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Note

Investigating the role of vitamin C in tomato through TILLING identification of ascorbate-deficient tomato mutants

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Abstract Besides being a model for fleshy fruits and *Solanaceae* species, tomato represents one of the main sources of ascorbate (vitamin C) in human diet in many parts of the world. Ascorbate fulfills various roles in plants due to its antioxidant potential and to its connection with other metabolic pathways e.g. cell wall biosynthesis. Among the functional genomic tools recently developed in tomato, EMS (ethyl methanesulfonate) mutant collections provide an opportunity for identifying allelic series of mutations in target genes by TILLING (Targeting Induced Local Lesions IN Genomes). We describe here the use of tomato EMS mutant collections in the miniature cv. Micro-Tom for the discovery of allelic variants in three ascorbate biosynthetic genes encoding the GDP-D-mannose pyrophosphorylase (GMP), the GDP-D-mannose epimerase (GME) and the GDP-L-galactose phosphorylase (GGP) respectively. We report on the discovery of several missense, truncation and splice junction mutations in these genes affecting plant ascorbate content to various levels, and show that several tomato mutant lines with strongly reduced ascorbate content undergo severe bleaching upon exposure to high light intensity.

Key words: Tomato, mutants, ascorbate, vitamin C, antioxidant, TILLING, GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose epimerase (GME), GDP-L-galactose phosphorylase (GGP).

Tomato (*Solanum lycopersicum*) is a member of the *Solanaceae* family that now represents the first cultivated fruit worldwide. As such, it has become a major source of health-beneficial compounds in human diet. One of the major micronutrient with proven health-benefit found in tomato is ascorbate or vitamin C (Fitzpatrick et al. 2012). At physiological pH, ascorbic acid (AsA) or Vitamin C exists as monoanion form called ascorbate. In vivo, the oxidation products of ascorbate are monodehydroascorbate (MDHA) and dehydroascorbate (DHA). The whole constitutes the ascorbate pool that can be more or less oxidized according to the redox state of the cell (Smirnoff 2000). In plants, ascorbate fulfills numerous roles, mainly through its antioxidant potential. Ascorbate acts as a scavenger of the free radicals generated by photosynthesis, cellular respiration, and abiotic stresses such as ozone and UV radiation (Conklin et al. 1996; Noctor and Foyer 1998; Smirnoff and Wheeler 2000). Ascorbate has also proven roles in plant development (Arrigoni and De Tullio 2002) and

stress tolerance in different species (Müller-Moulé et al. 2003; Yamamoto et al. 2005).

Although plants are the main source of vitamin C in the human diet, the major AsA biosynthetic pathway or Smirnoff-Wheeler pathway was described very recently in 1998 in plants (Wheeler et al. 1998). It involves the conversion of D-mannose into ascorbate via a succession of L-galactose containing intermediates namely GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, and L-galactono-1,4-lactone (Figure 1). Clear evidence for this pathway was given by the characterisation of the ascorbate-deficient *vtc1* mutant. Originally identified by screening *Arabidopsis* EMS-mutants for ozone sensitivity, *vtc1* was later shown to be defective in the GDP-D-mannose pyrophosphorylase activity (Conklin et al. 2000). Other *Arabidopsis* EMS ascorbate-deficient mutants have been used in the recent years to decipher the Smirnoff-Wheeler pathway, including the *vtc4* mutant (Galactose phosphatase; Conklin et al. 2006) and the *vtc2/vtc5* mutants (GDP-L-

galactose phosphorylase; Dowdle et al. 2007).

In recent papers, our group showed that the RNAi inactivation of the tomato galactonolactone dehydrogenase (GalLDH), the last enzyme of the major ascorbate biosynthetic pathway in plants, alters plant growth without affecting plant ascorbate content (Alhagdow et al. 2007). In contrast, silencing the GDP-D-mannose epimerase (GME) catalyzing the first committed step of ascorbate biosynthesis reduced plant ascorbate content by up to 50%. This study highlighted the existing link between ascorbate biosynthesis, cell wall biosynthesis and tomato fruit firmness (Gilbert et al. 2009). It was later shown that GME was crucial for proper synthesis of the cell wall constituent RhamnoGalacturonan II (RGII) polymer, and hence for plant growth (Voxeur et al. 2011). Additional experiments followed by transcriptome and metabolome meta-data analyses further indicated that, besides the direct connections between ascorbate and cell wall metabolic pathways, both pathways may share common regulations (Garcia et al. 2009). Stevens et al. (2008) also demonstrated the major role played by ascorbate in tomato fruit postharvest shelf-life by showing that allelic variation in monodehydroascorbate reductase (MDHAR), a key enzyme of the ascorbate recycling pathway, was correlated with fruit firmness losses due to chilling injury.

