Candidate Genes and Quantitative Trait Loci Affecting Fruit Ascorbic Acid Content in Three Tomato Populations

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Fresh fruit and vegetables are a major source of ascorbic acid (vitamin C), an important antioxidant for the human diet and also for plants. Ascorbic acid content in fruit exhibits a quantitative inheritance. Quantitative trait loci (QTL) for ascorbic acid content have been mapped in three tomato populations derived from crosses between cultivated tomato varieties (*Solanum lycopersicum* accessions) and three related wild species or subspecies. The first population consists of a set of introgression lines derived from *Solanum pennellii*, each containing a unique fragment of the wild species genome. The second population is an advanced backcross population derived from a cross between a cultivated tomato and a *Solanum habrochaites* (formerly *Lycopersicum hirsutum*) accession. The third population is a recombinant inbred line population derived from the cross between a cherry tomato line and a large fruited line. Common regions controlling ascorbic acid content have been identified on chromosomes 2, 8, 9, 10, and 12. In general, the wild alleles increased ascorbic acid content, but some improvement could also be provided by *S. lycopersicum*. Most QTLs appeared relatively stable over years and in different environments. Mapping of candidate genes involved in the metabolism of ascorbic acid has revealed a few colocations between genes and QTLs, notably in the case of a monodehydroascorbate reductase gene and a QTL present in two of the populations on chromosome 9 (bin 9-D), and a previously mapped GDP-mannose epimerase and a QTL on chromosome 9 (bin 9-J).

Fresh fruit and vegetables are the principal source of ascorbic acid (vitamin C) for humans, primates, and a few other mammals and passerines who are unable to synthesize this vitamin because of mutations in the enzyme catalyzing the final step of its biosynthesis, L-gulono-1,4-lactone dehydrogenase. The vitamin has numerous properties, including as an antioxidant and an enzyme cofactor, for example in collagen synthesis (Arrigoni and De Tullio, 2002). Ascorbic acid is also an essential compound for plants, having a primary role as an antioxidant, preventing oxidative stress as well as playing a part in plant development and hormone signaling (Pastori et al., 2003), the activation of the cell cycle (Potters et al., 2002), and possibly cell wall loosening during cell expansion or fruit ripening (Fry, 1998).

In plants, the major ascorbic acid biosynthesis pathway involves activated forms of the sugars GDP D-Man, GDP L-Gal, and L-Gal before L-galactono-1,4-lactone is finally derived and converted to L-ascorbic acid (Fig. 1; Wheeler et al., 1998; Valpuesta and Botella, 2004; Wolucka et al., 2005). The identification of low ascorbic acid (vtc) mutants in Arabidopsis (*Arabidopsis thaliana*; Conklin et al., 2000) has helped to confirm the intermediates of the pathway and the essential role of enzymes such as GDP-Man pyrophosphorylase (GMP; vtc1; Conklin et al., 1999) and L-Gal-1-P phosphatase (vtc4; Conklin et al., 2006). An alternative pathway has been proposed that uses GDP gulose and L-gulose instead of the corresponding Gal sugars (Wolucka et al., 2005), and in strawberry (*Fragaria* spp.), a third pathway has been identified involving the conversion of D-GalUA to L-ascorbic acid via L-galactono-1,4-lactone (Agius et al., 2003). Hormones such as methyl jasmonate may also have a role in the induction of ascorbate biosynthesis, as this hormone has been shown to induce the expression of both GDP-Man 3′,5′-epimerase and a putative L-gulono-1,4-lactone dehydrogenase gene in tobacco (*Nicotiana tabacum*) Bright-Yellow 2 cells (Wolucka et al., 2005). A final possible biosynthetic route for ascorbic acid may use myoinositol as the initial substrate; ascorbate levels increased 2- to 3-fold in Arabidopsis lines overexpressing the myoinositol oxygenase gene (Lorence et al., 2004).

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Figure 1. Major synthesis and recycling pathways for ascorbic acid in plants. HK, Hexokinase; PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannose mutase; GPP, Gal-1-P phosphatase (vtc4); GDH, Gal dehydrogenase. Mapped genes appear in gray circles, genes mapped in this study are starred. Genes in white circles have not yet been mapped.
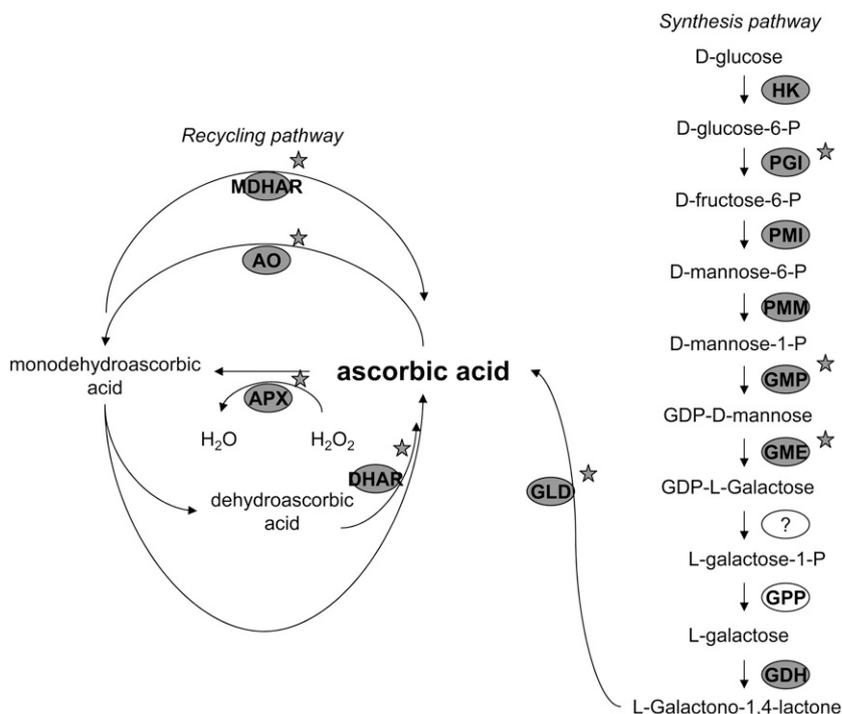
A recycling pathway also exists for ascorbic acid; because of its role as an antioxidant, reduced ascorbate is oxidized into an unstable radical (monodehydroascorbate), which dissociates into ascorbate and dehydroascorbate, the latter representing the second oxidized form. Dehydroascorbate is also unstable and rapidly degrades so the ascorbate pool can be depleted if the oxidized forms are not recovered by two reductases: monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR; Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). Modulation of DHAR activity may control the levels of ascorbate in tissues. Overexpression of this enzyme in tobacco increased ascorbic acid levels 2- to 4-fold (Chen et al., 2003) and significantly increased the ascorbate redox state (Chen et al., 2003; Kwon et al., 2003). The regulation of ascorbate levels in cells is therefore tightly controlled by the level of synthesis and recycling as well as degradation (Pallanca and Smirnoff, 2000; Green and Fry, 2005) and the transport of this molecule within the cell (Horemans et al., 2000), although little is known about the details of the latter two processes.

