



Pectic enzymes as potential enhancers of ascorbic acid production through the D-galacturonate pathway in Solanaceae



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ABSTRACT

The increase of L-Ascorbic Acid (AsA) content in tomato (*Solanum lycopersicum*) is a common goal in breeding programs due to its beneficial effect on human health. To shed light into the regulation of fruit AsA content, we exploited a *Solanum pennellii* introgression line (IL12-4-SL) harbouring one quantitative trait locus that increases the content of total AsA in the fruit. Biochemical and transcriptomic analyses were carried out in fruits of IL12-4-SL in comparison with the cultivated line M82 at different stages of ripening. AsA content was studied in relation with pectin methylesterase (PME) activity and the degree of pectin methylesterification (DME). Our results indicated that the increase of AsA content in IL12-4-SL fruits was related with pectin de-methylesterification/degradation. Specific PME, polygalacturonase (PG) and UDP-D-glucuronic-acid-4-epimerase (UGlCAE) isoforms were proposed as components of the D-galacturonate pathway leading to AsA biosynthesis. The relationship between AsA content and PME activity was also exploited in *PMEI* tobacco plants expressing a specific PME inhibitor (PMEI). Here we report that tobacco *PMEI* plants, altered in PME activity and degree of pectin methylesterification, showed a reduction in low methylesterified pectic domains and exhibited a reduced AsA content. Overall, our results provide novel biochemical and genetic traits for increasing antioxidant content by marker-assisted selection in the Solanaceae family.

1. Introduction

Solanum lycopersicum represents an important dietary source of antioxidants and other bioactive compounds. Indeed, tomato, being the second most consumed vegetable in the world, is one of the main sources of plant antioxidants [1]. Among these antioxidants, L-Ascorbic Acid (AsA) is highly bioavailable in tomato; therefore, a regular intake of small amounts of tomato fruits can increase cell protection from DNA damage induced by oxidant species [2]. In humans, AsA shows significant ability as electron donor and potent antioxidant; it protects against oxidation of LDL by different types of oxidative stress and inhibits LDL oxidation by vascular endothelial cells [2,3]. Recently, it has also been demonstrated that insufficient intakes of AsA lead to scurvy and/or low immunity [4]. In plants, AsA has an important role for the detoxification of reactive oxygen species and as an enzymatic cofactor,

and participates in stress response, plant development, senescence, defence, division, electron transfer and fruit ripening [4–7]. The possibility of enhancing the nutritional quality of cultivated tomato by increasing the content of AsA in the fruit has received considerable attention in the last few years [8]. Indeed, AsA content in commercial tomato fruits, ranging from 10 to 20 mg 100 g⁻¹ fresh weight (FW), is relatively low compared to other fruit types [9]. This is also due to the artificial selection exerted during tomato domestication that resulted in the loss of about 75% of the chemical diversity of wild relatives [8,10]. Plants produce AsA through several biosynthetic pathways and, recently, the architecture of the different ascorbate pathways in *Solanum lycopersicum* has been reconstructed by using a bioinformatics approach [8]. It is generally accepted that the prevalent AsA biosynthetic pathway, known as the Smirnoff-Wheeler pathway, uses GDP-mannose proceeding through L-galactose [1,9,11]. A side branch of the L-

Abbreviations: Ara, arabinose; AsA, ascorbic acid; BR, breaker; CG, candidate gene; CW, cell wall; DHA, dehydroascorbate; DME, degree of pectin methylesterification; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; GlcA, glucuronic acid; HGA, homogalacturonic acid; LDL, low-density lipoprotein; Man, mannose; MIOX, myo-Inositol oxigenase; MG, mature green; MR, mature red; MDHA, monodehydroascorbate; PME, pectin methylesterase; *PMEI*, pectin methylesterase inhibitor; PG, polygalacturonase; Rha, rhamnose; UGlcAE, UDP-D-glucuronic-acid-4-epimerase; Xyl, xylose

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galactose pathway through GDP-gulose has been recently suggested, as well as alternative pathways through D-galacturonate and myo-inositol [1,11,12]. In the D-galacturonate (D-GalA) pathway, first described in strawberry, AsA could be synthesized through the reduction of D-GalA, derived from pectin de-methylesterification by pectin methylesterases (PMEs) required for pectin degradation by endopolygalacturonases (PGs) [9,11]. The expression of PGs and PMEs has been previously associated to the D-GalA pathway for ascorbate biosynthesis; however, the relationship between PME activity and AsA production has not been yet elucidated [13]. The D-GalA pathway could be also stimulated by UDP-glucuronate epimerisation catalyzed by UDP-D-glucuronic-acid-4-epimerase (UGlCAE) [9,11], most likely, by increasing the galacturonic acid content [14].

In plants, the recycling pathway can also contribute to the regulation of AsA accumulation; since, once formed, the reduced form of ascorbate is rapidly oxidized to monodehydroascorbate radicals (MDHA) and dehydroascorbate (DHA) that can be enzymatically reduced back to AsA [6,15,16]. However, the genetic engineering of genes encoding enzymes involved in the recycling pathway led to contrasting results [8]. Finally, the involvement of a class of transporter (Nucleobase Ascorbate Transporter, NAT) in the mechanisms of AsA accumulation was hypothesized, due to the inability of the negatively charged form of AsA to diffuse through lipid bilayers at physiological pH [5,8,17].

Several strategies have been recently carried out to improve the content of antioxidants in tomato fruits. The use of wild genetic resources as a potential source of genetic and biochemical diversity for the improvement of the cultivated tomato quality was taken into account [18,19]. Wild introgression populations were previously generated to effectively re-introduce unused genetic variation from wild species into cultivated varieties and also to facilitate the detection of favorable wild alleles controlling the trait under study [20,21]. The *Solanum pennellii* IL population, which consists of 76 lines with different and defined wild chromosomal segments in the genetic background of the cultivated variety M82, was recently used for discovery of QTLs associated with agronomically interesting traits [20,22–24]. In our laboratory, one *Solanum pennellii* in *Solanum lycopersicum* introgression sub-line of the region 12-4 (IL12-4-SL) was previously selected harbouring one QTL at the bottom of chromosome 12 that increases the content of AsA in tomato fruits [25]. In this study transcriptomic, metabolic and biochemical analyses were carried out in fruits of the IL12-4-SL sub-line and of the cultivated line M82. We identified a PME, a PG and a UGlCAE as enzymes involved in the D-GalA pathway leading to AsA biosynthesis in tomato fruits of the introgression line. Exploiting tobacco plants overexpressing a PME inhibitor, we observed that tobacco PME inhibitor plants, altered in PME activity and degree of pectin methylesterification, showed a reduction in low methylesterified pectic domains and exhibited a reduced AsA content. Overall our results demonstrate the participation of PME activity and HG methylesterification in ascorbate biosynthesis.

