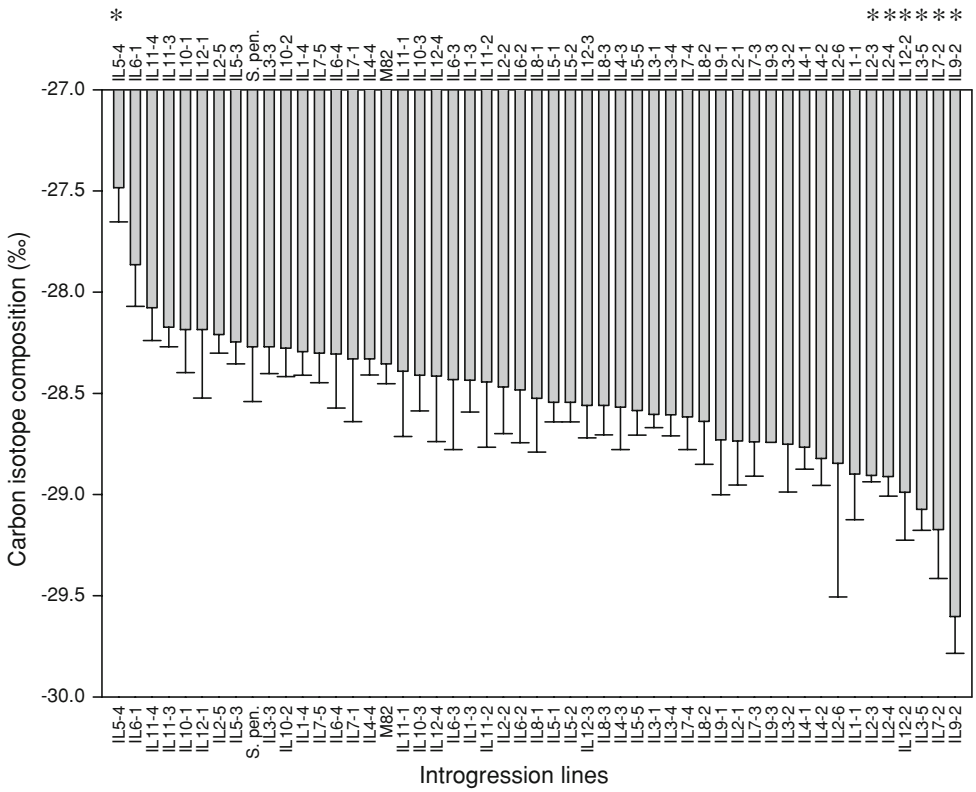

Fig. 2 Carbon isotope composition (δ^{13}C) values of introgression lines (*IL*) derived from *Solanum lycopersicum* cv. M82 and *S. pennellii* accession LA716. *Significantly different from M82 at $P = 0.05$

within the IL5-4 segment (QWUE5.1) was chosen for fine-mapping and NIL development. IL5-4 carries a *S. pennellii* fragment of about 12 cM from chromosome 5 (Tanksley et al. 1992; Eshed and Zamir 1994; Pan et al. 2000; http://sgn.cornell.edu/). Neither of the two introgression lines (IL5-3 and IL5-5) overlapping IL5-4 differed in δ^{13}C from M82. Thus, QWUE5.1 appeared to be located in the IL5-4 unique region flanked by RFLP markers TG351 and CD78, denoted Bin 5G in Fig. 1.

Development of PCR-based markers in the IL5-4 region

Most of the molecular markers previously mapped onto the unique introgression chromosome fragment in IL5-4 were RFLP markers derived from cDNA clones. Due to the time required to score each marker and the amount of DNA necessary, RFLP markers are poorly suited for marker-assisted selection. Six RFLP markers previously mapped in this region plus four flanking markers were selected to be converted into PCR markers (Fig. 1). Our strategy was to design markers anchored in two different exons within the cognate gene in order to increase the likelihood of amplification across the two species while also capturing variation within the intervening intron(s). This was done, where possible, by inspection of alignments between the sequenced RFLP probes, expressed sequence tags from tomato, and homologous genes from Arabidopsis using information obtained from http://sgn.cornell.edu and http://www.arabidopsis.org.