Tomato is not only a major crop but a model for fruit development with a wealth of data available at physiological and genetic levels. Research on this species is set to continue with the current genome sequencing project (Mueller et al., 2005). The plant lends itself to studies on fruit architecture, ripening, and all aspects of fruit quality; for this reason, different populations have been created and evaluated (Eshed and Zamir, 1995; Causse et al., 2002). Improvement of vitamin content in species of agronomic interest is also cited as an important criterion and wild species are a source of

variability for improving this and other fruit traits. Indeed, wild tomato accessions are rich in ascorbic acid, a quality that has been lost in many commercial varieties, which contain up to 5 times less ascorbic acid, although small-fruited varieties are richer in this vitamin than are standard varieties (Stevens, 1986).

Tomato fruit characteristics (size and composition) usually exhibit quantitative variation controlled by several genes, more or less influenced by the environment. Molecular markers allow the dissection of such quantitative traits into discrete quantitative trait loci (QTL), which can be located on a genetic map (Tanksley, 1993; Saliba-Colombani et al., 2001). Many QTL studies have been performed for fruit traits in tomato and reveal more than 30 QTL regions involved in the variation of fruit size (Grandillo et al., 1999) or soluble solid content (Fulton et al., 2002). The existence of a QTL in a chromosomal region means that at least one polymorphic locus is segregating in this region and is responsible for part of the trait variation. The characterization of QTLs may be attempted either through positional cloning (Frary et al., 2000) or through the candidate gene approach, which consists in looking for genes segregating around a locus, which are putatively responsible for the variation of a trait (Pflieger et al., 2001).

This study presents the genome location of QTLs for ascorbic acid detected in three populations derived from crosses involving three different species (or subspecies) related to the cultivated tomato. The first population (IL-pen) consists of a set of introgression lines derived from *Solanum pennellii*, each containing a unique fragment of the wild species genome. This population has already been used for mapping

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candidate genes and QTLs for carotenoids (Liu et al., 2003), fruit weight and composition in sugars and acids (Causse et al., 2004), antioxidant compounds (Rousseaux et al., 2005), volatile aromas (Tadmor et al., 2002), or various metabolites (Schauer et al., 2006). The second population (BC-hab) is an advanced backcross population derived from a cross between a cultivated tomato and a *Solanum habrochaites* (formerly *Lycopersicum hirsutum*) accession. The third population (RIL-cherry) is a recombinant inbred line population derived from the cross between a cherry tomato line and a large fruited line (Saliba-Colombani et al., 2001). In some cases, the QTL analysis was repeated over several years, enabling the stability of the QTLs to be assessed. Most of the known genes of the ascorbic acid biosynthesis or recycling pathway were mapped and colocations between the genes and QTLs analyzed. A candidate gene approach, using the genes of the biosynthesis and recycling pathways, has therefore been used as a first step toward identifying the key genes underlying the QTLs for ascorbic acid.

RESULTS

Genetic Variation of Ascorbic Acid Content

The variation of ascorbic acid content in whole fruit was evaluated in the three segregating populations: IL-pen, BC-hab, and RIL-cherry ("Materials and Methods"). The three wild accessions had ascorbic acid contents higher than the *Solanum lycopersicum* lines when expressed relative to fresh weight (Table I). When expressed as a percentage of dry matter weight, the ascorbic acid content of PI24 (*S. habrochaites*) remained higher than that of the cultivated accession Ferum, but the ascorbic acid content of Cervil was lower than that of Levovil. The ascorbic acid content per dry matter weight of the LA716 accession *S. pennellii* was over double that of M82. Figure 2 shows the distributions of ascorbic acid content in the three populations. In IL-pen, a continuous variation was observed, with a maximum for IL9.1.3, which showed a maximum ascorbic acid content double that of M82 (Table II; fresh weight value). In BC-hab, the two sets of lines grown in the same greenhouse conditions over two successive years had the same range of variation, and the ascorbic acid content of the 17 lines that were

grown both years were highly correlated ($r = 0.83$; see "Materials and Methods"). The RIL-cherry population showed the largest range of ascorbic acid concentrations of the three populations. The ranges of variation were consistent with the proportion of wild species genome in each population (about 4% in IL-pen, 21% in BC-hab, and 50% in RIL-cherry). Several transgressive lines were observed in each population.

QTL Mapping

Figure 3 summarizes the QTL locations detected in the three populations. Each IL line contains a unique genome fragment of the wild species LA716 (*S. pennellii*) in the genome of M82, an *S. lycopersicum* accession, dividing the genome into 107 bins (Pan et al., 2000). In IL-pen, the ascorbic acid content of 17 lines was different from that of M82, defining 12 QTLs (Table II). For nine QTLs, the *S. pennellii* allele increased the ascorbic acid content, in contrast to the three others. The QTL effects ranged from 20% lower than M82 to 100% higher. Eight lines that showed the greatest effects were grown during two other trials, one in the greenhouse, the other in the field. Figure 4 shows the variation of the introgression effect over the three trials. Three QTLs were detected during the three trials, four were detected during two trials, and the QTL on bin 10-D was only detected in 2000. Although under very different conditions, the autumn greenhouse trial did not give results that were very different from the field trials.

The BC-hab population was grown for 2 years. The correlation between ascorbic acid content during the two trials for the 17 families grown in common was highly significant ($r = 0.83$); thus, QTLs were first detected on each dataset separately and then on the whole dataset containing normalized data from all the families. Five chromosome regions showed an effect on ascorbic acid (Table III). QTLs on chromosome 9 and 11 were only detected in 2001, and the QTL on chromosome 10 was only detected for ascorbic acid expressed as a percentage of dry matter. For most of the QTLs, the *S. habrochaites* allele increased ascorbic acid content, except on chromosome 2. The QTL on bin 8-B showed the strongest and most repeatable effect.

Six QTLs were detected in the RIL-cherry population (Table IV). The cherry allele had a positive effect

Table I. *Characteristics of the parental lines for the three populations and range of variation in each progeny for ascorbic acid content*
Mean values are shown with SD in parentheses.