Among the various genes encoding enzymes from the ascorbate biosynthetic pathway shown in Figure 1, we are mostly interested in the GDP-D-mannose pyrophosphorylase (GMP), the GDP-D-mannose epimerase (GME) and the GDP-L-galactose phosphorylase (GGP). The GME catalyzes the first committed step of the ascorbate pathway and, with GGP, plays a key role in regulating ascorbate biosynthesis in plants (Bulley et al. 2012; Linster and Clarke 2008). In addition, GMP and GME are crucial for the synthesis of activated forms of D-mannose and L-galactose that are precursors of both AsA and key cell wall components such as mannans and rhamnogalacturonans (Gilbert et al. 2009; Keller et al. 1999; Voxeur et al. 2011). All three genes are thus likely targets for modulating ascorbate and cell wall biosynthesis in tomato, and therefore for studying the connection of the ascorbate and cell wall biosynthesis pathways and the development of the sensorial and nutritional quality of the fruit (Fitzpatrick et al. 2012).

To modulate plant ascorbate content and explore its relationships with genetic and environmental factors, in planta silencing or over-expression of genes involved in ascorbate biosynthesis, recycling or regulation through stable genetic transformation is an efficient strategy. As an example, stable over-expression in tomato and in strawberry of the newly discovered GDP-L-galactose phosphorylase (VTC2/VTC5) with the GDP-mannose

The Smirnov-Wheeler pathway of L-ascorbic acid in plants and links with cell wall polysaccharide synthesis

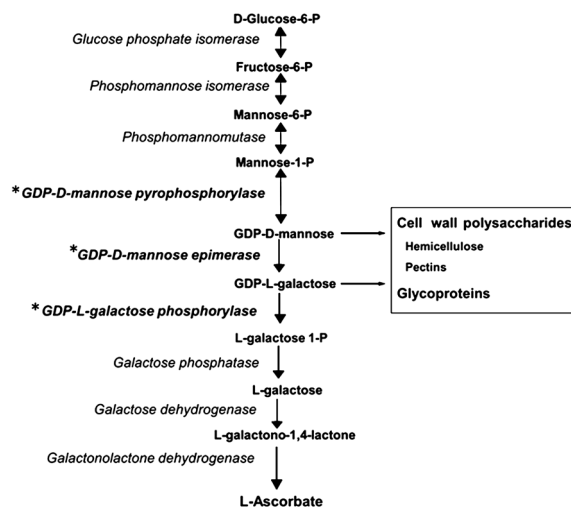


Figure 1. L-Ascorbic acid biosynthesis pathway and connection with cell wall metabolism in plants. In the Smirnov-Wheeler pathway, the carbon skeleton (D-Man 1-P) is synthesized from D-glucose via hexose phosphate intermediates. The activated sugars GDP-D-mannose and GDP-L-galactose are precursors of both ascorbic acid and cell wall polymers and are involved in glycoprotein biogenesis. TILLING screening of the Micro-Tom EMS mutant collections was done for the three enzymes indicated in bold with a star (*): GDP-D-mannose pyrophosphorylase, GDP-D-mannose epimerase and GDP-L-galactose phosphorylase.

epimerase (GME) led to two to six-fold increases in ascorbate levels (Bulley et al. 2012). Recently published results also highlighted how the functional analysis of various target genes in tomato can benefit from the availability of allelic series of mutations identified in tomato EMS mutant collections by TILLING (Di Matteo et al. 2013; Gady et al. 2012; MacAlister et al. 2012). Indeed, EMS mutagenesis is well adapted to tomato, in contrast to other gene inactivation approaches such as the T-DNA insertional mutagenesis commonly used in *Arabidopsis* that involves tedious and lengthy steps of plant transformation and requires large mutant populations to reach saturation mutagenesis in tomato (Emmanuel and Levy 2002).