2. Materials and methods

2.1. Plant material

The *S. pennellii* in *S. lycopersicum* introgression line IL12-4-B, renamed here IL12-4-SL, was obtained as described in Ruggieri et al. [25]. The cultivated genotype M82 (accession LA3475) was kindly provided by the Tomato Genetics Resources Centre (<http://tgrc.ucdavis.edu/index.cfm>). The genotypes IL12-4-SL and M82 were grown in an experimental field located in Acerra (Naples, Italy), with three replicates per genotype (10 plants per replicate). Fruits were collected at Mature Green (MG, 40 days post anthesis), Breaker (BR, 45 days post anthesis) and Mature Red (MR, 55 days post anthesis) stages.

Seeds and columella were subsequently removed, and fruits were chopped, ground in liquid nitrogen by a blender (FRI150, Fimar) to a fine powder, and kept at -80°C until the subsequent metabolic,

molecular and enzymatic analyses.

Transgenic tobacco plants overexpressing a PME inhibitor from *Actinidia chinensis* (AcPMEI), obtained as described in Lionetti et al. [26], and non-transformed *Nicotiana tabacum* plants (cv. SR1) were grown in a greenhouse in controlled conditions at the Department of Agricultural Science at the University of Naples (Naples, Italy). Plants were grown in 20 cm pots containing a 1:1 mixture of medium-sandy soil and compost with three replicates per genotype. Leaves from transgenic and non-transgenic plants were collected, ground in liquid nitrogen and stored at -80°C until subsequent analyses.

2.2. Determination of tomato fruit firmness

Firmness of fruits at MG, BR and MR stages collected from M82 and IL12-4-SL was measured by using a penetrometer PCE-PTR200 with a surface needle of 3.1- or 9.6-mm². Per each sample three biological replicates (three fruits per replicates) were analysed. At least three punctures from opposite sites per fruit were performed.

2.3. Ascorbic acid determination

Both reduced ascorbic acid (reduced AsA) and total ascorbic acid (AsA + DHA, total AsA) determinations were carried out by a colorimetric method [27] with modifications reported by Rigano et al. [28]. Briefly, 500 mg of frozen powder from tomato fruits at three ripening stages or from tobacco leaves were extracted with 300 μl of ice cold 6% TCA (trichloroacetic acid). The mixture was vortexed, incubated for 15 min on ice and centrifuged at 14,000 rpm for 20 min at 4°C . For reduced AsA evaluation, to 20 μl of supernatant, 20 μl of 0.4 M phosphate buffer (pH 7.4) and 10 μl of double distilled (dd) H₂O were added. Then, 80 μl of color reagent solution were added. This solution was prepared by mixing solution A (31% (w/v) H₃PO₄, 4.6% (w/v) TCA and 0.6% (w/v) FeCl₃) with solution B (4% (w/v) 2,2'-Dipyridyl). For total AsA test, to 20 μl of sample, twenty microliters of 5 mM dithiothreitol in 0.4 M phosphate buffer (pH 7.4), were added to reduce the oxidized ascorbate, and the mixture was incubated for 20 min at 37°C . Ten microliters of *N*-ethyl maleimide (NEM; 0.5% (w/v) in water) were added, mixed, and left for 1 min at room temperature. Eighty microliters of color reagent were added as previously described for reduced AsA. Both the final mixtures for the tests of reduced and total AsA were incubated at 37°C for 40 min and measured at 525 nm by using a NanoPhotometer™ (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in nmol of AsA according to the standard curve, designed over a range of 0–70 nmol; values were then converted into mg 100 g⁻¹ of fresh weight (FW). Results were expressed as the mean value \pm SD ($n = 3$). Differences among analysed genotypes were determined by using SPSS (Statistical Package for Social Sciences) Package 6, version 15.0. Significant different levels were determined by comparing mean values through a factorial analysis of variance (ANOVA) with Duncan post hoc test at a significance level of 0.05. The comparison between the genotypes at each maturation stage was carried out by a Student's *t*-test. The percentage of variations of each parameter in IL12-4-SL compared to M82 was calculated by using the following formula:

$$\text{Increase or Decrease (\%)} = \left[\frac{\text{value of tested genotype} - \text{value of M82}}{\text{value of M82}} \right] \times 100$$

2.4. Identification and expression of candidate genes

The search for candidate genes (CG) mapping in the region 12-4-SL of chromosomes 12 and potentially associated with ascorbate metabolism was conducted by exploring the annotations and the Gene Ontology terms of genes included in the region [1,8]. The expression of

CGs in the introgression lines fruit compared to that in M82 was verified by Real-Time PCR amplification. Total RNA was isolated from tomato fruit at different ripening stages of lines M82 and IL12-4-SL by using the TRizol[®] reagent (Invitrogen, Carlsbad, CA., U.S.A.) and treated with RNase-free DNase (Invitrogen, Carlsbad, CA., U.S.A.) according to manufacturer instructions (Invitrogen). Total RNA (1 µg) was treated by the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany). For each RT-PCR reaction, 1 µl of cDNA, diluted 1:10, was mixed with 12.5 µl SYBR Green PCR master mix (Applied Biosystems, Foster City, CA., U.S.A) and 5 pmol each of forward and reverse primers (Supplementary material Table S1) in a final volume of 25 µl. The reaction was carried out by using the 7900HT Fast-Real Time PCR System (Applied Biosystems, Foster City, CA., U.S.A). The amplification program was carried out according to the following steps: 2 min at 50 °C, 10 min at 95 °C, 0.15 min at 95 °C and 60 °C for 1 min for 40 cycles. In order to verify the amplification specificity, the amplification program was followed by the thermal denaturing step (0.15 min at 95 °C, 0.15 min at 60 °C, 0.15 min at 95 °C) to generate the dissociation curves. All reactions were run in triplicate for each of the three biological replicates and a housekeeping gene coding for the *elongation factor 1-α* (*Ef 1-α*; *Solyc06g005060*) was used as reference gene [1]. The expression levels relative to the reference gene were calculated using the formula $2^{-\Delta CT}$, where $\Delta CT = (CT_{RNA_{target}} - CT_{referenceRNA})$ [29]. Comparison of RNA expression was based on a comparative CT method ($\Delta\Delta CT$) and the relative expression was quantified and expressed as RQ (relative quantities) calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (CT_{RNA_{target}} - CT_{referenceRNA}) - (CT_{calibrator} - CT_{referenceRNA})$ [30,31]. M82 in each ripening stage was selected as calibrator for the genes *Solyc12g098340* and *Solyc12g096730*. For the gene *Solyc12g098480* IL12-4-SL BR was used as calibrator. Results were expressed as the mean value \pm SD (n = 3). Differences of expression of candidate genes among samples were determined by using SPSS (Statistical Package for Social Sciences) Package 6, version 15.0. Significant different levels were determined by comparing mean values through a factorial analysis of variance (ANOVA) with Duncan post hoc test at a significance level of 0.05. The comparison between the genotypes at each maturation stage was carried out by a Student's *t*-test.