| Accession	Ascorbic Acid	Ascorbic Acid	Fruit Weight	Dry Matter Weight	Progeny Ascorbic Acid Range
	mg/100 g fresh weight	*mg/g dry weight*	*g*	*%*	*mg/100 g fresh weight*
LA716 (*S. pennellii*)	70.7 (1.7)	6.8 (0.19)	3	10.4 (0.14)	10.8 (1.32)–27.3 (2.18)
M82 (*S. lycopersicum*)	13.6 (1.61)	2.42 (0.25)	61 (3.10)	5.60 (0.28)	
PI24 (*S. habrochaites*)	24.93 (2.83)	1.82 (0.31)	2 (0.21)	13.70 (0.58)	8.2 (0.91)–24.7 (2.78)
Ferum (*S. lycopersicum*)	10.67 (2.60)	1.47 (0.28)	122 (13.2)	7.26 (0.56)	
Cervil (*S. lycopersicum* cv *cerasiforme*)	23.68 (2.65)	2.24 (0.21)	7 (0.30)	10.59 (0.54)	12.5 (0.33)–29.4 (9.11)
Levovil (*S. lycopersicum*)	14.01 (1.21)	2.61 (0.13)	125 (6.21)	5.35 (0.28)	
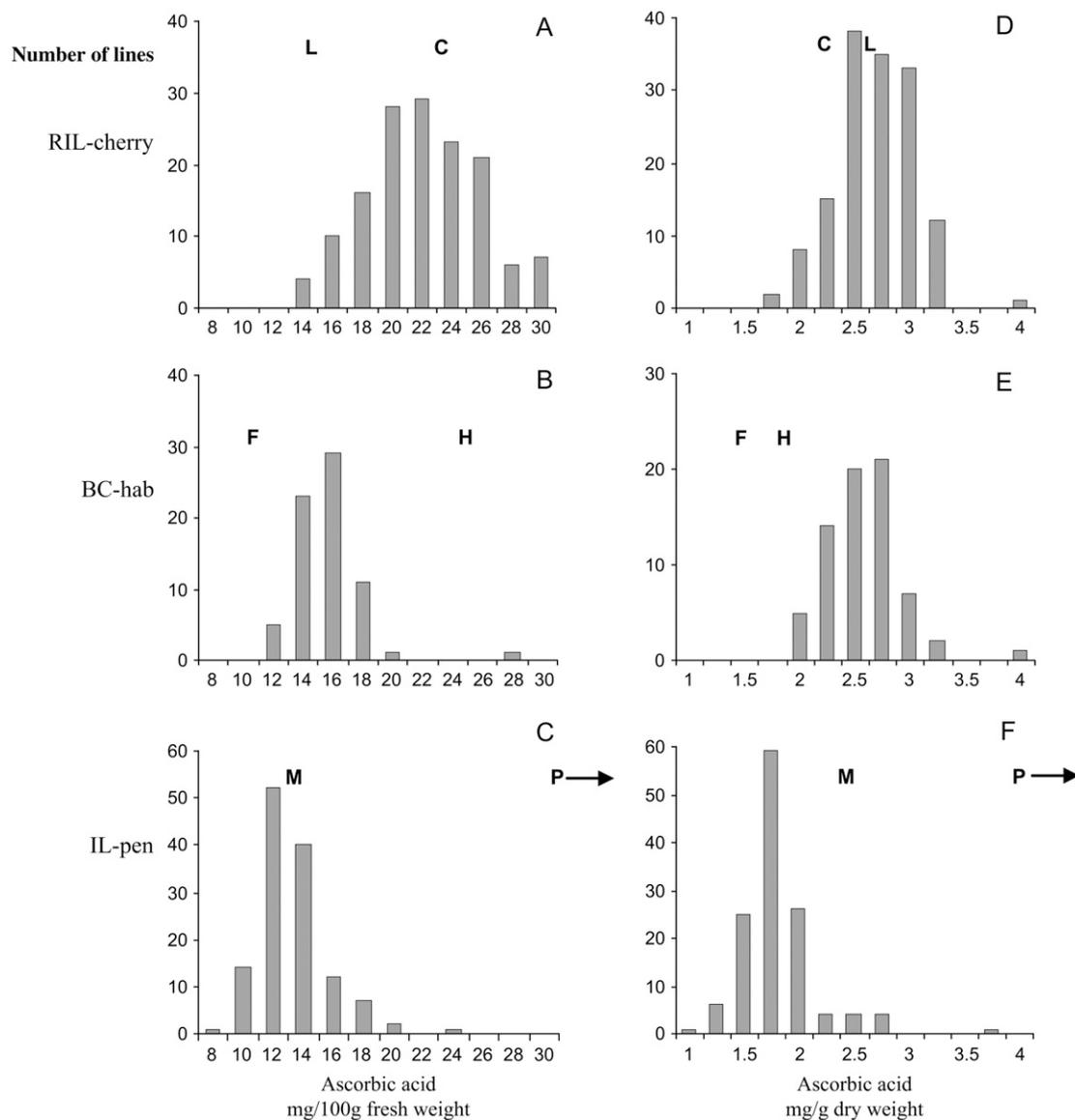
Figure 2. Distribution of ascorbic acid content in the three populations studied. Histograms (A–C) in comparison with fruit fresh weight and histograms (D–F) in comparison with fruit dry weight. A and D, A total of 144 recombinant inbred lines from a cross between a cherry tomato line and an *S. lycopersicum* accession. B and E, The 130 BC2 families derived from the backcross of *S. habrochaites* and *S. lycopersicum*. C and F, Seventy-five introgression lines of *S. pennellii*. The mean value of the parents is indicated (L, Levovil; C, Cervil; F, Ferum; H, *S. habrochaites* PI24; M, M82; P, *S. pennellii* LA716). The ascorbic acid content of *S. pennellii* fruit is marked with an arrow as being off the *x* axis of the graph (at 71 mg/100 g fresh weight and 6.8 mg/g dry weight).

for the four QTLs expressed in percentage fresh weight, while the reverse was true for two QTLs detected for ascorbic acid expressed as a proportion of dry matter weight only. Only one QTL on bin 8-D was detected for ascorbic acid as a proportion of both fresh and dry matter.

Relationship with Dry Matter Content and Fruit Weight QTLs

Table V shows the correlations between ascorbic acid content and sugar content, dry matter weight, titratable acidity, and fruit weight. Correlations were different from one population to another. For instance, ascorbic acid content was highly correlated to sugar content in the RIL-cherry population, moderately in the IL-pen population, and the correlation was not significant in BC-hab. The same trend was observed with dry matter weight, and correlations with fruit weight or acidity were low in the three populations.

For the genotypes that were repeatedly sown over several years (17 BC-hab families and eight IL-pen), a larger variation over years was detected for dry matter weight and sugar content than for ascorbic acid, with a significant year × genotype interaction for the first two traits but not for ascorbic acid content (data not

Table II. *QTLs detected for ascorbic acid (expressed relative to fresh weight or to dry matter weight) in the IL-pen population*

The bin corresponds to the chromosome fragment (http://www.sgn.cornell.edu). *, The ILs for which significant differences were detected. ns, Not significant. The parental species whose allele increased the trait is indicated (L, *S. lycopersicum*; P, *S. pennellii*). Mean, SD, and effect (percentage difference between the IL and M82) correspond to the 2000 trial. When two or more ILs were involved, the highest value is indicated. QTL stability indicates when the QTL was confirmed during the trials in 2002 and 2003. na, Lines that were not repeated. The presence of a QTL for dry matter weight (dmw) or fruit weight (fw) detected in the same trial in the bin is indicated with the allele whose effect increases the trait. Mean values are shown with SD in parentheses.

