

Dissecting a QTL into Candidate Genes Highlighted the Key Role of Pectinesterases in Regulating the Ascorbic Acid Content in Tomato Fruit

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Abstract

Tomato (*Solanum lycopersicum*) is a crucial component of the human diet because of its high nutritional value and the antioxidant content of its fruit. As a member of the Solanaceae family, it is considered a model species for genomic studies in this family, especially since its genome has been completely sequenced. Among genomic resources available, *Solanum pennellii* introgression lines represent a valuable tool to mine the genetic diversity present in wild species. One introgression line, IL12-4, was previously selected for high ascorbic acid (AsA) content, and a transcriptomic analysis indicated the involvement of genes controlling pectin degradation in AsA accumulation. In this study the integration of data from different "omics" platforms has been exploited to identify candidate genes that increase AsA belonging to the wild region 12-4. Thirty-two genes potentially involved in pathways controlling AsA levels were analyzed with bioinformatic tools. Two hundred-fifty nonsynonymous polymorphisms were detected in their coding regions, and 11.6% revealed deleterious effects on predicted protein function. To reduce the number of genes that had to be functionally validated, introgression sublines of the region 12–4 were selected using species-specific polymorphic markers between the two *Solanum* species. Four sublines were obtained and we demonstrated that a subregion of around 1 Mbp includes 12 candidate genes potentially involved in AsA accumulation. Among these, only five exhibited structural deleterious variants, and one of the 12 was differentially expressed between the two *Solanum* species. We have highlighted the role of three polymorphic pectinesterases and inhibitors of pectinesterases that merit further investigation.

THE TOMATO (*Solanum lycopersicum*) is one of the three most important vegetable crops in the world, with 161 million tons of production in 2012 (FAOSTAT, <http://faostat.fao.org/>). The fruit is used as fresh produce or canned and is processed into a number of forms such as paste, juice, sauce, and powder. It is well established that tomato is a crucial component in the human diet because of the nutritional value and antioxidant content of its fruit, which may help to protect against cancer and cardiovascular diseases (Friedman, 2013).

Tomato is also considered a model species for genetic studies in plants, especially among the Solanaceous species, because of its relatively small genome size (950 Mbp), short generation time, routine transformation technology, and numerous genetic and genomic resources (Barone et al., 2008). As a result of the high synteny present among the genomes of various species belonging to the Solanaceae family, tomato is considered a reference species for genomic studies (Mueller et al., 2005). Molecular studies performed on tomato, potato, pepper, eggplant, and tobacco have demonstrated that these species exhibit a high level of microsynteny and gene order (Wu and Tanksley, 2010). As a reference genome for these Solanaceae species, tomato was completely sequenced in 2012 (Tomato Genome Consortium, 2012).

Genetic and genomic resources available for tomato include introgression lines (IL), which carry homozygous regions of wild genome in the cultivated *S. lycopersicum*

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Abbreviations: AsA, ascorbic acid; DETC, differentially expressed tentative consensus; GO, gene ontology; IL, introgression line; PCR, polymerase chain reaction; PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; SNP, single nucleotide polymorphism; TC, tentative consensus.

genetic background (Eshed and Zamir, 1995). These lines represent an extremely useful tool to capture the genetic diversity existing among wild species and the cultivated tomato. Among IL populations that have been generated from various wild species (Chetelat and Meglic, 2000; Monforte and Tanksley, 2000; Francis et al., 2001; Canady et al., 2005; Finkers et al., 2007), the most exhaustive is the *S. pennellii* population, which consists of 76 introgression lines or sublines (Eshed and Zamir, 1995). It was recently very precisely defined at both the genetic and transcriptomic levels by high-density genotyping of all the ILs (Chitwood et al., 2013). This population allowed the identification of QTLs for many agronomically interesting traits (Lippman et al., 2007). When this genetic resource was combined with genomic resources, such as transcriptomic and metabolomic platforms, candidate genes for the control of quantitative traits were also identified (Baxter et al., 2005; Schauer et al., 2006; Perez-Fons et al., 2014). In our laboratory, this approach has been exploited to identify candidate genes that control AsA content in the tomato fruit of the introgression line IL12-4, which carries one QTL that increases AsA (Rousseaux et al., 2005; Di Matteo et al., 2010). We have utilized a Combinatrix array, including more than 20,000 tomato tentative consensus (TCs), to investigate variation of gene expression in the fruit of introgression line IL12-4 compared with the cultivated M82 genotype. This comparative transcriptomic analysis led to identification of 29 differentially expressed TCs and to the hypothesis that in introgression line IL12-4 the genes controlling pectin degradation play a role in tomato fruit AsA accumulation (Di Matteo et al., 2010). Similar relationships have been reported for *Arabidopsis thaliana*, grape, and strawberry (Agius et al., 2003; Cruz-Rus et al., 2010, 2011).

The aim of the present study was to employ transcriptomic- and genomic-data integration to identify candidate genes controlling AsA accumulation in fruit that could be introgressed from the wild *S. pennellii* into cultivated tomato varieties. Following the release of the complete tomato genome sequence, we had the opportunity to clearly locate on tomato chromosomes the previously identified 29 TCs and to better understand possible interactions among them. In the current study, these TCs were annotated and mapped on the latest release of the tomato sequenced genome (SL2.50), and we focused on a number of candidate genes mapping in the region 12-4. Finally, to better refine the number of candidates, we selected sublines of IL12-4 and evaluated them for AsA content. This allowed us to hypothesize that three pectinesterases and inhibitors of pectinesterases are potential candidates for increasing AsA content in IL12-4 tomato fruit. These findings provide a valuable tool for improving the nutritional content of tomato and a focus for our future investigations.