Mutagenesis by EMS generates point mutations, mainly C to T and G to A transitions, distributed at random over the whole genome. Their frequency varies with the species, the physiological state of the EMS-treated seeds and the EMS dose. As a consequence, large allelic series can be obtained with a limited number of highly mutagenized mutant lines. In tomato, various frequencies have been reported for the different tomato EMS mutant collections published to date, ranging from 1 mutation/322 kb (0.7% EMS) to 1 mutation/574 kb (1% EMS) in the Red Setter cultivar (Minoia et al. 2010), 1 mutation/732 kb in the TPAADASU cultivar (Gady et al. 2009) and 1 mutation/574 kb in the M82 cultivar

(Piron et al. 2010). In the Micro-Tom EMS mutant collections used for this study, the mean mutation frequency reported for the collection generated in Japan ranges between 1 mutation/1710 kb (0.5% EMS) and 1 mutation/737 kb (1% EMS) (Okabe et al. 2011) while estimated mutation frequency from the INRA Bordeaux collection ranges between 1 mutation/125 kb and 1 mutation/663 kb (Dan et al. 2007; unpublished results). Only half of the point mutations in the coding region of a gene are likely to affect the function of the protein (Greene et al. 2003), e.g. by introducing nonsense or splice junction mutations (5%) or non-synonymous nucleotide changes that do result in amino-acid changes with altered amino-acid properties (45%).

Increasing the size of the mutant population screened therefore increases the chance to isolate allelic variants of interest such as splice junction, non-sense or non-conservative missense mutations. To increase the Micro-Tom EMS mutant resources available for TILLING identification of unknown mutations in ascorbate biosynthetic genes, we therefore joined our efforts within the Tsukuba-INRA (TIL) joint lab. Three tomato genes were targeted: the *SIGMP2* gene (SGN-U568548) encoding the GDP-D-mannose pyrophosphorylase (GMP), the *SIGME1* (SGN-U581327) encoding the GDP-D-mannose epimerase and the *SIGGP2* gene (SGN-U579800) encoding the GDP-L-galactose phosphorylase. Selection of the genes was done based on their expression patterns in the fruit, regulation by light (Massot et al. 2012) and differential expression in a AsA-deficient GME-silenced tomato transgenic line (Gilbert et al. 2009).

Identification of unknown mutations by TILLING in Micro-Tom EMS mutant collections from the University of Tsukuba (Japan) and INRA Bordeaux (France) was done essentially as previously described (Okabe et al. 2011) using *Endo1* endonuclease and LI-COR DNA analyzer. By screening 3,052 Micro-Tom mutants (Japan) for mutations in exonic regions of the three genes, three *Slgmp2*, one *Slgme1* and six *Slggp2* mutant lines were identified. The mutations with possible effects on the functions of the encoded proteins were further confirmed by sequencing. These included two mutations in *SIGMP2*, one mutation in *SIGME1* and two mutations in *SIGGP2*. Further screening of 7,296 mutant lines (France) for mutations in the *SIGGP2* gene additionally produced six confirmed mutations for that gene, among which one was a nonsense mutation (Figure 2). Point mutations with likely strong effect on protein function were identified among the *Slgmp2* and *Slggp2* mutant lines identified (Figure 2). One of the *Slgmp2* mutant lines displayed a nonsense mutation causing premature termination of the protein in the N-terminal region while the second mutant exhibited a non-synonymous change of the neutral amino-acid glycine to the charged amino-

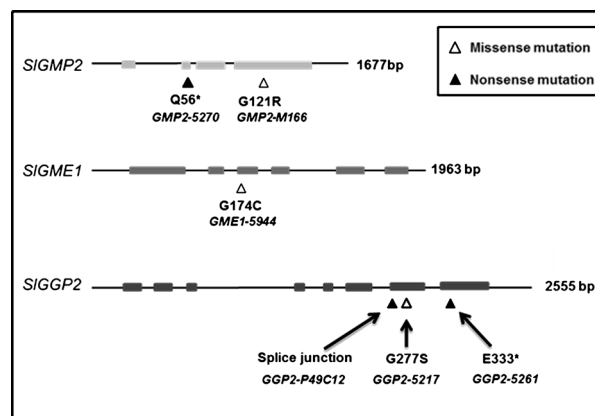


Figure 2. Representation of exonic mutations in the *SIGMP2* (SGN-U568548), *SIGME1* (SGN-U581327) and *SIGGP2* (SGN-U579800) genes. Predicted size of the coding DNA sequence is indicated on the right for each gene. Grey boxes represent the exons. Missense mutations are indicated by open triangles and nonsense mutations by black triangles. Amino acid changes (e.g., glycine to arginine G121R) are also indicated (star symbol correspond to stop codon). Name of the mutant line is indicated below.