Bin	IL	Allele Increasing Ascorbic Acid Content	Ascorbic Acid Fresh Weight			Ascorbic Acid Dry Weight		Colocation with QTL for Dry Matter Weight, fw
			Mean	Effect	Stability	Mean	Effect	
			mg/100 g	*% M82*		*mg/g*	*% M82*	
	M82 (control)	–	13.6 (1.61)	–	–	2.42 (0.25)		
1-J	1.4	P	17.08 (1.06)	25.7*	2002*	2.76 (0.31)	13.2 ns	QTL for dmw (P), fw (P)
"	1.4.18	"	15.98 (1.85)		na	2.54 (0.37)		
2-F	2.3	P	18.27 (1.25)	34.4*	2002/2003*	3.07 (0.27)	26.1*	QTL for dmw (P)
2-K	2.6	L	10.95 (1.33)	−19.4*	na	1.90 (0.21)	−21.5*	QTL for fw (L)
3-A	3.1	L	10.88 (0.89)	−19.0*	na	1.98 (0.12)	−18.3 ns	–
4-F	4.3.2	L	10.81 (0.39)	−20.4*	na	2.09 (0.23)	−14.1 ns	–
5-G	5.4	P	12.55 (2.74)	7.7 ns	na	1.86 (0.44)	−23.4*	QTL for dmw (P), fw (P)
8-B	8.1.1	P	17.40 (2.51)	28.1*	2003*	3.14 (0.63)	28.4*	QTL for dmw (P)
"	8.1	"	17.03 (1.41)		na	2.67 (0.17)		
9-D	9.1.3	P	27.33 (2.18)	101.0*	2002/2003*	3.96 (0.37)	63.7*	QTL for dmw (P)
"	9.2.5	"	16.90 (1.35)		2002/2003*	2.64 (0.34)		
"	9.2	"	17.19 (2.21)		na	2.54 (0.26)		
9-J	9.3.2	P	16.22 (1.87)	19.4*	na	2.78 (0.32)	14.9 ns	QTL for fw (L)
10-D	10.2	P	17.25 (0.55)	27.0*	na	2.59 (0.27)	6.7 ns	QTL for dmw (P)
11-B	11.1	P	17.22 (2.44)	26.7*	2002/2003*	2.69 (0.44)	10.9 ns	QTL for dmw (P)
"	11.2	"	16.14 (2.45)		na	2.59 (0.24)		
12-B	12.1	P	17.81 (1.39)	31.1*	2002*	3.00 (0.39)	23.0*	–

shown). This could explain why fewer QTLs were detected for ascorbic acid when expressed as a proportion of dry matter weight.

Candidate Gene Mapping

Genes associated with the ascorbic acid biosynthesis or recycling pathway are obvious candidates for QTLs of ascorbic acid content. Thirteen loci corresponding to eight of the known genes of the enzymes of the pathway and vtc2 (an unknown protein involved in ascorbic acid regulation; Fig. 1; Table VI) have been mapped, and their locations are presented in Figure 3 along with the QTLs identified for ascorbic acid in the different populations. In another study, DHAR and ascorbate oxidase (AO) genes from tomato have been cloned and 15 loci involved in tomato ascorbic acid biosynthesis and metabolism mapped (Zou et al., 2006), eight of which are complementary to genes mapped in this study, thus giving an exhaustive view of the genes involved in ascorbic acid metabolism. We have mapped additional loci corresponding to GDP-Man epimerase (GME1), ascorbate peroxidase (APX; putative peroxisomal form), MDHAR1 and MDHAR3, DHAR1, GMP2, and the tomato vtc2 homolog (Jander et al., 2002). For five other genes, we confirmed their map locations, as presented in Zou et al. (2006) apart from in the case of AO. For this last gene, we have mapped single band RFLP polymorphisms from two independent clones (one covering the coding region and one covering both coding and untranslated regions).

Colocation of QTLs and Candidate Genes

A few loci were mapped in regions where QTLs were located. A colocation found with a QTL present in two of the three populations concerns the MDHAR3 gene and a QTL on chromosome 9 (bin 9-D, QTL identified in both IL-pen and RIL-cherry). Other colocations might exist for GMP2 and the QTL covering the bin 9-E and galactono-1,4-lactone dehydrogenase (GLD) and the QTL on the bin 10-E, although the QTLs span a large region. Another interesting colocation exists for the gene GME2 mapped by Zou et al. (2006) and a QTL found in all three populations covering the bin 9-J.

It is worth bearing in mind that assuming a random distribution of candidate genes on the genome, the number of colocations expected by chance would be 4.7, as 21 loci were mapped in 19 bins, four of which carried QTLs for ascorbic acid. Assuming a random distribution of candidate genes, 0.018 candidate genes per centimorgan were expected (21/1,155 cM) for the 258 cM covered by the QTLs, thus 4.7 (258 × 0.018) colocations were expected if the colocations were distributed at random. This number is not different from the four colocations observed.

