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ORIGINAL PAPER

Identification of *Solanum habrochaites* loci that quantitatively influence tomato fruit ripening-associated ethylene emissions

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Abstract The phytohormone ethylene is essential for ripening of climacteric fruits such as tomato. While many of the genes responsible for ethylene synthesis and perception have been identified, the regulatory network controlling autocatalytic climacteric ethylene synthesis is not well understood. In order to better understand the regulation of ripening-associated ethylene, we have exploited the genetic variation within *Solanum* Sect. *Lycopersicon.* In particular, we have used a near-isogenic population of *S. habrochaites* introgression lines to identify chromosome segments affecting ethylene emissions during ripening. *S. habrochaites* fruits produce much larger quantities of ethylene during ripening than do cultivated *S. lycopersicum* tomatoes. A total of 17 segments were identified; 3 had emissions more than twice the level of the tomato parent, 11 had less than a twofold increase and 3 had significantly reduced emissions at one or more ripening stages. While several of these segments co-segregate with known ethylene-related genes, many do not correspond to known genes. Thus, they may identify novel modes of regulation. These results illustrate the utility of wild relatives and their introgression lines to understand regulation of fruit ripening-related processes.

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Introduction

The plant hormone ethylene controls many aspects of growth and development as well as interaction with the environment, including biotic and abiotic stresses (Abeles et al. 1992). Ethylene is synthesized in a two-step process from *S*-adenosylmethionine by the enzymes ACC synthase (ACS) and ACC oxidase (ACO). ACS is generally accepted to be the rate-limiting step in ethylene synthesis (Cameron et al. 1979; Yang and Hoffman 1984). The role of ethylene in mediating ripening of many fruits is particularly well established, based on large part in studies of tomato. The tomato is a climacteric fruit; at the onset of ripening, a large, autocatalytic burst of ethylene synthesis coincides with increased respiration. Blocking either ethylene synthesis or perception prevents ripening (Klee et al. 1991; Oeller et al. 1991; Picton et al. 1993; Wilkinson et al. 1995). Immature fruits produce very low but measurable levels of ethylene. This synthesis, referred to as system 1 ethylene, is relatively constant and is suppressed by exposure to exogenous ethylene. Upon maturation, fruits enter the autocatalytic system 2 phase of ethylene synthesis. This synthesis is the consequence of induction of two ACC synthase genes, *ACS2* and *ACS4*, which are ethylene-inducible specifically during ripening (Rottmann et al. 1991; Barry and Giovannoni 2007). What triggers the transition from system 1 to system 2 ethylene is not well understood. Exposure of immature fruits to ethylene does not induce ripening but does hasten its onset, indicating that the fruit has a mechanism for monitoring cumulative exposure to ethylene (McMurchie et al. 1972;

Yang 1987). Much of our knowledge about ethylene action comes from genetic studies of the model plant *Arabidopsis thaliana* (Wang et al. 2002). Its small size and rapid generation time make it an ideal plant for mutant screens and mutants altered in aspects of the ethylene biosynthesis and response have been invaluable tools for elaborating the signaling pathway for the hormone.

While Arabidopsis is an excellent model system for identifying the genes mediating ethylene synthesis and signal transduction, its fruit anatomy and physiology is vastly different from that of the fleshy, climacteric fruit of *Solanum lycopersicum*, the domesticated tomato. Because of these differences and the singular importance of ethylene to tomato fruit ripening, one would expect tomato to have evolved unique mechanisms to regulate ethylene synthesis during ripening. One approach for elaborating the pathways regulating ethylene-mediated fruit ripening is to exploit natural variation within the genus. There are several sexually compatible species within *Solanum* Sect. *Lycopersicon* with large variations in fruit physiology and ripening (Grumet et al. 1981; Taylor 1986). Wild relatives have been a rich source of many traits that have been introgressed into the domesticated tomato (Eshed and Zamir 1995; Foolad et al. 2002; Frary et al. 2004; Fridman et al. 2004). In a survey of ripening behavior of fruits from wild relatives of tomato, Grumet et al. (1981) identified *S. habrochaites* LA1775 (then, *Lycopersicon hirsutum*) as having particularly unusual properties. The fruits remain green and hard upon ripening. However, fruits exhibit a maturation-associated burst of ethylene synthesis, producing approximately tenfold higher levels than any other species, including the red-fruited climacteric ripening species. This highly unusual pattern of ripening and ethylene synthesis suggested that *S. habrochaites* may be a good source of genes that modify ethylene-dependent ripening in tomato. We therefore initiated screens of *S. habrochaites* germplasm for genes that modify the patterns of ethylene synthesis during ripening. We exploited a set of near-isogenic introgression lines (NILs) derived from LA1777 (Monforte and Tanksley 2000a) to identify multiple chromosome segments that potentially define quantitative trait loci (QTLs) with altered patterns of ethylene emissions.