Materials and Methods

Materials

Plant materials used in this study consisted of one *S. pennellii* in *S. lycopersicum* introgression line (IL12-4, accession LA4102) and the cultivated genotype M82 (accession LA3475). IL12-4 was previously selected in our laboratory for its higher AsA fruit content in comparison to the cultivated genotype (Di Matteo et al., 2010). The introgression subline IL12-4-1 (accession LA4103) was added to the plant material used in trials performed to estimate AsA content in the fruit. All the accessions were kindly provided by the Tomato Genetics Resources Centre (<http://tgrc.ucdavis.edu/index.cfm>). In addition, we evaluated for AsA content four IL12-4 sublines selected from F_2 genotypes previously obtained through inter-crossing of two IL lines (Sacco et al., 2013). The parental genotypes M82 and IL12-4 were grown under greenhouse conditions in 20-cm pots containing a 1:1 mixture of medium-sandy soil and compost for three consecutive years in a completely randomized design (three replicates for each genotype). The four selected sublines were grown in open-field conditions in a randomized complete block design with three replicates per genotype and 10 plants per replicate. In all trials, fruits were collected at the red ripe stage by pooling several berries from each plant. Seeds and columella were subsequently removed, and the fruit was ground in liquid nitrogen and stored at -80°C until analyzed. AsA levels were measured using the method described by Di Matteo et al. (2010).

Bioinformatic Analyses

Bioinformatic analyses were performed on 21,550 tomato TCs available at the Institute for Genome Research database Gene Index Release 11.0 (21 June 2006). These TCs were mapped on tomato chromosomes by blasting the probes used to design the Combinatrix chip against the WGS Chromosomes database (SL2.50) of the Sol Genomics Network (<http://solgenomics.net/>). Only results that showed expectation values between 0 and 1.0E^{-6} were considered. All the TCs associated with significant matches of the blast search were annotated with Blast2GO (version 2.7.2, <http://www.blast2go.org/>; Conesa and Gotz, 2008). We selected the introgression region 12-4 (from around 63 Mbp onward) for further analysis. An enrichment analysis using a Fisher's exact test was performed on the 460 genes of the region IL12-4 to compare the frequencies of gene ontology (GO) terms with those of the whole tomato genome. This statistical tool as implemented in Blast2GO estimates the differences of GO terms between two sets of sequences by computing a *p*-value that allows determining the significance of associations between the two categorizations. In addition, a false discovery rate control was applied to limit the number of false discoveries.

Within this region, we examined GO terms and annotations to find the Solyc loci corresponding to the candidate genes mapping to the region spanning from 74

Table 1. List of cleaved amplified polymorphic sequence markers used to dissect the introgression region 12-4 of the tomato sequenced genome (SL2.50).

Marker code	Literature marker†	Position in chromosome 12	Primer sequence (5'-3')‡	PCR§ product size	Restriction enzyme	M82 fragment size	IL12-4 fragment size
M1		bp 64217027	F: TGTTACCTGCCTCATCTGTATAAG R: ATTTGAAGACCTCTCCAGAACATTGG	bp 500	HaeIII	230+200+70	200+300
M3	At5g38530	65214240	F: TCGCACAGCCAACCTGCTG R: ACTCCTCACCGATAACGGTCTGATG	482	MseI	56+426	56+241+186
M4	C2_At3g17000	66500546	F: TTCCGATATTGATGAGCCTTCC R: TCGTGGACCCCTGATTGCAAATTG	1194/1000	—	—	—
M7		65642990	F: GGTGCAACAGGCAACTGAA R: AACTCAAAGCAAGGACACCG	516	AluI	170+346	516
M8		66072827	F: TGTTCAACTGCTGCAACGAT R: AGGGATGTTTGCTGGAGGA	771	TaqI	578+193	771
M9		64628259	F: GTAGTTCTTATGCTAGCGC R: ACTACACTCGCGAAGGAACA	608	AluI	414+194	608
M10		64920606	F: GGAACCAAACCTGTGCAAGT R: TCAGCTGCAAGAGGACATGA	636	TaqI	193+442	636
M11		65858413	F: TGCTTGGGTGACTAGTGGG R: CCTCCCTCAACTGTTCCCAA	639	HaeIII	21+189+429	21+618
M12	TG367	62093659	F: CCATGTGAACACGGGTCAT R: GAGAGTCAGAGTCAGAGAACGA	569	AluI	29+90+427+23	29+517+23
M13	T0801	63005704	F: GAGCCGGAAGGTATGATTGA R: GACCTTGTTGCTAGGGCATGT	589	RsaI	66+524	66+340+184
M17		63126136	F: GCTCAAGAAATTGGTGTCCC R: GCACCTGGCTCTCTTCCC	622	RsaI	351+271	622
M18	CD2	66885787	F: CCTATGCTGCTGGACTCTT R: ACTCCAGCAAGACATCCCAA	699	ScaI	699	339+360
M19		64745235	F: TGACCTCTGACGTTCACT R: TCGAGTAGAACGAGCACCTA	594/—	—	—	—

† Alseekh et al., 2013.

‡ F, forward; R, reverse.

