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# Towards the characterization of regulators involved in the metabolism of ascorbic acid in tomato: Impact of environmental conditions on plant adaptation

Paul Deslous

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Par **Paul DESLOUS**

Towards the characterization of regulators involved in  
the metabolism of ascorbic acid in tomato

**Impact of environmental conditions on plant adaptation**

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« La plupart des hommes ont de la poésie une idée si vague que ce vague même de leur idée est pour eux la définition de la poésie »

**Paul Valéry**

« Most men have an idea of poetry so vague that this vague of their idea is for them the definition of poetry. »

**Paul Valéry**



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**à Pierre DESLOUS**



## RESUME

### Vers la caractérisation des régulateurs impliqués dans le métabolisme de l'acide ascorbique chez la tomate

L'acide ascorbique (AsA, vitamine C) est l'un des composés parmi les plus importants chez les eucaryotes. Il joue un rôle majeur dans les défenses anti-oxydantes, en réponse aux stress biotiques et abiotiques, principalement en éliminant les EROs (Espèces Réactives de l'Oxygène), et plus particulièrement chez les plantes lors de la photosynthèse. En raison de son potentiel antioxydant élevé, l'AsA représente un critère important de la qualité nutritionnelle des végétaux, et plus particulièrement des fruits. Certains mammifères, y compris l'Homme, ont perdu la capacité à synthétiser l'AsA et doivent donc avoir un apport quotidien par la consommation de fruits et légumes. Outre sa valeur bénéfique pour la santé, une augmentation de la teneur en AsA du fruit aurait un effet bénéfique pour la qualité post-récolte et la résistance aux pathogènes. Par conséquent, comprendre la régulation du métabolisme de l'AsA, afin d'améliorer les espèces d'intérêt agronomique, est une question importante en matière de sélection variétale pour de nombreuses espèces de fruits charnus. En tant que premier fruit consommé au monde, la tomate est une espèce végétale majeure, et constitue un modèle d'étude du développement et de la qualité des fruits charnus. La biosynthèse de l'AsA chez les plantes a été établie par Smirnoff *et al.* (1998), et nommée la voie du L-Galactose. Cependant, les mécanismes de régulation restent méconnus. De nombreux facteurs environnementaux, tels que la température et la lumière, affectent le contenu en AsA dans les plantes en modulant l'expression de gènes et certaines activités enzymatiques. Par exemple, la conservation des fruits à basse température constitue le stress le plus important lié à la phase de post-récolte. *In planta*, la lumière est la principale source d'EROs produits lors de la photosynthèse, processus crucial pour le développement des plantes, et donc de stress oxydatif. Pour mieux comprendre ces régulations chez les plantes et leurs impacts sur la qualité des fruits, une collection de tomates EMS hautement mutagénisée (cv. Micro-Tom) a été criblée pour identifier des mutants dont les fruits seraient enrichis en AsA (notés AsA+). Cette stratégie de génétique directe associant le criblage à une approche de cartographie par séquençage a permis d'identifier de nouveaux gènes liés au caractère AsA+. L'un des mutants, noté P21H6, présentait un enrichissement en AsA de 2 à 4 fois supérieur à celui du WT, et fut le premier à être génétiquement caractérisé. Il correspond à une mutation KO du gène Solyc05g007020 codant pour une nouvelle classe de photorécepteurs sensible à la lumière bleue appelée PLP (PAS/LOV Protéine). Cette PLP est un régulateur négatif de la synthèse d'AsA chez la tomate. La première partie de la thèse a permis de confirmer le rôle de S/PLP dans le phénotype AsA+ du fruit par une approche de mutagenèse dirigée et réaliser sa caractérisation fonctionnelle. Nous avons

démontré que *S/PLP* interagissait avec *S/GGP* (GDP-L-galactose phosphorylase), une enzyme clé de la voie du L-galactose, sous contrôle de la lumière bleue, et que cette interaction avait lieu dans le cytoplasme et le noyau. Nos résultats renforcent l'hypothèse que GGP joue un rôle central dans la biosynthèse de l'AsA et suggèrent un nouveau mécanisme de régulation par la lumière bleue de l'activité GGP, en plus de son rôle métabolique. La deuxième partie de la thèse porte sur la caractérisation génétique d'un second mutant EMS de tomate, appelé P17C5. Ce mutant présentait la plus forte augmentation du taux d'AsA dans les fruits (5 à 10 fois le WT). Outre le phénotype AsA+, le mutant P17C5 présentait un phénotype de parthénocarpie (fruit sans graine) rendant difficile la génération d'une population en ségrégation, nécessaire à la suite de l'étude. Grâce à un croisement avec le cultivar de Tomate M82, la mutation causale a pu être identifiée. Elle correspond à une mutation dominante dans un uORF cis-régulateur en amont du gène *S/GGP*. Ce résultat confirme le rôle clé de la GGP dans la voie du L-galactose. Des études préliminaires en relation avec le phénotype de parthénocarpie du fruit suggèrent un problème de stérilité male associée aux processus de développement du pollen. Enfin, dans l'étude de la qualité des fruits après la récolte, des expériences de stress froid effectuées avec les fruits du mutant PAS/LOV semblent démontrer que l'augmentation de la teneur en AsA améliore la durée de conservation et la capacité de maturation des fruits. Dans l'ensemble, nos résultats confirment la position clé de la protéine GGP dans la voie de biosynthèse de l'AsA. De plus, les deux mutants enrichis en AsA caractérisés dans ce travail de thèse sont un matériel végétal précieux pour comprendre le rôle de la GGP dans la régulation de la biosynthèse de l'AsA ainsi que dans l'étude de l'effet de l'AsA sur la qualité du fruit et ses caractéristiques après la récolte.

**Mot clefs:** Acide ascorbique ; Tomate ; GDP-L-galactose phosphorylase ; Mutants EMS ; Lumière ; Stérilité

## ABSTRACT

### **Towards the characterization of regulators involved in the metabolism of ascorbic acid in tomato**

Ascorbic acid (AsA, vitamin C) is one of the most important biochemical in living organisms. It has a leading role in antioxidant defences, in response to biotic and abiotic stresses and mainly by eliminating ROS (Reactive Oxygen Species) notably in plants during photosynthesis. Due to its high antioxidant potential, AsA represents an important trait of nutritional quality in fruits and vegetables. Few mammals, including humans, have lost the capacity to synthesize AsA and therefore must have a daily intake of fruits and vegetables. In addition to its beneficial health value in fruit consumption, increasing fruit AsA content would likely affect postharvest quality and resistance to pathogens. Thus, understanding the regulation of AsA accumulation in order to improve crop species of agronomical interest is an important issue in plant breeding for many fleshy fruit species. Tomato stands for the model in fleshy fruit development and quality and, is also a major crop plant species, as being the first fruit consumed in the world. The plant AsA biosynthesis was established by Smirnoff *et al.* (1998), namely the L-galactose pathway, but little is known about regulatory mechanisms. Many environmental factors, like chilling stress and light stress, affect AsA levels in plants by modulating gene expressions and enzyme activities. The storage of fruits at low temperature is the most important stress related to the post-harvest phase. During photosynthesis, that is crucial for plant development, light is the main origin of ROS production and so oxidative stress. To get a better understanding of the regulation of AsA level in plants and its impact on fruit quality, a highly mutagenized EMS tomato collection (cv. Micro-Tom) was screened for fruit AsA-enriched (AsA+) mutants. This forward genetic strategy which combined the screening with a mapping-by-sequencing approach, had allowed to identify new genes related to the AsA+ trait. One of the mutant named P21H6, displayed an AsA-enrichment 2 to 4 fold that of the WT, and was the first to be genetically characterized. It corresponded to a KO mutation in the Solyc05g007020 gene encoding a new class of photoreceptor involved in blue light sensing named PLP (PAS/LOV Protein). This PLP is a negative regulator of AsA accumulation in tomato. The first part of the PhD work was to confirm the role of the PLP in the AsA+ plants using a directed mutagenesis strategy and to undertake its functional characterization. We demonstrate that *S/PLP* interacts with *S/GGP* (GDP-L-galactose phosphorylase), a key enzyme of the L-galactose pathway, under blue light control and that this interaction takes place in the cytoplasm and the nucleus. Our results strengthen the central role of GGP in the AsA biosynthesis and suggest a new regulation mechanism by blue light of the GGP function in addition to its metabolic activity. The second part of the PhD was the genetic characterization of a second

EMS Tomato mutant, named P17C5. This mutant displayed the highest increase of fruit AsA level (5 to 10 times the WT). Besides the AsA+ phenotype, the P17C5 mutant displayed a parthenocarpic-like phenotype (seedless fruit) making difficult the generation of a segregant population necessary for further mapping. Thanks to the crossing with M82 tomato cultivar, the causal mutation was identified. It corresponded to a dominant mutation in a cis-acting open reading frame (uORF) upstream of the GGP gene. This result confirms the key role of GGP in the L-galactose pathway. Preliminary studies related to the parthenocarpic phenotype suggest a problem of male sterility associated with pollen development processes. Finally, in the study of the post-harvest fruit quality, chilling stress experiments carried out with the P21H6 fruits seem to demonstrate that increasing AsA content improve the fruit shelf life and its maturation capacity. Taken as a whole, our results confirmed the key position of the GGP protein in the AsA biosynthesis pathway. In addition, the two AsA-enriched mutants characterized in this PhD work are precious plant material for deciphering the role of GGP in the regulation of AsA and for studying the effect of AsA on fruit quality and post-harvest traits.

**Keywords:** Ascorbic acid; Tomato; GDP-L-galactose phosphorylase; EMS Mutants; Light signal; Sterility



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# ABBREVIATION LIST

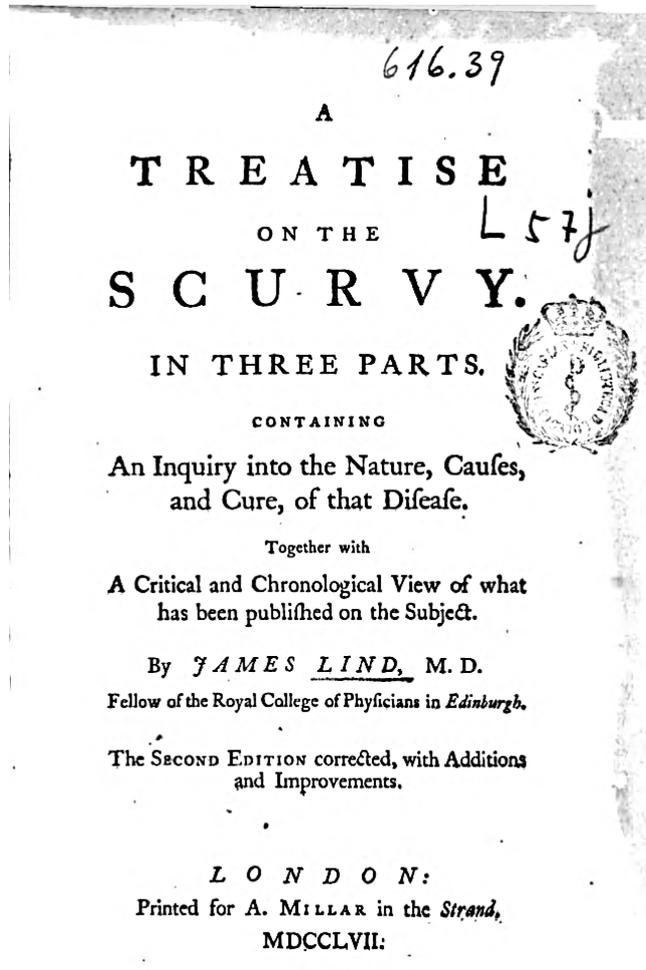
|       |   |
|-------|---|
| ACC   | 1-Aminocyclopropane-1-Carboxylate         |
| AMR1  | Ascorbic Acid Mannose Pathway Regulator 1 |
| AO    | Ascorbate Oxidase                         |
| APX   | Ascorbate Peroxidase                      |
| AsA   | Ascorbate                                 |
| At    | Arabidopsis Thaliana                      |
| ATP   | Adenine Triphosphate                      |
| BY    | Bright Yellow                             |
| CAT   | Catalase                                  |
| COP9  | Constitutive Photomorphogenesis 9         |
| CRY   | Cryptochrome                              |
| CSN5B | COP9 Signalosome Subunit 5                |
| DHA   | Dehydro-L-Ascorbate                       |
| DHAR  | Dehydro-Ascorbate Reductase               |
| DKG   | 2, 3-Diketo-L-Gulonic Acid                |
| DNA   | Deoxyribonucleic Acid                     |
| DRE   | Drought-Responsive Element                |
| EMS   | Ethylmethane Sulfonate                    |
| ERF   | Ethylene Response Factor                  |
| FAO   | Food And Alimentation Organization        |
| FKF1  | Flavin-Binding Kelch Repeat F-Box 1       |
| FW    | Fresh Weight                              |
| GDH   | L-Galactose Dehydrogenase                 |
| GGP   | GDP-L-Galactose Phosphorylase             |
| GLDH  | L-Galactono-1,4-Lactone Dehydrogenase     |
| Gm    | Glycine Max                               |
| GME   | GDP-D-Mannose-3,5-Epimerase               |
| GMO   | Genetically Modified Organisms            |
| GMP   | GDP-D-Mannose-Pyrophosphorylase           |

|                             |  |
|-----------------------------|--|
| GPP                         | L-Gal-1-P Phosphatase                                      |
| GR                          | Glutathione Reductase                                      |
| GSH                         | Reduced Glutathione  |
| GSSG                        | Oxidized Glutathione                                       |
| H2O2                        | Hydrogen Peroxide  |
| Insp3                       | Inositol (1,4,5)-Triphosphate                              |
| KO                          | Knock Out  |
| LKP2                        | LOV Kelch Protein 2  |
| LOV                         | Light Oxygen Voltage                                       |
| MDHA                        | Monodehydro-L-Ascorbate                                    |
| MDHAR                       | Monodehydro-Ascorbate Reductase                            |
| MYB                         | Myeloblastosis   |
| NADP                        | Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form |
| NADPH                       | Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form  |
| NLS                         | Nucleus Localization Signal                                |
| O <sub>2</sub>              | Singlet Oxygen   |
| O <sub>2</sub> <sup>-</sup> | Superoxide Anion   |
| ODD                         | 2-Oxo-Acid-Dependent Dioxygenase                           |
| OH <sup>-</sup>             | Hydroxyl-Radical   |
| Oxa                         | Oxalate  |
| PAS                         | Per-ARNT-SIM   |
| PHT                         | Phosphate Transporter                                      |
| PHY                         | Phytochrome  |
| PLP                         | PAS/LOV Protein  |
| PMI                         | Phosphomannose Isomerase                                   |
| PMM                         | Phosphomannomutase   |
| PR                          | Pathogenesis-Related                                       |
| PS                          | Photo-System   |
| QNP                         | Non-Photochemical Quenching                                |

|         |  |
|---------|--|
| RNA     | Ribonucleic Acid                           |
| ROS     | Reactive Oxygen Specie                     |
| SI      | Solanum Lycopersicum                       |
| SOD     | Superoxide Dismutase                       |
| SVCT    | Sodium-Dependent Vitamin C Transporters    |
| Thro    | Threonate                                  |
| TILLING | Targeting Induced Local Lesions In Genomes |
| UVR     | Ultraviolet Resistant                      |
| VDE     | Violaxanthin De-Epoxidase                  |
| VTC     | Vitamin C Defective                        |
| WT      | Wild-Type                                  |
| ZTL     | Zeitlupe                                   |



# **INTRODUCTION**



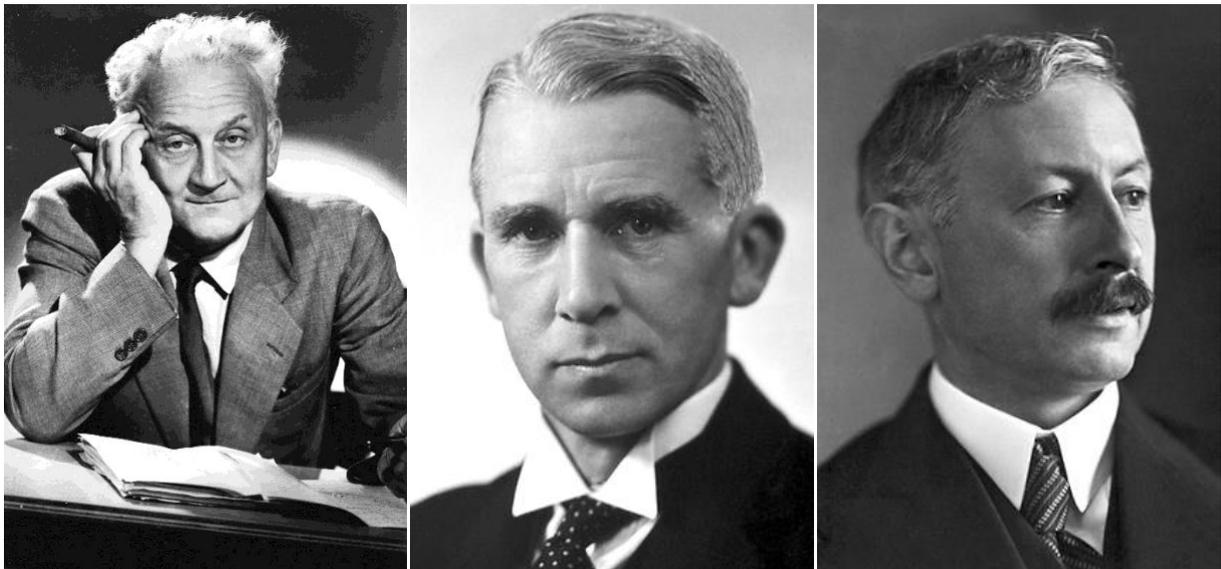
**Figure 1:** James Lind (1716-1794) and his treatise on the scurvy. The Scottish naval surgeon was behind the first prospective comparative trial.