Materials and methods

Growth of plant materials

A total of 39 NILs derived from a cross between *S. lycopersicum* and *S. habrochaites* (Monforte and Tanksley 2000a) as well as the two parents, *S. lycopersicum* E6203 (LA4024) and *S. habrochaites* LA1777, were analyzed for ethylene emissions. NILs were chosen to cover as much of the *S. habrochaites* genome as possible based on the published information (Monforte and Tanksley 2000a). Certain areas of the genome are not represented in the NILs, presumably due to reproductive behavior and/or pollen viability (Bernacchi and Tanksley 1997; Monforte and Tanksley 2000a).

Plants were grown in randomized, replicated plots over multiple seasons in a greenhouse in Gainesville, Florida or in a field at Live Oak, Florida. Field plants were grown using standard commercial practices in raised plastic mulched beds. Greenhouse plants were grown in 3 Gal pots containing Metro-Mix 500 that were watered twice a day and supplemented with Osmocote 14-14-14 and Osmocote 15-9-12 Sierra Mix slow release fertilizer.

Seeds were obtained from the C. M. Rick Tomato Genetics Resource Center (Davis, CA, USA) and the plants were grown in the field or in the greenhouse as previously described (Mathieu et al. 2008). Fruits were collected from 3 to 12 plants per line, in a various number depending on the stage in which the harvest occurred. Fruits per plant ranged from 1 to 30 fruits per plant, per stage and per growing season.

Ethylene measurements

Fruit ethylene was evaluated over several trials (seasons and locations): fall 2005 greenhouse (GH) University of Florida in Gainesville (UF), spring 2006 field (Live Oak, FL, USA), spring 2006 GH UF, fall 2006 GH UF, and fall 2007 GH UF. Fruits were harvested, left for 1 h in the lab, and then sealed in 500 ml jars for 1–2 h. 1.0 ml of airspace was withdrawn and injected for analysis on a Hewlett Packard 5890 Series II gas chromatograph equipped with a Unibead A80/100 6′ × 1/8″ SS HP 5890 C column (Alltech, Deerfield, IL USA). Ethylene values are expressed as volume (nl) of ethylene per weight of the fruit (g) per time (h). For each season, between two and six plants of each line were grown, with an average of three per line. Each data point represents an average of 20 individual fruits per time point per line. In the first season, tomatoes were collected at breaker (Stage 2) and red (Stage 6) stages. The lines displaying ethylene production differing by at least 50% from the control were repeated in the following seasons with fruits collected at Stages 2 and 6 as well as turning (Stage 3), pink (Stage 4) and early red (Stage 5). The stages were determined by USDA guidelines. Additional lines showing overlap with positive NIL insertions were added to validate and/or localize the locus imparting the phenotype. Further validation for each potential QTL identified in the different seasons was provided by analysis of ethylene production in fruits harvested at Stage 2 and monitored daily up to 8 days following harvest over several seasons. This analysis was performed to exclude false positives related to errors in staging due to irregular fruit pigmentation.

LA1777 fruits were harvested every other day from 48 to 66 days after pollination (DAP) and ethylene measured as described above. To minimize environmental influences to the developmental time course, flowers were hand-pollinated at 2-day intervals over an extended period. Fruits were collected and ethylene measurements performed on a single day.

Statistical analysis

The datasets for all seasons and sites were combined. Statistical analysis was performed as previously presented (Mathieu et al. 2008). Ethylene evolution data were first normalized by log2 transforming the values. In order to deal with non-homogenous data, two-way ANOVA analysis was performed to test whether there were significant differences in ethylene evolution among the NILs, as well as the effect of different trials. Then, for each stage or day, each NIL was compared to the control line (LA4024) using the post hoc Dunett's test to determine if ethylene evolution in an NIL was significantly different from the control line. The resulting raw *P* values were also corrected for multiple tests using the Benjamini and Hochberg false discovery rate (FDR) test. All the statistical analyses were performed using the SAS program (SAS Institute, Cary, NC, USA). The complete dataset is provided in Tables 1S and 2S.

Recombinant isolation and NIL validation

In order to accurately map the introgressed fragments containing candidate genes affecting ethylene synthesis, the insertions of the LA1777 genome into LA4024 genome were checked using a series of Cleaved Amplified Polymorphic Sequences (CAPS) markers. To overcome the genetic heterogeneity within the LA1777 population, DNA from several plants was extracted separately with the CTAB method (Sambrook et al. 1989) and then pooled together and amplified by PCR. For all the other lines several plants were tested independently. The Tomato-EXPEN 2000 (Fulton et al. 2002) and EXPIMP 2008 (Gonzalo and van der Knaap 2008) maps were used as references (http://www.sgn.cornell.edu/). Where possible, marker sequences within introns or untranslated portions of the predicted mRNAs were used. Primers were as described at the website at SOL Genomics Network (SGN) (http://www.sgn.cornell.edu/) or designed with the Genefisher online program (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/) (Giegerich et al. 1996).

PCR was performed as previously described (Dal Cin et al. 2007) with the protocol reported for *Taq* polymerase from Amersham Bioscience (Piscataway, NJ, USA). The amplicons were sequenced with the primers used for amplification. Polymorphic sequences suitable for CAPS mapping were identified using the CAPS designer program available at SGN (http://www.sgn.cornell.edu/).