§ PCR, polymerase chain reaction.

cM to 120 cM of chromosome 12. Single nucleotide polymorphisms (SNPs) between the wild and the cultivated candidate genes were identified in the coding regions by exploiting the variants available at the site <http://www.tomatogenome.net> for both the *S. lycopersicum* Heinz reference genome and the *S. pennellii* acc. LA0716. The potential effects of these polymorphisms on the protein sequences in terms of neutral or deleterious effects were predicted with the PROVEAN protein tool (publicly available from the J. Craig Venter Institute at http://provean.jcvi.org7seq_submit.php). Finally, for two genes showing deleterious amino acidic substitutions or insertion/deletion (Solyc12g099410 and Solyc12g099190), a preliminary prediction on the structural and functional consequences of these variations was obtained by using an in silico analysis on the I-TASSER server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>; Roy et al., 2010). Using the PLACE program (<http://www.dna.affrc.go.jp/htdocs/PLACE/>; Higo et al., 1999), regulatory elements, such as sequence-specific DNA-binding transcription factors, were analyzed in a 1264-bp promoter region of one selected candidate gene (Solyc12g098340). This

program performs a search in a dedicated database of motifs found in plant *cis*-acting elements derived from previously published reports.

Molecular Marker Analysis

Total genomic DNA was extracted from leaves using the PureLink Genomic DNA Kit (Invitrogen). Markers belonging to the wild introgression region 12-4 were designed with the Sol Genomics Network database. Primer pairs used for these analyses are listed in Table 1. Polymerase chain reaction (PCR) DNA amplification was performed in a 25-μL reaction volume containing 50 ng DNA, 1.5 mM MgCl₂, 0.2 mM of each deoxyribonucleotide triphosphate, 1.0 mM primer and 1.25 U Taq polymerase (Invitrogen). PCR amplicons were purified and then sequenced by an ABI Prism 3130 system using Big-Dye 3.1 Terminator cycle sequencing reactions (Applied Biosystems). Discriminating restriction enzymes were identified with dCAPS Finder 2.0 Software (<http://helix.wustl.edu/dcaps/dcaps.html>). The restriction endonuclease reaction was made in a 20-μL reaction volume containing 5 μL PCR product, 2 μL 10× buffer, and 1 μL of

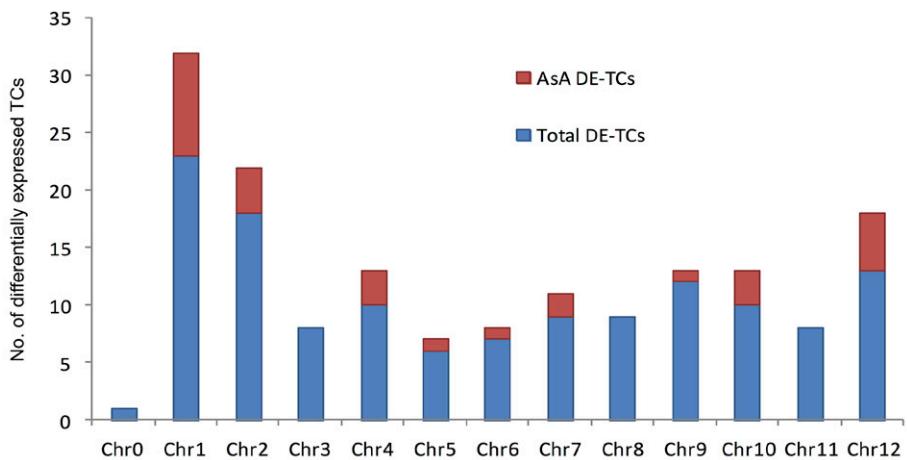


Figure 1. Distribution on the twelve tomato chromosomes of the 29 tentative consensuses (TCs) (AsA differentially expressed tentative consensus, DE-TCs) used in the model describing the higher AsA level in IL 12-4 fruit with respect to the total differentially expressed TCs (DE-TCs) detected through the transcriptional analysis of IL12-4 and M82 red ripe fruit reported by Di Matteo et al. (2010).

the selected restriction enzyme (10 U/ μ l). Digested fragments were separated by electrophoresis on agarose gel 2% in tris-borate-EDTA.

RESULTS AND DISCUSSION

Significant advances have been made in the sequencing and annotation of the tomato genome since our laboratory first reported the transcriptional profiling of the introgression line IL12-4 (Di Matteo et al., 2010). In addition to the expanding genomic resources, a number of “omics” tools have become available for tomato (Kusano and Fukushima, 2013). These allow for the linkage of phenotypic traits to genomic sequences, transcripts, or proteins, as demonstrated in species such as *Arabidopsis*, legumes, and cereals (Lysenko et al., 2009; Borrell et al., 2014; Feuillet et al., 2012; Pratap et al., 2014). These tools present an opportunity to better interpret results previously obtained from the transcriptional analysis performed to identify candidate genes controlling AsA content in tomato fruit. Utilizing these tools, we have developed a novel strategy based on data mining and experimental analyses.