## **VITAMIN C - ASCORBIC ACID**

### Historical Background: Let us go back a bit in time

As for most vitamins, ascorbic acid (Vitamin C) was discovered because of the diseases caused by its deficiency. Already around 1550 BC, the first descriptions of scurvy were mentioned in medical "Ebers Papyrus", presenting anatomic notions, pathologic cases and treatments. During their time, Aristotle and also Hippocrates formally described the symptoms: "The patient smells foully from the mouth, the gums separate from his teeth, and blood flows his nostrils. Sometimes also ulcers break out on his legs - and while some heal, others develop- his colour is dark, and his skin is thin" (L473 Hippocrates VI. Diseases, Internal Affections). It is only in the early of the fifteenth century, with the great maritime explorations, that the disease reappears causing the death of thousands of men. In 1747, a young doctor, James Lind, embarks on a ship of the British fleet to monitor the health status of the sailors (Fig. 1). He determines a correlation between a vegetable-deficient diet and scurvy appearance. James Lind set up the first clinical experiment on humans by defining six groups, each one with a different treatment: vinegar, garlic, cider, lemon associated to orange juice, sulfuric acid and seawater. Seven days later, seafarers eating citrus fruits shows some signs of healing. This experiment established for the first time a link between vitamin C and the prevention of some diseases. Lind published these results in his book "A treatise of the scurvy" in 1753 (Fig. 1). It was only forty years later, in 1790, that Gilbert Blane imposed the consumption of lemon juice on English ships, so that the incidence of scurvy became minor.

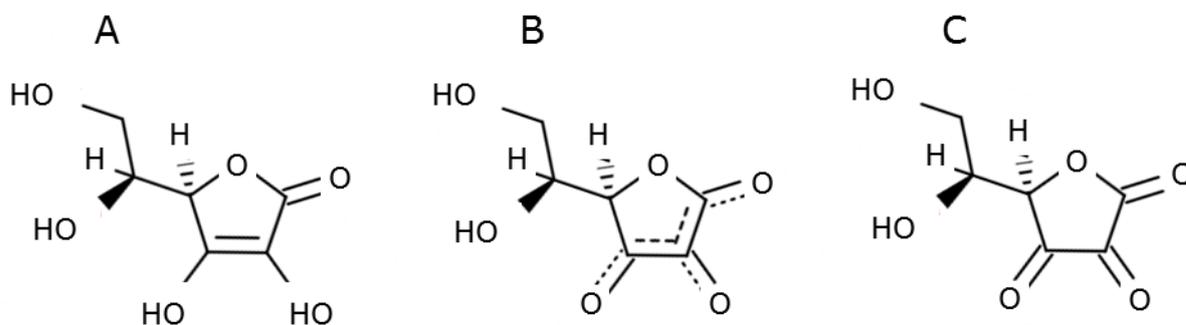
In the beginning of twentieth century, chemists discovered that the deficiency in only one molecule is responsible for scurvy. Then, in 1912, Kazimierz Funk defined the name "Vitamin" to group all the vegetal molecules that are essential for human health, and Drummond named the anti-scorbutic agent "water soluble C" which will give "Vitamin C". From 1928, during almost ten years, research on Vitamin C really took off with no less than two Nobel prizes. First, in 1928, Albert Szent Györgyi who worked on redox potential in both animals and plants, isolated a hexuronic acid displaying a strong antioxidant potential. He identified this molecule as the famous Vitamin C and named it according to the contraction of "anti-scorbutic": Ascorbic Acid. Szent Györgyi won the Nobel Prize in



**Figure 2:** Photos of three Nobel laureates in 1937 for their work on vitamin c. From left to right: Albert Szent Györgyi (Medicine Nobel), Sir Walter Norman Haworth and Paul Karrer (Biochemistry Nobel).

Medicine in 1937 (Fig. 2). The same year, Sir Walter Norman Haworth and Paul Karrer shared the Nobel Prize in chemistry for their research on carbohydrates and vitamin C (Fig. 2). The former, based on the newly identified structure of the vitamin C, succeeded to synthesize the molecule. It can be noted that, the process for the synthetic production of ascorbic acid has been named according to a Swiss researcher Tadeusz Reichstein, who independently get the same results one year earlier. Surprisingly, it was not until 1998 that the first vitamin C biosynthetic pathway was described in plants (Wheeler *et al.*, 1998).

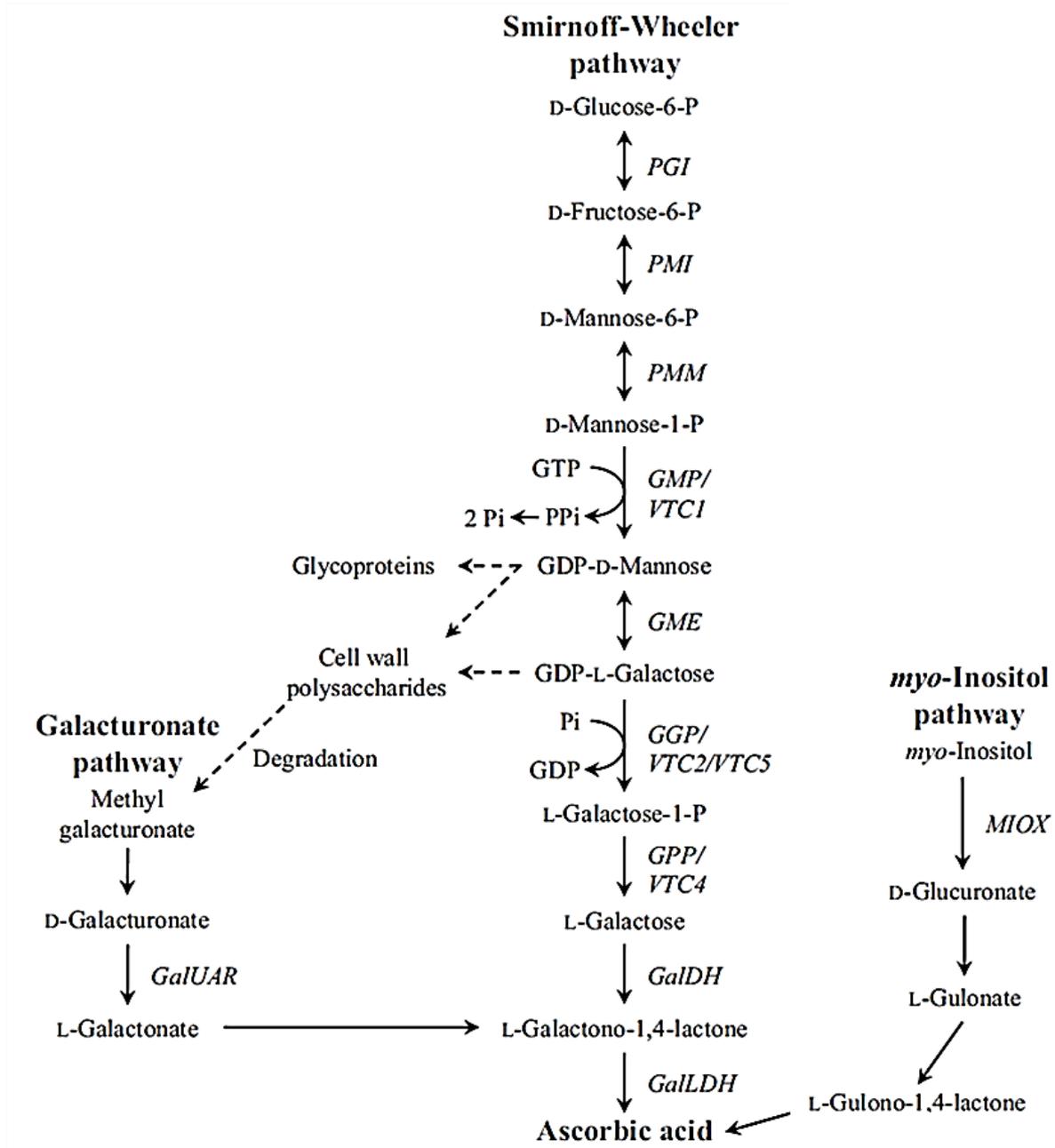
Research on animal catabolism of ascorbate has been developed a lot throughout twentieth century. Since 1957 and the works of J.J. Burns, we know that some animals have lost their capability to synthesize Ascorbic acid (Burns, 1957; Lachapelle & Drouin, 2011). Indeed, evolution has made them lose an active L-gulono- $\gamma$ -lactone oxidase which catalyses the last step of the biosynthetic pathway. This phenomenon could be explain by the harmful production of H<sub>2</sub>O<sub>2</sub> during the enzymatic reaction. Thereafter, numerous studies have tried to demonstrate a positive link between vitamin C content in food and/or plasma and health benefits (e.g., the prevention of cardiovascular disease, cancer, and other diseases). But, despite biochemical evidences linking ascorbate to molecular events associated with oxygen sensing, redox homeostasis, and carcinogenesis, the only proven mechanism remains in the prevention of scurvy (Fitzpatrick *et al.*, 2012).



**Figure 3:** L-ascorbic acid molecule ( $C_6H_8O_6$ ). **A.** Reduced form, ascorbate can act as a powerful antioxidant by releasing two  $H^+$  and two  $e^-$ , enabled by the carbon-carbon double bond. Its reductive function causes its oxidation to dehydroascorbate (**C.** DHA) through an intermediate form mono-dehydroascorbate (**B.** MDHA) which can act as electron donor or acceptor.

## Chemical Description of Vitamin C

The vitamin C is the usual name when referring to its nutritional properties. The molecule, L-ascorbic acid ( $C_6H_8O_6$ ; *L-threo-hex-2-enono-1, 4-lactone*) is a derivative from hexose metabolism. This organic compound displays antioxidant and acidic properties because of the ionisation power ( $pK_a=4.7$  and  $11.7$ ) and electron-donor capability of its "ene-diol" group. While "ascorbic acid" refers to acidic form, "ascorbate" names the alkaline form, and both can be oxidized or reduced. Its molecular weight is  $176.12 \text{ g.mol}^{-1}$  and it dissolves in water up to  $0.33 \text{ g.ml}^{-1}$ , but reduced ascorbate is very unstable in solution and rapidly oxidized (DHA: dehydro-L-ascorbate) by air and light. There is a third transitional semi-reduced or mono-oxidized form (MDHA: monodehydro-L-ascorbate). All these forms constitute the pool of vitamin C in the cell (Fig. 3). The recycling by enzymatic reactions with the monodehydro-ascorbate reductase (MDHAR) or through glutathione metabolism, contributes to renew this pool. In humans, these processes are partial which explains the need for a permanent intake of vitamin C. When a part of DHA is not reduced, it is irreversibly hydrolysed in 2, 3-diketogulonic acid and then degraded in erythrulose, oxalate, and threonate (Green and Fry, 2005).



**Figure 4:** Ascorbic acid biosynthesis pathways in plants. The abbreviations indicate as follows: PGI Phosphoglucose isomerase; PMI Phosphomannose isomerase; PMM Phosphomannomutase; GMP GDP-D-mannose pyrophosphorylase; GME GDP-D-mannose 3,5-epimerase; GGP GDP-L-galactose phosphorylase; GPP L-galactose-1-P phosphatase; GalDH L-galactose dehydrogenase; GalLDH L-galactono-1,4-lactone dehydrogenase; MIOX Myo-inositol oxygenase; GalUAR D-galacturonate reductase. The enzymes of VTCs, which were identified from the AsA-deficient vtc (vitamin C) mutant lines, are also shown (adapted from Suekawa *et al.*, 2017).

## Ascorbic Acid Metabolism

### **i. Biosynthesis**

In animals, a unique ascorbic acid biosynthesis pathway has been early elucidated by using radiolabelled molecules and biochemistry assays. In the fifties, Isherwood *et al.* (1953) proposed a similar anabolic scheme between animal and plant pathway, with just a difference for D-galacto to L-galacto transition. It was not until 1998 that Smirnoff highlights a main specific ascorbate biosynthetic pathway in plants, so named the L-galactose pathway (Fig. 4). Since then, some studies have hypothesized additional alternative pathways (Smirnoff *et al.*, 2001; Ishikawa *et al.*, 2006). Among them, only the galacturonate and myo-inositol pathways are relevant. Interestingly, a connexion between ascorbate biosynthesis and several compounds involved in the cell wall construction has also been described (Fig. 4) (Wheeler *et al.*, 1998).

#### **The L-galactose pathway (Smirnoff-Wheeler)**

The L-galactose pathway is the first and the only one to have been demonstrated for synthesizing ascorbate in higher plants. In the first study (Wheeler *et al.*, 1998) - basing on previous results in animals - radiolabelling experiments was used to confirm the role of the L-galactono-1, 4-lactone, the L-galactose and the D-mannose as precursors of ascorbate. Moreover, they identified some enzymes involved: the GDH (L-galactose dehydrogenase), and the GME (GDP-D-mannose-3, 5-epimerase). The other activities have also been isolated and characterized for the majority: the GMP (GDP-D-mannose-pyrophosphorylase), the GPP (L-Gal-1-P phosphatase), the PMI (Phosphomannose isomerase) and the PMM (Phosphomannomutase) (Conklin *et al.*, 1999; Conklin *et al.*, 2006). These studies were based on biochemical experiments using ascorbate-deficient *Arabidopsis thaliana* mutants. The last enzyme to be identified is GGP (GDP-L-galactose phosphorylase) by sequence similarity analyses and enzymatic assays (Linster *et al.*, 2007).

All these reactions are localized in the cytosol, except for the GLDH located in the inner membrane of the mitochondria and which involves a transfer of electrons *via* the cytochrome c (Siendones *et al.*, 1999; Bartoli *et al.*, 2000) it seems that this interaction is important to coordinate both ascorbate and energetic metabolisms. The first six steps are not specific to the ascorbate synthesis. The hexokinase and the phosphoglucose isomerase



which catalyse the synthesis of fructose-6-phosphate, are mostly involved in the glycolysis. Furthermore, all the compounds until GDP-L-galactose are precursors for the polysaccharides synthesis of the cell wall. That makes GGP the first specific enzyme in the ascorbic acid biosynthesis (Laing *et al.*, 2007; Bulley *et al.*, 2009; Bulley *et al.*, 2012).