Scoring of CAPS markers was done by first amplifying the sample and then digesting it with 1.0 U of the appropriate restriction enzyme. Details concerning primer sequences, annealing temperature of the PCR, restriction enzymes used in the digestion reactions and fragment pattern for LA4024 and LA1777 are presented in Table 3S.

The same procedure was followed for genes encoding ACS and ACO (Table 1).

Recombinants of LA4005 containing smaller segments of the *S. habrochaites* genome were obtained by crossing to the *S. lycopersicum* parent, LA4024, and screening 150 F2 plants. This screen was performed with CAPS markers: C2_At5g07960, C2_At4g24690, T1049. The original LA4005 contains two *S. habrochaites* segments, one on chromosome 3 and another on chromosome 6. An F2 line lacking the chromosome 3 segment was designated LA4005-1. The screen identified three distinct recombinants within the chromosome 6 segment. These lines were further mapped using all of the markers shown in Fig. 4. Each line was crossed to LA4024 and 90 F2 plants for each recombinant were rescreened for further recombination events. No further recombinants were identified. Homozygous lines containing the new *S. habrochaites* chromosome 6 segments were designated LA4005-2, LA4005-3 and LA4005-4.

Results

Ethylene biosynthesis in *S. habrochaites*

Previous work on *S. habrochaites* LA1775 indicated that this accession has a highly unusual ripening pattern, producing tenfold higher levels of ethylene in the absence of obvious external signs of ripening (Grumet et al. 1981). We took advantage of a NIL population (Monforte and Tanksley 2000a) created from a cross between *S. habrochaites* LA1777 and the tomato variety E6203 (LA4024) to study the phenotype. Each individual line contains one or more segments of *S. habrochaites* chromosomes introgressed into LA4024. Taken together, most of the *S. habrochaites* genome is represented in the population. As a first step in characterizing the material, we examined the ripening behavior of the *S. habrochaites* LA1777 parent to determine if its ripening behavior is comparable to LA1775. Fruits from LA1777 and LA4024 were collected at 2 and 1 day intervals, respectively for ethylene measurements. Ethylene emissions in the two lines displayed a major difference (Fig. 1). While LA4024 fruits produced a burst of ethylene associated with climacteric ripening that peaked at 48 DAP, the peak of LA1777 ethylene emissions did not

Table 1 CAPS markers for ethylene-related genes

Gene	Acc no.	CAPS information						
		Primer F	Primer R	Length	T (°C)	Enzyme	LA4024	LA1777
ACS1	U72389	GCTACAAATGATGGTCATGGGGAA	TTTCAGCTCCTTCAACAGTGCA	500	54	ApoI	200 + 300	150
ACS2	X59139	GGCTACTAATGAAGAGCATG	TCTAACTCTTCCTCCTCTTG	400	54	DpnII	400	300 + 100
ACS3	L34171	CTATTGTCAGAGAAAGCCACGTGT	GATAGTATGGGGTAGGAAGGAGA	600	55	AluI	210	150
ACO3	Z54199	ACGAGCAATGGATCGATG	ACATCCGAGTCCCCATCTG	500	54	AluI	500	150 + 350

List of genes, accession number (Acc no.), sequences of the forward (F) and reverse (R), primers (5′–3′), product length, annealing temperature (T, °C), restriction enzyme and band patterns in bp for LA4024 and LA1777

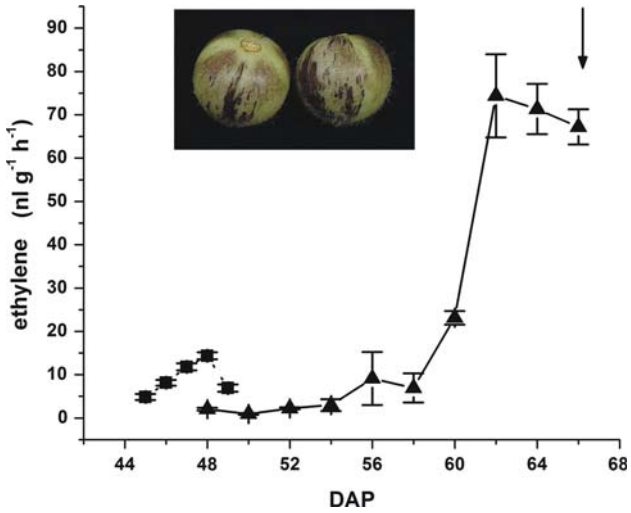

Fig. 1 Ethylene emissions from *S. habrochaites* and *S. lycopersicum* fruits. Fruits from *S. lycopersicum* LA4024 (*square*) and *S. habrochaites* LA 1777 (*triangle*) were harvested at 1 and 2-day intervals, respectively after pollination (DAP) and ethylene emissions were determined as described in "Materials and methods". The *arrow* indicates abscission of *S. habrochaites* LA 1777 fruits. *Bars* indicate standard error. The *inset* shows a picture of ripe *S. habrochaites* fruits harvested at 62 DAP

occur until 62 days. Maximum emissions were approximately fivefold greater than those of LA4024 (74.4 vs. 14 nl/g per h). In contrast to LA1775, the large ethylene peak at 62 days was accompanied by visible signs of ripening; fruits became less green, pronounced anthocyanin spots were visible at ripening, and fruits that abscised at day 66 presented diffused cracking (Fig. 1). Based on these differences, we went forward with a screen for ripening-associated loci affected in ethylene emissions.