Mapping of Differential Expressed TCs

The 29 differentially expressed TCs (DE-TCs) selected from a previous transcriptional profiling study (Di Matteo et al., 2010) allowed the development of a model that could explain the higher AsA content in the IL12-4 fruit. Of these differentially expressed TCs, four were related to AsA pathways, six to hormone metabolism, five to glycolysis and the Calvin cycle, and three to glutathione metabolism. In addition, six DE-TCs involved in stress responses and three in plastid metabolism were considered linked to the model. Two additional TCs not differentially expressed in the microarray analysis, but identified by quantitative reverse transcription PCR analysis and annotated as a β -glucosidase and a β -glucuronidase precursor, were also used to refine the

model. Our previous study hypothesized the involvement of these 29 DE-TCs in specific biological processes based on the automatic annotation performed by the Blast2GO software using information available at the time of publication. In the present work the 29 DE-TCs were remapped and reannotated (Table S1) using the new tomato genome assembly SL2.50 and the annotation ITAG SL2.40. The updated genome was recently released utilizing a fluorescence in situ hybridization (FISH) and optical mapping approach that allowed improved arrangement of scaffolds (Shearer et al., 2014). Figure 1 represents the distribution of the 29 DE-TCs on the twelve tomato chromosomes with respect to the distribution of all the TCs present on the Combinatrix chip used for the transcriptomic analysis. Nine DE-TCs mapped to chromosome 1 and five to chromosome 12, whereas three or four mapped to each of chromosomes 2, 4, and 10. Only one or two mapped to chromosomes 5, 6, 7, and 9. No DE-TCs mapped to chromosomes 3, 8, and 11. It is noteworthy that on chromosome 1, besides the nine DE-TCs identified, three additional genes related to the AsA biosynthetic and recycling pathways were detected. One L-ascorbate oxidase (Solyc01g008830), one guanosine diphosphate-mannose 3',5'-epimerase (Solyc01g097340) and one L-galactose dehydrogenase (Solyc01g106450) co-localized close to Solyc01g008080, Solyc01g097920, and Solyc01g106780, respectively. When other genes from various AsA pathways identified in tomato were examined (Ioannidi et al., 2009; Cronje et al., 2012), other clusters were found with genes differentially expressed in IL12-4 (data not shown). Of the five DE-TCs mapping to chromosome 12, only one (corresponding to Solyc12g098340) belongs to the D-galacturonate biosynthetic pathway, which contributes to AsA accumulation in tomato fruit (Badejo et al., 2012). In our previous study we hypothesized that this pectinesterase was one of the key enzymes in the model proposed to explain the higher AsA content observed in IL12-4. Therefore, to verify if other candidate genes also co-localized with

this pectinesterase on chromosome 12, we investigated all genes mapping to region 4 of chromosome 12 using information available from the completed tomato genome sequencing project.

Identification of Candidate Genes in the Region 12-4

The first step to identify new candidate genes was to define the introgressed region limits. For this purpose, we took into account the results recently reported by Chitwood et al. (2013) but updated them with the newly released version of the tomato genome (i.e., SL2.50). We established that the region spans from Solyc12g088820 (at around 64 Mbp) to Solyc12g100360 (at around 67 Mbp). After looking specifically at the 460 genes mapping between these borders, 32 genes were identified that might have a function related to AsA biosynthesis and accumulation (Table S2). One group includes genes that are involved in the mechanisms of AsA recycling, such as two apoplastic L-ascorbate oxidases (Solyc12g094460, Solyc12g094470), one glutathione dehydrogenase (Solyc12g094430), and three peroxidases (Solyc12g089360, Solyc12g089370, Solyc12g096530). L-ascorbate oxidase controls the redox status of extracellular AsA (Pignocchi and Foyer, 2003; Sanmartin et al., 2003). This enzyme is considered to be involved in the first step of the AsA degradation pathway in the apoplast (Green and Fry, 2005); however, only up to 10% of AsA is transported to the apoplast (Noctor and Foyer, 1998). Glutathione dehydrogenase (or dehydroascorbate reductase) is one of the main enzymes that recycle oxidized forms of ascorbate. Different studies have highlighted the increase in AsA or redox ratios via the overexpression of the monodehydroascorbate reductase and dehydroascorbate reductase. These studies have shown that AsA increased 2- to 4-fold in photosynthetic tissue, 1.5 to 6-fold in potato tubers and maize kernels (Goo et al., 2008; Naqvi et al., 2009), and 1.6-fold in fleshy fruit such as tomato (Haroldsen et al., 2011).

The second group of identified genes includes those for a glyceraldehyde 3-phosphate dehydrogenase (Solyc12g094640), a diphosphate-fructose-6-phosphate 1-phosphotransferase (Solyc12g095760), and a phosphofructokinase (Solyc12g095880). All of these enzymes are involved in carbohydrate metabolism, and sugar metabolism has been correlated with AsA content in previous studies (Causse et al., 2004; Stevens et al., 2007). The role of sugars as substrates in AsA biosynthesis is well documented (Hanson and Smeekens, 2009).

The third group of interest contains genes involved in AsA biosynthetic pathways alternative to that of Smirnoff-Wheeler, which is the most represented in plants (Valpuesta and Botella, 2004). Among the genes of this group there are three pectinesterases (Solyc12g098340, Solyc12g099230, and Solyc12g099410) and three polygalacturonases (Solyc12g096730, Solyc12g096740, Solyc12g096750). These enzymes are involved in the degradation of cell wall polymers, the

first step of the D-galacturonic pathway. Other genes belonging to this group are involved in the D-glucuronic pathway, such as a UDP-D-glucoronate 4-epimerase (Solyc12g098480) and 10 glucosyltransferases, and in the myo-inositol pathway, such as a myo-inositol oxygenase (Solyc12g098120). These findings are also supported by GO enrichment analysis of the IL12-4 region. The enrichment in this region of a number of genes involved in sugar metabolism and in the various AsA pathways (Figure S1) demonstrates that the wild alleles present in the region 12-4 play a significant role in increasing AsA production in the IL12-4.