DISCUSSION

Variation of Ascorbic Acid Levels in the Populations

Ascorbic acid levels in the three populations exhibited the typical distribution of quantitative traits that
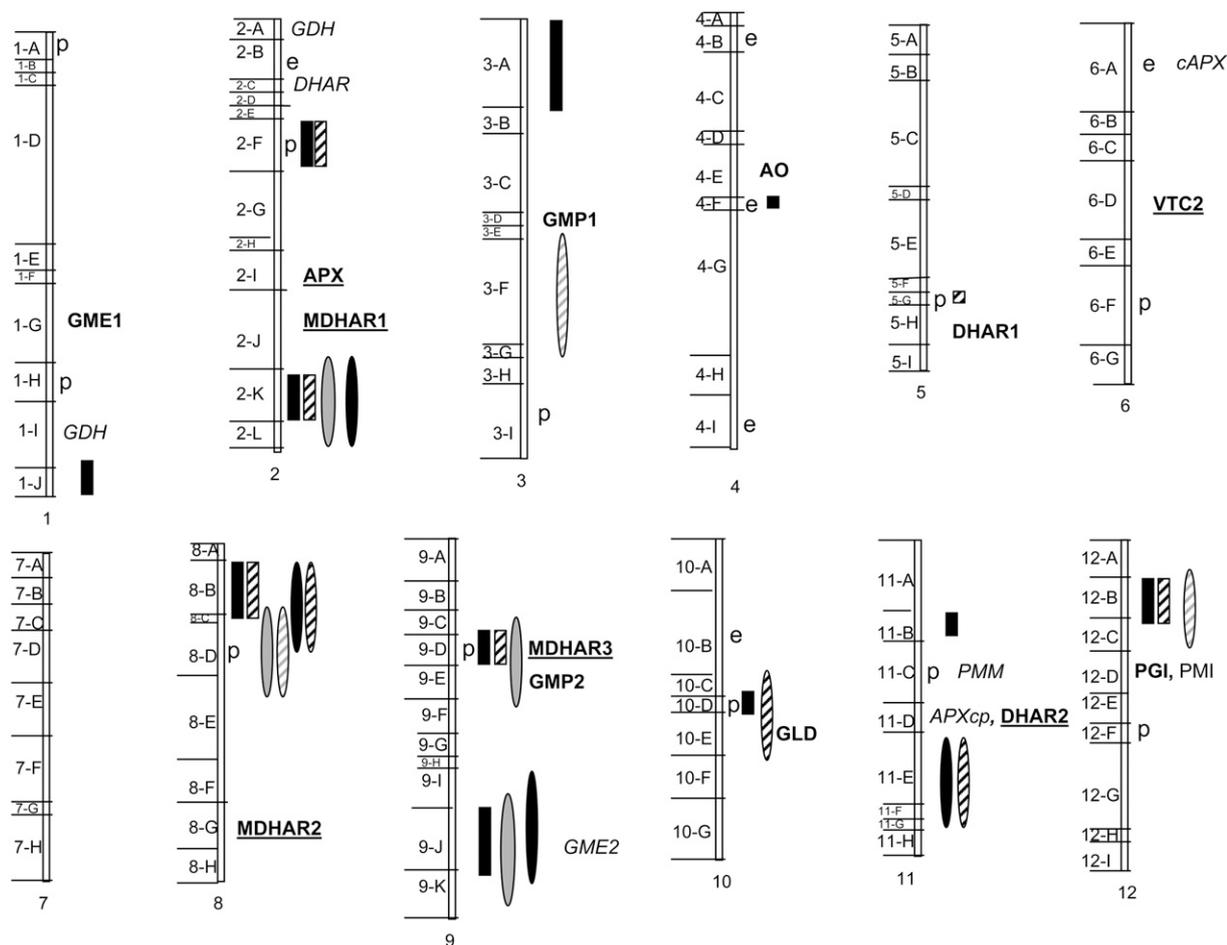
Figure 3. QTL and candidate gene map: summary of the QTL location detected in the three populations. Bins are shown on the left of the chromosomes, as presented in Pan et al. (2000). Gene locations are indicated close to their corresponding bin. Gene codes are detailed in Table VI. Genes in bold type were mapped in this study; genes mapped by PCR are underlined. The genes in italics were mapped by Zou et al. (2006). QTLs are on the right of the chromosomes; squares correspond to QTLs detected in IL-pen, dark ovals to QTLs detected in BC-hab, and pale ovals to QTLs detected in RIL-cherry. Solid color indicates ascorbic acid QTLs with respect to fresh weight; hatched QTLs indicate ascorbic acid QTLs with respect to dry weight. On the right of the chromosomes, p and e indicate the bin locations of QTLs for DHA content detected by Schauer et al. (2006) on IL-pen, where the wild species alleles increase (p) or decrease (e) DHA content.

are controlled by several QTLs. To our knowledge, no null mutants have been identified for ascorbic acid content in fruit, and only four mutant loci have been identified in Arabidopsis after an ozone screen (Conklin et al., 2000). Of these mutants, vtc1 corresponds to a mutation in the GMP gene (Conklin et al., 1999), and the positional cloning of the vtc2 mutation led to an unknown protein. The vtc4 mutant has been recently shown to encode L-Gal-1-P phosphatase, a plant ascorbic acid biosynthetic enzyme (Conklin et al., 2006). These mutants contain reduced levels of ascorbic acid, and no null mutants are found, presumably because plants without ascorbate would not be viable. After screening 118 M82 tomato mutant families, a few mutants with reduced or increased fruit ascorbic acid content compared to M82 have been identified (Stevens et al., 2006). Fruits from the 118 families contained between 6.1 and 31.4 mg ascorbic acid/100 g

fresh weight, which is similar to the range of natural variation observed in the populations studied here.

Improvement of ascorbic acid content may be a target for tomato breeders. Improvement of vitamin content in species of agronomic interest is cited as an important criterion (Agius et al., 2003; Davuluri et al., 2005; Paine et al., 2005). The total ascorbate pool measured in tomatoes in this study reflects what a consumer would encounter when eating a fresh tomato from these lines, providing the time between harvest and consumption was not too great. Any processing of these tomatoes would change the amount of ascorbic acid available to the consumer. The presence of several transgressive lines has revealed the potential use of the wild relatives to improve this trait. In some populations, some positive correlations were shown with sugar content and low correlations with fruit weight, which may help the
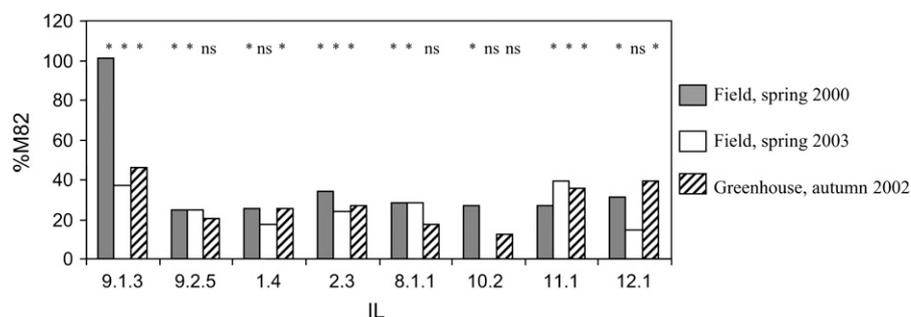
Figure 4. Variation of the effect of *S. pennellii* introgression (% M82) for eight lines repeated over 3 years in field trials in Spring 2000 (gray), Spring 2003 (white), and in the greenhouse in Autumn 2002 (hatched). Lines significantly different from M82 are indicated by a star (*P* < 0.05); ns, Nonsignificant according to a Dunnett test.

breeding process. Furthermore, improved antioxidant content may be associated with improved fruit post-harvest properties; for example, in apple (*Malus domestica*), increased flesh browning is associated with the presence of a less reduced ascorbic acid pool (Davey et al., 2006).

QTLs for Ascorbic Acid Are Common to Several Populations

The QTLs detected in BC-hab and RIL-cherry covered large intervals spread over several bins, but most of the QTLs detected in these two populations were located in regions where QTLs were also mapped in IL-pen. Bins 9-D, 10-D, and 12-B carried QTLs detected in two populations, and QTLs were detected in three populations on bins 2-K, 8-B, and 9-J. It is noticeable that on bin 2-K, the positive alleles are provided by the *S. lycopersicum* parent in two populations. The QTL on bin 9-D had a very strong effect, as it doubled the ascorbic acid content; however, this effect was only detected in IL-pen in 1 year and the effects seen in the other years were more moderate. This QTL may therefore be under environmental control. Several QTLs could be linked in this region, as the lines carrying bin 9-D (IL9.1, 9.1.3, 9.2, and 9.2.5) had an ascorbic acid increase of 9.4%, 101%, 26.5%, and 24.4%, respectively.