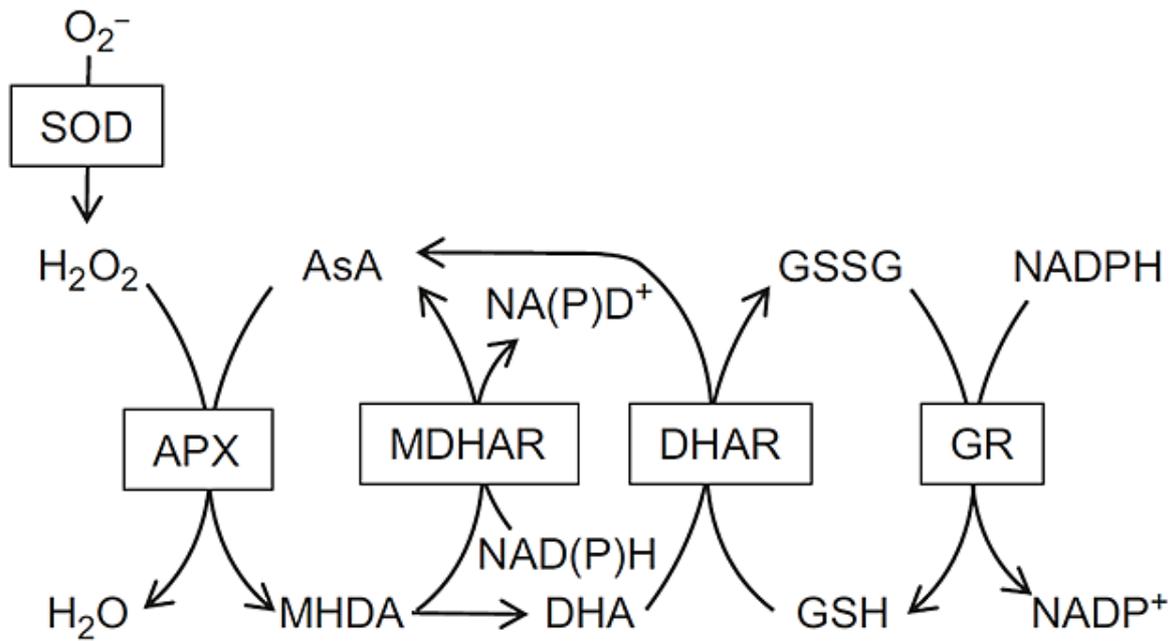
### **The D-galacturonate pathway**

Although L-galactose pathway is predominant in all the higher plants, there is a possibility that other alternative pathways may be tissue or specie specific: here is the case of the D-galacturonate pathway.

It had been demonstrated that the D-Galacturonic acid methyl ester could be directly converted to L-ascorbic acid, without involving the reactions of the hexose monophosphate pathway (Isherwood *et al.*, 1953). The alternative D-galacturonate pathway has been shown in strawberry when the gene coding for the involved enzyme, a NADPH-dependent D-galacturonate reductase was identified (Agius *et al.*, 2003). In tomato, the input of D-galacturonate improves the ascorbate content in extract of red ripe fruits (Badejo *et al.*, 2012). The activities of the two enzymes associated with this pathway (GalUR and aldonolactonase) are detectable in the insoluble fractions of fruits suggesting that this path is dependent on the stage of development. However, this does not occur in green fruits. Finally, protists which are capable of photosynthesis, use the D-galacturonate pathway for ascorbate synthesis (Ishikawa and Shigeoka, 2008).

### **The myo-inositol pathway**

The possibility that an animal biosynthetic pathway of AsA is present in plants has never been ruled out. Overexpression in lettuce and tobacco of the gene encoding a rat L-gulonono-1,4-lactone oxidase induces an increase in ascorbate content of approximately seven-fold (Jain and Nessler, 2000). More recently, ectopic expression of this gene in Arabidopsis mutant (*vtc*) leaves restores AsA levels (Radzio *et al.*, 2003). In animals, the substrate used for this enzyme, is L-gulonono-1, 4-lactone. It is interesting to note that the mutant *vtc1* Arabidopsis produces very little of this intermediate. The myo-inositol pathway uses myo-inositol which is accumulated under stress conditions (Zhu *et al.*, 2014). This osmolite is converted to D-glucuronic acid by myo-inositol oxygenase (Brown *et al.*, 2006). Thus, these data suggest that under some conditions the presence of an alternative pathway can compensate for the L-galactose pathway.



**Figure 5:** The ascorbate-glutathione cycle and superoxide dismutase in plants. APX ascorbate peroxidase, AsA ascorbate, DHA dehydroascorbate, DHAR dehydroascorbate reductase, GR glutathione reductase, GSH reduced glutathione, GSSG oxidized glutathione, MDHA monodehydroascorbate, MDHAR monodehydroascorbate reductase, SOD superoxide dismutase. (Satoshi *et al.*, 2017)

However, this alternative route has not been completely demonstrated and some data go against these assumptions. Thus, the *Arabidopsis vtc2/vtc5* double mutant is unable to grow without addition of exogenous ascorbate (Dowdle *et al.*, 2007). This suggests that there would be no other way than the Smirnoff-Wheeler one to fill this ascorbate deficiency.

## ii. Recycling: redox state

Ascorbate has a leading role in antioxidant defences, in response to biotic and abiotic stresses, mainly by eliminating ROS (Reactive Oxygen Species) produced during photosynthesis. It is for this reason that the reduced ascorbate pool is constantly renewed. The <sup>14</sup>C-ascorbate has been used to estimate a turnover rate of approximately 2 to 3% per hour in *Arabidopsis* leaves (Conklin *et al.*, 1997). The recycling system must therefore be a well-oiled mechanism.

### Oxidation

This ability of ascorbic acid to neutralize free radicals, is driven either by directly interacting with radicals, or by enzymatic reactions involving an ascorbate oxidase (AO), or an ascorbate peroxidase (APX) (Kärkönen *et al.*, 2017; Maruta *et al.*, 2016). These two enzymes lead to the production of MDHA (Fig. 5).

For instance, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is reduced in H<sub>2</sub>O by APX which uses reduced ascorbate as electron donor (Fig. 5) (Noctor and Foyer, 1998). APX belongs to a multigenic family, since there are nine genes in *Arabidopsis*, eight in rice, and seven in tomato. These APX families are further classified into three subfamilies according to subcellular localization: chloroplast, cytosol, and microbody isoenzymes. Chloroplastic isoenzymes are stroma-soluble (sAPX) and thylakoid membrane-bound (tAPX) forms.

On his side, AO also participate in ascorbate oxidation consuming ½ O<sub>2</sub> to form one H<sub>2</sub>O molecule. Its apoplastic localization has been confirmed using knock-down mutants which display a more reduced apoplastic ascorbate pool (Fotopoulos *et al.*, 2006; Yamamoto *et al.*, 2005). AO activity would be related to cell expansion and hormone and redox signalling in the apoplast (Garchery *et al.*, 2013). Other evidences have shown that



decreasing AO activity leads to noticeable effects during abiotic and biotic stress treatments (De Tullio *et al.*, 2007).

## Recycling

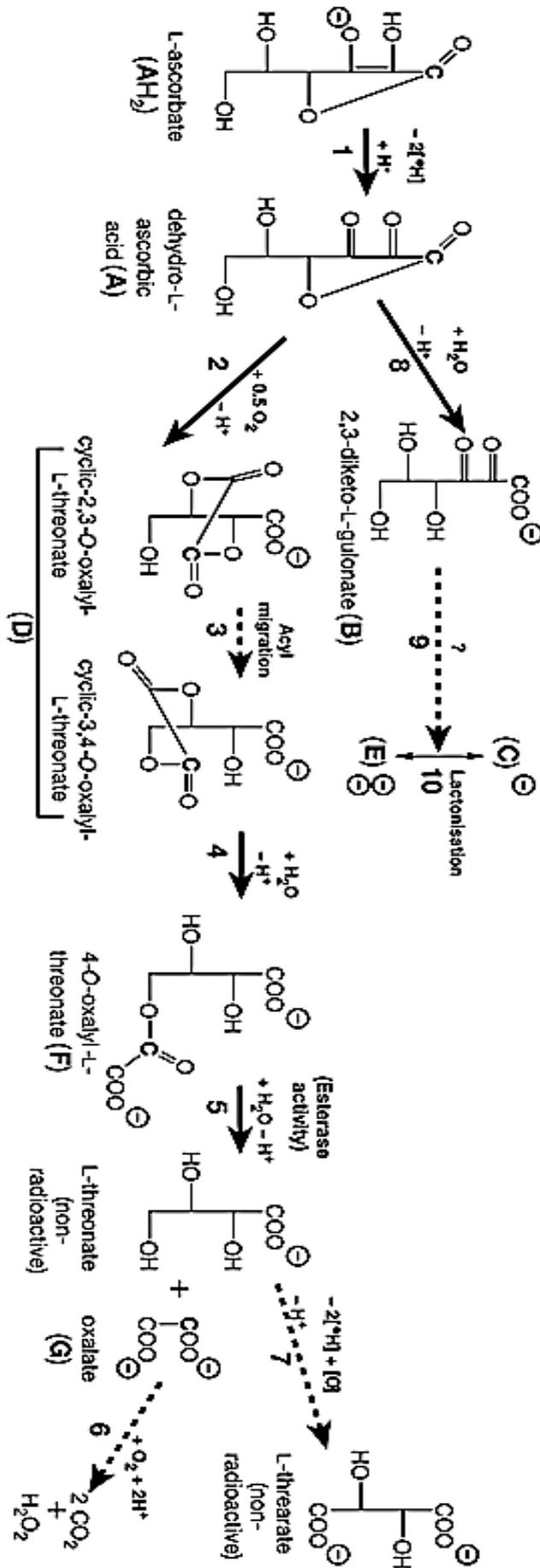
The ascorbate recycling system involves either a direct reduction of MDHA through the photosystem I (PSI) in thylakoids, either the activities of a monodehydro-ascorbate reductase (MDHAR) or a dehydro-ascorbate reductase (DHAR).

The products of detoxification with ascorbate are MDHA and DHA (Bielski *et al.*, 1981). The MDHA is supported by a MDHAR which reforms operational ascorbate with NADH as preferential co-factor (Fig. 5). Although MDHAR is mainly considered in ascorbate metabolism, some evidences have shown that it has a wide substrate specificity and could be multifunctional. Indeed, MDHAR can also reduce phenoxy radicals and more surprisingly in bioremediation area. MDHAR has been identified as responsible for the phytotoxicity of the explosive 2, 4, 6-trinitrotoluene (TNT) by reducing it in mitochondria (Johnston *et al.*, 2015).

When the activity of MDHAR is not sufficient, there is a disproportionation of MDHA to form one DHA and one molecule of ascorbate (Fig. 5). The di-oxidized DHA form is reduced in ascorbate through a DHAR using glutathione (GSH) as cofactor; if not, DHA is degraded in 2, 3-diketo-L-gulonic acid (DKG) (Deutsch, 1998). The reduction of DHA by GSH is quite slow under physiological conditions but there are multiple isoforms of GSH-dependent DHARs which are localized in almost all cell compartments, *i.e.* the cytosol, the chloroplasts, the mitochondria and the peroxisomes (Jiménez *et al.*, 1998; Hossain and Asada, 1984). Once the DHAR reaction, the oxidized GSSG is recycled in GSH by the glutathione reductase (GR) (Fig. 5).

### iii. Degradation

The *in vivo* mechanisms causing the ascorbate catabolism are not well known. The degradation of ascorbate was proposed to occur in apoplasm of the cells from DHA (Green and Fry, 2005), but it cannot be excluded that this way or others may also exist in additional compartments. In most plants, the degradation of DHA induces the formation of two products: oxalate (OxA) and L-threonate (ThrO). In some cases, such as grapes, ascorbate can also be degraded *via* L-idonate to L-threonate and then L-tartrate



**Figure 6:** Enzymatic and non-enzymatic degradation of vitamin C in the apoplasm of the rosebush. All reactions can proceed non-enzymatically except steps 6 and 7. (Green and Fry, 2005)

(Green and Fry, 2005). *In-vitro* models have shown that the degradation of DHA can be performed enzymatically or non-enzymatically leading to the irreversible formation of DKG (Fig. 6). According to the experiment, in low oxidation conditions, DHA can be either oxidized to oxalic ester, oxalate, and threonate, or hydrolysed to DKG (Parsons and Fry, 2012). In the presence of H<sub>2</sub>O<sub>2</sub>, DHA which is unstable at physiological pH, is also hydrolysed in DKG which is spontaneously oxidised to non-identified products. It must be noticed that the conditions leading to the formation of these products are not necessarily identical to those occurring *in vivo*. Those products of ascorbate catabolism are likely to play a metabolic role. Present in higher plants, oxalate is involved in osmoregulation and control of calcium concentration (Nakata, 2012). Similarly, the tartare is a very important compound for the establishment of the organoleptic characteristics of the grape berry (Debolt *et al.*, 2007). Although the intermediates of this pathway have been identified, the enzymes involved are not characterized.



Kakadu plum (*Terminalia ferdinadiana*)  
**Up to 5 g/100g FW**



Camu-camu (*Myrciaria dubia*)  
**Up to 3 g/100g FW**



Acerola (*Malpighia emarginata*)  
**Up to 2g/100g FW**

**Table 1.** Ascorbate levels <sup>a</sup> and production volume <sup>b</sup> of major global commodity crops <sup>c</sup>

|                | Ascorbate<br>(mg/100 g FW) | Production<br>(tonnes × 1000) |
|----------------|----------------------------|-------------------------------|
| Rice, paddy    | 0.2                        | 738                           |
| Wheat          | 0.2                        | 671                           |
| Barley         | 0.6                        | 134                           |
| Apples         | 4.6                        | 76                            |
| Maize          | 4.8                        | 873                           |
| Onions, dry    | 6.4                        | 83                            |
| Watermelons    | 8.1                        | 105                           |
| Bananas        | 8.7                        | 102                           |
| Tomatoes       | 10                         | 162                           |
| Potatoes       | 11.4                       | 365                           |
| Sweet potatoes | 17.1                       | 108                           |
| Cassava        | 20                         | 269                           |
| Soybeans       | 29                         | 241                           |
| Kiwi           | 50                         | 2500                          |

<sup>a</sup> According to USDA food composition database

<sup>b</sup> According to FAO

<sup>c</sup> Excluded from the table; sugarcane and sugar beet, as they are used for processed sugar; and the FAO food category vegetable, fresh as this includes a range of crops with differing ascorbate levels (but generally >50 mg ascorbate/100 g fresh weight).

## Regulation: Variability *In Planta*

### **i. Genetic variability**

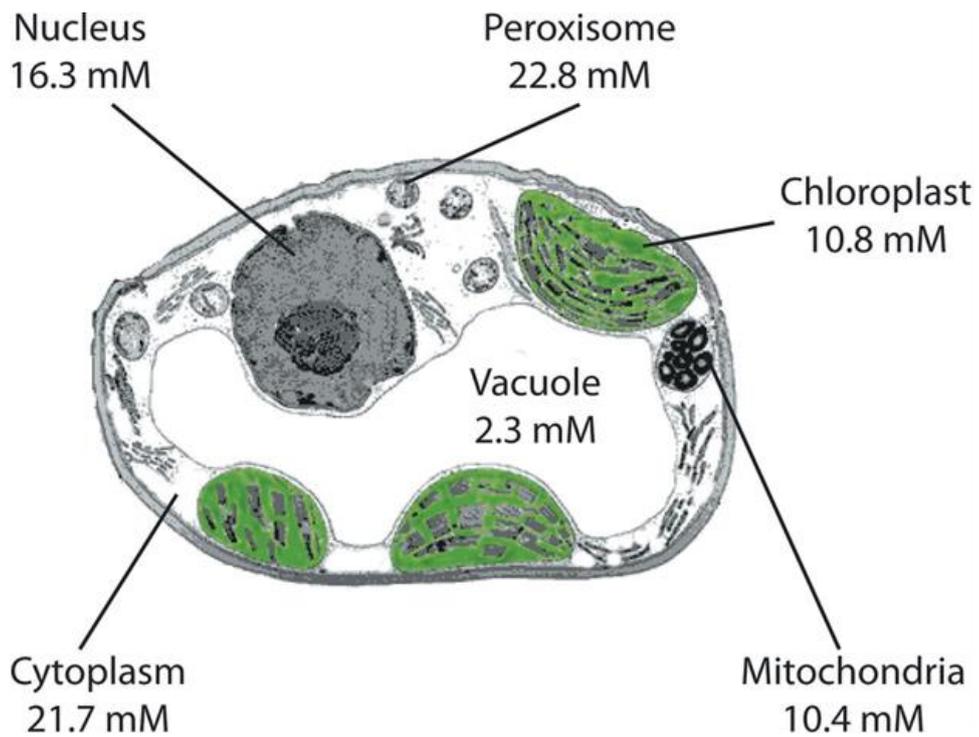
In plants, there is a great variability in ascorbic acid content according to the genus and the species. During a very long time, a common misconception was that citrus fruits (50 mg/100g FW) are the richest fruits in vitamin C. It does not count on two fruits from South America namely camu-camu (*Myrciaria dubia*) and acerola (*Malpighia emarginata*), accumulating ascorbic acid respectively up to 3 g and 1 g per 100 g of fresh material. Today, the champion is the Kakadu plum (*Terminalia ferdinandiana*) which is well known by Australian aboriginal tribes for its medicinal properties. Its vitamin C content can reach more than 100 times that of oranges (~5% of fruit FW). Among the common commercially available fruits, the kiwifruit can be considered the best source of vitamin C in daily consumption (Table 1).