Beside the pectinesterase corresponding to Solyc12g098340, which was highly overexpressed in the IL12-4 (21.69-fold), all the other candidate genes mapping in the region 12-4 were not differentially expressed between the wild and the cultivated genotypes. Therefore, we hypothesized that the structural differences could play a role in increasing AsA levels in the introgression line 12-4. We examined those genes to determine if they contribute to this increase.

Detection of Polymorphisms in Candidate Genes

Recently, the 150 Genome Consortium made publicly available the structural variation browser for many cultivated tomato genotypes and its wild relatives (www.tomatogenome.net/VariantBrowser). This tool also includes recently published SNPs and InDels for studying variations associated with introgressions and breeding events (Causse et al., 2013) and for detecting intraspecific polymorphisms in the model tomato cultivar 'Micro-Tom' (Kobayashi et al., 2014). It is therefore possible to find SNPs/InDels between the reference genome Heinz and the accession LA0716 of *S. pennellii* in every gene annotated in the framework of the whole sequenced tomato genome. We used this tool to detect structural variations in the list of the 32 candidate genes that map to the introgressed region and might be involved in increasing AsA content in the fruit of IL12-4. Among these candidate genes, only the UDP-D-glucuronic acid epimerase (Solyc12g098480) did not show any polymorphism, possessing neither SNPs nor InDels (Table 2). Considering the other 31 genes, only eight InDels were detected with predicted deleterious effect on the related *S. pennellii* proteins as estimated by the software PROVEAN. In two cases the InDels caused a predicted frame shift in the protein translation, and in two cases caused alternative stop codons. Out of 519 identified SNPs, the number of SNPs per gene varied from a minimum of 1 for Solyc12g099210 to a maximum of 36 for Solyc12g094470. We found an average of 16.2 SNPs per gene, and approximately 50% of detected SNPs (250/519) were nonsynonymous. A total of 29 variants were predicted to cause a deleterious effect by PROVEAN, in that the amino acid substitutions observed might have an impact on the biological function of *S. pennellii* proteins compared with the cultivated *S. lycopersicum*. Functionally relevant variations were observed in 18 genes belonging to the three groups described in the previous

Table 2. Polymorphisms detected in the 32 candidate genes for AsA control that map in the introgression region 12-4.

Solyc ID [†]	No. of InDels	No. of SNPs [‡]	No. of nonsynonymous SNPs	No. of PROVEAN deleterious variants
Solyc12g089080	1	9	8	2
Solyc12g089360	0	24	9	1
Solyc12g089370	0	9	2	0
Solyc12g089380	0	9	3	0
Solyc12g094430	0	7	2	0
Solyc12g094460	0	22	7	1
Solyc12g094470	0	36	9	2
Solyc12g094640	0	12	1	0
Solyc12g095760	0	9	2	0
M10				
Solyc12g095880	0	13	7	0
Solyc12g096080	1	23	15	1
Solyc12g096530	1	18	7	1
Solyc12g096730	0	15	7	2
Solyc12g096740	0	23	11	1
Solyc12g096750	0	9	2	1
Solyc12g096820	1	20	13	0
Solyc12g096830	1	17	12	1
Solyc12g096870	0	28	17	3
Solyc12g097080	2	18	11	5
Solyc12g098120	0	9	3	1
M11				
<i>Solyc12g098340</i>	0	16	13	0
<i>Solyc12g098480</i>	0	0	0	0
<i>Solyc12g098580</i>	0	24	12	1
<i>Solyc12g098590</i>	0	12	6	0
<i>Solyc12g098600</i>	0	23	12	1
<u>Solyc12g099190</u>	1	15	12	1
<u>Solyc12g099200</u>	0	5	2	0
<u>Solyc12g099210</u>	0	1	0	0
<i>Solyc12g099230</i>	0	14	4	0
<i>Solyc12g099410</i>	0	32	15	1
<i>Solyc12g099680</i>	0	32	18	3
<i>Solyc12g099730</i>	0	15	8	0
Total polymorphisms	8	519	250	29
Total genes showing polymorphisms	7	31	31	18

[†] The position of markers M10 and M11 representing the upper border of the two selected sublines is also included. In italics: pectinesterases; underlined: pectinesterase inhibitors. For details see Table S2.

[‡] SNP, single nucleotide polymorphism.

paragraph. Polymorphisms were also detected in one pectin methylesterase inhibitor (Solyc12g099190), which might in some way interact with the pectinesterases present in this or other genomic regions. Indeed, it has been shown that pectin methylesterase activity is regulated by endogenous inhibitor proteins (pectin methylesterase inhibitors, PMEIs) in kiwi fruit (Balestrieri et al., 1990), pepper, broccoli, wheat (An et al., 2008; Peaucelle et al., 2008; Hong et al., 2010; Zhang et al., 2010), and *Arabidopsis* (Raiola et al., 2004; Juge, 2006; Jolie et al., 2010).