2.5. Determination of PME activity

PME activity was calculated as previously described [32] with some modifications. Briefly, total protein extracts were obtained by homogenizing tomato fruits at different ripening stages in the presence of 20 mM NaOAc, 1 M NaCl, 0.02% Sodium Azide, protease inhibitor 1:100 v/v (pH 4.7) (P8849, Sigma-Aldrich, St. Louis, MO, U.S.A.). One mL of extraction buffer per g of tissue was used. The homogenates were shaken for 3 h at 4 °C, centrifuged at 15,000 x g for 20 min, and the supernatant collected. Protein concentration was determined in the supernatants using Bradford protein assay method (Bradford reagent, Sigma-Aldrich, St. Louis, MO, U.S.A.) and bovine serum albumin as standard [33]. PECTOPLATE was prepared with 0.1% (w/v) of apple pectin (molecular weight range 30000–100000 Da; 70–75% esterification; 76282, Sigma-Aldrich, St. Louis), 1% (w/v) SeaKem[®] LE agarose (Lonza, Basel, Switzerland, Catalog no: 50004E), 12.5 mM citric acid and 50 mM Na₂HPO₄, pH 6.5. The gel was cast into 120 mm square petri dishes (50 ml per plate) and allowed to polymerize at room temperature. Wells with a diameter of 4 mm were obtained with a steel cork borer and equal amounts of protein samples (1 µg of total protein in 20 µl) were loaded in each well. Plates were incubated at 30 °C for 16 h, and stained with 0.05% (w/v) Ruthenium Red (R2751; Sigma-Aldrich, St. Louis) for 30 min. Plates were incubated at 30 °C for 16 h, and stained with 0.05% (w/v) Ruthenium Red (R2751; Sigma-Aldrich, St. Louis, MO, U.S.A.) for 30 min. The plates were de-stained by several washes with water and the diameter of the fuchsia-stained haloes, resulting from de-methylesterification of pectin, were measured with

Image J software <https://imagej.nih.gov/ij/download.html> [34]. Using the “Analyze” and “Set Scale” windows in Image J, a measurement scale was set by revealing a line of 100 mm drawn on the plate with the tool “Straight”. The haloes areas were surrounded using the “Oval” tool and the values obtained using “Analyze” then “Measure”. The PME activity in the protein extracts was calculated by using a standard curve obtained with known amounts of commercially available PME from orange peel (P5400; Sigma-Aldrich, St. Louis, MO, U.S.A.). Enzymatic data were subjected to ANOVA statistical analyses and means were compared using the Tukey HSD test ($p \leq 0.05$) by using SygmaPlot software. The comparison between the genotypes at each maturation stage was carried out by a Student's *t*-test.

2.6. Determination of DME and monosaccharide composition of CW

The extraction of Alcohol Insoluble Residue (AIR) was performed from tomato pericarp tissues as previously described [35]. De-starched AIR (2 mg) was saponified in 30 µl H₂O and 10 µl of 1 M NaOH. The solution was incubated at room temperature for 1 h and neutralized afterwards with 10 µl HCl. After centrifugation at 14,000 x g for 10 min, aliquots of 10 and 20 µl, respectively, of the supernatant were loaded in microtiter plates (96-well COD.9018, Costar, Cambridge, MA, U.S.A.) and the volume was adjusted to 50 µl. Fifty µl of Alcohol oxidase (Sigma, St. Louis, MO, U.S.A.) was added to each well (0.03 units in 0.1 M sodium phosphate, pH 7.5) and this mixture was incubated at room temperature for 15 min on a shaker. Thereafter, 100 µl of a mixture containing 0.02 M 2,4-pentanedione in 2 M ammonium acetate and 0.05 M acetic acid were added to each well. After 10 min of incubation at 68 °C, samples were cooled on ice and the absorbance was measured at 412 nm in a microplate reader (ETI-System reader; Sorin Biomedica Cardio S.p.A., Saluggia, Italy). The methanol (MeOH) amount was estimated as previously described [36]. The degree of methylesterification was expressed as MeOH to uronic acid molar ratio (%).

Two mg of saponified AIR were incubated in 200 µl of 2 M Trifluoroacetic acid (TFA) at 121 °C. After 1 h, 200 µl of isopropanol was added and the mixtures were evaporated at 40 °C with a stream of N₂ gas. This step was repeated twice and samples were dried at room temperature overnight. The TFA hydrolyzed monosaccharides were suspended in 200 µl of water. The monosaccharide composition of de-starched and TFA hydrolysed AIR was determined by HPAEC-PAD using a PA20 column (Dionex, CA, USA). Peaks were identified and quantified by comparison to a standard mixture of Rhamnose, Arabinose, Fucose, Galactose, Xylose, Mannose, Galacturonic Acid and Glucuronic Acid (Sigma-Aldrich, St. Louis, MO, U.S.A.). The crystalline cellulose was determined as previously described [37].

Results were expressed as the mean value \pm SD (n = 3). Differences among analysed genotypes were determined by using SPSS (Statistical Package for Social Sciences) Package 6, version 15.0. Significant different levels were determined by comparing mean values through a factorial analysis of variance (ANOVA) with Duncan post hoc test at a significance level of 0.05. The comparison between the genotypes at each maturation stage was carried out by a Student's *t*-test. The percentage of variations of each parameter compared to M82 was calculated by using the following formula:

$$\text{Increase or Decrease (\%)} = [(value \text{ of tested genotype} - value \text{ of M82}) \times value \text{ of M82}^{-1}] \times 100$$

2.7. Extraction of chelating agent-soluble solids (ChASS) and immunodot assay

ChASS were extracted from 10 mg of AIR for 4 h at 70 °C in a buffer containing 50 mM Tris-HCl, 50 mM ammonium oxalate, and 50 mM *trans*-1,2-cyclohexanediaminetetraacetic acid (CDTA) (pH 7.2). The

extracts were dialyzed against four changes of deionized water, and lyophilized. The immunodot-blot procedure was performed according to Willats et al. [38]. Equal amounts of ChASS were applied as 1 μ l aliquots to nitrocellulose membrane (0.45 μ m pore size; Bio-Rad, Hercules, CA, USA) in dilution series as indicated and allowed to air dry. Filters were incubated for 1 h in 5% (w/v) milk protein (MP; Bio-Rad) in PBS pH 7.8 (MP-PBS), and probed for 1.5 h with primary JIM5 monoclonal antibodies (purchased from PlantProbes, Paul Knox Cell Wall Lab, University of Leeds, Leeds, UK) diluted 1:20 in 3% MP-PBS. After extensive washes in PBS, arrays were incubated with anti-rat secondary antibody conjugated to horseradish peroxidase (Amersham, UK) diluted 1:1000 in MP-PBS buffer. After washing in PBS, arrays were developed using 4-chloro-1-naphthol (25 ml distilled water, 5 ml MeOH containing 3 mg mL⁻¹ 1–4-chloro-1-naphthol and 25 μ l of 40% (v/v) H₂O₂). JIM5 signals were quantified using Image J software <https://imagej.nih.gov/ij/download.html> [34].