In the case of tomato species, strong differences can also be observed between wild and domesticated cultivars. In general, wild tomato species display higher ascorbate content (Galiana-Balaguer *et al.*, 2006). For example, wild *Solanum pennellii* displays around 50 mg/100g FW compared to commercial *Solanum lycopersicum* with 20mg/100g FW (Stevens *et al.*, 2007). This study also showed that it is possible to increase ascorbate content in medium size fruits by introducing wild alleles by breeding (Stevens *et al.*, 2007).

### **ii. Spatiotemporal variability**

#### **Distribution at the plant level**

Although ascorbic acid biosynthesis takes place in all plant tissues (mainly in leaves, stems, fruits and meristems), there is a wide difference in term of ascorbate amount between all plant organs (Sanmartin *et al.*, 2000). As expected, the acid ascorbic biosynthesis is more important in photosynthetic organs. Then, plant organs can be classified in two groups: the source organs where biosynthesis is the strongest (leaves, stems) and the sink organs (fruits, tubers, roots, meristems). At the plant level, ascorbate can be localized in companion cells, in screened tubes and in xylem vessels, indicating that ascorbate can be transported into the plant through vascular tissues (Zechmann, 2011). Autoradiography studies using <sup>14</sup>C-AsA showed a movement of radiolabelled ascorbate



**Figure 7:** Subcellular distribution of ascorbate in photosynthetic *Arabidopsis thaliana* rosette leaves (accession Col-0). The ascorbate content was determined by an immuno-labelling method under conditions of high light intensity ( $700 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) (Gest, Gautier, *et al.*, 2013).

through the phloem, from source organs to sink organs (Franceschi, 2002; Tedone *et al.*, 2004). Thus, the long-range transport of ascorbate may be necessary in order to complete the *in situ* synthesis performed by the sink organs. However, in the fruits, the contribution of this transport in the final AsA content remains to be determined precisely although Massot *et al.* (2009) demonstrated that in tomato a non-negligible (70%) part of AsA pool found in the fruit is produced by the fruit itself.

The high oxidation stress caused by photosynthesis explain the need to have a strong antioxidant production capacity in green tissues (del Carmen Cordoba-Pedregosa *et al.*, 2003). Studies have shown that *Arabidopsis* leaves incubated with L-galactose have an increase of ascorbate synthesis from 3 to 7 times, while incubation of sink tissue does not induce a significant increase (Franceschi, 2002). There is also a difference in term of ascorbate amount in the organs themselves. Generally, they display a decreasing gradient of ascorbate between the outer and inner tissues. For instance, tomato pericarp has a higher ascorbate content than the gel and the seeds (F. Wokes *et al.*, 1943); the skin and the pulp of the tomato fruit being the richest tissues (Chandra *et al.*, 2012).

### **Subcellular distribution**

From its production at the level of the outer membrane of mitochondria, ascorbic acid is distributed throughout the whole cell compartments. Electron microscopy methods have been used to locate ascorbate in mitochondria, chloroplasts, nucleus, peroxisomes, cytosol, vacuoles, apoplast, and along endoplasmic reticulum membranes (Fig. 7) (Gest, Garchery, *et al.*, 2013). The omnipresence of ascorbate makes sense considering all the physiological processes in which it is directly or indirectly involved: development, growth but also the protection of the plant. The redox status of ascorbate - depending on the compartment and the environmental conditions - implies a signal that can modulate the expression of genes and some enzymatic activities. In the case of light stress, the ascorbate content increases in most cell compartments. Surprisingly, the largest difference is observed in the vacuoles (+ 395%). This effect highlights an essential role in the direct or indirect detoxification of H<sub>2</sub>O<sub>2</sub> produced in chloroplasts and transported into vacuoles. Similarly, in chloroplasts and mitochondria, ascorbate is detected in the stroma and matrix, whereas during light stress it is found in the crests and lumen of thylakoids. These observations are consistent with the non-photochemical quenching capacity (NQP) of ascorbate, reducing the formation of ROS by heat dissipation, when high light saturates the capacity of the photosystems.

The necessary and rapid response of ascorbate according to external signals requires a very efficient transport system. Transport through membranes can be achieved through a passive or active system. In the case of ascorbate, it cannot diffuse simply

| Enzyme   | Target plant       | Gene                     | Gene source             | Type of genetic manipulation | Ascorbate content  | Phenotypic changes  |
|----------|--------------------|--------------------------|-------------------------|------------------------------|--|---|
| GMP      | Tobacco            | <i>GMPase</i>            | Tomato                  | Overexpression               | 2.0–4.0-fold increase<br>0.61–0.75-fold decrease                             | Increased tolerance to temperature stress<br>22.4%–34% decreases in myoinositol content                           |
| VTC4/IMP | <i>Arabidopsis</i> | <i>VTC4</i>              | –                       | T-DNA knockout               |  | Slow seed germination under control conditions<br>Slightly hypersensitive to ABA and NaCl during seed germination |
| GLDH     | Tobacco            | <i>RrGalLDH</i>          | <i>Rosa roxburghii</i>  | Overexpression               | 2.1-fold increase  | Enhanced tolerance to salt stress   |
| MDHAR    | Tobacco            | <i>AtMDAR1</i>           | <i>Arabidopsis</i>      | Overexpression               | Up to 2.2-fold increase  | Enhanced tolerance to ozone, salt and PEG stresses  |
|          | Tobacco            | <i>Am-MDAR</i>           | <i>Avicennia marina</i> | Overexpression               | Up to 2.0-fold increase  | Increased tolerance to salt stress  |
|          | Tobacco            | <i>DHAR-OX</i>           | <i>Arabidopsis</i>      | Overexpression               | Up to 1.3-fold increase  | Increased tolerance to AI stress  |
| DHAR     | Tobacco            | <i>DHAR</i>              | <i>Arabidopsis</i>      | Overexpression               | 1.9–2.1-fold increase  | Enhanced tolerance to ozone, drought and salinity   |
|          | Tobacco            | <i>DHAR</i>              | Wheat                   | Overexpression               | 2.1-fold increase  | Increased ozone tolerance and NPR   |
|          |                    |                          | Tobacco                 | Antisense downregulation     | 0.29-fold decrease   | Substantially reduced stomatal area and low NPR   |
|          | Tobacco            | <i>DHAR</i>              | Human                   | Overexpression               | No significant change  | Enhanced tolerance to low temperature and NaCl  |
|          | Tobacco            | <i>tAPx</i>              | Tobacco                 | Overexpression               | No change  | Increased tolerance to MV and chilling stresses under light conditions  |
|          | <i>Arabidopsis</i> | <i>HvAPX1</i>            | Barley                  | Overexpression               | –  | Increased tolerance to salt stress  |
|          | <i>Arabidopsis</i> | <i>OsAPXa and OsAPXb</i> | Rice                    | Overexpression               | –  | Increased tolerance to salt stress  |
| APX      | Tobacco            | <i>CAPOA1</i>            | Pepper                  | Overexpression               | –  | Increased plant growth  |
|          | Tobacco BY-2 cells | <i>cAPX</i>              | <i>Arabidopsis</i>      | Antisense downregulation     | No change  | Increased tolerance to MV stress<br>Increased tolerance against heat and salt stresses                            |
|          | Tobacco            | <i>StAPX</i>             | Tomato                  | Overexpression               | –  | Improved seed germination   |
|          | Rice               | <i>OsAPXa</i>            | Rice                    | Overexpression               | –  | Increased spikelet fertility under cold stress  |
|          | Rice               | <i>Osapx2</i>            | Rice                    | Overexpression               | –  | Enhanced stress tolerance   |
|          |                    |                          |                         |                              |  | Sensitive to abiotic stresses   |
|          | Alfalfa            | <i>Osapx2</i>            | Rice                    | Overexpression               | –  | Increased salt resistance   |
|          | Tomato             | <i>cAPX</i>              | Pea                     | Overexpression               | –  | Enhanced tolerance to UV-B and heat stresses  |
|          | Tomato             | <i>cAPX</i>              | Pea                     | Overexpression               | –  | Enhanced tolerance to chilling and salt stresses  |
|          | Tomato             | <i>LetAPX</i>            | Tomato                  | Antisense downregulation     | No significant change  | Transgenic plants photosynthetically less efficient and sensitive to chilling stress                              |
| AO       | Tobacco            | <i>AAO</i>               | Cucumber                | Overexpression               | No change  | Plants become susceptible to ozone  |
|          | Tobacco            | <i>AAO</i>               | Cucumber                | Overexpression               | No change  | Increased drought tolerance due to reduced stomatal conductance   |
|          | Tobacco            | <i>AAO</i>               | Tobacco                 | Overexpression               | –  | Severe inhibition of germination and seed yield under high salinity   |
|          | Tobacco            | <i>AAO</i>               | Tobacco                 | Antisense downregulation     | –  | Increased tolerance to salt stress  |
|          |                    |                          |                         |                              |  | Increased seed yield under salt stress  |
|          | <i>Arabidopsis</i> | <i>AAO</i>               | –                       | T-DNA knockout               | Increased tolerance to salt stress<br>Increased seed yield under salt stress |   |
| MIOX     | Rice               | <i>OsMIOX</i>            | Rice                    | Overexpression               | No change  | Increased drought tolerance   |
| AMR1     | <i>Arabidopsis</i> | <i>AMR1</i>              | –                       | T-DNA knockout               | 2.0–3.0-fold increase  | Increased ozone tolerance   |

**Table 2:** Diverse studies carried out in plants, or mutants presenting a modification of AsA content in relation to stress.

**References:** Wang et al. 2011, Torabinejad et al. 2009, Liu et al. 2013a, Eltayeb et al. 2007, Kavitha et al. 2010, Yin et al. 2010, Eltayeb et al. 2006, Chen and Gallie 2005, Kwon et al. 2003, Yabuta et al. 2002, Xu et al. 2008, Lu et al. 2007, Sarowar et al. 2005, Ishikawa et al. 2005, Sun et al. 2010a, Sato et al. 2011, Zhang et al. 2013, Guan et al. 2012, Wang et al. 2006, Wang et al. 2005, Duan et al. 2012b, Sanmartin et al. 2003, Fotopoulos et al. 2008, Yamamoto et al. 2005, Yamamoto et al. 2005, Duan et al. 2012a, Zhang et al. 2009

through the lipid bilayer mainly because of its negative charge at physiological pH. Fortunately, DHA which is a neutral molecule with greater hydrophobicity, can diffuse easily (Horemans *et al.*, 1997). Nevertheless, the transport of ascorbate and DHA is essentially mediated by a facilitated diffusion system and/or active transport. These transport systems are not clearly identified in plants, unlike animals for which sodium-dependent vitamin C transporters (SVCTs) have been widely characterized (Daruwala *et al.*, 1999; Tsukaguchi *et al.*, 1999). These proteins belong to a family of nucleobase-ascorbate transporter (NAT) also present in bacteria and higher plants (Koning and Diallinas, 1999). Recent homology studies have identified 12 NAT orthologues in the *Arabidopsis thaliana* genome. Of these, the At5g62890 gene has a conserved region similar to mammalian SVCTs (Li and Schultes, 2002), but the involvement of this protein in the transport of ascorbate remains to be studied.

In plants, studies have been carried out on isolated organelles and protoplasts, but also according to the similarities with mammals. They reveals clues on the modalities of the transport. For example, absorption of ascorbate in isolated vacuoles exhibits kinetics that suggest a saturation mechanism, and indicate the absence of a specific transporter (Rautenkranz *et al.*, 1994). When considering the transport of DHA in animals, there is a substantial role for a glucose transporter. Interestingly, the addition of a glucose transport inhibitor such as cytochalasin B or genistein blocks the uptake of DHA and glucose into mitochondria isolated from tobacco BY-2 cells (Szarka *et al.*, 2004). Besides, Horemans *et al.*, (2003) proposed the presence of a transporter in the plasma membrane through which the apoplastic DHA will be exchanged with the cytological ascorbate. Unfortunately, the protein or gene associated with such a transport activity has not been identified. Recently, a phosphate transporter protein belonging to the PHT4 family has been described as an ascorbate transporter located in the chloroplast envelope (Miyaji *et al.*, 2015). Apart from that, other mechanisms involved in the transport of ascorbate between the different cellular compartments have not yet been revealed.

### **iii. Environmental impact**

Environmental factors have a strong influence on the chemical composition of horticultural species (B. P. Klein and Perry, 1982). Biotic or abiotic stresses increase ROS production which can be toxic for the cells resulting in membrane peroxidation, damages to DNA, denaturation of proteins the all leading to cell death. However, there is no evidence of direct modulation of the ascorbate content related to biotic stress (Table 2). The responses of ascorbate to pathogenic infection are presented in the section that deals with its biological roles.

| Protein name   | Proposed mechanism  | Fold change in ascorbate with gene over-expression | Fold change in ascorbate with reduced gene expression |
|----------------|---|--|---|
| <b>ERF98</b>   | Transcription factor (TF) promoting l-gal pathway gene expression | 1.7  | 0.65–0.8  |
| <b>HD-ZIP1</b> | TF promoting l-gal pathway gene expression                        | 1.3–1.6  | Little effect in leaves<br>0.7 in fruit               |
| <b>VTC3</b>    | Post-transcriptional mechanism                                    | 1.0  | 0.4   |
| <b>CSN5B</b>   | Post-translational control of GMP amount                          | Not tested   | 1.3   |
| <b>CSN8</b>    | Unknown   | Not tested   | 1.7   |
| <b>AMR1</b>    | Degradation of a transcription factor                             | 0.4  | 2.0   |
| <b>CML10</b>   | Interacts with PMM and promotes activity                          | Not tested   | 0.5–0.75  |
| <b>KONJAC</b>  | Stimulation of GMP activity                                       | 1.4  | 0.4   |