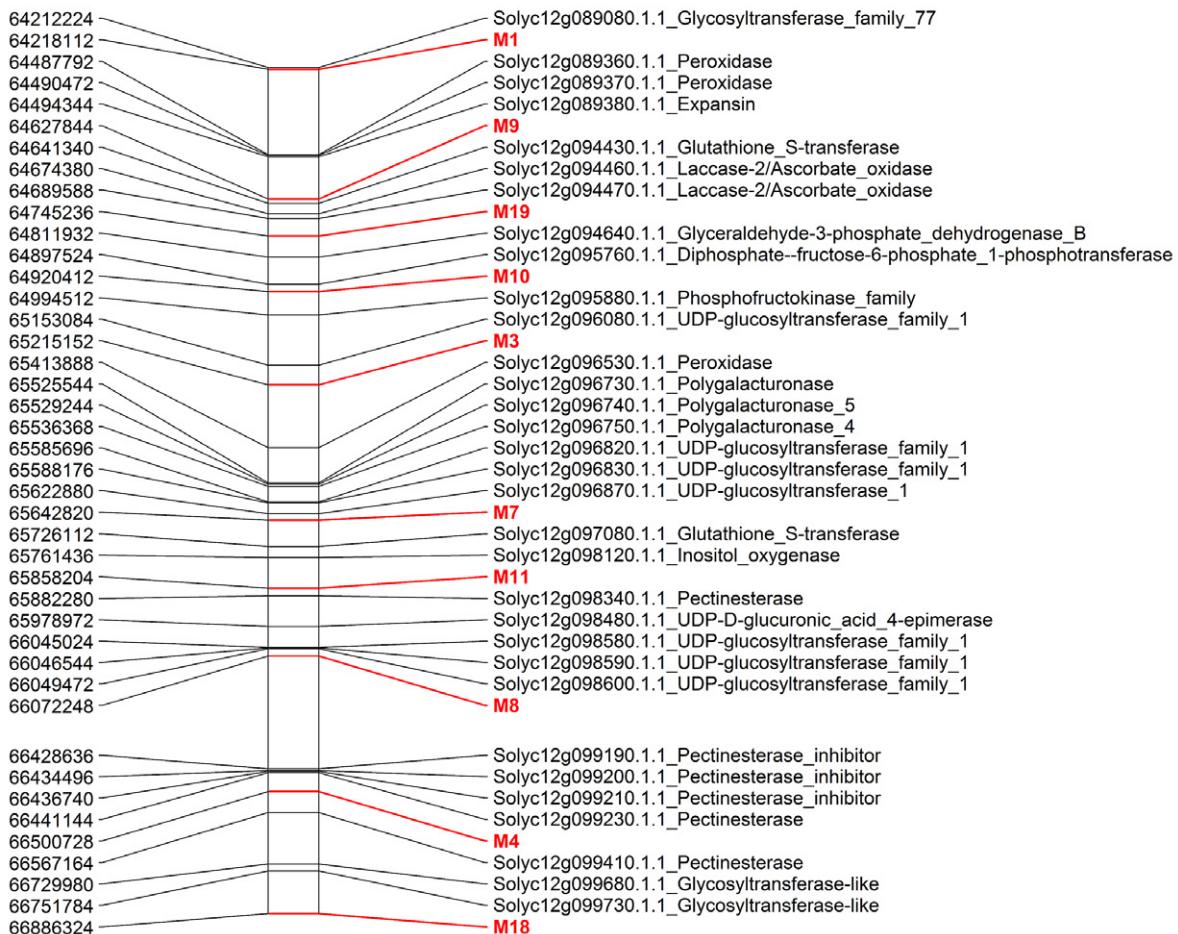
Recently, Reca and coworkers (2012) reported that the expression of a Solyc PMEI in tomato was correlated during ripening with that of PME-1 (pectin methylesterase-1), the major fruit-specific PME isoform. This evidence allowed us to hypothesize that a modified functionality between pectinesterases of IL12-4 and M82 located in the region 12-4 could also be due to the structural variation that the pectinesterase inhibitor Solyc12g099190 exhibited in IL12-4 with respect to the cultivated form.

To further decrease the number of candidate genes to focus on, we decided to split the IL12-4 into various sublines that carry the introgression on chromosome 12 with a reduced size with respect to the wild region of the IL12-4. These sublines would allow us to further dissect the positive QTL for AsA, which was detected on this region. It is expected that the obtained sublines could exhibit different fruit AsA contents, depending on the genes present in the specific wild genomic region.

Selection of Introgression Sub-lines

Additional sublines were searched for among the F₂ progenies previously obtained in our laboratory (Sacco et al., 2013). These materials were screened with *S. pennellii*-specific markers more or less distributed on the top (M1), at the center (M3), and on the bottom (M4) of the 46-cM introgressed region. The primers and restriction enzymes used in this screening are listed in Table 1. This analysis allowed us to define for each genotype whether the introgression region was present at the homozygous or heterozygous condition or absent. We found seven genotypes that exhibited a heterozygous region 12-4. Self-crossed progenies from these genotypes were then screened with 10 new markers specific for the region 12-4 and mapping at an average distance of 0.23 Mbp. The positions of these markers with respect to the putative candidate genes identified in this study are shown in Figure 2. This analysis allowed four IL12-4 sublines to be identified. The chromosomal location and size of these sublines are shown in Figure 3 with respect to the other sublines of the region 12-4 publicly available. When we started our research activity, the only introgression subline of IL12-4 publicly available was the IL12-4-1 selected by Eshed and Zamir (1995). Two additional sublines (IL12-4-2 and IL12-4-3) in this region were later generated by Alseekh et al. (2013). The IL12-4-1 line carries the wild genome in the very distal end of chromosome 12, described as bin 12-I by Pan et al. (2000). This subline was evaluated in our laboratory for AsA fruit content, and it exhibited a level comparable to that of M82, thus demonstrating that wild genes localized in the upper part of introgression 12-4 are required for increasing AsA. Therefore, we hypothesized that the key genes for AsA are located in bins G and H of the introgression 12-4. The first subline identified in our work (IL12-4A) carries the wild introgression starting from marker M10 to the end of the chromosome (from around 64.9 Mbp forward) and spans a region of approximately 2.1 Mbp, thus disrupting bin G and reducing the number of candidate genes from 32