Identification of NILs with altered fruit ethylene emissions

In order to identify regions of the *S. habrochaites* genome with altered ethylene emission patterns, a set of lines providing maximum coverage of the genome were selected and grown in a greenhouse as described in "Materials and methods". Ethylene emissions were measured at Stages 2 and 6 (Breaker and Red Ripe, respectively) for each line. A number of possible loci were identified. These lines as well as NILs containing overlapping segments of *S. habrochaites* DNA were repeatedly grown over multiple seasons to validate and delimit the positions of each locus. Statistical analysis was performed on the combined results for all seasons on the different stages (2–6). Lines with corrected *P* value less than 0.05 were scored as having a QTL affecting ethylene synthesis. Twenty-six lines were identified as having significantly different ethylene emissions, of which seven lines produced at least twice the amount of ethylene of the control: LA3922, LA3923, LA3935, LA3937,

LA3944, LA3945, LA3969 and LA4005. Overall, no line emitted as much ethylene as LA1777. These results are presented in Table 1S and summarized in Fig. 2.

All of the lines that were at least 20% different from the control were further investigated by measuring daily ethylene emissions starting at Stage 2 (breaker). Statistical analysis was performed independently for each day (Table 2S). In this way, we were able to identify a total of 17 chromosomal regions potentially corresponding to QTLs. Of these, 3 had emissions more than twice the level of LA4024, 11