**Table 3:** Various proteins that have been shown to affect ascorbate accumulation (reviewed in Bulley et al. 2016)

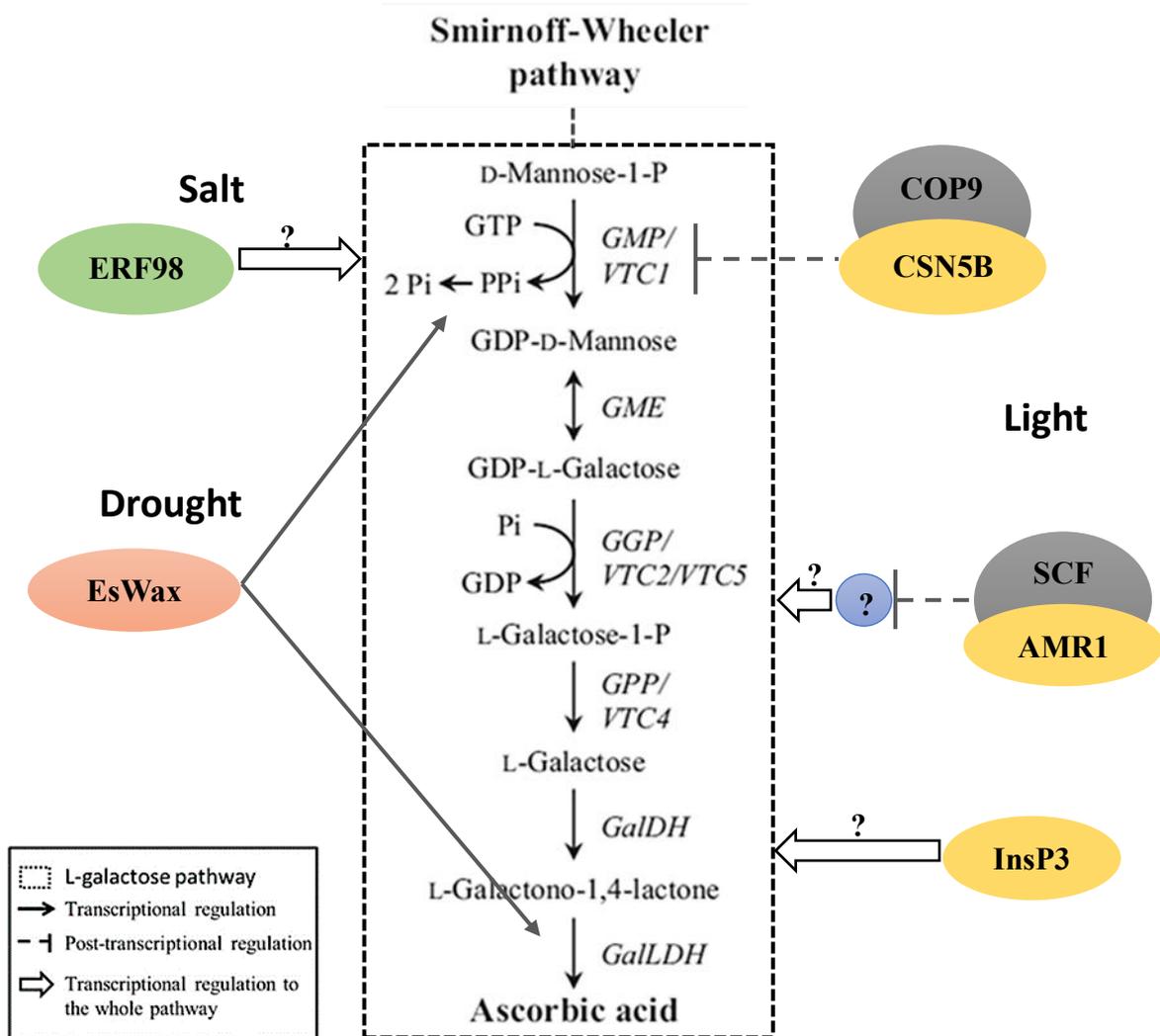
Although biosynthesis or recycling pathways are well characterized, mechanisms involved in their regulation are relatively unknown. However, recent studies conducted in *Arabidopsis thaliana* have identified abiotic stresses responsible for a variation in the amount of ascorbate, and the corresponding molecular regulators. These are able to act at the transcriptional and post-transcriptional level on the main ascorbate biosynthesis pathway (Table 3).

## Salt

Salt stress increases the ascorbic acid content in sorghum (Telesiński *et al.*, 2008). An ethylene response factor (ERF) has been identified to mediate this regulation. Proteins belonging to the ERF family have an important role in transcriptional regulation during growth and developmental processes, but also in the response to environment (Aharoni, 2004; Zhang *et al.*, 2004). ERF98 acts as a positive regulator on the main biosynthetic pathway (Zhang *et al.*, 2012)(Table 3). Thus, overexpression of ERF98 in *Arabidopsis thaliana* causes an increase in ascorbate content, and on the contrary a decrease in erf98 deficient mutants. In the erf98 mutants, the sensitivity to salt stress is higher, indicating that ERF98 may be involved in the salt stress response by stimulating ascorbate synthesis. Expression levels of the genes involved in the L-galactose pathway as well as some enzymes involved in the recycling of ascorbate (APX3, APX6, ChIDHAR, CytDHAR and GR1) increase in the transgenic lines over-expressing ERF98 and they decrease in the knock-out mutants. However, only VTC1 seems to be really affected in erf98 deficient mutants. Analyses of the promoter show that ERF98 would be able to interact with that of VTC1 and VTC2 through a DRE motif. ERF98 could act directly on the transcriptional activity of genes and thus modulate the ascorbate pool (Fig. 8).

## Drought

*Eutrema salsugineum*, a model species for the analysis of abiotic stresses, allowed the identification of an R2R3-MYB type transcription factor called EsWAX1 (Zhu *et al.*, 2014). This factor is involved in drought resistance. The ectopic expression of EsWAX1 in transgenic plants of *Arabidopsis thaliana* significantly increases the amount of cuticular waxes but also the ascorbate content. In these transgenic lines, the expression of VTC1, GLDH and MIOX4 is stimulated, whereas those of the enzymes involved in the AsA-GSH cycle are not affected. It therefore seems that EsWAX1 is able to regulate ascorbate synthesis by modulating the expression of VTC1, GLDH and MIOX4 genes in dry conditions (Fig. 8). Nevertheless, the molecular mechanisms involved remain unknown.



**Figure 8:** Regulatory elements involved in the control of the main ascorbate biosynthetic pathway.

**Salt** stress induces the expression of ERF98, which in turn stimulates the expression of VTC1. However the action of ERF98 on the other enzymes of the pathway remains to be determined. During **drought**, EsWAX1 is also able to stimulate VTC1 and GLDH, to induce an increased resistance of the plant. **Light** inhibits the expression of AMR1, which will inhibit the expression of enzymes in the biosynthetic pathway through the action of an unknown intermediate protein. InsP3 is a secondary messenger in the light signal transduction capable of inducing ascorbate synthesis but the molecular mechanisms are still unknown. In the dark, CSN5B acts at the post-transcriptional level where it induces the degradation of VTC1 in order to inhibit ascorbate synthesis. (Adapted from Wang et al. 2013)

## Oxygen and Temperature

In the same way, oxygen influences the metabolism of ascorbate. The activity of AO is stimulated in *Cucurbita pepo* varieties cultivated in the dark in response to an increase of oxygen, whereas it decreases during germination under conditions of hypoxia (De Tullio *et al.*, 2007). Reuther and Nauer (unpublished data 1972, Lee & al., 2000) showed that mandarins grown at high temperatures (30-35°C at day, 20-25°C at night) have lower ascorbate levels than grown under cooler temperatures (20-22°C at day, 11-13°C at night).

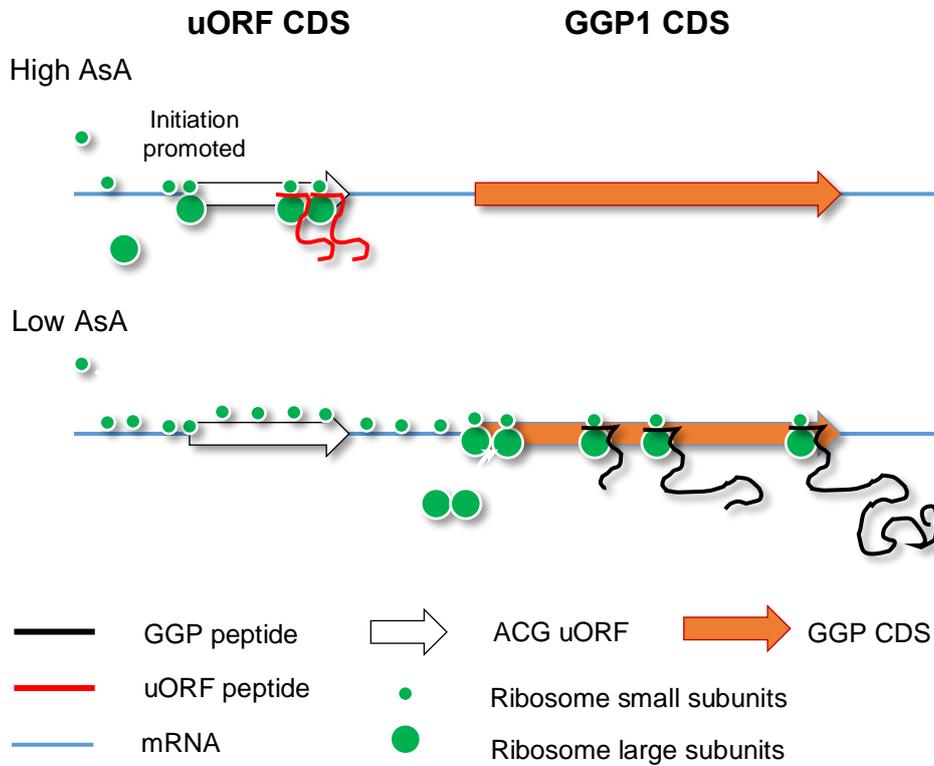
## Light

Among the climatic factors that significantly influence the accumulation of ascorbate, light holds a special place. It is not an essential element in the synthesis, nevertheless light quantity and intensity during the growth of the plant have some influence on the quantity of ascorbate produced (Fig. 8). Because of its great importance, the light factor will be treated in a separate part.

## Intake for cultivation

Cultivation conditions also modulate the ascorbate pool. Today, the demand for alternative farming methods limiting the use of fertilizers and synthetic pesticides is growing. There is a notable difference between the antioxidant levels in plants under conventional growing conditions and those grown under more environmental friendly conditions (Lee and Kader, 2000). In the case of tomato, ascorbate levels appear to be higher in organic tomatoes. These observations are controversial in the fruit but remain significant in the leaves (Caris-Veyrat *et al.*, 2004; Woese *et al.*, 1997; Worthington, 2001). In the same way, the use of nitrogen fertilizers has been showed to lead to a decrease in ascorbate content in many potato and cabbage cultivars (Lisiewska and Kmiecik, 1996).

Conversely, the use of potassium fertilizer induces an increase in the ascorbate pool (Nagy, 1980). Several hypotheses have been proposed to explain the impact of nitrogen fertilizers on fruits and vegetables. It seems that beyond its impact on leaf expansion, this type of fertilizer increases the amount of NO<sub>3</sub> in the plant (Mozafar, 2008). Other practices such as folding and lowering fruit load influence the nutritional composition and size of the fruit. Similarly, chemicals such as pesticides and growth regulators have important effects. Thus, the application of gibberellins induces an approximately 20% increase in ascorbate content in green tea (Liang *et al.*, 1996).



**Figure 9:** Simplified scheme for ribosome interaction with the GGP mRNA at the uORF and CDS. According to the model, **high ascorbate** promotes recognition of the uORF ACG start codon, while under **low ascorbate**, the ribosomes skip the uORF and start translation at the main GGP CDS.

## iv. GGP/VTC2: the key enzyme

GGP/VTC2 enzyme, which is now considered as the limiting enzyme of L-galactose pathway, is also the last to have been identified (Laing *et al.*, 2007; Dowdle *et al.*, 2007; Linster *et al.*, 2007). Despite debates about the nature of the reaction it catalyses, it is now accepted that it is a GDP-L-galactose phosphorylase (Linster *et al.*, 2008). It supports the first committed step in ascorbate biosynthesis in plants, converting GDP-L-galactose to L-galactose-1-phosphate. Also named *GGP/VTC2* - according to *vtc* Arabidopsis mutants - the encoded protein belongs to the HIT (Histidine triad) protein superfamily. The knock-out *vtc2* mutants display a drastic decrease in ascorbic acid while keeping a residual amount of it. That makes sense with the activity of VTC5 which is encoded by a homologous gene of *GGP/VTC2*. The *VTC5* gene has a strong sequence homology with *GGP/VTC2* but it is 100 to 1000 less expressed (Dowdle *et al.*, 2007; GAO *et al.*, 2011). Furthermore, the seedlings of double mutant *vtc2/vtc5* are lethal and do not go beyond the germination stage, only exogenous addition of ascorbate to the medium could rescue the plantlets. Both GGP/VTC2 and VTC5 are strongly involved in the regulation of ascorbate biosynthesis. A lot of studies have shown a positive effect of light on the expression of these genes. GGP/VTC2 gene was constitutively expressed throughout the plant growth and development, with the significant higher expression abundance in green tissues than that in roots.

At cellular level, fluorescent fusion protein have highlighted that GGP/VTC2 would be localized in both cytoplasm and nucleus (Müller-Moulé, 2008). The unexpected localization for a metabolic enzyme indicates that GGP might also act as a dual-function protein: a regulatory factor as well as a catalysing enzyme. Using a computer sequence analysis software a putative Nuclear Localization Sequence (NLS) sequence was found in the GGP peptide and this could explain the nuclear localization. However, according to different software used the presence of the NLS sequence could not be confirmed, suggesting the involvement of another partner to be translocated to the nucleus.

### Auto-regulation

Recently it has been shown that ascorbate could regulate itself the activity of GGP/VTC2. The *in vivo* amount of AsA would feed back on the translation of the GGP/VTC2 (Laing *et al.*, 2015). This regulation is possible *via* an upstream open reading frame (uORF) present in the 5 untranslated region (UTR) of the GGP mRNA. The uORF is initiated by a non-canonical start site and produces a cis-acting polypeptide which controls the translation of the GGP in an AsA-dependent way (Fig. 9). While the mechanism allowing



ascorbate to initiate uORF transcription remains unclear, it has been hypothesized that this cis-acting polypeptide blocks the translation of GGP/VTC2 by stalling the ribosomes. The expression of a mutated uORF results in an ascorbate accumulation to nearly twice the level of wild-type plants (Laing *et al.*, 2015). Furthermore, using luciferase-based assay, these authors observed that an increase in ascorbate leads up to 90% reduction in the translation of the protein. Being highly conserved in dicotyledonous species such as kiwi, apple, tobacco and tomato (Laing *et al.*, 2015), the uORF could be therefore an attractive biotechnological target for ascorbate bio-fortification in crop species.



## Biological Roles in Plants

### **i. Plant development: from seed to fruits**

It is very complex to demonstrate a direct correlation between ascorbate and a particular physiological event. Indeed, due to its strong link with the stress response, the amount of ascorbic acid is modulated by a very large number of processes in plants, and *vice versa*.

#### **Plant growth**

The studies of vitamin C defective (*vtc*) mutants have allowed to report the clearest cues about the role of ascorbate in plant growth. Most of the results are only observations that attempt to describe a role for the AsA without being able to describe a specific mechanism. However, ascorbic acid seems to be indispensable for plant living. Indeed, the *vtc2/vtc5* double mutants, which are unable to synthesize ascorbate, cannot develop beyond germination (Dowdle *et al.*, 2007).

At cellular level, RNAi-SIGME2 lines, with a decrease in ascorbate of approximately 50%, show a severe delay in tomato plant and fruit growth (Gilbert *et al.*, 2009). This makes sense with the participation of GME - the last non-specific enzyme of the L-galactose pathway - in the biosynthesis of parietal compounds in some specific tissues. The pool of AsA and the redox status would be linked to cellular expansion through common reactions to the cell wall. Like other examples, in RNAi *SIGa/LDH* tomato lines, cell expansion decreases resulting in lower leaf and fruit size than in WT (Alhagdow *et al.*, 2007). Moreover, the degradation of ascorbate in oxalic acid (Loewus *et al.*, 1987) contributes to trap calcium ions in the apoplasm; and thus increases the extensibility of the cell wall by decreasing the cross-links between the polygalacturonate chains (Lin and Varner, 1991). The enzymes associated with the recycling of AsA also mediate parietal plasticity and thus the cell expansion. The ascorbate oxidase induces the formation MDHA and DHA, which elements directly affect cell expansion (Smirnoff, 1996). For instance, when MDHA is reduced, it would play the role of electron acceptor *via* cytochrome b, thus allowing the increase of electron transport through the membrane. This depolarization of the membrane will activate H<sup>+</sup>-ATPase (proton transport to apoplasm) which stimulates cell expansion (Rayle and Cleland, 1992). On his side, DHA could inhibit structural protein binding with hemicellulose and polygalacturonate (Lin and Varner, 1991).