REGION 12-4



Figures 2. Mapping of the 32 candidate genes and of polymorphic molecular markers (M) in region 4 of chromosome 12. On the left, the position in bp of each gene/marker on the chromosome is also reported. Markers in red are from the present work.

to 23. Consequently, this subline lost the wild alleles for a group of genes including most of those involved in the AsA recycling pathway and some glucosyltransferases. The other three sublines (overall coded IL12-4B) carry the same wild region spanning from marker M11 (at around 65.8 Mbp) to the end of the chromosome; this segment covers a 1.2-Mbp region, which disrupts bin H, and consequently only includes 12 candidate genes. Therefore, this group of sublines, IL12-4B, lost the wild alleles for one peroxidase and the cluster of polygalacturonases. In the wild regions of both sublines IL12-4A and IL12-4B, genes annotated as unknown proteins were also mapped, and their potential involvement in the control of AsA level in tomato fruit could not be excluded. Functional characterization would be required to determine if they are potential candidates in controlling AsA content.

Analysis of AsA content in the fruit of our sublines IL12-4A and IL12-4B revealed levels of AsA significantly higher than in the control genotype M82 (Fig. 4). They exhibited a range of AsA from 35.23 mg 100 g⁻¹ fresh wt. (IL12-4A) to 42.91 mg 100 g⁻¹ fresh wt. (IL12-4B3), which are higher than the 32.50 mg 100 g⁻¹ fresh wt. of M82 and comparable to the 37.07 mg 100 g⁻¹ fresh wt. of IL12-4. These results indicate that wild alleles of genes responsible

for the higher AsA content are shared by the four sublines, and thus that the 12 genes mapping in their common region spanning from M11 onward represent the best candidates responsible for the observed phenotype.

Therefore, considering candidate genes mapping in the region between markers M11 and M18 (Fig. 2), we highlighted a main role for pectinesterases in increasing AsA levels in the fruit of IL12-4. This prediction is in accordance with results reported by Badejo et al. (2012) on the contribution of the alternative D-galacturonate pathway to AsA accumulation in red ripe fruit of the cultivar Micro-Tom. In particular, two different mechanisms involving pectinesterases might be active in IL12-4. The first would involve a higher expression of the pectinesterase Solyc12g098340, and a preliminary analysis of its promoter found 29 SNPs and four InDels in the 1264-bp upstream region of the gene. These differences were predicted to modify the number and typology of the *cis*-elements motifs present in the promoter of the wild *S. pennellii* compared to the reference (Heinz). Interestingly, we identified an ethylene responsive element 257 bp upstream of the start codon present only in the promoter of the IL12-4 sequence. This enhancer element mediates ethylene-induced activation and is involved in