3. Results

3.1. IL12-4-SL fruits exhibit a high content of ascorbic acid and pectic polysaccharides during ripening

Ascorbic acid content was monitored in fruits at three ripening stages of the cultivated line M82 and of the *Solanum pennellii* introgression line IL12-4-SL grown in open fields (Fig. 1). In both tomato lines, the content of total AsA (AsA + DHA) and of the reduced form of AsA (reduced AsA) increased from mature green (MG) to breaker (BR) stage before declining when the fruits reached the mature red (MR) stage (Fig. 1). At the MG stage, the content of total AsA was higher in the sub-line IL12-4-SL compared to the cultivated line M82. At the BR stage, an increase of 49.1% of total AsA in IL12-4-SL compared to M82 and of 62.4% of the reduced form of AsA was observed. Finally, at the MR stage, an increase of total AsA of 32.9% in the introgression line compared to M82 and an increase of 47.3% of the reduced form of AsA were detected. These results indicate that the positive QTL for AsA in the region 12-4-SL [25] influences total AsA content during fruit ripening and modulates the content of the reduced AsA form only in intermediate and advanced phases of fruit maturation.

Due to the involvement of pectin components in the D-galacturonate pathway, we explored the possibility that the differences in AsA content observed in IL12-4-SL and M82 fruits could be related to a different composition of cell wall matrix polysaccharides. As preliminary analysis, the total sugars content in CW matrix of fruits of M82 and of IL12-4-SL was evaluated by HPLC analyses (Figs. 2). The monosaccharides content was significantly higher in IL12-4-SL compared to M82 at the MG and MR stages while not significant differences were observed at the BR stage.

The monosaccharides composition analysis indicates that the galacturonic acid (GalA) was the highest represented sugar in tomato

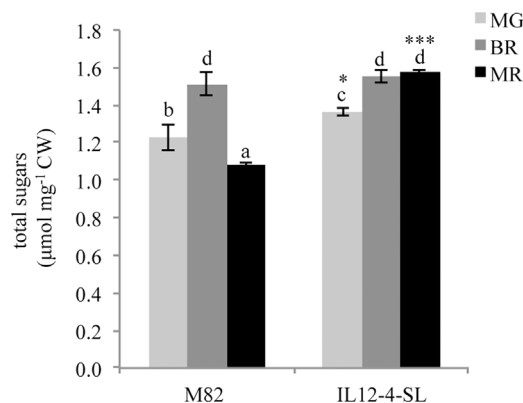


Fig. 2. Content of total sugars measured in CWs of fruits of the introgression line IL12-4-SL and in M82 at different ripening stages (mature green, MG; breaker, BR; mature red, MR). Total sugars are expressed as μ mol mg⁻¹ CW. Values are means \pm SD of three independent experiments (n = 3). Values with different letters are significantly different (p < 0.05; ANOVA). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each ripening stages (*p < 0.05, *** p < 0.001; Student's t-test).

fruits, while the fucose (Fuc) showed the lowest concentrations in all the analysed samples (Fig. 3; Table 1). In the line M82, GalA increased in BR fruits compared to MG fruits before decreasing again at the red ripe stage. Interestingly, in IL12-4-SL the amount of GalA did not undergo a decline and, on the contrary, increased constantly at BR and MR stages. The same trend of accumulation observed in M82 and IL12-4-SL for the GalA was registered for the monosaccharides rhamnose (Rha), xylose (Xyl) and mannose (Man). Instead, the content of galactose (Gal) and arabinose (Ara) decreased at the MR stage compared to BR and MG stages in both lines. At the MG and MR ripening stages, a higher content of Ara and Gal was registered in IL12-4-SL compared to M82. At the MR stage a higher content of Fuc (50%), Rha (50%), Xyl (26%), GalA (44%), and GlcA (53%) was detected in the line IL12-4-SL compared to M82. These results indicate a high content of pectic polysaccharides in the introgression line. The higher GalA content in IL12-4-SL compared to M82 correlates with the higher AsA content observed in the same line. These evidences could imply a higher substrate availability in the introgression line for the D-galacturonate pathway leading to AsA biosynthesis.

3.2. Candidate genes coding for pectic enzymes are related to AsA biosynthesis in tomato fruits

In order to identify the candidate genes controlling AsA content in the *Solanum pennellii* introgression line, we explored the wild region 12-4-SL that covers a region of 1.8 Mbp and carries 180 wild genes [25]. Based on the *S. lycopersicum* genes of the different ascorbate pathways recently identified by using a bioinformatics approach [8] and by

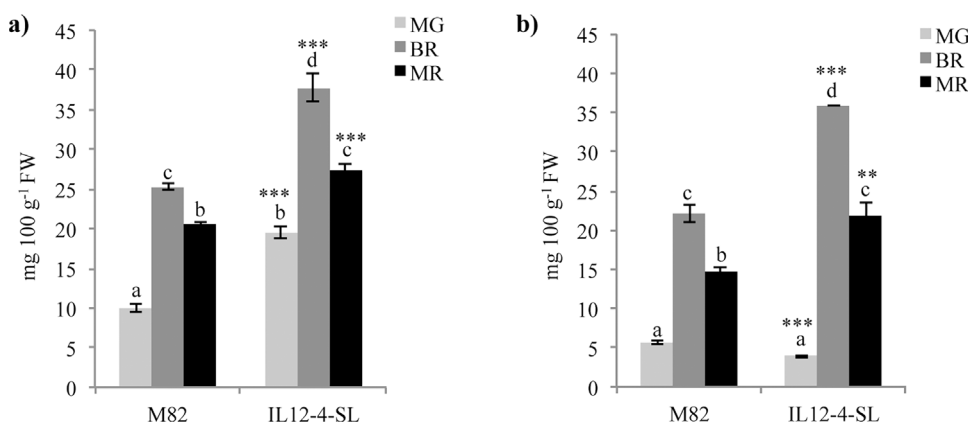


Fig. 1. Content of total AsA (a) and of the reduced form of AsA (b) calculated in fruits of the introgression line IL12-4-SL and in M82 at different ripening stages (mature green, MG; breaker, BR; mature red, MR). Total and reduced ascorbic acid are expressed as mg 100 g⁻¹ FW. Values are means \pm SD of three independent experiments (n = 3). Values with different letters are significantly different (p < 0.05; ANOVA). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each ripening stage (**p < 0.01, *** p < 0.001; Student's t-test).