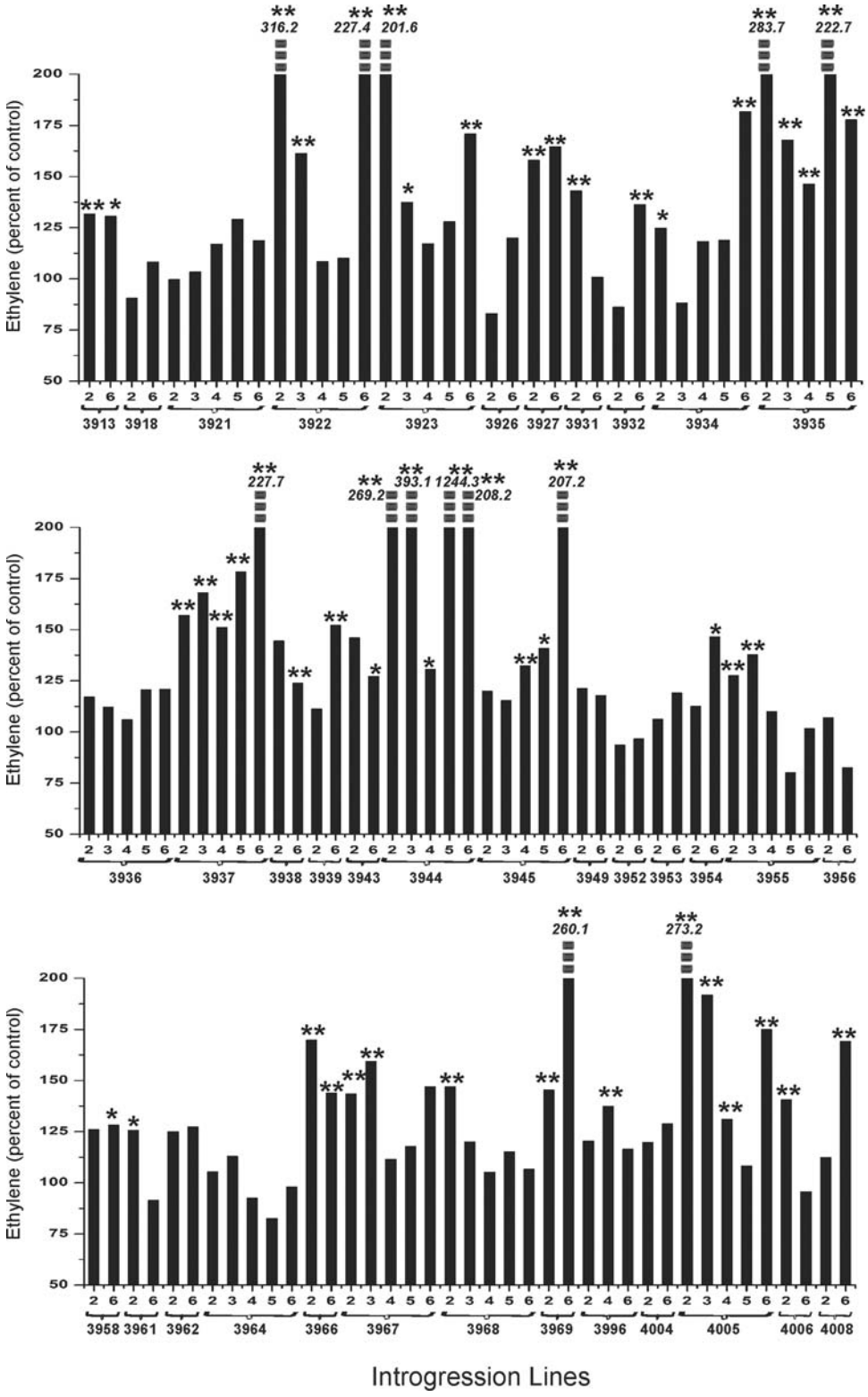

Fig. 2 Ethylene emissions of fruits from NILs. Values are presented as percent of control (LA4024) for each line at the indicated ripening stages (1–6). *$P < 0.05$ and ** corrected $P < 0.05$. For lines in excess of 200% of control (*dashed columns*) the actual values are indicated

had less than a twofold increase and 3 had significantly reduced emissions at one or more ripening stage (Table 3; Table 2S). There was significant variation among the group in the patterns of ethylene difference between the line and the control (Table 2; Figure S1). Some lines exhibited large differences throughout ripening; some were different mainly at the early stages; in some the differences were higher at the later stages of ripening.

Some of the QTLs exhibited fruit-related phenotypes that may or may not be related to ethylene over-production. Locus 2B at the bottom of chromosome 2, defined by NILs 3922 and 3923, exhibits altered carotenoid synthesis during ripening (Mathieu et al. 2008). Fruits are yellow in appearance but otherwise normal in ripening. The gene associated with this carotenoid deficiency has yet to be determined. Locus 4C at the bottom of chromosome 4, defined by NILs 3935 and 3937, has extensive fissures in the fruit cuticle. The gene responsible for this abnormality, *Cwp1*, has been identified (Hovav et al. 2007). Fruits containing *Cwp1*, in addition to cuticle scarring, are susceptible to water loss. The high ethylene associated with locus 4C may be due to the stress associated with *Cwp1*.

Table 2 Summary of loci affecting ripening-associated ethylene synthesis

Segment	Lines	Pattern	Max dif (%)	SE
1	LA3913	H2	150	8.7
2A	LA3921, LA3924	H1	178	17.7
2B	LA3922, LA3923	H3	144	11.9
3A	LA3925	L3	81	9.8
3B	LA3927	H1	189	7
4A	LA3932	H2	139	7.6
4B	LA3934, LA3936	H3	188	19.9
4C	LA3935, LA3937	H1	227	15.4
5	LA3939, LA3943	H3	145	7.3
6	LA3944, LA3945, LA4005	H1	338	13.6
8A	LA3952	L2	74	12.8
8B	LA3955	H2	148	26.6
10A	LA3961, LA3962, LA3966	H3	208	5.9
10B	LA3964	L1	81	9.5
11	LA3958, LA3967, LA3996	H1	166	6.4
12A	LA3968	H2	158	18.5
12B	LA3969	H3	158	6.3

Each locus was identified with a number indicating the chromosome and a letter from the top to the bottom of the chromosome. Loci were classified as having either high (H) or low (L) ethylene synthesis. Tested lines containing each chromosome segment are indicated. Subcategories were characterized by the ratio of NIL to control through ripening: constant across ripening (1), decreasing (2), increasing (3). The maximum statistically significant difference from control during fruit ripening is reported (max dif, %) as well as the standard error (SE)

Improved mapping of NILs

Although the LA1777 NIL population has been very useful to the research community, many of the introgressed segments were mapped with a minimal number of markers. To improve the utility of the population to the community, we used CAPS markers to improve the mapping of the recombination break points for certain NILs (Table 3S). A total of 12 lines were mapped with greater precision (Table 3). These more precise end points were incorporated into the map in Fig. 3. It should be noted that at this time we can neither eliminate the existence of multiple loci within an introgressed DNA segment nor can we eliminate the possibility that there are additional small, unmapped portions of *S. habrochaites* DNA in some lines.

The chromosome 6 locus exhibits the single largest effect on ethylene synthesis. In order to more precisely localize the QTL, we identified recombinants that reduced the size of the IL segment. Approximately, 600 F2 seedlings from the cross of LA4005 to LA4024 were screened and several independent recombinant lines identified (Fig. 4). Phenotypic characterization of the recombinants allowed us to place the QTL between approximately 53 and 63 cM. It should be noted that LA4005 has two *S. habrochaites* segments, located on chromosomes 3 and 6. The recombinants shown in Fig. 4 contain only the indicated chromosome 6 segments.