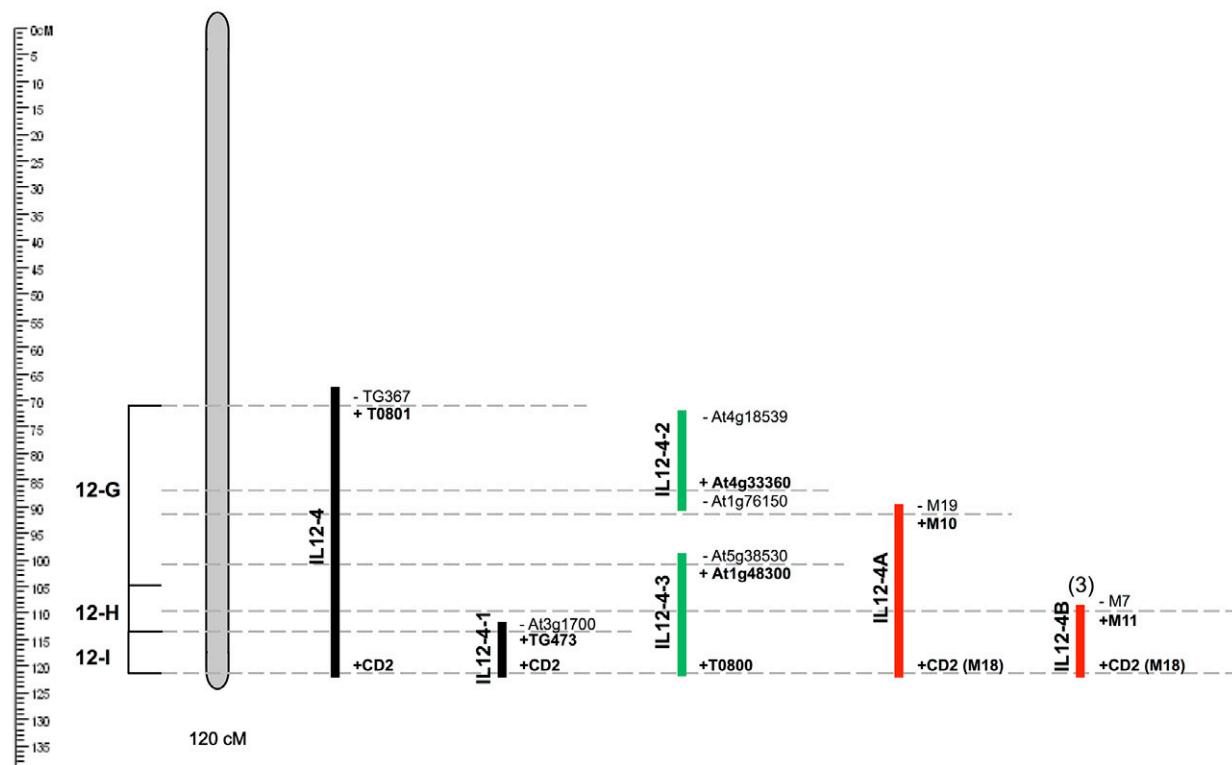


Figure 3. Position of the introgression line IL12-4, three sublines from the literature (IL12-4-1, IL12-4-2, IL12-4-3), and our selected sublines (IL12-4A and IL12-4B) with respect to the chromosome 12. For each line, markers delimiting the borders (+, wild; -, cultivated) and bins are reported. Markers coded "M" are from the present work. The number in parenthesis above subline IL12-4B represents the number of genotypes sharing the same marker pattern.

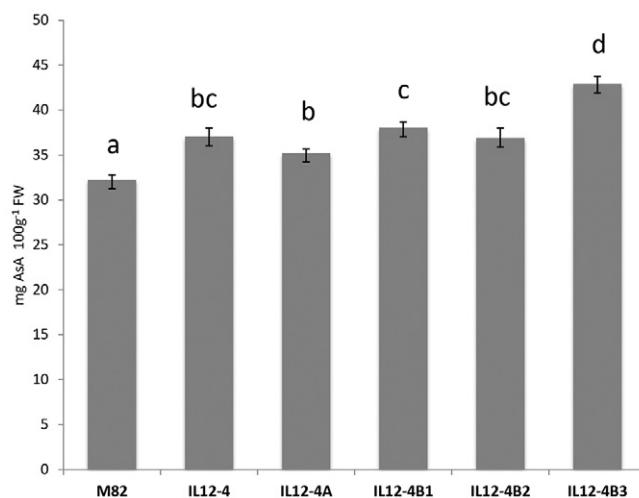


Figure 4. Evaluation of AsA content in the four IL12-4 sublines compared with the parental genotypes M82 and IL12-4. Values with different letters are significantly different ($p < 0.005$, Duncan test).

different processes, including senescence-related expression (Itzhaki et al., 1994), fruit ripening (Montgomery et al., 1993), ethylene stress-induced expression (Tapia et al., 2005), and circadian regulation (Rawat et al., 2005). It could also play a relevant role in pectinesterase expression of the IL12-4 line. The second mechanism might be

ascribed to the structural deleterious polymorphisms found between the two species in the pectinesterase Solyc12g099410 and in the inhibitor Solyc12g099190. Although the InDel detected in the inhibitor produces changes in the N-terminal portion of the inhibitor protein, this is a region not directly involved in the PME/PMEI interaction, as described by Di Matteo et al. (2005). However, by using the I-TASSER software some predicted alterations of the secondary structure were surveyed that could potentially affect folding and functionality of the protein. These potential alterations merit further investigation. Functional validation of the implication of these three novel candidate genes in increasing AsA would better define their role, and the selected sublines exhibiting higher AsA will be extremely useful to this purpose.

Conclusions

The integration of different "omics" approaches initially led to the identification of 32 putative candidate genes in the wild region 4 of chromosome 12 that might be involved in the increase of AsA content previously observed in the *S. pennellii* IL12-4 fruit. Among these genes, only one pectinesterase showed differential expression between IL12-4 and M82, whereas structural variations that have potential impacts on the biological function of related proteins were detected in 18 candidate genes.

Thirteen polymorphic markers between *S. pennellii* and *S. lycopersicum*, designed using the recently available 150 Genome Consortium browser, were used to dissect the wild region in subregions and to select two IL12-4 sublines (IL12-4A and IL12-4B). The evaluation of AsA content in tomato fruit of these sublines led to identification of three priority candidate genes mapping in the wild region they share and exhibiting either deleterious structural variants or a differential expression. Finally, our findings support the hypothesis that pectinesterases have a crucial role in increasing AsA in the fruit of subline IL12-4. This increase is probably either through the transcriptional regulation of a polymorphic promoter in case of Solyc12g098340, or through an altered interaction between one pectinesterase and one inhibitor due to their structural polymorphisms, as could be the case with Solyc12g099190 and Solyc12g099410. A lower or null functional interaction between these two proteins in IL12-4 might explain a higher rate of pectin degradation that could increasingly feed the AsA biosynthetic flux through the D-galacturonate pathway. The validation of these hypotheses, as well as the involvement of other candidate genes present, including those up till now classified as “unknown protein,” will require additional functional studies to be definitively supported. These efforts will probably be successful thanks to the continuously evolving of the genomic resources available for tomato.

Supplemental Information Available

Supplemental information is included with this article.

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