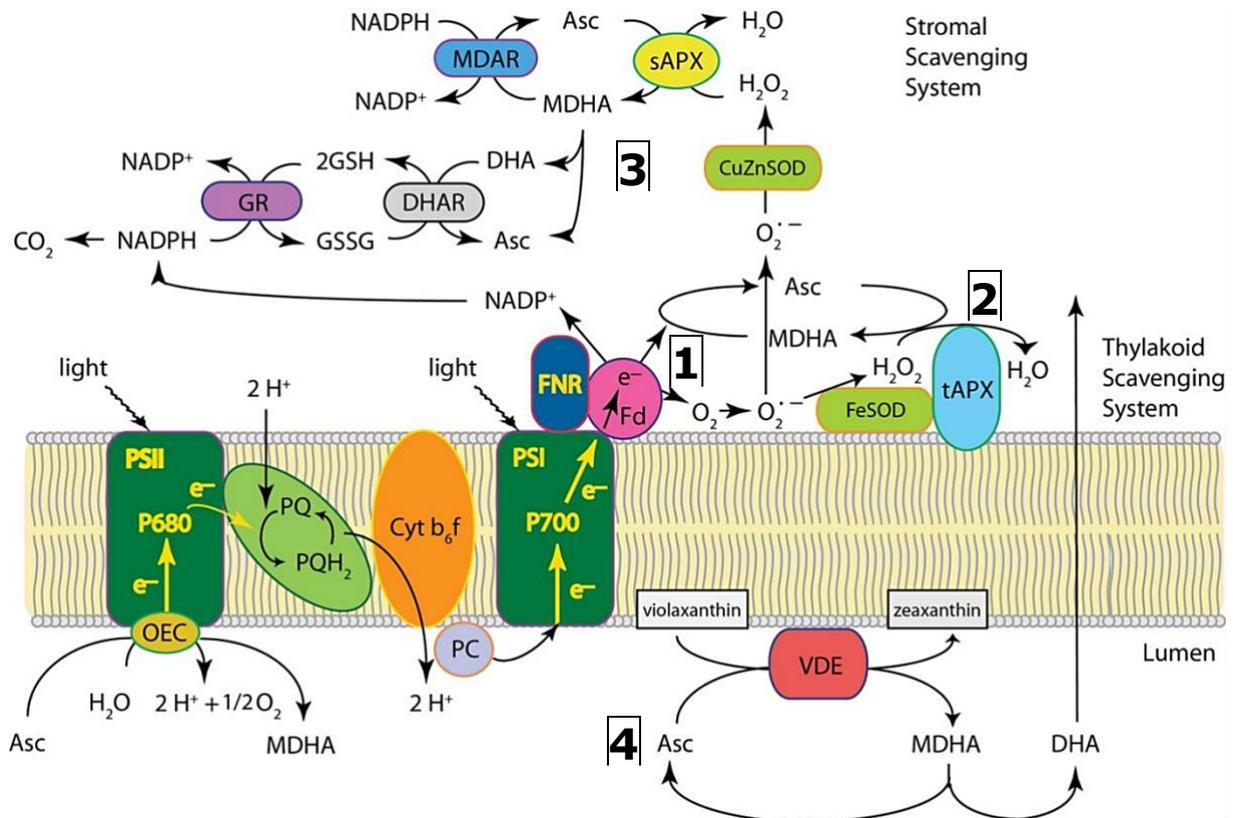


## Flowering

The transition between vegetative and reproductive phases is a crucial step for the survival of flowering plants. These processes are controlled by many endogenous but also exogenous parameters, such as the stage of plant development and environmental signal: photoperiod, vernalisation, intensity of light, water and nutrients (Bernier, 1993; Bernier and Périlleux, 2005). Studies carried out on ascorbate-deficient mutant *vct1*, *vct2*, *vct3* and *vct4* in the model plant *Arabidopsis thaliana*, showed that under "long day" culture conditions (16h of light), these mutants exhibit an early flowering relative to the WT (Conklin and Barth, 2004). Similarly, increasing the ascorbate content by the addition of L-galactono-1, 4-lactone, the direct precursor of ascorbate in the biosynthetic pathway, causes a delay of about five days. These observations suggest an inhibitory effect in triggering floral induction. Furthermore, the molecular data showed that at the vegetative apex of *Arabidopsis thaliana*, ascorbate has an antagonistic effect on gibberellins, which activate the expression of floral development genes such as LEAFY (Blázquez *et al.*, 1997). The ascorbate-induced regulation analysis shows a high expression of these genes in the *vct* deficient mutants. Thus, ascorbate acts as an endogenous signal that influences floral induction by modulating the expression of blooming genes and thereby affecting the signalling processes of phyto-hormones (Kotchoni *et al.*, 2008).

## Co-factor

Due to its properties, ascorbic acid has a link with a very large number of molecular processes. One of the main functions of ascorbate in metabolism is to act as a cofactor for mono- or dioxygenase that contain iron or copper in their active site (De Tullio *et al.*, 1999; Arrigoni and De Tullio, 2002). The role of ascorbate is to maintain the metal ion of the active site in reduced form, thereby increasing the activity of the enzyme (Sanmartin *et al.*, 2000). In plants, for example, ascorbate is used as a coenzyme for an iron dioxygenase, which participates in the post-translational modification of the cell wall proteins. Ascorbate can also modulate iron uptake and release by ferritin (Fry, 1998). In a more developmental aspect, ascorbate is a cofactor of the enzymes 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) and GA2-oxidase, which are involved in the synthesis of phyto-hormones such as ethylene and GA respectively (Smirnoff, 1996). Similarly, it serves as an enzymatic cofactor for violaxanthin deoxidase (VDE) which catalyzes the conversion of violaxanthin to zeaxanthin. The xanthophyll cycle is important for the protection of the reaction centers of photosynthesis (see chapter Photosynthesis). Ascorbate also allows the regeneration of  $\alpha$ -tocopherol (vitamin E) from tocopheryl radical (Asada, 1999).



**Figure 10:** Role of ascorbate recycling in photosynthetic-related processes.

**1.** Transfer of electrons from ferredoxin (Fd) at PSI to  $O_2$  generating superoxide ( $O_2^{\cdot-}$ ) constitutes the Mehler reaction. **2.** A thylakoid membrane-associated iron-containing superoxide dismutase (FeSOD) and ascorbate peroxidase (tAPX) detoxify  $O_2^{\cdot-}$  generated at PSI as part of the thylakoid scavenging system. **3.** A stromal ascorbate peroxidase (sAPX), CuZnSOD, MDAR, DHAR, and GR constitute the stromal scavenging system. **4.** While AsA can donate electrons to the photosystems and is used as a cofactor for Violaxanthin de-epoxidase (VDE), no AsA recycling system is present in the thylakoid lumen. Luminal MDHA disproportionates non-enzymatically into AsA and DHA, which is transported to the stroma (Gallie, 2013).

PSII, photosystem II; OEC, oxygen-evolving complex; P680, specialized chlorophyll dimer; PQ, plastoquinone; cyt b<sub>6</sub>f, cytochrome b<sub>6</sub>f complex; PC, plastocyanin; PSI, photosystem I; P700, specialized chlorophyll dimer; FNR, Fd-NADP<sup>+</sup> reductase.

## ii. Photosynthesis

Photosynthesis is responsible for the transformation of light energy into chemical energy, *i.e.* the synthesis of organic matter. The photoenergy is absorbed by a series of oxidation-reduction reactions to be transmitted to the reaction centres of the photosystems: PSI and PSII, respectively located in chloroplast stroma and thylakoid lumen. The process results in the production of energy in the form of ATP and NADPH. These organic molecules are then combined with dark-assimilated carbon dioxide (CO<sub>2</sub>) to produce carbohydrates.

The photosynthetic activity is limited by the rate of assimilation of CO<sub>2</sub>, and so when the lighting is too important, the electron transport chain is saturated. In this case, the photosystems cannot evacuate the electronic charges resulting from this excess of photons. This dysfunction results in the production of ROS by transfer of these electrons to oxygen molecules. The pool of ascorbate - important in chloroplasts (20-300mM) - increases with light intensity. At the acceptor site of PSI, ferredoxin (FD) rather than reducing the pool of NADP<sup>+</sup> can reduce molecular oxygen to superoxide anion. This superoxide anion is then reduced by the SOD in hydrogen peroxide which is oxidized by the APX in H<sub>2</sub>O. This water-water cycle is called the Mehler reaction (Wituszyńska and Karpiński, 2013). The produced MDHA can be directly reduced in ascorbate by the PSI and/or by normal ascorbate/GSH cycle (Ivanov and Edwards, 2000). MDHAR bound at the thylakoid level, uses reduced FD as an electron donor to regenerate ascorbate (Apel and Hirt, 2004).

Through its reducing power, ascorbate also plays a role in the regulation of non-photochemical quenching (NPQ) processes (Smirnoff, 2000). This phenomenon allows the dissipation of excess excitation energy to harmless heat under high light conditions. Plants can improve this capacity converting the orange pigment violaxanthin to antheroxanthin and then the pale-yellow pigment zeaxanthin. The reaction is catalysed by the violaxanthin de-epoxidase (VDE) which uses ascorbate as co-factor (Hager and Holocher, 1994). Similarly, the study of mutants with a higher ascorbate content, such as the *miox4* mutant, reveals ascorbate as a limiting factor for VDE activity (Toth *et al.*, 2011). In addition to its involvement in ROS detoxification, ascorbate would therefore have a role in the direct protection of the integrity of the photosystems.



### **iii. Oxidative stress (Post-harvest)**

Reactive oxygen species (ROS) are derived from aerobic normal oxygen metabolism in chloroplasts, mitochondria, and peroxisomes. These became chemically reactive by the presence of unpaired valence electrons. Among the ROS there are the superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^\cdot$ ) and oxygen singlet ( $O_2$ ). The amount of these ROS species significantly increases during abiotic and biotic stresses, causing damage to the cells and therefore to the plant. That is oxidative stress. ROS can attack cell membrane by lipid peroxidation, and also by altering the conformation of the proteins. When they reach the nucleus, ROS can disturb DNA replication leading to mutations and, in some cases, cancer. Many processes are able to detoxify the ROS, either through enzymatic reaction or by the involvement of antioxidant molecules. The first case is represented by the superoxide dismutase (SOD), and the catalase (CAT) (Fig. 5). The former catalyses the partitioning of the superoxide ( $O_2^-$ ) radical into dioxygen ( $O_2$ ) or hydrogen peroxide ( $H_2O_2$ ), while the latter catalyses the decomposition of hydrogen peroxide to water ( $H_2O$ ) and oxygen. Thus, SOD and CAT are essential in the response against ROS.

Ascorbic acid (vitamin C), Tocopherol (vitamin E) and Glutathione are all antioxidant molecules working against ROS. Glutathione acts indirectly to detoxify cell by recycling DHA in ascorbate. Process involving vitamin E takes place mainly in cell membrane, interacting with lipid free radicals (LOO) to give a neutral species and a reactive tocopherol which can be recycled by ascorbate or glutathione. Ascorbate interacts enzymatically and non-enzymatically with ROS (Noctor and Foyer, 1998; Foyer and Noctor, 2011). The redox status model is based on this interaction ROS/ascorbate, which is responsible for a signal in the cell. The ascorbate peroxidase (APX) uses the ability of the reduced form of ascorbate to give a proton, in order to convert the  $H_2O_2$  in water. This reaction results in the production of MDHA. From there, all the enzymes involved in the recycling of ascorbate, participate to its potential of detoxification.

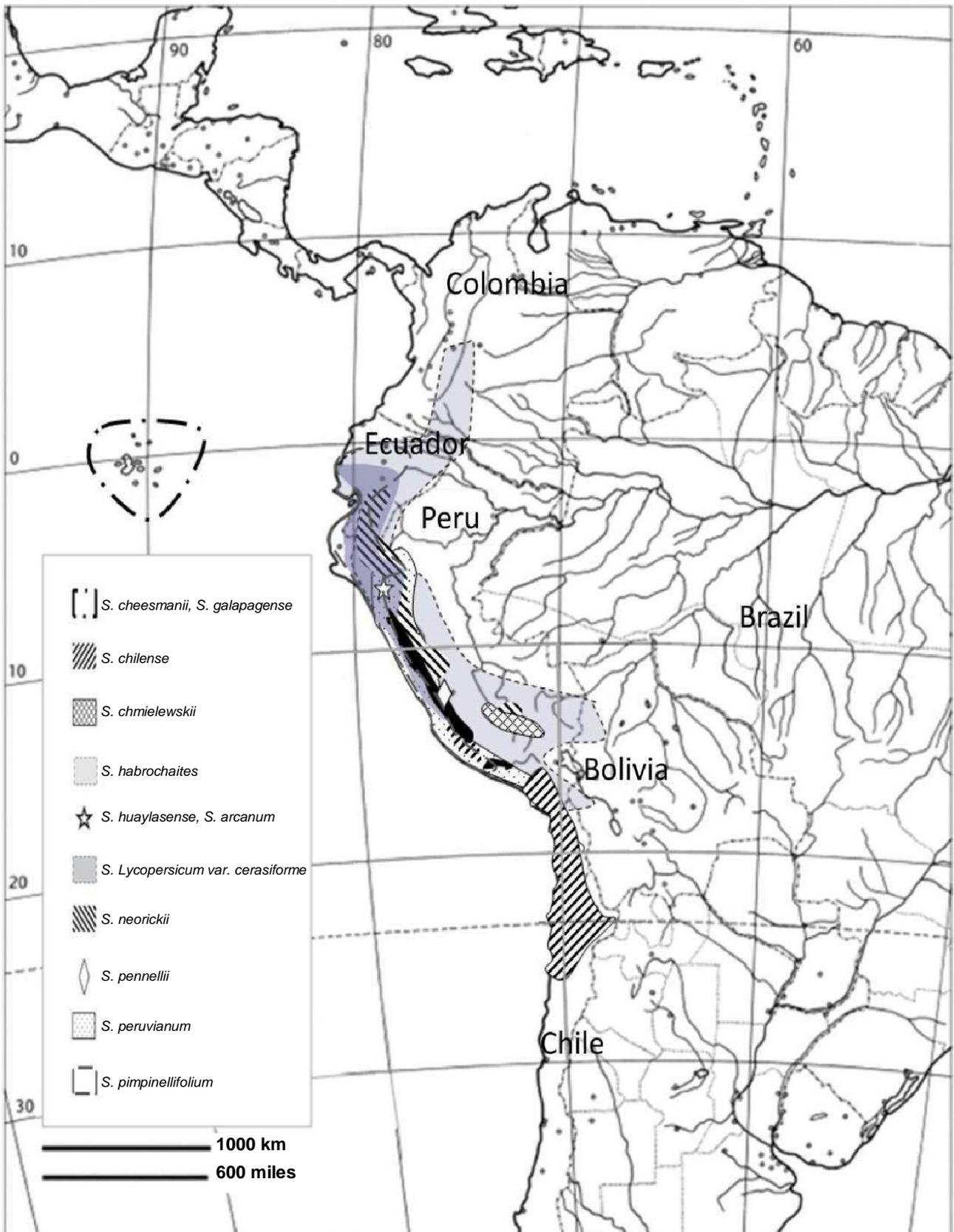
### **iv. Pathogenic infection**

The plant displays mechanisms protecting itself from the activation of signalling pathways responsible for the synthesis of particular proteins (Pathogenesis-Related PR, Heat-Shock-Protein HSP, Late Embryogenesis Abundant Proteins LEAP), or the stimulation of antioxidant systems. Ascorbate plays an important role in these two processes. Indeed,



ascorbate is not only an essential antioxidant, it is also a cofactor for the activity of a 2-oxo-acid-dependent dioxygenase (2ODD). The 2ODD family is responsible for the synthesis of a wide range of secondary metabolites including hormones (Arrigoni and De Tullio, 2002). Hormones are fundamental compounds for the transmission of the signal and consequently the setting up of an adapted response. *Arabidopsis vtc* mutants deficient in ascorbate are thus sensitive to abiotic stress while they show resistance to pathogens (Pastori, 2003; Barth, 2004; Colville and Smirnoff, 2008).