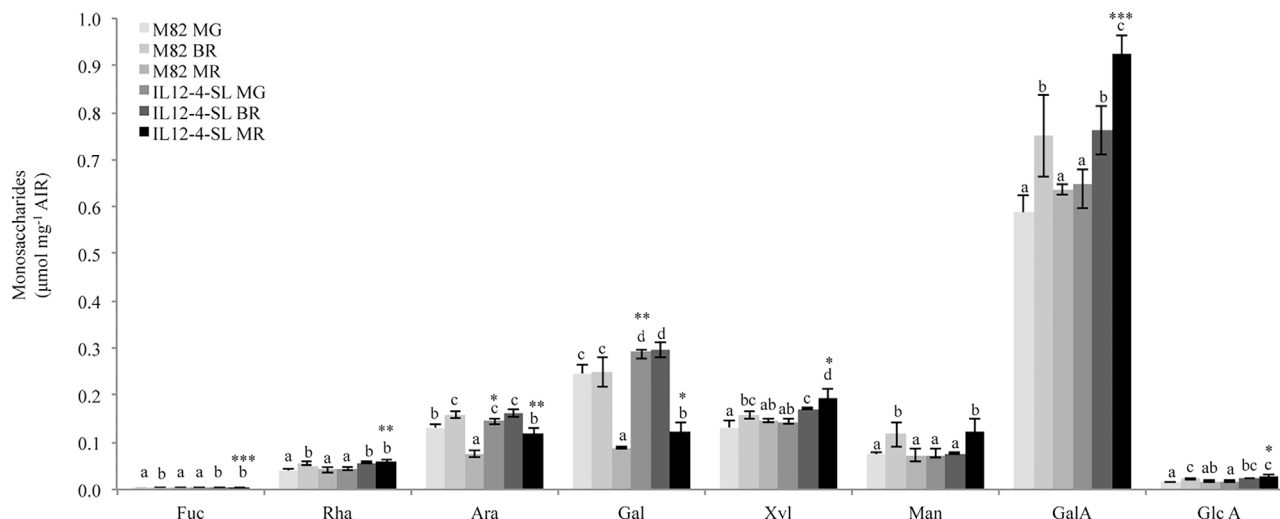


Fig. 3. Monosaccharide composition of matricial CW polysaccharides in fruits of the introgression line IL12-4-SL and in M82 at different ripening stages (mature green, MG; breaker, BR; mature red, MR). The monosaccharides fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), xylose (Xyl), mannose (Man), galacturonic acid (GalA) and glucuronic acid (GlcA) are expressed as $\mu\text{mol mg}^{-1}$ AIR. Values are means \pm SD of three independent experiments ($n = 3$). Values with different letters are significantly different ($p < 0.05$; ANOVA). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each ripening stages (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t -test).

Table 1

Monosaccharide composition of matricial cell wall polysaccharides in fruits of the introgression line IL12-4-SL and in M82 at different ripening stages (mature green, MG; breaker, BR; mature red, MR). The monosaccharides fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), xylose (Xyl), mannose (Man), galacturonic acid (GalA) and glucuronic acid (GlcA) are expressed as $\mu\text{mol mg}^{-1}$ AIR. Values are means \pm SD ($n = 3$). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each ripening stage (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t -test).

| Means | | | | | | | | |
|---------------------|-----------|----------|----------|----------|---------|--------|-----------|---------|
| | Fuc | Rha | Ara | Gal | Xyl | Man | GalA | Glc A |
| M82 MG | 0.0018 | 0.0405 | 0.1312 | 0.2465 | 0.1317 | 0.0734 | 0.5878 | 0.0149 |
| M82 BR | 0.0027 | 0.0551 | 0.1579 | 0.2498 | 0.1583 | 0.1167 | 0.7499 | 0.0231 |
| M82 MR | 0.0021 | 0.0418 | 0.0751 | 0.0883 | 0.1471 | 0.0724 | 0.6368 | 0.0173 |
| IL12-4-SL MG | 0.0020 | 0.0444 | 0.1473* | 0.2929** | 0.1420 | 0.0716 | 0.6487 | 0.0159 |
| IL12-4-SL BR | 0.0025 | 0.0568 | 0.1612 | 0.2958 | 0.1714 | 0.0769 | 0.7618 | 0.0223 |
| IL12-4-SL MR | 0.0031*** | 0.0601** | 0.1182** | 0.1243* | 0.1938* | 0.1207 | 0.9238*** | 0.0269* |
| Standard deviations | | | | | | | | |
| | Fuc | Rha | Ara | Gal | Xyl | Man | GalA | Glc A |
| M82 MG | 0.0001 | 0.0026 | 0.0083 | 0.0173 | 0.0153 | 0.0067 | 0.0349 | 0.0015 |
| M82 BR | 0.0003 | 0.0042 | 0.0062 | 0.0319 | 0.0093 | 0.0270 | 0.0858 | 0.0017 |
| M82 MR | 0.0001 | 0.0063 | 0.0064 | 0.0033 | 0.0044 | 0.0145 | 0.0109 | 0.0023 |
| IL12-4-SL MG | 0.0002 | 0.0019 | 0.0046 | 0.0044 | 0.0091 | 0.0150 | 0.0304 | 0.0039 |
| IL12-4-SL BR | 0.0002 | 0.0031 | 0.0089 | 0.0154 | 0.0024 | 0.0038 | 0.0516 | 0.0005 |
| IL12-4-SL MR | 0.0003 | 0.0029 | 0.0137 | 0.0189 | 0.0215 | 0.0294 | 0.0388 | 0.0051 |

exploring the annotations and the Gene Ontology terms of genes included in the introgressed region, we identified five candidate genes involved in the ascorbate biosynthetic pathway out of 180 genes mapping in the region 12-4-SL. As previously reported, none of the identified candidate genes belongs to the main Smirnoff-Wheeler pathway [25]. Three candidate genes coding for pectic enzymes linked to the D-GalA biosynthetic pathway of AsA were identified: the *PMEs* isoforms *Solyc12g098340*, *Solyc12g099230* and *Solyc12g099410* [5]. A *myo-Inositol* oxigenase (*MIOX*) (*Solycg098120*) and a *UGlcAE* (*Solyc12g098480*) genes, which code for enzymes involved in the Glucuronate/*myo*-inositol pathway, were also present in the introgressed region.

The expression of the selected candidate genes in fruits of M82 and IL12-4-SL at the three ripening stages was analysed using real-time q-PCR. The *PME* genes *Solyc12g099230* and *Solyc12g099410* and the *MIOX* gene were not expressed in tomato fruits at any ripening stages (data not shown) and then were not further investigated. The *UGlcAE* gene was expressed only at the MG and BR stages in fruits of the line

IL12-4-SL (Fig. 4), while the gene was not expressed in M82 fruits during ripening.

Different expression patterns of the *SolyPME* (*Solyc12g098340*) transcripts were identified in M82 and IL12-4-SL tomato lines. While in M82 the *SolyPME* expression decreased during ripening, in IL12-4-SL the expression significantly increased in the BR stage but declined in the MR stage. A higher expression of the *SolyPME* was demonstrated in the introgression line compared to M82 at the BR and MR stages (Fig. 4). By examining the region immediately outside the 12-4-SL region, we identified three *PGs* isoforms (*Solyc12g096730*, *Solyc12g096740* and *Solyc12g096750*). In order to establish their possible contribution in AsA biosynthesis, their expressions were analysed in M82 and in IL12-4-SL fruits during ripening. The lack of expression of *Solyc12g096740* and *Solyc12g096750* in tomato fruits (data not shown) indicates that these genes cannot be involved in the regulation of AsA content in this organ. Instead, the relative expression of *SolyPG* (*Solyc12g096730*) peaked at BR stage and then declined at MR stage in both M82 and IL12-4-SL genotypes. A higher expression of the *SolyPG*