All markers were successfully amplified from at least one of the two parents with the exception of CD78. We developed six co-dominant STS markers (STS-CT80-1, STS-CT80-2, STS-T1777, STS-TG351-1, STS-TG351-2, and STS-T1541-1) that differ in amplicon size between the two parents. We also developed four co-dominant CAPS markers (CAPS-TG60, CAPS-CHS3, CAPS-TG69 and CAPS-T1584) that are monomorphic in size but have one or more polymorphic restriction sites (Table 1), and three dominant markers (STS-CHS3, STS-TG413 and STS-T1541-2) that amplify a band from M82 but not from IL 5-4. Since the size polymorphism for STS-CT80-1 is only 12 bp, which is difficult to detect on an agarose gel, it was scored using a Li-Cor DNA Analyzer. STS-CT80-1 may also be scored as a CAPS marker since there are at least five restriction sites that differ between the two alleles (Table 1). The lack of amplification for CD78 may be due to the presence of a large intron within the targeted regions; introns larger than 1,500 bp are not uncommon in the tomato genome (Bai et al. 2004).

We obtained additional markers in the region using AFLP. Seven-hundred *Pst*I/*Mse*I primer pairs were screened using IL5-4 and M82, and informative primer pairs that amplified polymorphic bands between the two parents were identified. Introgression lines IL5-3, IL5-4

and IL5-5 were then screened with the informative primer pairs and 13 primer pairs were identified that amplified polymorphisms unique to IL5-4.

Marker-assisted development of a single-chromosome-fragment-recombination (SCFR) population

The F2 population was screened to identify individuals with different genotypes at markers STS-TG351 and CAPS-TG69, indicating a recombination breakpoint within the interval. In total, approximately 2,000 plants were analyzed and 41 recombinants were identified. Thus, the recombination frequency in the target region was about 1%. The same two markers had a recombination frequency of 9% in the EXPEN-2000 F2 population (http://sgn.cornell.edu), suggesting considerable suppression of recombination when using the IL5-4 introgression line. Seed were obtained from 29 of the recombinants. The breakpoints of the corresponding 29 recombination events were not evenly distributed among intervals. The number of breakpoints falling into each interval varied from 1 to 7 with the largest number in the interval between AT.CGT114 and SSR49. Using genetic distances derived from the EXPEN-2000 F2 population and assuming that breakpoints were midway between markers, the size of the introgression fragments varied from 1.1 cM in IL5-4-6 and IL5-4-18 to 11.4 cM in IL5-4-3 (Fig. 3). The mean introgression fragment length was about 6.3 cM

Fifteen F3 plants derived from each F2 heterozygous recombinant plant were analyzed with STS-TG351 and CAPS-TG69 and two to five homozygous recombinants were recovered from each family. In general, segregation did not deviate from Mendelian expectation. These homozygous recombinants represent 29 single-chromosome-fragment-recombinant (SCFR) lines that each contains a molecular marker-defined introgression chromosome fragment from *S. pennellii*.

To more precisely locate the recombination breakpoints, 29 markers were used to genotype the SCFR plants: 10 AFLP markers within bin 5G, five AFLP markers within bin 5H, one AFLP marker within bin 5-F, the nine STS and CAPS markers developed above, one STS (C2-At3g55360) and one CAPS marker (C2-At4g12590) previously mapped on chromosome 5, and two SSR markers (SSR49 and SSR590) (http://soldb.cit.cornell.edu).