acid arginine (G121R). Among the three *Slggp2* mutant lines, one was a splice junction mutant and a second was a nonsense mutant, both mutations leading to the truncation of the protein. Additional non-synonymous but potentially milder exonic mutations were identified in the *Slgme1* mutant (glycine to cysteine G174C) and in one *Slggp2* mutant (glycine to serine G277S).

To test whether induced mutations within the targeted isoforms of GMP, GME and GGP could alter vitamin C accumulation in the mutant lines, total ascorbate content of fully expanded leaves was measured in the corresponding homozygous mutants as previously described (Gilbert et al. 2009). As shown in Figure 3, significant reductions in AsA content were observed in 4 mutants out of the 6 examined. While the missense mutations *Slgmp2* G121R (line GMP2-M166) and *Slggp2* G277S (line GGP2-5217) did not alter significantly leaf AsA content of the mutant plants, moderate (~35 to 40%) reductions were observed in the *Slgmp2* Q56* (line GMP2-5270) and *Slgme1* G174C (line GME1-5944) mutants and strong reductions (~95%) were observed in the two *Slggp2* truncation and splice junction lines (GGP2-5217 and GGP2-P49C12-2 respectively). Because the *SIGMP2* gene belongs to a tomato multigene family, the moderate AsA reduction in the *Slgmp2* truncation (line GMP2-5270) is likely explained by functional redundancy between these genes. Same explanation may hold true for the GME1-5944 line, though the mild ascorbate phenotype observed in this line may also result from the creation of a weak allele by the missense mutation. More unexpected is that, despite the expression of two genes encoding GDP-L-galactose phosphorylase in tomato (Massot et al. 2012), the two independent *Slggp2* truncation and splice junction

mutant lines have only ~5% residual ascorbate content in the leaves (Figure 3). This result indicates that the two *GGP* genes found in tomato are not functionally redundant and are unable to complement each other in the conditions studied.

In normal growth conditions in the greenhouse, no obvious phenotypic alteration of the various homozygous mutant plants was observed. In order to check whether the changes in leaf ascorbate content induced by the most important ascorbate reductions have physiologically relevant effects in the plants, we submitted in May–June 2011 the *Slgmp2* truncation mutant (GMP2-5270 line; ~40% reduction in AsA content), the *Slgme1* misense mutant (GME1-5944; ~35% reduction in AsA content) and a *Slggp2* truncation mutant (GGP2-5261 line; ~95% reduction in AsA content) to high light intensity. Towards this end, the plants were transferred outside of the greenhouse between 9 h to 15 h UT (Universal Time) on a sunny day and exposed to sun light. After this high light treatment, plants were transferred back to

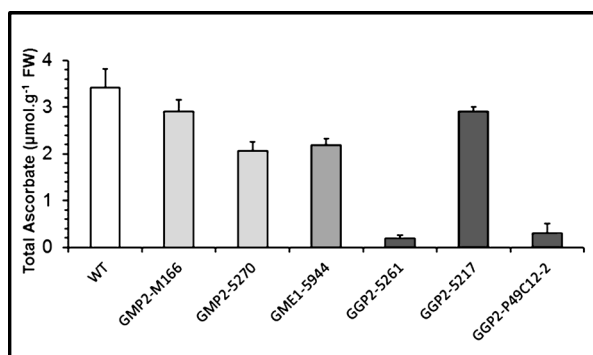


Figure 3. Selected mutants were assayed for total ascorbate content in fully expanded leaves of wild type and homozygous mutant plants. Names of the mutant lines are indicated on the X-axis. Data represent means \pm SE ($n=4$).