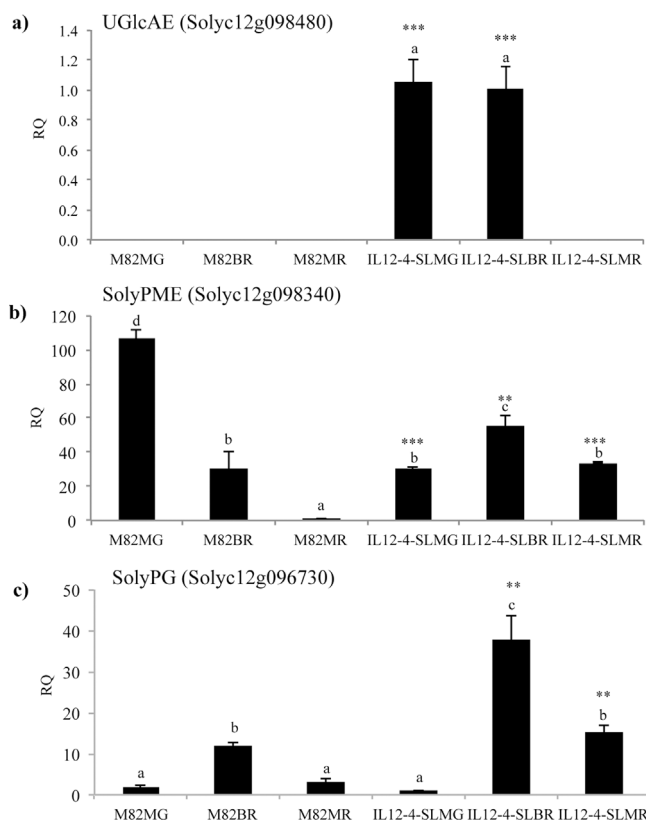


Fig. 4. Expression of genes coding for pectic enzymes possibly involved in AsA biosynthesis during tomato fruit ripening. Relative RNA accumulation of the genes *UglcAE* (*Solyc12g098480*) (a), *SolyPME* (*Solyc12g098340*) (b) and *SolyPG* (*Solyc12g096730*) (c) in fruits of the introgression line 12-4-SL and in M82 at different ripening stages (MG, mature green; BR, breaker; MR, mature red). Values are means \pm SD of three independent experiments ($n = 3$). Values with different letters are significantly different ($p < 0.05$; ANOVA). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each ripening stages (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's *t*-test).

was demonstrated in the introgression line compared to M82 at the BR and MR stages (Fig. 4). Interestingly, the kinetics and the levels of co-expression of *SolyPME* and *SolyPG* genes correlate with the AsA accumulation observed in IL12-4-SL during ripening.

3.3. IL12-4-SL displayed an increased PME activity and a decreased degree of methylesterification compared to M82 during fruit ripening

The next step was to evaluate if the increased *SolyPME* expression in the introgression line results in an altered PME activity and pectin methylesterification during ripening. PME activity and degree of pectin methylesterification (DME) were quantified at different ripening stages in IL12-4-SL and M82 fruit (Fig. 5). In both tomato lines, PME activity significantly increased at BR and MR stages. A significantly higher PME activity was registered in IL12-4-SL compared to M82 at the BR and at the MR stages. Consistently, the pectin DME was lower in IL12-4-SL compared to M82 in the last two stages of ripening [36,39]. The increase of PME activity and the lower DME also influenced the binding of JIM5 antibody raised against low-methylesterified pectin (Fig. 5c). Accordingly, JIM5 epitopes appeared increased in IL12-4-SL at BR and MR stages of ripening compared with M82 fruits. These results show for the first time a relationship between a higher PME activity, a lower degree of methylesterification and a higher AsA content in IL12-4-SL compared to M82 in advanced phases of tomato fruit ripening.

Fruit firmness was also tested in M82 and in IL12-4-SL at the different ripening stages (Supplementary material Table S2). Analyses demonstrated that fruit firmness did not change significantly between

M82 and IL12-4-SL during ripening.

3.4. Transgenic tobacco plants overexpressing a pectin methylesterase inhibitor exhibit a high content of AsA

PME activity is post transcriptionally regulated by proteinaceous PME inhibitors [40]. PMEIs typically inhibit plant PMEs by forming a noncovalent, specific and high affinity 1:1 complex with the enzyme [41] and their expression in plants is used as an efficient biotechnological tool for reducing the overall PME activity *in vivo* [42–45].

To find a functional and direct link between PME activity and AsA production, the AsA content was determined in previously generated and characterised transgenic tobacco plants overexpressing a pectin methylesterase inhibitor (AcPMEI) [26]. Tobacco transgenic PMEI plants, PMEI 3.2 and PMEI 9.4, exhibited a reduction of PME activity and an increased degree of methylesterification compared to non-transformed plants [26]. A reduction of pectin de-methylesterification in leaves of both PMEI plants compared to non-transformed (WT) plants was also revealed using JIM5 antibody (2.6 and 2.9 fold changes in PMEI3.2 and PMEI9.4 plants, respectively, compared to WT plants in 0.4 μ g samples) (Fig. 6a) [36,38,39]. A decrease of total AsA (1.2 and 1.3 fold changes in PMEI3.2 and PMEI9.4 plants, respectively, compared to the non-transformed tobacco plants) and a decrease of the reduced form of AsA (1.2 and 1.7 fold changes in PMEI3.2 and PMEI9.4 plants compared to WT, respectively) were registered (Fig. 6 b, c). These results indicate that in PMEI overexpressing tobacco plants a reduced level of PME activity and of low-methylesterified pectin correlate with a reduced AsA content, supporting the role of PME activity in AsA biosynthesis.

4. Discussion

Thanks to the novel genetic and genomic resources now available for tomato, genes controlling the AsA pathway were recently elucidated [8]. The Smirnoff-Wheeler is considered the main pathway in plant tissues, however the D-GalA pathway, whose precursors derive from degradation of CW polymers, is emerging as an alternative pathway for AsA biosynthesis [13]. This pathway, firstly reported in strawberry, provides as an intermediate the D-galacturonate, a main component of pectin [13]. Badejo et al. [5] demonstrated that the D-GalA pathway may contribute to increasing the ascorbate level in ripening tomato fruits and that the fruit could switch between different sources for AsA supply depending on the ripening stage [5]. Our evidence, showing a lower amount of galactose in the MR stage compared to the former ripening stages in the tomato lines, suggests that the biosynthesis and the maintenance of the final AsA pool at later stage of fruit ripening could rely on alternative pathways, such as the D-GalA pathway [8]. This hypothesis is also supported by the inactivation of the genes related to the main AsA biosynthetic pathway and to the mechanisms of oxidized AsA recovery demonstrated previously at late ripening stages [8]. However, up to date, the involvement of the D-GalA pathway in controlling the AsA pool during tomato fruit maturation is still under debate.