Linkage analysis integrated all 29 markers in one linkage map. Apart from ACC.C70 which was proximal to TG351, all other markers were mapped in the interval between STS-TG351 and CAPS-TG69. Because recombination events were limited in the target region, 10 sets of markers cosegregated, dividing the target region into 9 intermarker intervals. Assuming the length of the whole interval between STS-TG351 and CAPS-TG69 is 9 cM, as in the EXPEN-2000 F2 population, the length of each intermarker interval, in the absence of recombination suppression ranged from 0.14 to 2.5 cM with an average length of 1 cM.

Mapping QTL for δ^{13}C in the SCFR lines

F3 plants were phenotyped in a number of independent experiments. In each experiment, the two parents showed a significant contrast in δ^{13}C ($P < 0.0001$). Averaged across experiments, the measured δ^{13}C value of M82 was 0.8‰ more negative than for IL5-4. The distribution of mean δ^{13}C in the F3 recombinant population was continuous over a range of 1.1‰ (Fig. 4). ANOVA showed significant differences among family means and recombination event

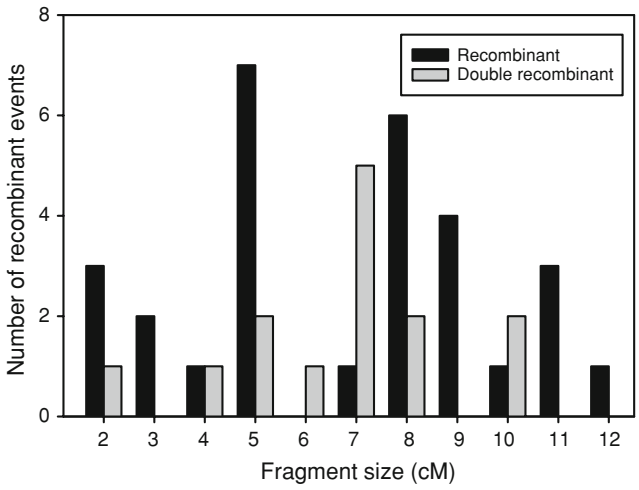

Fig. 3 Frequency distribution of *S. pennellii* chromosomal fragment sizes in F3 recombinants and F5 double recombinants

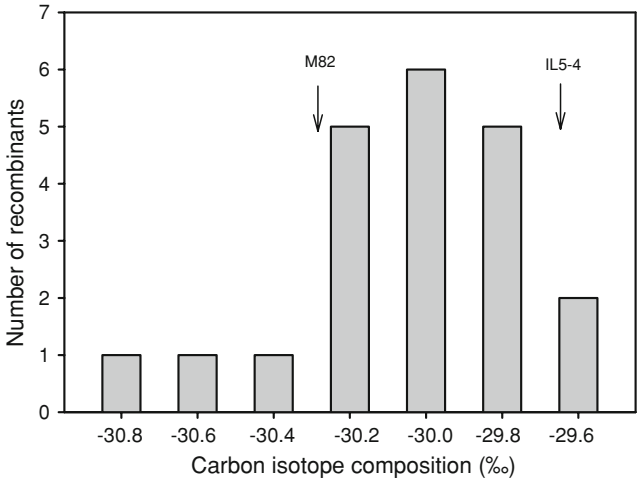

Fig. 4 Frequency distribution for mean carbon isotope composition in a single-chromosome-fragment–recombinant population derived from *S. lycopersicum* cv. M82 × IL5-4 and the parents. Two to seven homozygous recombinants were identified in each F2:F3 family

means, confirming that the recombinants differed in the presence or absence of at least one QTL.