In general, during a pathogens infection, the plant puts up a hypersensitive response, inducing rapid death of cells near the point of infection. Ascorbate regulates the production of defence proteins called pathogenesis-related (PR). For instance, in ascorbate-deficient mutant *vtc1*, protein PR are activated (Pastori, 2003). That can be explained by a state of stress of the plant. Treatment with high doses of ascorbate alleviates the severity of symptoms during RNA virus infection but is unrelated to elimination of ROS. This indicates that ascorbate increases plant resistance to pathogens through a specific mechanism that does not use ROS as a signal. Conversely, the abundance of ascorbate in plants infected with herbivorous insects increases their susceptibility. Indeed, ascorbate works as a phage-stimulant for many insects that are likely to use it for their metabolism. The ascorbate content in leaves of different tomato cultivars correlates positively with the survival of a species of Colorado potato beetle (*Leptinotarsa decemlineata*). Nevertheless, the impact of ascorbate in this type of response is complex. It is used both for the metabolism of the insect and the plant (Goggin *et al.*, 2010).



**Figure 11:** Geographic distribution of wild species in Solanum section Lycopersicon (Bauchet and Causse, 2012).

# TOMATO - MORE THAN A FRUIT, A MODEL

## From Origins to Domestication

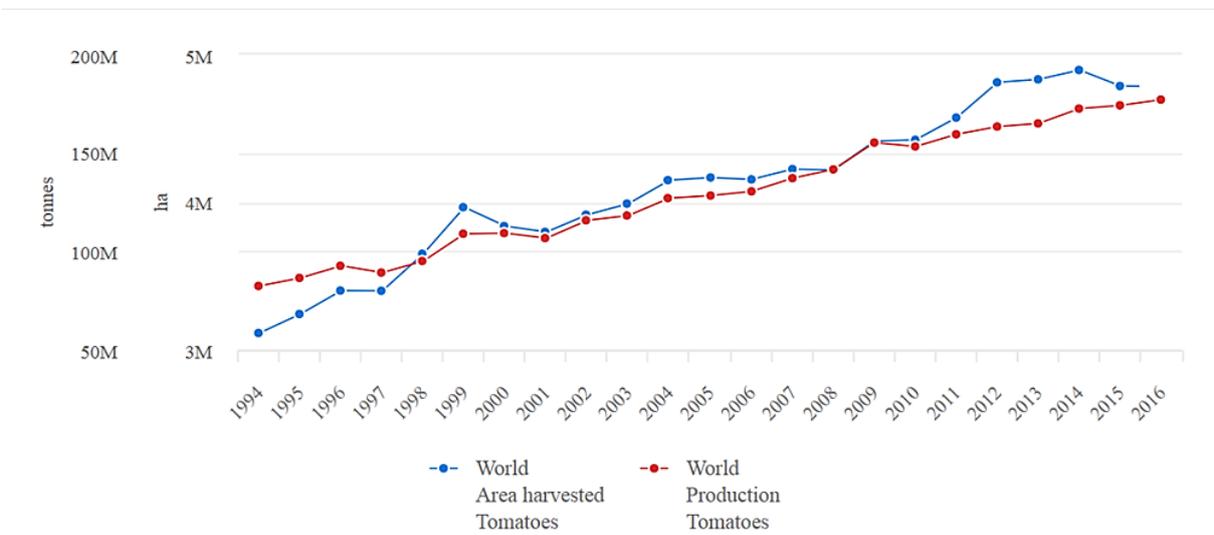
Wild tomato species are native to western South America in the Andean region (Fig. 11). It is part of the products introduced to Europe in the early 16th century by the Spanish conquistadors. Although location of wild species origin is well established, the precise starting point of its domestication has not yet been clearly elucidated. Indeed, since the end of the 19th century, two hypotheses have emerged: the Peruvian and Mexican hypotheses. First in 1886, DeCandolle in its "Origin of cultivated plants" proposed the Peruvian origin of tomato domestication. His conclusions were based on a study combining botany, archeology and paleontology, history and philology (reviewed in Peralta & Spooner, 2006). Among the facts noted, the names "mala peruviana" and "pomi del Peru" used in Europe to refer to the tomato, and the localization of its closest ancestor: the wild cherry tomato. In 1948, some clues came to discuss this theory. The most important is that there is no evidence of pre-Colombian tomato cultivation in South America, while there is in Mexico. Secondly, the name "tomato" comes from the Mexican Nahua word "tomatl" which refers to "plants bearing globous and juicy fruit" (Bauchet and Causse, 2012). Based on this, Jenkins proposed a Mexican origin of domestication. As the two hypotheses are not conclusive, it is possible that domestication appeared independently in the two regions. Recently, a study based on SNP analysis has hypothesized a 3-steps model: a pre-domestication of the tomato in the Andean region (Peruvian), followed by a complementary domestication in Mesoamerica (Mexican) and then finally its introduction and cultivation in Europe (Blanca *et al.*, 2012).

At that time, the tomato fruit was considered toxic and mainly used as an ornamental and medicinal plant. During the 18th century, the first steps of the tomato breeding allow to select varieties improved for the resistance to pathogens, the size, the colour and the taste of the fruits. Up to now, advances in selection and biotechnology programs have made it possible to target more and more morphological characters, in order to adapt to environmental conditions and consumption. Despite its benefits, this evolution has greatly contributed to the reduction of genetic diversity.



In 1753, the Swedish naturalist Linnaeus -founder of the modern system of the binomial nomenclature- classified tomatoes in the genus *Solanum* as *Sl. lycopersicum*. After more than 200 years of debate and controversy, the use of molecular data allowed in 2006 to confirm the classification of Linnaeus. *Solanum lycopersicum* like several thousand botanical varieties, including the large-fruited tomato *Solanum lycopersicum* var. *esculentum*, from which derive almost all cultivars on the market, and finally the *Solanum lycopersicum* var. *cerasiform*, the cherry tomato, which is the only wild variety found outside of South America (Rick, 1986). The different varieties of tomato are characterized according to the mode of plant growth that can be determined or undetermined, but also according to the colour, the size and shape or the colour of the fruit. Cultivar collections are maintained in various countries to preserve genetic resources (California: TGRC, Taiwan: AVRDC, France: INRA).

1994 - 2016



**Figure 12:** Production/Yield quantities of Tomatoes in World

## A Model Species

### **i. Agro-economic importance**

The tomato is - after the potato - the most consumed vegetable in the world, either fresh or after processing. It is cultivated under all latitudes in very varied conditions (climates, production methods), which demonstrates a great original plasticity and testifies to the efficiency of the work of the breeders. The main producing countries are China (50 million tons), India (17.5 million tons), the United States (13.2 million tons) and Turkey (11.35 million tons). France is only 32nd in the world with an annual production of nearly 600 thousand tons in 2014 (FAOstat).

Another factor demonstrating the utility of studying tomato is its nutritional importance. Indeed, everybody is aware of the recommendation: "five fruits and vegetables a day". Actually, some fruits or vegetables are not recommended such as fruits rich in fructose, unwell metabolized by the body, which causes peaks of glycaemia and accumulation of fats in the cells. One of the particularities of the tomato is to be nutritionally rich but very low caloric (15 kcal per 100 g MF). It contains little carbohydrate (less than 20 kcals per 100 g MF), so it is particularly recommended for overweight or diabetes. Tomato also has a significant amount of antioxidants (carotenes, vitamin C, vitamin E, lycopene and polyphenol) involved in the prevention of cancer and cardiovascular disease. In the same way, it is rich in minerals (potassium, magnesium and phosphorus), fibers and water (95% of the MF).

### **ii. Food losses from field to plate: Post-harvest injuries**

Food and Alimentation Organization (FAO) indicates that 30 to 50% of global food production would be lost from the field to the consumer plate. At the time of harvest when the products have reached a sufficient maturity to be marketed, fruits and vegetables are very sensitive to biotic and abiotic stresses. Losses can be caused either because of a climatic stress, or by attacks of bio aggressors. These losses are difficult to quantify, because they are very random and variable from one year to another.



Although the greatest risk of damage during the production is in the field, storage and transportation remain critical steps for the conservation of the products. Fresh fruits and vegetables usually are not or not very storable. Perishability depends on the physiology of the products. At 4 ° C, the apple can be stored for up to 8-12 months in a controlled atmosphere, while for raspberry the shelf life is only 2 days. For vegetables, the shelf life ranges from 5 days maximum for leafy vegetables, about 1 week for zucchini and tomatoes, and 2 to 3 weeks for cabbage and carrots.

There are losses at the packing and shipping station for several reasons. Products that do not conform to the specifications (over-maturity, defects in size or appearance etc.) are immediately discarded. Then, during storage of products in the cold room, conservation diseases are likely to develop. For products intended for the fresh market, losses may occasionally be significant at this stage because of a temporary imbalance between supply and demand, or because of overproduction for very perishable products (strawberry, peach, etc ...) or many massive imports (tomato, peach, etc ...).

Improving product conservation requires coordinated research in genetics and agronomy that implements new tools and systems approaches. Research has also to take into account the agroecology goals. The extension of the shelf life also involves the development of technological innovations such as modelling and simulation (predictive microbiology), new physical or biological processes (fermented products) from food preparation and active packaging or indicators of quality / freshness.

## **i. Genetic tools**

Based on these agro-economic aspects, tomato (*Solanum lycopersicum*) is a model for the study of processes associated with the development of fleshy fruits. It has also many biological advantages. Tomato shows ease of cultivation according to its short development cycle, an easy crossing ability and a culture throughout the year. In parallel with the increase in available biological material and the development of technologies, "omics" databases have been improved with many genetic resources, molecular and biochemical availability (Robertson and Labate, 2006; Rodriguez *et al.*, 2011). The major advance was few years ago, when the genome of *Solanum lycopersicum* cv. "Heinz 1706" was sequenced and annotated (Tomato Consortium 2012, Aoki *et al.*, 2013). This genome



**Figure 13:** Genetically modified (GM) and natural cultivar tomatoes. From left to right: Moneymaker (parental control), GM (56B), GM (7C), Aichi First, Alisa Craig, M82, Micro-Tom and Rutgers (2011 Tadayoshi Hirai)

has a size of about 900 Mb, distributed over 12 chromosomes, of which more than 75% is heterochromatin largely devoid of genes. Last year, was released the third version of the tomato genome (SL3.0) and version ITAG3.20 of the annotation. It lists 30,868 genes with almost than 5000 novel genes reported.

### **Micro-Tom**

The tomato cultivar Micro-Tom was released in 1989 by J. Scott and B. Harbaugh for home gardening purposes and launched as a genetic tool by the Avraham Levi group in 1997. It was produced by crossing the cultivars Florida Basket and Ohio (Scott and Harbaugh, 1995). Miniature phenotype of Micro-Tom, bearing small fruits, is caused by two major recessive mutations: dwarf (*d*) and miniature (*mnt*). The dwarf phenotype is explained by a mutation affecting brassinosteroid biosynthesis in vegetative tissues (Marti, 2006; Nomura *et al.*, 2005). It has also been suggested that the cultivar has a mutation in the *SELF-PRUNING* (*SP*) gene, leading to its determinated growth (Benfey and Chua, 1990).

Micro-Tom brings together all the criteria necessary for a model system in tomato research. Its small size, about 15 cm in height, allows dense cultivation of up to 1,357 plants per square meter in case of genomic screening, and around 115 plants per square meter for physiological and molecular studies requiring the generation of seeds. Interestingly, mature fruits can be harvested 70 to 90 days after sowing (Emmanuel and Levy, 2002). Micro-Tom grows normally under artificial light. The small size of Micro-Tom allows experiments in confined and controlled environments that comply with safety regulations for genetically modified organisms (GMO). In addition, a highly efficient transformation procedure for Micro-Tom using *Agrobacterium* has been established (Sun *et al.*, 2006). This contributes to establish Micro-Tom as a model genomics system, concomitantly with the development of 'Micro-Tom'-based genomic resources, including transposon tagging lines (Meissner *et al.*, 2000), ethylmethane sulfonate (EMS)- and  $\gamma$  ray-mutagenized lines (Saito *et al.*, 2011), near-isogenic lines (Carvalho *et al.*, 2011) and a full-length cDNA collection (Aoki *et al.*, 2010).

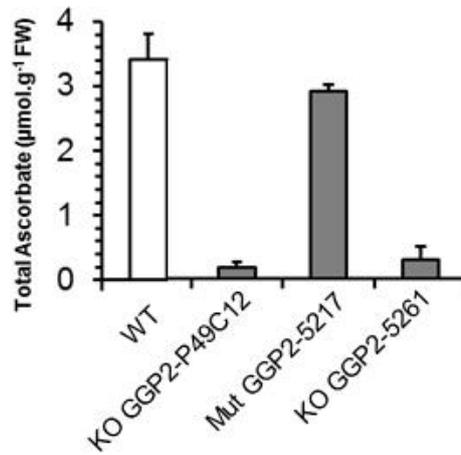
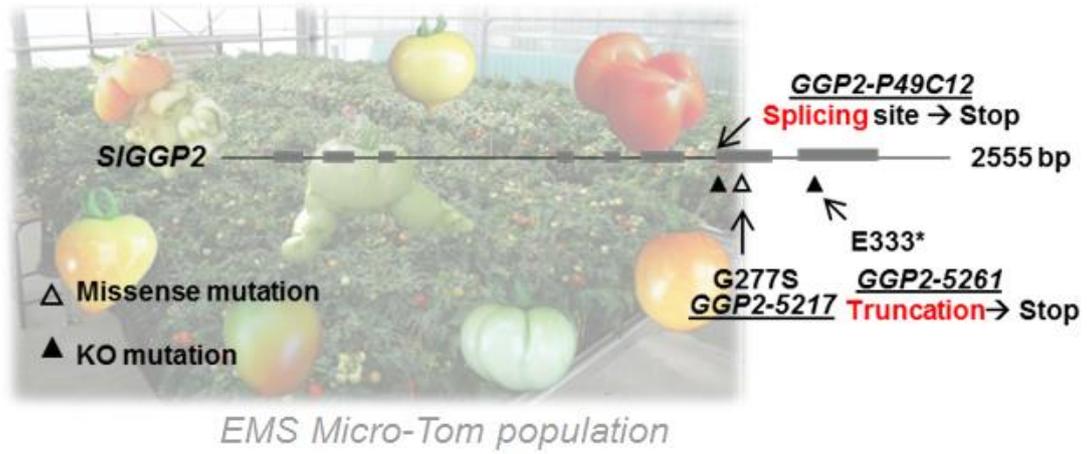


## MicroTom Ascorbate Mutants

### **i. EMS mutant library**

Since the MicroTom cultivar has become a model in functional genomics in tomato, the interest of developing the most diversified genetic resources has grown steadily. The reasons to go towards a highly-mutagenized EMS (Ethyl MethaneSulfonate) mutant collection was i) to manage with the need for high throughput functional analysis of target genes, ii) to get a new source of genetic diversity that can be used for the isolation of genes underlying traits of interest, iii) to produce allelic series including strong and weak alleles that can be used for mutation breeding of plant and fruits with improved traits. EMS mutation densities display a wide range of variation from one mutation per Mb in barley (Caldwell *et al.*, 2004) to one mutation per 170 kb in Arabidopsis (Greene *et al.*, 2003) and one mutation per 25 kb in the hexaploid wheat (Slade *et al.*, 2005).

At INRA Bordeaux, two mutant populations were generated using 1% EMS solution to obtain M1 plants as described by Okabe *et al.* (2011). For one of these, a second round of mutagenesis was performed on M2 seeds, in order to increase the mutation frequency. The whole M2 families -once and twice mutagenized- representing around 8000 accessions were collected for DNA isolation. In addition, a complete phenotypic analysis starting from the seed germination up to ripe fruits, was done with the 3500 M2 (most mutagenized) families and integrated in a phenotypic mutant database called MicroTom Mutant Database (MMDB, Just *et al.* unpublished data and not yet publicly searchable). This database corresponds to more than 30,000 annotations for more than 150 phenotypic categories. Mutagenesis by EMS generates point mutations, mainly C to T and G to A transitions, distributed randomly over the whole genome. The mean mutation frequency reported for the combined collections generated in Bordeaux ranges between 1 mutation/125 kb and 1 mutation/663 kb. At the same period another EMS mutant collection in the MicroTom was also created by Pr. H. Ezura from University of Tsukuba (Japan), and in this Japanese population the estimated mutation frequency ranges between 1 mutation/1710 kb (0.5% EMS) and 1 mutation/737 kb (1% EMS).