The *Solanum pennellii* introgression line IL12-4-SL carrying a QTL for high AsA content in the red ripe fruits was previously obtained [25]. Comparative transcriptomic analysis of IL12-4-SL and M82 in red ripe fruits identified differences in the expression of pectin – related genes suggesting that AsA accumulation in the introgression line could be achieved through the L-galacturonic pathway [25]. To deepen this concept, in this paper we focused on pectin metabolism in relation with AsA accumulation during fruit ripening of IL12-4-SL compared with the cultivated M82 genotype. The content of total and reduced ascorbic acid in this introgression line was compared to M82 at the three different fruit ripening stages. In both tomato lines, the AsA pool increased over time, peaking at BR stage and then decreasing. This pattern is similar to that observed in other tomato genotypes and in other fruit type

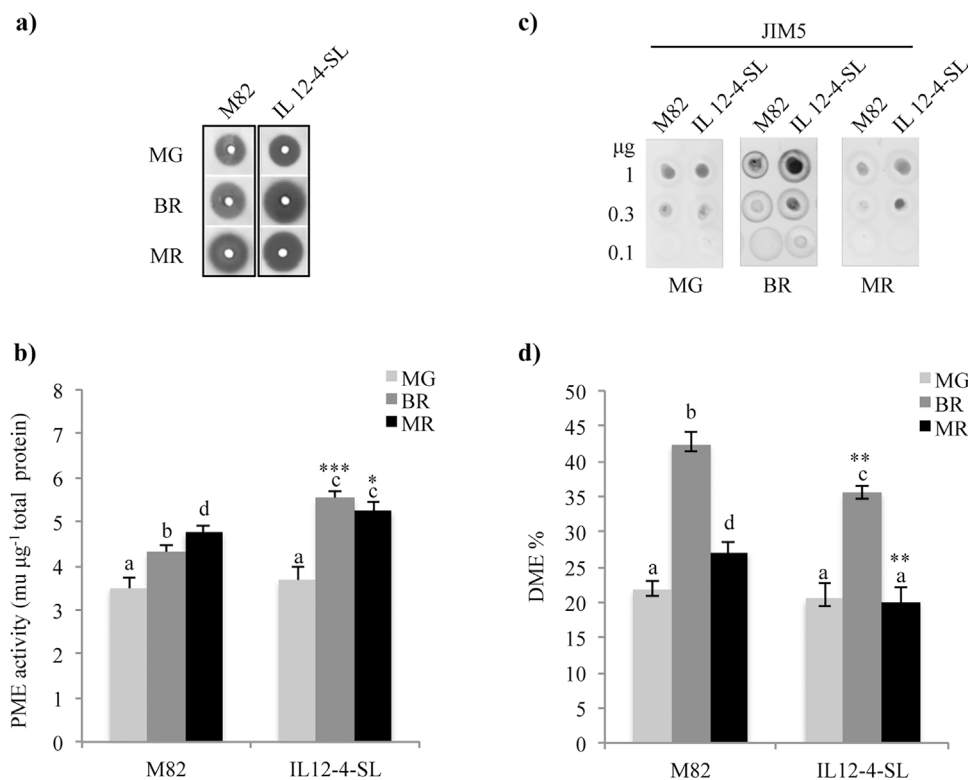


Fig. 5. PME activity and degree of pectin methylesterification in tomato fruits of the introgression line IL12-4-SL and of M82 at different stages of ripening. a) Radial gel diffusion assay showing PME activities in proteins extracted from tomato fruits; b) Quantification of PME activity using image J software; c) Immunodot analysis on pectin fractions detected by using JIM5 monoclonal antibody. The indicated amounts of ChASS fractions were applied as 1 µl aliquots in a 3-fold dilution series to nitrocellulose membrane; d) Degree of pectin methylesterification in tomato fruits of the introgression line IL12-4-SL and of M82 at different stages of ripening. Values are means ± SD of three independent experiments (n = 3). Values with different letters are significantly different (p < 0.05; ANOVA). Asterisks indicate statistically significant differences of IL12-4-SL compared to M82 in each ripening stages (*p < 0.05, **p < 0.01, ***p < 0.001; Student's t-test).

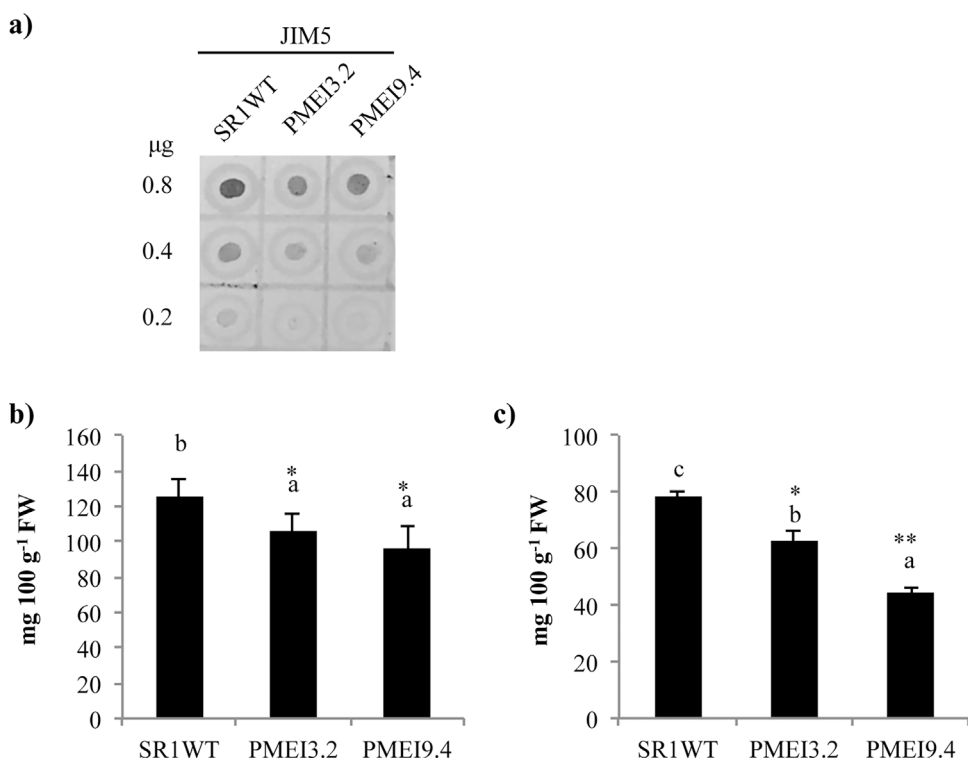


Fig. 6. Immunochemical characterization of isolated leaf pectins using the anti-homogalacturonan monoclonal antibody JIM5 and content of total and reduced AsA determined in leaves of the transgenic tobacco plants PME13.2 and 9.4 and of WT (cv. SR1). a) Immunodot assay indicating amounts of ChASS fraction applied as 1 µl aliquots in a 2-fold dilution series to nitrocellulose membrane. A representative array is shown. b) Total and c) reduced AsA expressed as mg 100 g⁻¹ FW. Values are means ± SD of three independent experiments (n = 3). Values with different letters are significantly different (p < 0.05; ANOVA). Asterisks indicate statistically significant differences of PME13.2 and PME19.4 compared to SR1 (*p < 0.05, **p < 0.01, ***p < 0.001; Student's t-test).

such as kiwi [16,46]. A higher content of total AsA in IL12-4-SL compared to M82 in all the ripening stages and of the reduced form of AsA at the BR and MR stages was detected. These results indicate that the positive QTL for AsA in the region 12-4-SL [25] influences the total AsA content during fruit ripening and modulates the reduced AsA form only in intermediate and advanced phases of fruit maturation.