Statistical analyses for each marker revealed that the effects of eight of the 11 loci on δ^{13}C were significant (Table 3). Based on our analysis, the δ^{13}C QTL is most tightly linked to the loci represented by markers STS-T1777 and SSR49. STS-T1777, which cosegregated with three other markers (STS-T1541, CGT.CTT180 and CGT.CTT130/129) and had the largest effect ($P < 0.0001$) explained 25.6% of the phenotypic variance for δ^{13}C. Genotypic means for the STS-T1777 locus were −30.18‰ (M82 homozygote), −29.79‰ (heterozygote), and −29.74‰ (IL5-4 homozygote); hence, the IL5-4 allele was dominant to the M82 allele ($d/a = 0.75$), and substitution of the IL5-4 allele made δ^{13}C less negative. SSR49 (which cosegregated with SSR590, CAG.CGT138 and C2_At4g12590) explained 23.4% of the phenotypic variance for δ^{13}C ($P < 0.0001$). Genotypic means for this locus were −30.17‰ (M82 homozygote), −29.74‰ (heterozygote), and −29.77‰ (IL5-4 homozygote). These results again suggest that QWUE5.1 is dominant ($d/a = 1.03$).

To further pinpoint the location of QWUE5.1, informative recombinants were analyzed. The breakpoints of critical recombinants are shown in Fig. 5. Both 5-4-21 and 5-4-8 have a breakpoint in the interval between SSR49 and CGT.CGT130. The mean δ^{13}C values of homozygous recombinants (genotype 1 and genotype 3), and heterozygous recombinants of 5-4-8 (genotype 4) were similar to that of M82, while the mean value of heterozygous recombinants of 5-4-21 (genotype 2) was similar to that of IL5-4. The introgression fragments in 5-4-5 (genotypes 7 and 8) and 5-4-28 (genotypes 5 and 6) span this interval and both homozygous and heterozygous recombinants expressed similar phenotype as IL5-4. Thus, QWUE5.1 appears to reside between the breakpoints of 5-4-8 and 5-4-21, an interval of less than 2.2 cM.

Table 3 Association of markers with carbon isotope composition, δ^{13}C, in the F3 population

Marker	R^2	*df*	*F*	Pr > *F*	*a*	Pr > *t*	*d*	Pr > *t*	|*d*/*a*|
ACC.C70	0.036	47	1.91	0.1600	−0.069	0.2094	−0.125	0.1748	1.81
STS-TG351	0.039	46	2.25	0.1174	−0.080	0.1480	−0.130	0.1599	1.63
TGC.C123	0.039	46	2.25	0.1174	−0.080	0.1480	−0.130	0.1599	1.63
STS-CHS3	0.080	40	4.53	0.0168	−0.144	0.0099	−0.096	0.3058	0.67
AG.CGA73/75	0.080	40	4.53	0.0168	−0.144	0.0099	−0.096	0.3058	0.67
CGA.CGA78	0.080	40	4.53	0.0168	−0.144	0.0099	−0.096	0.3058	0.67
CGA.CTGA73/76	0.080	40	4.53	0.0168	−0.144	0.0099	−0.096	0.3058	0.67
STS-CT80	0.115	42	6.55	0.0034	−0.171	0.0020	−0.111	0.2189	0.65
AG.GCAG120/122	0.115	42	6.55	0.0034	−0.171	0.0020	−0.111	0.2189	0.65
CAPS-CT60	0.115	42	6.55	0.0034	−0.171	0.0020	−0.111	0.2189	0.65
AGC.GCAG120/122	0.115	42	6.55	0.0034	−0.171	0.0020	−0.111	0.2189	0.65
AT.CGT114	0.115	42	6.55	0.0034	−0.171	0.0020	−0.111	0.2189	0.65
CGT.CGT130	0.162	40	9.39	0.0005	−0.196	0.0003	−0.115	0.1878	0.59
SSR49	0.234	30	16.09	<0.0001	−0.203	<0.0001	−0.210	0.0199	1.03
SSR590	0.234	30	16.09	<0.0001	−0.203	<0.0001	−0.210	0.0199	1.03
CAG.CGT138	0.234	30	16.09	<0.0001	−0.203	<0.0001	−0.210	0.0199	1.03
C2_At4g12590	0.234	30	16.09	<0.0001	−0.203	<0.0001	−0.210	0.0199	1.03
STS-T1777	0.256	28	20.30	<0.0001	−0.224	<0.0001	−0.169	0.0514	0.75
STS-T1541	0.256	28	20.30	<0.0001	−0.224	<0.0001	−0.169	0.0514	0.75
CGT.CTT180	0.256	28	20.30	<0.0001	−0.224	<0.0001	−0.169	0.0514	0.75
CGT.CTT130/129	0.256	28	20.30	<0.0001	−0.224	<0.0001	−0.169	0.0514	0.75
CAPS-T1584	0.192	26	10.53	0.0004	−0.211	0.0001	−0.018	0.8690	0.09
C2_At3g55360	0.192	26	10.53	0.0004	−0.211	0.0001	−0.018	0.8690	0.09
CAG.CTA220	0.192	26	10.53	0.0004	−0.211	0.0001	−0.018	0.8690	0.09
ACC.CTC49	0.192	26	10.53	0.0004	−0.211	0.0001	−0.018	0.8690	0.09
AAC.CTT99	0.192	26	10.53	0.0004	−0.211	0.0001	−0.018	0.8690	0.09
AA.CTC179	0.192	26	10.53	0.0004	−0.211	0.0001	−0.018	0.8690	0.09
ACC.CTT99	0.192	26	10.53	0.0004	−0.211	0.0001	−0.018	0.8690	0.09
CAPS-TG69	0.166	22	5.25	0.0137	−0.156	0.0079	0.241	0.1214	1.54
STS-TG413	0.166	22	5.25	0.0137	−0.156	0.0079	0.241	0.1214	1.54