Colocations with QTLs for Fresh or Dry Matter Weight or Fruit Weight

We found more QTLs when expressing ascorbic acid relative to fresh weight (19 QTLs) than relative to dry matter weight (13 QTLs), and only nine QTLs were common to both traits. Among the QTLs relative to fresh weight, nine colocalized with dry matter content QTLs and could thus be related to a difference in dry matter. In spite of the lower number of QTLs for ascorbic acid per dry matter content, four QTLs remained colocated with dry matter content QTLs. All of these QTLs but one (bin 5-G) had the same positive alleles. Only six QTLs for ascorbic acid were found in common regions with QTLs for fruit weight, but the positive alleles were common in only three (bin 1-J, 2-K, and 12-BC) and opposite on bins 5-G and 9-J, suggesting fortuitous colocations.

Effect of the Environment on QTLs

Although the fruit ascorbic acid content is highly influenced by the environment (Toor et al., 2006), QTLs for ascorbic acid appeared quite stable across environments, as among the eight ILs that were grown in three trials (two in the field and one in the greenhouse in autumn), seven were detected under at least two conditions. The experimental conditions for measuring

Table III. *QTLs detected for ascorbic acid (expressed relative to fresh weight or to dry matter weight) in the BC-hab population*

The bin corresponds to the chromosome fragment on the IL-pen map where the confidence interval of the QTL mapped. The parental species whose allele increased the trait is indicated (L, *S. lycopersicum*; H, *S. habrochaites*). Data represent the dataset on which the QTL was observed (2001, 79 families; 2002, 68 families; m, normalized data from 130 families). The average and SD of the families according to their genotypes (LL or HH) are indicated. R^2 is the variation explained by the QTL and log of the odds (LOD), the probability associated with the most likely location of the QTL detected by interval mapping. The presence of a QTL for dry matter weight (dmw) or fruit weight in the same trial in the bin is indicated. Mean values are shown with SD in parentheses. ns, Not significant.

Bin	Marker	Allele	Ascorbic Acid Fresh Weight					Ascorbic Acid Dry Weight				Colocation with QTL for dmw, Fruit Weight
			Data	Mean LL	Mean HH	R^2	LOD	Mean LL	Mean HH	R^2	LOD	
				mg/100 g		%		mg/g		%		
2-KL	CT274	L	2002	12.93 (2.21)	10.9 (2.54)	21	3.46	1.69 (0.32)	1.44 (0.33)	19	3.15	
			m	12.58 (2.36)	10.94 (2.17)	12	3.78	1.71 (0.34)	1.50 (0.29)	–	ns	
8-BCD	TG45	H	2001	12.13 (2.22)	13.98 (4.26)	22	3.07	1.69 (0.31)	1.99 (0.61)	35	7.33	
			2002	11.81 (1.67)	14.46 (3.06)	31	5.59	1.53 (0.17)	1.94 (0.47)	30	4.22	
			m	11.92 (2.04)	13.90 (3.15)	30	6.02	1.52 (0.28)	1.81 (0.48)	20	5.17	
9-IJ	TG8	H	2001	12.26 (2.67)	12.57 (1.79)	15	2.65	1.71 (0.38)	1.75 (0.26)	–	ns	QTL for dmw (H)
10-CDE	PGAL	H	m	12.33 (2.37)	12.19 (2.52)	–	ns	1.56 (0.35)	1.76 (0.33)	9.4	2.37	
11-EFG	I2	H	2001	12.08 (2.08)	13.40 (3.93)	24	2.23	1.68 (0.27)	1.88 (0.62)	28	4.33	

Table IV. *QTLs detected for ascorbic acid (expressed relative to fresh weight or to dry matter weight) in the RIL-cherry population derived from the cross between a cherry tomato line S. lycopersicum cv cerasiforme (C) and a large fruited line (L)*

The bins correspond to the chromosome fragment on the IL-pen map where the confidence interval of the QTL mapped. The parental species whose allele increased the trait is indicated (L, S. lycopersicum; C, cerasiforme). The average and SD of the families according to their genotype (CC or LL) are indicated. R^2 is the variation explained by the QTL and LOD, the probability associated with the most likely location of the QTL detected by interval mapping. The presence of a QTL for dry matter weight (dmw) or fruit weight (fw) in the same trial in the bin is indicated. Mean values are shown with SD in parentheses. ns, Not significant.

Bin	Marker	Effect	Ascorbic Acid Fresh Weight				Ascorbic Acid Dry Weight				Colocation with QTL for dmw, fw
			Mean CC	Mean LL	R^2	LOD	Mean CC	Mean LL	R^2	LOD	
			mg/100 g		%		mg/g		%		
2-KL	CT274	C	22.38 (3.71)	19.90 (3.48)	22	5.78	2.61 (0.42)	2.48 (0.34)	–	ns	QTL for fw (L), dmw (C)
3-FG	CT85	L	20.58 (4.01)	21.64 (3.46)	–	ns	2.44 (0.37)	2.64 (0.37)	9	2.31	QTL for fw (L)
8-CDE	CT245	C	21.77 (3.44)	20.15 (4.10)	14	3.11	2.58 (0.28)	2.49 0.47)	10	2.14	–
9-DE	CT32	C	21.79 (3.91)	20.16 (3.48)	8	2.45	2.53 (0.33)	2.57 (0.46)	–	ns	QTL for dmw (C)
9-JK	TG8	C	21.42 (3.48)	20.71 (3.79)	7	2.16	2.57 (0.39)	2.52 (0.38)	–	ns	–
12-BC	CT120	L	21.00 (3.80)	21.35 (3.63)	–	ns	2.44 (0.37)	2.64 (0.39)	7	2.20	QTL for fw (L)

the trait may be subject to variation. Indeed, analysis of the same IL-pen population grown over 2 years in Israel for a range of fruit metabolites showed 17 QTLs for oxidized ascorbic acid (dehydroascorbate; Fig. 3), of which six were common with our study (Schauer et al., 2006). A reason for the differences with our study, apart from the fact that here total ascorbic acid QTLs (i.e. reduced + oxidized forms) have been identified, may be due to the sample chosen, as our quantification was performed on whole fruit and Schauer et al. (2006) studied peeled pericarp. We have detected higher concentrations of ascorbic acid in the locular jelly than the pericarp for M82 and Levovil at the red ripe stage (R. Stevens and M. Causse, unpublished data). Also, there are larger concentrations of ascorbic acid in the skin compared to the rest of the pericarp (P. Baldet, unpublished data). Of course, the environment may also play a role. In a recent study of the IL-pen population (Rousseaux et al., 2005), the ascorbic acid content (total ascorbate) was analyzed over 2 years and QTLs were found in four bins (3-F, 5-E, 10-B, and 12-G). Only one (3-F) was common with our study. Rousseaux et al. (2005) did not detect any ascorbic acid in *S. pennellii* fruits, although this wild species has been shown to be a good source of the vitamin (Schauer et al., 2005). Our results show a concentration of 71 mg/100 g fresh weight. These differences could be explained because of different extraction methods; samples in the Rousseaux et al. (2005) study were ground fresh on ice, and not in liquid nitrogen like in this study, prior to being frozen. Rousseaux et al. (2005) mention in their discussion that on grinding in liquid nitrogen, *S. pennellii* ascorbate levels are restored to 1.9 mg/g dry weight, which is still slightly lower that our value of 6.8 mg/g dry weight. Other explanations for the differences seen may include the environment (central California versus Southeastern France). There is also the difficulty that as *S. pennellii* fruits remain green, it is difficult to evaluate when they are ripe and will therefore have accumulated a maximum amount of ascorbic acid.