Genes located within NILs

The approximate map positions of many genes involved in ethylene synthesis and perception have been previously identified (Giovannoni et al. 1999). To identify whether any obvious candidate genes are localized to the NIL segments associated with the chromosome segments, we developed CAPS markers that distinguish between the two parents for several ACS and ACO genes (Table 1) and determined whether the *S. habrochaites* or *S. lycopersicum* locus was present. A bioinformatic analysis on the tomato genome was also performed for genes that have been precisely mapped by the genome sequencing program. The mapping results indicated that several *ACS* and *ACO* genes are linked to the identified chromosome segments (Fig. 3). Linked genes included *ACS3* (2B), *ACS5* (8A), *ACO4* (2A) and *ACO1* (12A). The other *ACO* gene involved in ripening, *ACO3* (Barry et al. 1996) was mapped to chromosome 7 by its presence in LA3985 but no QTL was linked to this gene. Notably, the two genes associated with *S. lycopersicum* fruit ripening ethylene synthesis, *ACS2* and *ACS4*, are not linked to any of the ethylene QTLs.

Table 3 Mapping points of recombination in NILs

Line	Chr	Start		End	
		Marker	cM	Marker	cM
LA3913	1	cLED-15-I5/C2_At3g04710	87/95	C2_At4g34700/TG27	160/165
LA3922	2	C2_At5g66530/ovate	87.5/89	C2_At1g67730/ND	143/ND
LA3923	2	C2_At3g02300/C2_At1g19690	111.8/113	C2_At1g67730/ND	143/ND
LA3924	2	C2_At4g21580/T1585	69/69	C2_At5g66530/ovate	87.5/87.5
LA3934	4	TG264/C2_At4g09010	75/83	C2_At1g75350/P33	120.5/121
LA3935	4	T1600/C2_At1g27530	85.5/88.3	TG498/ND	135.7/ND
LA3936	4	T1600/C2_At1g27530	85.5/88.3	C2_At1g46480/SSR146	101.5/102.2
LA3937	4	C2_At1g46480/SSR146	101.5/102.2	TG498/ND	120.5/121
LA3944	3	TG324/C2_At1g28530F	4.6/21	T1388/cLPT-2-E21	47/61
LA3944	6	C2_At1g22850/C2_At1g73885	46/48	C2_At1g65700/ C2_At5g22620	73/74
LA4005	3	TG324/C2_At1g28530	4.6/21	T1388/cLPT-2-E21	47/61
LA4005	6	C2_At1g22850/C2_At1g73885	46/48	C2_At1g65700/ C2_At5g22620	73/74
LA3945	6	T1188/TG178	3/10	C2_At4g24690/C2_At1g24360	62.5/62.7
LA3945	10	C2_At3g09740/T1521	71/76	TG233/ND	86//ND
LA3967	11	TG497/CT269	0/11		

CAPS markers were used to determine the break points in the indicated lines. Positions are reported as cM and refer to the Tomato-EXPEN 2000 map (http://www.sgn.cornell.edu/). Each point is comprised by two CAPS markers the positions of which are reported in cM. In each case the first marker listed for start points and the second marker for end points identifies the presence of *S. lycopersicum* DNA while the other paired marker identifies *S. habrochaites* DNA. *ND* Not determined (chromosome end point)

Dominance effects

To provide further insight into the identified loci, some of the lines were crossed to LA4024 and fruits from the hybrid F1 plants were examined for ethylene emissions (Table 2S). QTL 2A was dominant, QTLs 2B, 6 and 11 produced intermediate levels of ethylene production while QTL 4C showed a recessive phenotype. The three QTLs that are co-dominant likely express gene products stimulating ethylene synthesis in a gene dosage-dependent manner. Such a phenotype would be consistent with, for example, higher expression of an *ACS* gene or to a difference in protein activity or stability. During the design of the CAPS markers, multiple differences between LA1777 and LA4024 were observed within open reading frames (data not shown). The recessive 4C locus may encode a gene product that acts to suppress ethylene synthesis.

Discussion

The wild relatives of tomato provide a rich genetic pool for variation in many different fruit traits (Grandillo et al. 1999), including color (Liu et al. 2003), composition (Rousseaux et al. 2005; Schauer et al. 2006), size (Lippman and Tanksley 2001) and volatile emissions (Tieman et al. 2006). The wild relatives mainly utilized are *S. pennellii* (Eshed and Zamir 1995) and *S. pimpinellifolium* (Tanksley et al. 1996; Grandillo and Tanksley 1996) whose introgression lines are well characterized both genetically and phenotypically. The *S. habrochaites* NILs have not been extensively exploited but are potentially very useful as a source of traits (Bernacchi et al. 1998; Mathieu et al. 2008; Monforte and Tanksley 2000b). *Solanum* Sect. *Lycopersicon* species are widespread throughout central and South America in a diversity of habitats. As a consequence of variation in form and habit, it is likely that different species have evolved different molecular mechanisms for controlling fruit growth, maturation and seed dispersal. By moving individual components of a complex regulatory framework out of their normal context, we can potentially uncover novel aspects of this framework. Here, we have exploited genetic diversity to elaborate the control of fruit development and ripening. Availability of a NIL population (Monforte and Tanksley 2000a) greatly facilitates this gene discovery process, although the large size of some introgressed DNA segments may mask some complex events (Causse et al. 2007).