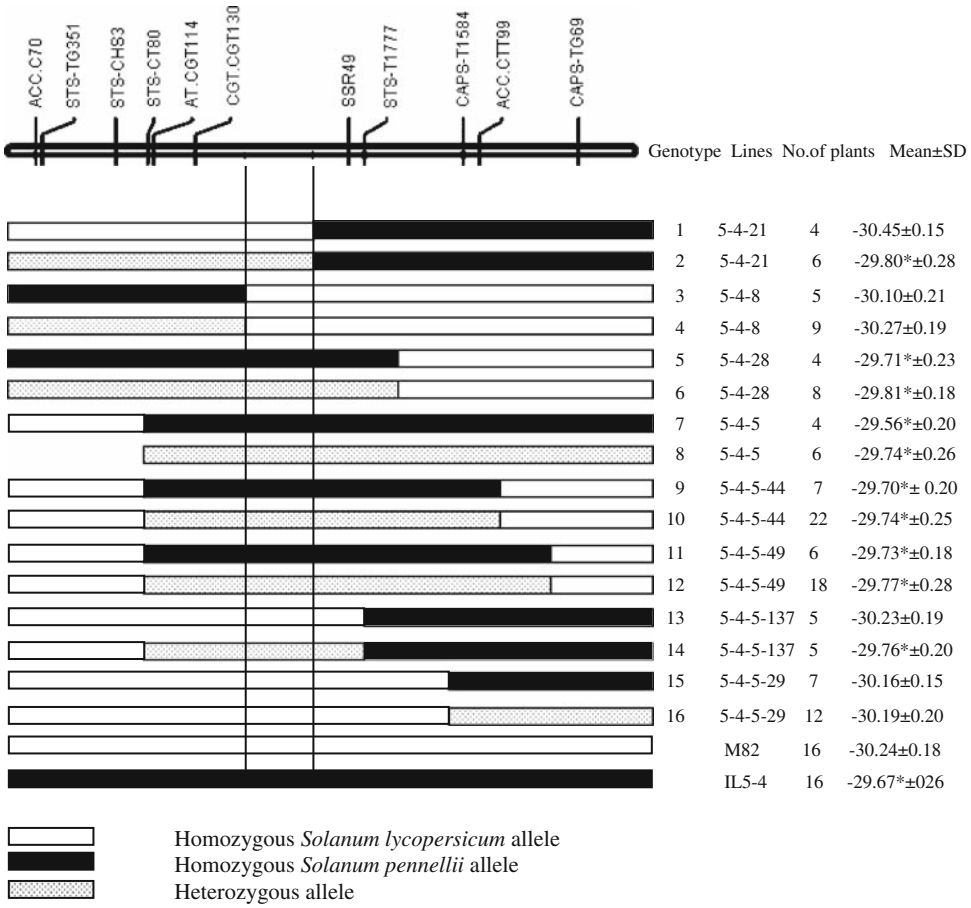

Fig. 5 Graphical genotypes and mean carbon isotope composition (± 1 standard deviation) phenotypes of near isogenic lines. QWUE5.1 was mapped to the interval bracketed by CGT.CGT130 and SSR49. Only one marker is shown at each locus. Genotypes 1–8 are F3s derived from a cross between IL 5-4 and M82, while genotype 9-16 are F5 double recombinants derived from genotype 8. *Significantly different from M82 at $P = 0.05$ using Student's t test

Development and phenotyping of near isogenic lines

Two of the 29 F3 heterzygous recombinants with a short introgression fragment carrying QWUE5.1, 5-4-5 and 5-4-28, were selfed and produced 915 and 210 F4 plants, respectively, and these were screened with family-specific markers (see Materials and methods). Nineteen and two new recombination events were identified in 5-4-5 and 5-4-28 progenies, respectively. The recombination frequencies were about 1% in the 5-4-5 region, and 0.5% in the 5-4-28 region.

Fourteen of the double recombinants produced seed and were characterized with molecular markers. The introgression fragment in these double recombinants ranged from 2 cM in 5-4-5-11 to 9.1 cM in 5-4-5-2 and 5-4-5-4 with a mean length of 6.4 cM (Fig. 3) Following screening, progeny carrying homozygous introgressions were used to found 14 NIL, each with a different introgression fragment.

Those NIL with breakpoints closest to QWUE5.1 on either side were characterized with respect to carbon isotope composition. The two NIL IL5-4-5-44 and IL5-4-5-49 had similar mean δ^{13}C values of −29.70 and −29.73‰, respectively. *Solanum lycopersicon* and IL5-4 had values of −30.24 and −29.67‰, respectively (Fig. 5). These results confirm that the location of QWUE5.1 previously derived from F3 data is correct.