Candidate Gene Mapping and Colocations

Together with the results of Zou et al. (2006), a complete set of enzymes for the synthesis and turnover pathways has been mapped. The only discrepancy is the difference in map location for the AO gene, which is surprising, as in both cases the same consensus sequence has been used. In this study, two independent clones have been mapped covering both coding and untranslated regions.

A colocation has been identified for the gene GME2 mapped by Zou et al. (2006) and the QTL on chromosome 9 (bin 9-J) found in the three populations. The enzyme GME is interesting because it forms a branch point from the major biosynthesis pathway with the alternative pathway via L-gulose and the synthesis of cell wall intermediates. It defines the committed step into ascorbic acid biosynthesis and is implicated as a control point for the carbon flux into the biosynthesis pathway in response to cell redox conditions and demand for cell wall biosynthesis (Wolucka and Van Montagu, 2003). Furthermore, feedback inhibition of the pathway is observed, apparently at the level of this enzyme. Therefore, GME could be a good candidate for the regulation of ascorbic acid levels (Wolucka and Van Montagu, 2003; Valpuesta and Botella, 2004).

A second interesting colocation involves a QTL for ascorbic acid on chromosome 9 with the MDHAR3 gene. This cDNA has been cloned from red ripe tomato fruit and shown to be expressed in fruit, and

Table V. *Correlations between ascorbic acid content and sugar content (sug), dry matter weight (dmw), titratable acidity (TA), and fruit weight (fw) in the three populations*

ns, No significant correlation.

Progeny	Vit C, sug	Vit C, dmw	Vit C, TA	Vit C, fw
IL-pen	0.36	0.45	ns	ns
BC-hab	ns	ns	−0.30	ns
RIL-cherry	0.65	0.63	0.20	−0.37

Table VI. *Candidate gene characteristics*

*, Primers and restriction sites used for PCR markers are as follows: MDHAR1, GGAACCAAAGAGGAGTATGAGGC and CGACGTG-TAAAAATCTTCCGTGGGG, *Pvu*II site. MDHAR2, GCCATATGCACCATATGAACGTCC and CCCTTGTTCCTTGTACCAATCAGG, *Taq*I site. MDHAR3, GGGCAAGTTGAAGAGGAGAAGGG and GGAAAAGTGGCAACATCACCC, *Hinf*I site. DHAR2, GGGCAAAGAACTGTCTTACAGCC and GCCACTTCCACAAGTCATTGGACC, size polymorphism. APX, CCTTACTGGCACCTGAGGTGCG and GCTCCACAAAGTATGAGTTGTC, *Ssp*I site. VTC2, GCTTATTACGTACAGTTGTATC and CCAGTTAGATAGACTTGGTTTAG, *Alu*I site. **, Mapping of two independent clones gave the same profiles.

Gene	Function	TC No.	Clone No.	Tomato Library	Marker	Bin
GMP1	GDP-Man pyrophosphorylase	TC99113	cLEM8J23	Immature green	RFLP**	3-D
		TC99112	cLEG62O22	Fruit/breaker fruit		
GMP2	GDP-Man pyrophosphorylase	TC110163	cLEM7F10	Immature green fruit	RFLP	9-E
GME1	GDP-Man epimerase	TC99842	cLEN10C18	Red ripe fruit	RFLP	1-G
GLD	L-Galactono-γ-lactone dehydrogenase	TC112703	cLHT24N6	*S. habrochaites*	RFLP**	10-E
		TC103695	cTOC18F20	Trichome/flower		
AO	Ascorbate oxidase	TC99795	cLEC67D22	Tomato callus	RFLP**	4-E
MDHAR1	Monodehydroascorbate reductase	EST263177 TC singleton SGN-U155072	cLES18B10	Tomato leaf	PCR*	2-J
MDHAR2	Monodehydroascorbate reductase	TC126092	cTOD20A21	Tomato flower, anthesis	PCR*	8-G
MDHAR3	Monodehydroascorbate reductase	TC124435	cTOC25K2	Tomato flower, 8 mm, preanthesis buds	PCR*	9-D
DHAR1	Dehydroascorbate reductase	TC116433	cLEI16N22	Tomato germinating seed	RFLP	5-H
DHAR2	Dehydroascorbate reductase	TC117375	cLEG59I24	Tomato breaker fruit	PCR*	11-D
APX	Ascorbate peroxidase (putative peroxisomal)	TC161616	cTOE3O17	Tomato crown gall	PCR*	2-I
VTC2	Unknown protein	TC153912	cTOD24H13	Tomato flower, anthesis	PCR*	6-D
PGI	Phosphoglucose isomerase	TC98796	cLEB8K2	Tomato shoot	RFLP	12-D

the mRNA levels were shown to be positively correlated with ascorbate content (Grantz et al., 1995). The authors suggest that this enzyme may contribute to maintaining levels of ascorbic acid for protection against oxidative stress arising from wounding. It is possible that turnover genes have a role in the regulation of ascorbate levels (e.g. under stress conditions), particularly as elevated expression of the other enzyme involved in ascorbate turnover, DHAR, has been shown in tobacco to enhance ascorbate content of plants (Chen et al., 2003).

In another study, mapping of QTLs for fruit color and comparison with the location of candidate genes involved in the well-characterized carotenoid biosynthetic pathway revealed the same number of cosegregations as would have been expected by chance alone. The candidate genes of the pathway tended to cosegregate with known mutations such as *yellow flesh* or *Det* (orange fruits; Liu et al., 2003), and the candidate gene approach was efficient for identification of these major loci but not for the quantitative variation of red color and the regulation of this process. It is worth noting that regulators of carotenoid content may often have nothing to do with the biosynthetic pathway; an example is the mutant *de-etiolated 1* that encodes a gene of the phytochrome signal transduction pathway. A mutation in this gene gives rise to high-lycopene tomatoes (Mustilli et al., 1999). The same may be true for ascorbic acid QTLs; the regulators of this pathway may be genes unrelated to the biosynthesis and metabolism of ascorbic acid. Conversely, mutants, as shown with the vtc mutants, have more chance of being mutated in a biosynthesis pathway gene, similar to the findings of the carotenoid study.