the greenhouse, and allowed to recover for 48 h before analysis of light effect. The light intensity exceeded 2000 $\mu\text{mol photons.m}^{-2}\text{s}^{-1}$ PAR (Photosynthetically active radiation) on average in the time course of the experiment whereas the light intensity in the greenhouse in our culture conditions never exceeded 1200 $\mu\text{mol photons.m}^{-2}\text{s}^{-1}$ PAR. As shown on Figure 4, the photo-oxidative stress imposed under natural solar radiation caused leaf surface discoloration in the *Slgmp2* and *Slggp2* ascorbate-deficient mutants, the intensity of which was related to the extent of ascorbate deficiency. In the most severely affected *Slggp2* mutant plant, most leaves showed strong bleaching and wilting and developed necrotic lesions while these symptoms were much less severe in the *Slgmp2* mutant. Interestingly, small leaves protected or shaded by the canopy created by the older ones, did not display this bleaching phenotype. In contrast, the *Slgme1* mutant in which the ascorbate level is similar to that of *Slgmp2* was not affected by such high light treatment (data not shown).

Exposure to intense solar radiation and elevated temperature likely provokes photo-oxidative stress, which in turn triggers damages in plant tissues (Müller-Moulé et al. 2003; Torres et al. 2006). Indeed, as an antioxidant, ascorbate participates through various mechanisms in the protection of plants from reactive oxygen species (ROS) produced as a result of excess absorbed light energy. Plants with constitutively low ascorbate pools (e.g., *Slgmp2* and *Slggp2* truncation mutants) are therefore less susceptible to adapt to high light, as shown for the *Arabidopsis vtc2* mutant deficient in GDP-L-galactose phosphorylase activity, which accumulates 10 to 30% of wild type ascorbate and undergoes bleaching and drying of the leaves, higher degrees of lipid peroxidation and photoinhibition in high light conditions (Müller-Moulé et al. 2003). Interestingly,

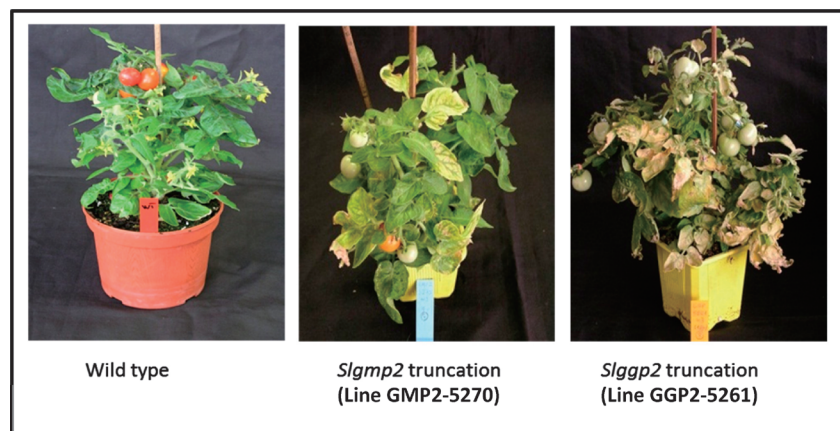


Figure 4. Light effect on ascorbate biosynthesis in ascorbate-deficient mutant plants. Homozygous plants for *Slgmp2* truncation mutation (GMP2-5270 line), *SlGGP2* truncation mutation (GGP2-5261 line) and wild type plants were submitted to high light stress as indicated in Materials and Methods. Mild or severe bleaching and necrosis can be observed on the more exposed leaves of the ascorbate-deficient mutants. Results from a representative experiment are shown.

the *Slgme1* mutant was able to withstand the stress imposed. Possible explanation is that the adaptation of *Slgme1* mutant to high light intensity may result from the up-regulation of ascorbate biosynthesis genes in the leaf in response to light (Massot et al. 2012) and therefore in ascorbate production. The up-regulation of either *SIGME1* or *SIGME2* and as a consequence of GME activity in the leaf would be sufficient to compensate for a weak missense mutation in *SIGME1*.

In conclusion, large Micro-Tom EMS mutant collections have been developed in both France and Japan. The large artificially-induced genetic variability available in these collections can be harnessed through TILLING for identifying allelic series for target genes. As shown in the present study for ascorbate biosynthetic genes, increasing the size of the mutant collection screened increases the possibility of identifying mutants of interest, which are valuable tools for deciphering the physiological role of candidate genes in the plant.

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