Discussion

Comparison of locations of PCR-based markers developed in this study with those of corresponding RFLP markers

In general, the relative order of CAPS and STS markers developed in this study was consistent with that of corresponding RFLP markers (Fig. 1). The exceptions are the locations of STS-T1777, CAPS-T1541 and STS-CHS3. The RFLP markers T1777 and T1541 were previously mapped proximal to SSR590 (Fig. 1). In this study, both STS-T1777 and CAPS-T1541 were distal to SSR590. In a previous study (Tanksley et al. 1992), CHS3 was located distal to CT80 and TG60, while STS-CHS3 was proximal to STS-CT80 and CAPS-TG60. The genetic map obtained in this study was based on a SCFR population in which each line had only one break point, so the relative ordering of markers obtained should be reliable.

Inheritance of δ^{13}C and genetic improvement of WUE in tomato

Carbon isotope composition (δ^{13}C) provides an indirect measure of the relative variance in leaf-level transpiration efficiency (exchange of CO_2 for water vapor) and WUE

among C3 species. The close correlation with WUE and high heritability make δ^{13}C an ideal surrogate for WUE. The recent release and success of two high WUE wheat cultivars, Rees and Drysdale, for production in rainfed wheat growing regions of Australia demonstrates that it is effective to breed water-use-efficient cultivars by screening δ^{13}C (Richards 2004).