CONCLUSION

A minimum of 23 ascorbic acid QTLs (19 when expressed relative to fresh weight and 13 to dry matter weight) covering 15 bins have been identified in this study, with a further 11 from Schauer et al. (2006). Thus, at least 30 QTLs exist for ascorbic acid content, a similar number to other quantitative traits. Furthermore, similar to studies on other fruit characteristics, common regions and, presumably, common genes exist in different populations, which control the variation of this character. The major regions controlling ascorbic acid content appear to be bins 2-K, 8-B, 9-D, 9-J, 10-D, and 12-B. In general, it is the wild alleles that increase ascorbic acid content, but some improvement can be also provided by *S. lycopersicum* accessions. Among the candidate genes mapped, colocations occur for MDHAR3 and bin 9-D, GME2 and bin 9-J, GLD and bin 10-E, and GMP2 and bin 9-E. QTL fine mapping is required in parallel with screening for differences in gene sequences and expression levels in QTL near isogenic lines (differing only for the region of the QTL) to confirm whether the identified genes are responsible for the QTLs observed. This is especially important for QTLs identified in RIL and backcross populations, as the positions of the QTLs are not very precise and can cover a large region.

MATERIALS AND METHODS

Plant Populations and Growing Conditions

The first population (IL-pen) consisted of 75 lines, each containing a single introgression fragment from *Solanum pennellii* LA716 in the genetic background of M82, a processing tomato variety with determinate growth (Eshed and Zamir, 1995). The population was first planted in the field in Avignon, France, in Spring 2000, in a three-block trial under irrigated conditions. Each block contained 70 lines (six plants per plot) and four plots of M82 as a control (lines IL1.3, IL3.3, IL6.2, IL6.2.2, and IL 6.3 were not planted). Fully ripe fruits were harvested and a total of 21 fruits per plot were used for analyses. Subsequently, eight lines (IL1.4, IL2.3, IL8.1.1, IL9.1.3, IL9.2.5, IL10.2, IL11.1, and IL12.1) were grown twice, first in a greenhouse trial in autumn (planted in August, 2002), then in the field (Spring, 2003) under the same conditions as the 2000 trial. Each plot consisted of six plants per line. Thirty fully ripe fruits were harvested per line, and five bulks of six fruits were analyzed.

The second population (BC-hab) consisted of 130 BC2 families derived from a cross between a single plant of the wild species *Solanum habrochaites* PI247087 (kindly provided by Dr. J.E. Thomas, Australia) and the *Solanum lycopersicum* accession Ferum bred at the Institut National de la Recherche Agronomique (France) for fresh-market use (indeterminate growth). The advanced backcross population was developed using Ferum as the recurrent parent. A total of 166 BC1 plants were grown and 130 plants were selected for fertility and fruit size to produce the BC2 generation. Then 130 BC2S1 families were used for phenotyping and molecular marker analysis. The population was studied in the greenhouse in Avignon in Spring for two consecutive years. The first trial was composed of 79 families and the second of 68 families (plus the Ferum line as a control repeated in six plots). Seventeen families were grown both years and allowed the genotype × year interaction to be tested. Each line was represented by six plants. Ripe fruits were harvested twice per week for 5 weeks, and each week one bulk of six fruits was analyzed per family, giving five bulks in total. The molecular map consisted of 217 markers, including 138 amplified fragment length polymorphism markers, 36 RFLP markers spread over the genome, 26 microsatellites (Smulders et al., 1997; Milbourne et al., 1998; Areshchenkova and Ganal, 2002; http://sgn.cornell.edu), 15 PCR markers, and two phenotypic markers (data not shown). The RFLP, PCR, and microsatellite markers allowed the map to be aligned with the high density molecular map for tomato (Tanksley et al., 1992). It covered about 80% of this reference map.

The final population (RIL-cherry) was composed of 144 recombinant inbred lines derived from the cross between a cherry tomato line, *S. lycopersicum* cv *cerasiforme* (Cervil) and a large fruited line (Levovil). The population was grown in the greenhouse in Spring and fruits harvested as described (Saliba-Colombani et al., 2001).

Total Ascorbic Acid Content Measurement

Ascorbic acid assays (total and reduced forms) were carried out using a spectrofluorometric method and values expressed as total ascorbate (ascorbic acid + dehydroascorbate) in mg/100 g fresh weight or mg/g dry matter weight. The method is based on the reaction of oxidized ascorbate (dehydroascorbate) with orthophenylenediamine producing a fluorescent quinoxaline and has been previously described (Deutsch and Weeks, 1965). This reagent is known to be highly specific for ascorbic acid (Tessier et al., 1996). A set of standards containing between 5 and 50 μg ascorbic acid/mL was prepared in extraction buffer (3% metaphosphoric acid, 25% methanol) and processed in the same way as the test samples. Tomato samples were ground in liquid nitrogen (powders were stored at −80°C if necessary), and 5 g of frozen tomato powder (nonlyophilized) were then mixed with 50 mL extraction buffer and incubated with 1 g of activated charcoal (Norit) for 10 min to oxidize the reduced ascorbate present in the sample. If the endogenous oxidized form in the sample was required, then a small quantity of extract was removed before addition of the activated charcoal. The mix was filtered and the samples were dialyzed then mixed with 3.7 M sodium acetate and 0.05% orthophenylenediamine dihydrochloride before incubation in a water bath at 37°C. The orthophenylenediamine reacted with the oxidized form of ascorbate, producing a fluorescent quinoxaline whose fluorescence at 435 nm was detected by a spectrofluorometer after excitation at 350 nm, at the rate of 30 samples/h. Results appear in the form of peaks and the concentration of ascorbic acid was calculated by reference to the standards.

Total Sugar Content and Titratable Acidity Determinations

Sugar content and titratable acidity were determined as described in SCAR Agro-Food Tomato Working Group (1991).

QTL Mapping Strategy

Statistical analyses were performed using the SAS package (SAS Institute, 1994). For the IL-pen population, QTLs were mapped by comparing each line value to the M82 average using a Dunnett test (α level, 0.05), as has been previously described (Causse et al., 2004). Results were expressed as a percentage difference compared to M82. For the BC-hab and RIL-cherry populations, QTLs were mapped by interval mapping according to the previously described procedure (Saliba-Colombani et al., 2001). After an initial analysis of QTLs on each set of data from BC-hab, QTLs were mapped on the whole dataset, with family data from each year normalized by using the mean and variance for the relevant year of the set of lines grown the 2 years.

Isolation and Mapping of Candidate Genes

The metabolism of ascorbic acid involves well-studied pathways. Functional candidate genes were therefore chosen from the enzymes known to be involved in ascorbate metabolism (Fig. 1) and mapped onto the introgression line population (IL-pen) as previously described (Causse et al., 2004). If cDNA clones were available, RFLP mapping was carried out using polymorphisms identified from DNA digested with common restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I). Otherwise, specific PCR primers were designed, PCR amplifications were carried out on the parent lines (*S. lycopersicum* and *S. pennellii*), products were sequenced to check that the desired gene had been amplified, and single nucleotide polymorphisms were identified and used to generate cleavage amplified polymorphism markers for use on the entire population. The list of cDNA clones mapped and The Institute for Genomic Research accession numbers are shown in Table VI. Genomic DNA isolation, digestion, and hybridization for RFLPs and amplified fragment length polymorphisms were carried out as described in Saliba-Colombani et al. (2001).

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