The amount of GalA in tomato fruits CWs was higher in IL12-4-SL compared to the cultivated line M82 at the MR stage. This could imply

that in IL12-4-SL a high amount of GalA precursors is available for AsA biosynthesis through the D-galacturonate pathway late during ripening [5,8,9,11,14].

The higher level of GalA in IL plants may result from an enhanced pectin biosynthesis or/and from a high pectin degradation [9,11,14]. Interestingly, a gene coding for a UGlcAE (*Solyc12g098480*) was expressed in the introgression line in the MG and BR stages, whereas its expression was not observed in M82 fruits. This pattern of expression

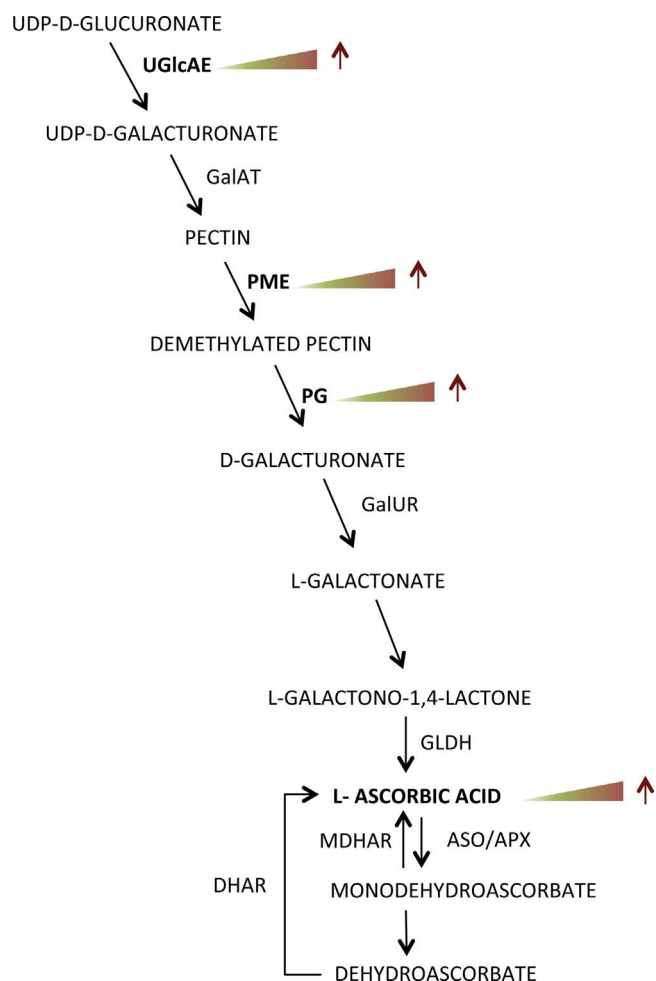


Fig. 7. Schematic model illustrating how an increased expression of enzymes of the alternative D-Galacturonate pathway can lead to an increased ascorbic acid production in Solanaceae. The enzymes (UGlcAE, PME and PG) whose expression increased with ripening in the genotype IL12-4-SL compared to M82 are indicated by a bold font and by a red arrow. UGlcAE, UDP-glucuronic-acid-4-epimerase; GalAT, galacturonyltransferase; PME, pectin methylesterase; PG, polygalacturonase; GalUR, D-galacturonate reductase; GLDH, L-galactonolactone dehydrogenase; MDHAR, monodehydroascorbate; DHAR, dehydroascorbate reductase; ASO, L-ascorbate oxidase; APX, L-ascorbate peroxidase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggests *UGlcAE* as a good candidate gene participating in the regulation of AsA concentration enhancing the D-galacturonate pathway in tomato during fruit ripening. The analyses of the 12-4-SL region allowed to identify also one *SolyPME* gene (*Solyc12g098340*) located in the introgressed region, and one endopolygalacturonase *SolyPG* gene (*Solyc12g096730*) located immediately outside the introgressed region. The expression of *SolyPME* and *SolyPG* was higher in IL12-4-SL compared to M82 at the BR and MR stages. A higher PME activity and a lower DME of pectins were found at the BR and MR stages in IL12-4-SL compared to M82. HG epitopes recognised by JIM5, known to be highly sensitive to PG digestion [38], are also increased in IL12-4-SL at late stages of ripening. Overall, these results suggest that an UGlcAE-dependent glucuronate epimerisation in the IL could lead to a higher pectin accumulation that, combined with an increased pectin de-methylesterification by *SolyPME* followed by pectin degradation mediated by *SolyPG*, participates to the higher AsA content at later stages of ripening through D-galacturonate pathway (Fig. 7). The higher PME activity and lower degree of pectin methylesterification detected at the BR and MR stages in IL12-4-SL compared to M82 well support our concept. Moreover, fruit firmness was similar in M82 and in IL12-4-SL indicating

that the increased expression of the *SolyPME* and *SolyPG* isoforms detected in the introgression line does not influence fruit texture, differently from other isoforms. Indeed, PME activity plays little role in tomato fruit softening during ripening [47]. For example, it was demonstrated that a lower PME activity in tomato plants silenced for the *pme2* gene displayed a higher pectin methylesterification but no changes in fruit softening during ripening compared to non-transformed plants [48,49]. In addition, the fruits of double antisense PE1 (pectin methylesterase 1) and PE2 (pectin methylesterase 2) tomato plants soften normally during ripening [50]. These data indicate that PME activity could play roles unrelated to softening during ripening and support our conclusions.

A functional link between PME activity and AsA content was explored exploiting transgenic tobacco plants overexpressing *AcPMEI* from *Actinidia chinensis* able to efficiently inhibit the tobacco PME isoforms [26]. Tobacco *AcPMEI* plants showed a reduced PME activity and a higher degree of pectin methylesterification compared to the non-transformed plants [26]. We found that the transgenic tobacco plants exhibited a related reduced level of JIM5 epitopes and a decreased content of total and reduced form of ascorbate compared to non-transformed plants.

Altogether, in this work we propose *SolyPME*, *SolyPG* and *UGlcAE* as possible candidate genes contributing to an increase of AsA production by affecting the alternative D-galacturonate pathway in tomato fruits. We found correlations between PME activity, level of pectin de-esterification and AsA production at later stage of tomato fruit ripening. Our results also indicate that the AsA biosynthesis through D-galacturonate pathway is a more general mechanism active in different tissues and different plant species. This knowledge provides novel tools for the development of new molecular markers for breeding and selection of new genotypes with a higher content of antioxidants. Due to the involvement of both PME activity and AsA production in plant resistance to disease [7,51–53] future experiments will be needed to investigate the possible role of these components in plant defense.

Contributions

MMR, AR, VL contributed to metabolic, biochemical and transcriptomic analyses and to draft the manuscript; DB and AB contributed to the conception of the experiment, to data analysis and interpretation and to draft the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.plantsci.2017.10.013>.

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