Understanding the inheritance of δ^{13}C is imperative for the development of plant cultivars with high WUE via selecting high δ^{13}C lines. Several studies have demonstrated that genetic variance in δ^{13}C can be attributed to nuclear factors, and QTL for δ^{13}C have been assigned to specific chromosomes in several different species, including barley (Teulat et al. 2002), cotton (Saranga et al. 2001) and rice (Price et al. 2002; Xu et al. 2004). Lambrides et al. (2004) reported a case in which high δ^{13}C and high transpiration efficiency in cultivated sunflower was cytoplasmically inherited. This may be because several genes associated with photosynthesis are encoded in the chloroplast genome and transmitted to progeny through the female parent. Rubisco activase is chloroplast encoded, so it is interesting to note that Zhu et al. (1998), using differential display in tomato, reported that a putative rubisco activase gene conditioned δ^{13}C.

Understanding the mode of gene action would assist in selecting the most suitable breeding strategies. There are several reports on modes of gene action of δ^{13}C, for example in wheat (Ehdaie and Waines 1994; Rebetzke et al. 2006), alfalfa (Johnson and Rumbaugh 1995), maritime pine (Brendel et al. 2002) and black spruce (Johnsen et al. 1999). Predominantly additive gene action, significant general combining ability, and moderate to high-narrow sense heritability were reported in these studies. Apart from additive gene effects, Rebetzke et al. (2006) also reported partial dominance and epistatic gene action in wheat using a half-diallel analysis. In another study in wheat (Ehdaie and Waines 1994) dominance was also detected, and the direction of dominance was toward less negative δ^{13}C. Similarly, predominantly additive gene effects for transpiration efficiency, photosynthesis rate and leaf conductance have been reported in wheat (Carver et al. 1989; Malik et al. 1999; Rebetzke et al. 2003).

In this study, a significant dominance effect was detected for QWUE5.1, suggesting that introgression and fixation of QWUE5.1 could not be readily achieved in selection of individual plants or lines with high δ^{13}C in early generations. Given that the use of δ^{13}C as a breeding target would be costly and inefficient, we propose that a more suitable approach would be to identify molecular markers for δ^{13}C and to use MAS to transfer QWUE5.1 into other cultivars.

MAS is a powerful tool to improve traits for which selection is not readily attainable with conventional methods. In this study, we have located a QTL for WUE in tomato, QWUE5.1, in an interval about 2.2 cM long. SSR49 and SSR590 are less than 2.2 cM from QWUE5.1. The additive effects of these markers on δ^{13}C are 0.203‰, and can be effectively used in MAS of QWUE5.1.

Potential applications of the SCFR population in tomato genomic studies

We have taken advantage of several desirable features of an IL population for QTL mapping, including a clean genetic background, well-defined mapping resolution, and high statistical power (Eshed and Zamir 1995; Fridman et al. 2004). Using this same IL population, Eshed and Zamir (1995) successfully identified twice as many yield-associated QTL as previously reported, and they further developed a set of sub-introgression lines that allowed them to identify three closely linked QTL for yield. This suggests that use of IL populations in QTL analysis can effectively eliminate variances that in other types of populations are not associated with QTL on the introgression fragment of interest. This improves the power to identify QTL.

The SCFR population developed in this study, which consists of 29 second generation and 14 third generation introgression lines, will be valuable for genetic improvement of other traits in tomato. Apart from the QTL for δ^{13}C, the unique introgression chromosome fragment in IL5-4 also harbors QTL for plant height, plant weight, fruit weight, total soluble solids, yield, and total soluble solids × yield (Eshed and Zamir 1995). It also contains a gene controlling self-pruning, and a gene for clear veins (C. Jones, U. C. Davis, personal communication). The homozygous SCFR lines developed in this study divide the target region into nine bins with an average genetic distance of 1 cM. These SCRF lines provide excellent materials to fine map genes/QTL, and the PCR markers developed in this study can be directly used to tag them in marker-assisted selection. Fine mapping and manipulating these genes/QTL in tomato breeding programs is of horticultural and economic importance.

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