Ethylene synthesis is essential for tomato fruit ripening. Upon maturation, system 2 ethylene synthesis autocatalytically increases by as much as 100-fold. Two ACC synthases, ACS2 and ACS4, account for most of the ethylene

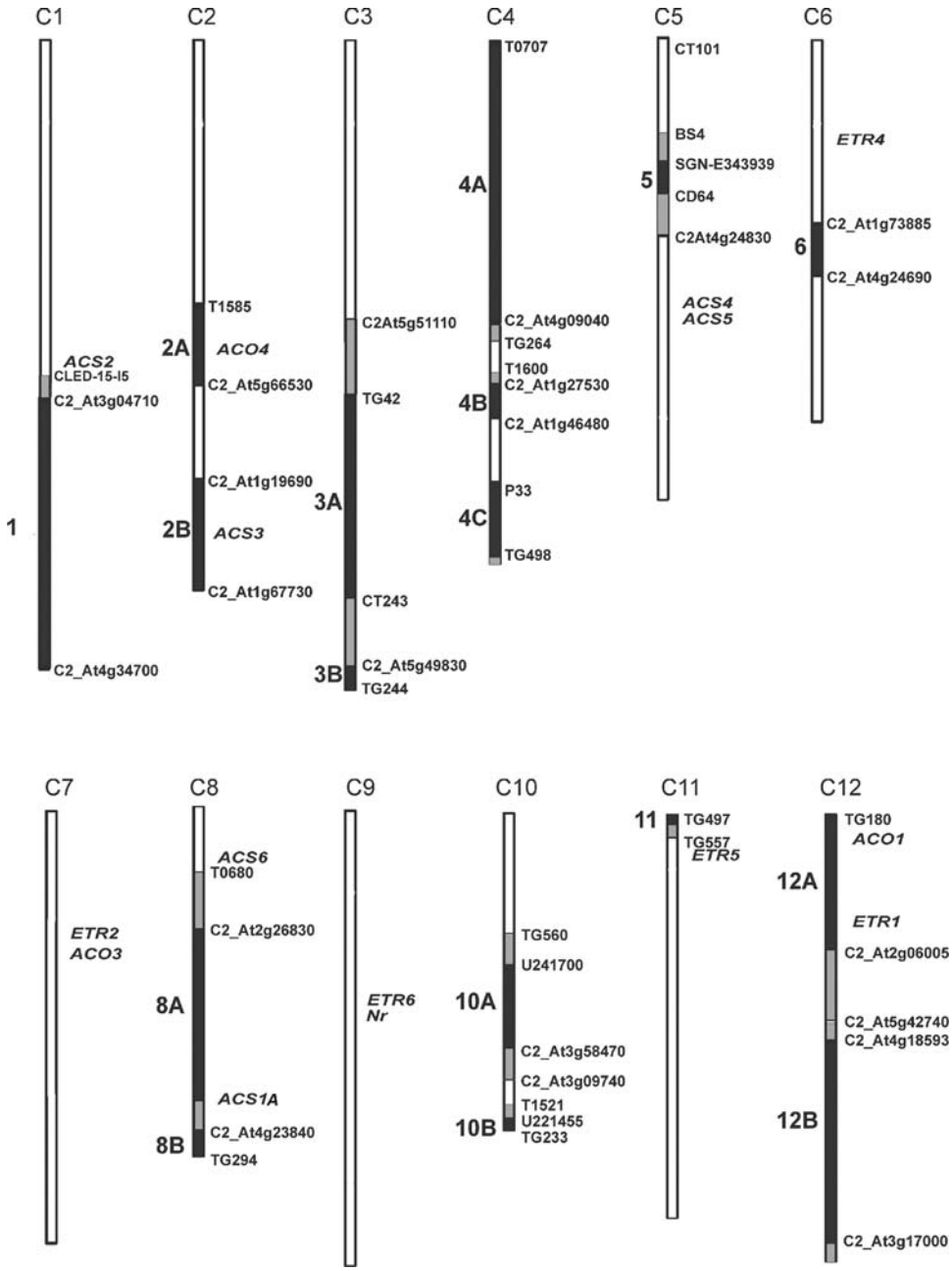

Fig. 3 Map positions of ethylene-associated QTLs. Distance in cM for each chromosome is depicted on the *left side*. The defined introgressed segment for each QTL is shown in *black*. The end points of each introgressed segment as defined by the closest marker present and absent, are indicated in *gray*. Markers used to define each end point are shown and the actual CAPS markers are defined in Table 3S. Map positions of known ethylene-related genes are indicated

synthesis (Rottmann et al. 1991; Nakatsuka et al. 1998). Precisely how the fruit initiates system 2 ethylene is not fully established. We have used the natural variation in the genus to identify candidate genes regulating ethylene synthesis during fruit ripening. Starting with the green-fruited *S. habrochaites*, we identified QTLs that alter fruit ethylene emissions. *S. habrochaites* was an obvious candidate to initiate such a search since the fruits make approximately sevenfold more ethylene at the peak of ripening than the fruits of *S. lycopersicum*. Thus, it was not surprising that we identified multiple QTLs that, when introgressed, cause significant increases in ethylene production. None of the NILs produce nearly as much ethylene (on a fresh-weight basis) as the *S. habrochaites* parent, although the NIL on chromosome 6 does cause over a threefold increase in fruit ethylene. The large number of QTLs that we identified indicates highly complex regulation of ethylene synthesis. It is also likely that there are compensatory mechanisms that prevent runaway ethylene synthesis in *S. lycopersicum* fruits at play. Understanding how the multiple genes interact to influence ethylene synthesis will provide invaluable information about the overall regulation of ethylene-mediated ripening. Since the approximate map positions of most of the currently known genes encoding biosynthetic and perception functions are known (Giovannoni et al. 1999), and many of the presently identified QTLs do

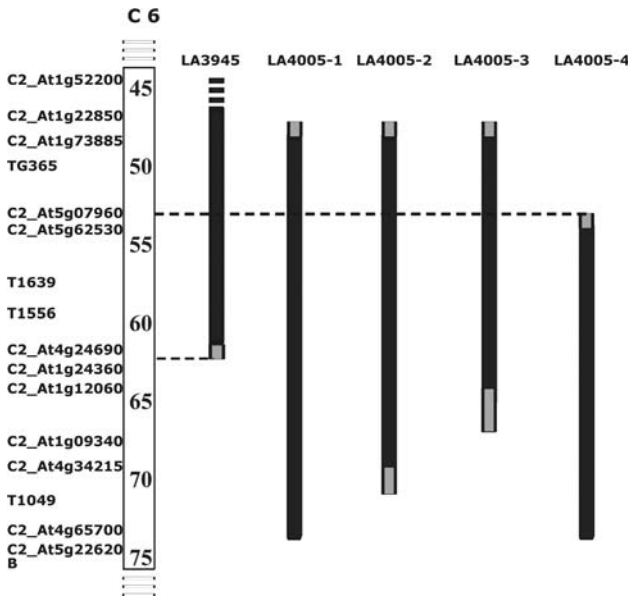

Fig. 4 Recombinant-mapping of QTL6. Recombinant lines obtained from crossing LA4005 to LA4024 as well as the overlapping LA3945 are presented (1–4). On the *left*, schematic representation of chromosome 6 with distance in cM is depicted. The introgressed segment is shown in *black*. The end points of each introgressed segment, as defined by the closest marker present and absent, are indicated in *gray*. Markers used to define each end point are shown and the actual CAPS markers are defined in Table 3S. Map positions for QTL 6 are delimited by *dashed lines*

not overlap with these genes, it is likely that unknown factors will emerge.

Several ethylene-related genes are located on the NIL bins containing the QTLs described here. Two obvious candidates, *ACS2* and *ACS4*, responsible for *S. lycopersicum* ripening-associated ethylene synthesis, do not co-segregate with any of the QTLs. Two other *ACS* genes, *ACS1* and *ACS3*, are present on the NIL segments that define locus 8A and locus 2B, respectively. Although ACO is not believed to be the rate-limiting step in ethylene synthesis, genes encoding *ACO1* and *ACO4* also co-segregate with the 12A and 2A loci, respectively. That none of the genes most responsible for ripening-associated ethylene are obvious candidates is not entirely surprising. High expression of one of the *S. habrochaites ACS* alleles not normally associated with fruit ripening is an obvious possible way to achieve higher ethylene emissions in one or more QTLs.

We have separately determined the timing of ripening in the same set of NILs (Dal Cin and Klee, manuscript in preparation). Higher levels of ethylene produced during ripening would not be expected to cause faster ripening. Our extensive work with reduced ethylene synthesis using ACC deaminase (Klee et al. 1991) or antisense *ACS2* constructs indicated that only when ethylene was reduced by 90% or more is there a delay in ripening (Klee, unpublished). This result indicates that in fruits of cultivated *S. lycopersicum* varieties, ethylene is made far in excess of what is needed for optimal ripening. Additional ethylene, therefore, would not be expected to accelerate the ripening process. However, higher ethylene production in immature fruits could initiate the ripening program earlier (Yang 1987). Several of the QTLs (2A, 4B, 4C, 6 and 11) had significantly higher ethylene emissions in immature fruits (data not shown). Of those QTLs, 4B and 4C, but not the others, ripened significantly earlier than control fruits (Dal Cin and Klee, manuscript in preparation).

In summary, we have identified multiple chromosome segments that significantly alter ethylene emissions during tomato fruit ripening. Many of these newly defined segments do not contain known genes involved in ethylene synthesis. Thus, they may identify novel modes of regulation. With an emerging genome sequence in combination with recombinants that reduce the NIL segments, identification of the causative genes should be possible in the near future.

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