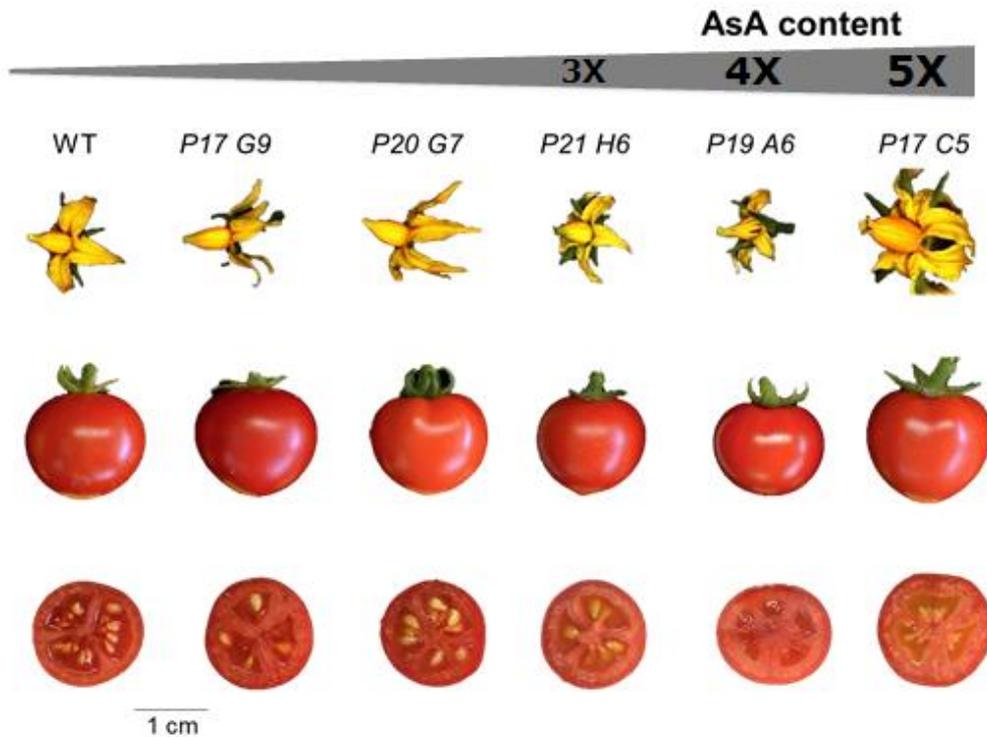
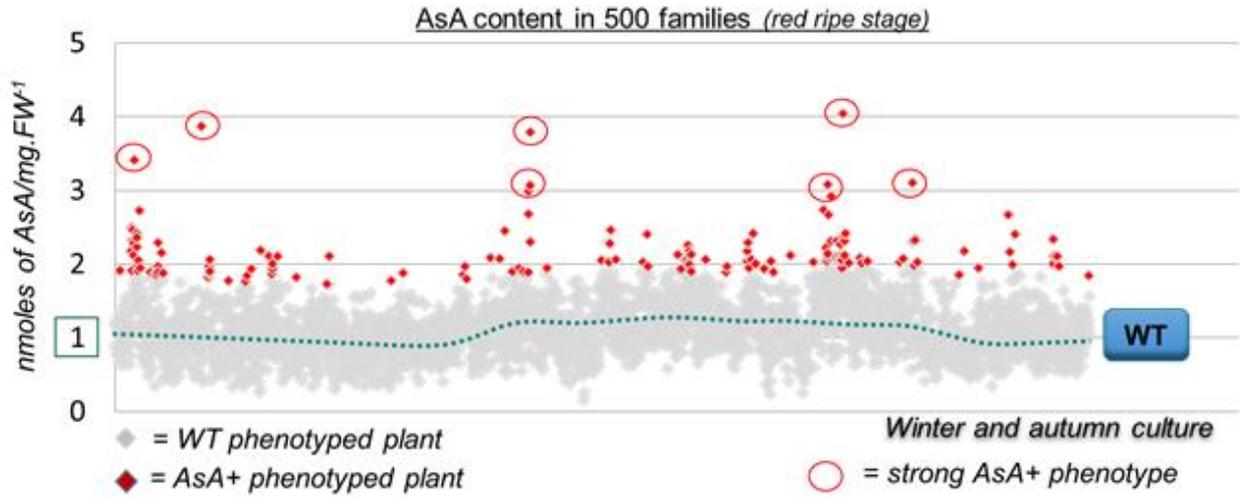


**Figure 14:** Isolation of allelic variants for *SIGGP1* gene using TILLING strategy

## ii. Reverse genetic: Ascorbate-deficient mutants

In the frame of functional characterisation of targeted gene, genetic transformation is useful and commonly used in *Arabidopsis* such as insertional T-DNA mutagenesis. This kind of technology is easily achievable in tomato but the whole process is time-consuming. Several months are usually necessary to obtain a generation and in the case of MicroTom this period represents about 6 months, which makes it difficult to apply for a large series of genes. Using the very high mutation frequency of the EMS population, TILLING (Targeting Induced Local Lesions IN Genomes) makes it possible to rapidly identify allelic series for a gene of interest.

This approach was used to study the function of genes involved in the modulation of ascorbate in relation with cell wall biosynthesis in tomato. The aim was to isolate in the EMS population from INRA-Bordeaux and Tsukuba ascorbate-deficient mutants, targeting the *SIGMP2* gene (Soly06g051270.2) encoding the GDP-D-mannose pyrophosphorylase, the *SIGME1* (Soly01g097340.2) encoding the GDP-D-mannose epimerase and the *SIGGP1* gene (Soly06g073320.2) encoding the GDP-L-galactose phosphorylase (Baldet et al., 2013, and Baldet et al. unpublished data). Several mutations was identified in MicroTom EMS mutant collection from Tsukuba and Bordeaux. Among the mutations isolated in the exonic region of the selected genes, some display point mutations that lead to the alteration of protein function. Two mutations in *SIGMP2*, one in *SIGME1* and three mutations in *SIGGP1*, confirmed by sequencing, were selected for further characterization. To determine the physiological effect of the mutations, ascorbate content was assayed in fully expanded leaves. The only ones to display a significant alteration were the two *Siggp1* truncation and splice junction lines, with ~95% decrease in ascorbate. This results confirm again the key role of GGP in ascorbate biosynthesis. Moreover, the two *GGP* homologous genes are also found in tomato (*SIGGP1* and *SIGGP2*). Moreover, this study revealed that the two proteins are not totally and functionally redundant since the KO of *SIGGP1* was not fully complemented by *SIGGP2*, which is about 100 fold less expressed than its counterpart in Tomato wild-type (Massot et al., 2012).



**Figure 15:** Forward genetic approach to identify AsA-enriched families.

Screening of the EMS Mutant population for AsA-enriched fruits (Top). Phenotype of the fruits and flowers of the AsA-enriched mutant (Bottom).

### iii. Forward genetic: Ascorbate-enriched mutants

At INRA-Bordeaux, the artificially generated population of MicroTom mutants, with a genetic and phenotypic variability far beyond the natural variation found in domesticated species, has allowed to go further the identification of allelic series of already known genes. Indeed, the screening of this population for a trait of interest, combined with a mapping by sequencing approach makes possible to reveal new genes underlying the phenotypic variations (Garcia *et al.*, 2016).

In the case of the study on ascorbic acid metabolism developed in the INRA group to which I belong, and prior to the start of my PhD, 500 tomato mutant families from the INRA EMS Micro-Tom tomato mutant collection were screened for high ascorbate fruit content. This tremendous phenotyping work on more than 6000 plants, resulted in the finding of five mutant families displaying a significant ascorbate content 2 to 5 times higher than the WT fruits. The morphological analysis of the vegetative and reproductive parts of the plants, showed no obvious phenotype difference in mutant families up to 3 times the WT: P17G9, P20G7, and P21H6 families. For the two other mutant families, P19A6 and P17C5, displaying an increase of ascorbate up to 5 times that of the WT, the fruits exhibited a low seed number and in extreme extend a parthenocarpic phenotype, meaning seedless fruits. The absence of seed production has been a big challenge in the frame of the mapping by sequencing approach. Despite that, two mutant families have been the focus of the attention and finally successfully characterized: the P21H6 and the P17C5 family mutants

#### **The P17C5 mutant family**

Among mutant families to have been isolated, P17C5 is the one with the higher ascorbate accumulation (up to 5 times the WT). Moreover, the segregation analysis performed in its progeny seemed to reveal that the responsible mutation is dominant, what is really significant and attractive for breeding programs. However, even being very original, the parthenocarpic phenotype made more complex the way to characterize the mutation. Observations revealed that the flowers was abnormally bigger than the WT, and preliminary analysis of the pollen germination rate also showed an obvious loss of fertility. As mentioned previously, the genetic characterization of the original mutant plant allowed to conclude that this mutation is dominant. Knowing that, it was possible to transfer the P17C5 mutation to another commercial genetic background, at the same time than to attempt to break the link between the AsA + phenotype and parthenocarpy. In that aim the ascorbate enriched P17C5 mutant was crossed with the *Solanum lycopersicum* cv M82



cultivar, a tomato variety known for both the fresh market and processing. This well-known variety displays morphological and genetic characteristics very different from that of the Micro-Tom cultivar. It has a bushy determinate port, growing up from 1 to 2 meters, and produces large fruits (~8-10cm in diameter compared to MicroTom of 3-4cm). From this first Out-Cross (OC), few seeds have developed after crossing. At the start of my PhD work, among the four OC<sub>1</sub>F<sub>1</sub> plants sown, only one produced fruits displaying a strong AsA+ phenotype, confirming the dominant nature of the mutation in the first mutant P17C5. However those AsA-enriched fruits were all parthenocarpic making impossible to generate the OC<sub>1</sub>F<sub>2</sub> population to perform the mapping by sequencing approach.

## **The P21H6 mutant family**

### *Genetic characterisation*

Even if it is not the EMS mutant family with the higher ascorbate accumulation, the P21H6 family displays a strong ascorbate enrichment in fruits (up to 3 fold increase) while having no obvious phenotypic alteration of the plant. Moreover, the BC<sub>1</sub>F<sub>2</sub> population resulting from the backcross of the mutant, showed a Mendelian segregation of the mutation as expected in the case of a recessive mutation. Because of the parthenocarpic issue in the other mutant families isolated, the P21H6 mutant line was the first to be selected for identifying the mutation through mapping-by-sequencing approach.

According to the chosen strategy, one homozygous ascorbate enriched P21H6 mutant was backcrossed with the WT MicroTom parent leading to hybrid F<sub>1</sub>. These F<sub>1</sub> plants were thereafter self-pollinated to generate a BC<sub>1</sub>F<sub>2</sub> segregant progeny. In the case of a recessive mutation the F<sub>2</sub> individuals showing the AsA-enriched phenotype carry the causal mutation as homozygous alleles. The BC<sub>1</sub>F<sub>2</sub> segregant population constituted of 440 plants was therefore phenotyped to define two bulks corresponding to the "AsA+" and to the "WT-like AsA" plants. The DNA from each bulk was prepared as a pool that was sequenced at a depth of 40x. Then, the sequencing data was supported by a bioinformatics workflow that basically allow to compare it to the Heinz reference genome, and that of MicroTom, in order to detect both EMS mutations and natural polymorphism along the 12 tomato chromosomes. In the case of the P21H6 mutant, only five SNPs were retained. They correspond to a linked group and are all located in the fifth chromosome.

Among the five SNPs, only two correspond to annotated genes. The first SNP appeared in the middle of the first intron of the *Solyc05g006660* gene, encoding an unknown protein. The second was detected in the fifth exon of the *Solyc05g007020* gene, and was present at the top of the SNPs plot. It corresponded to a G to A transversion leading to the appearance of a STOP codon in the protein sequence and consequently to a



non-functional protein. At this stage, all the evidences suggested that the SNP in the *Solyc05g007020* gene was likely to be the causal mutation for the ascorbate-enriched phenotype in P21H6 mutant. Indeed, this point mutation was the only one to have a potential direct effect in the coding region of a referenced gene. Interestingly, the gene encoded a LOV-containing protein, named PAS/LOV protein or PLP, which would be a blue light sensitive protein. However, additional genetic analyses remained necessary to unequivocally link the trait-of-interest to the mutation.



# **LIGHT - THE DARK SIDE OF ASCORBATE**

## Description

### **i. Some physical aspects of light**

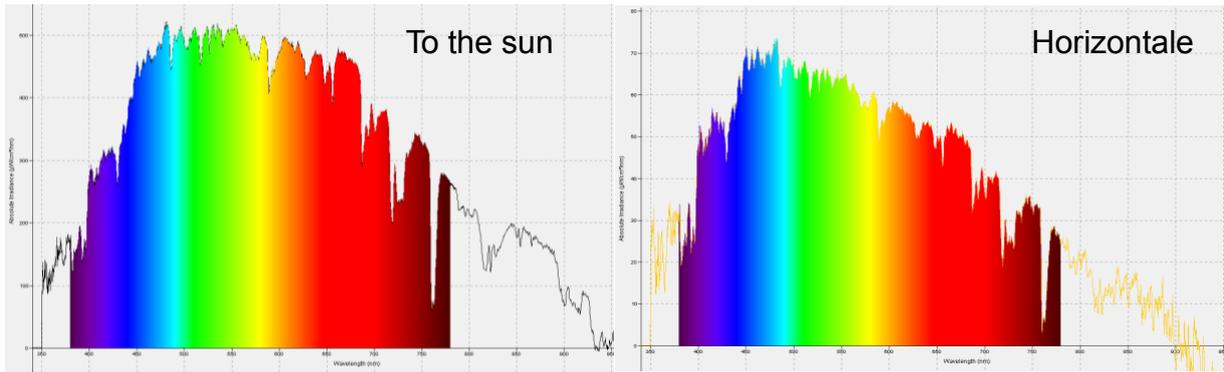
Natural light source is provided by the sun which illuminates the earth's surface. The solar radiation received brings together all the colours to compose the white light spectrum. All organisms living on earth are adapted to this light. For instance, plants particularly use blue (440 nm) and red (660 nm) wavelengths for photosynthesis as well as to control their physiological processes during development.

### **ii. Light and plants**

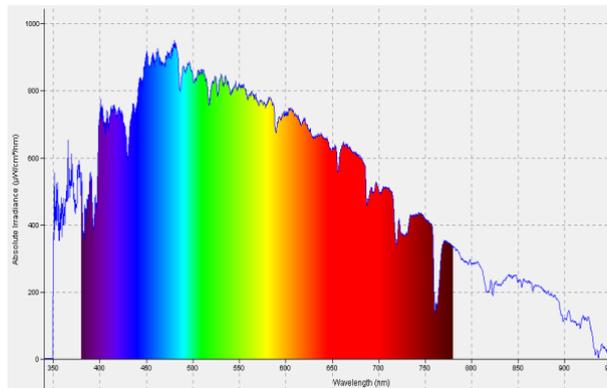
The energy contained in a photon in the visible spectrum, 40–60 kcal mol<sup>-1</sup>, is sufficiently large to easily drive chemical processes such as electron transfer, formation or rupture of a covalent chemical bond, or isomerization about a double bond, if suitably harnessed. Thus, light can readily influence chemistry. Light is an essential parameter in plant life. Indeed, plants are autotrophic organisms producing their own nutrients from atmospheric CO<sub>2</sub>, using water and minerals from the soil.

More precisely, plants are photoautotrophic: through the photosynthetic process, they depend on light as source of energy to synthesise carbohydrates. All green organs, mainly leaves, have the capacity to implement photosynthesis. At the chloroplast level, blue and red wavelengths are intercepted by collecting antennas as chlorophyll complexes. The luminous radiations are then considered as a flow of photons carrying energy which is capable of exciting the chlorophyll molecules. The energy is absorbed by a series of oxidation-reduction reactions to be transmitted to the reaction centres of the photosystems namely PSI and PSII. This allows the production of ATP and NADPH, which are necessary for the unfolding of the last so-called carboxylation phase. This last step allows to produce

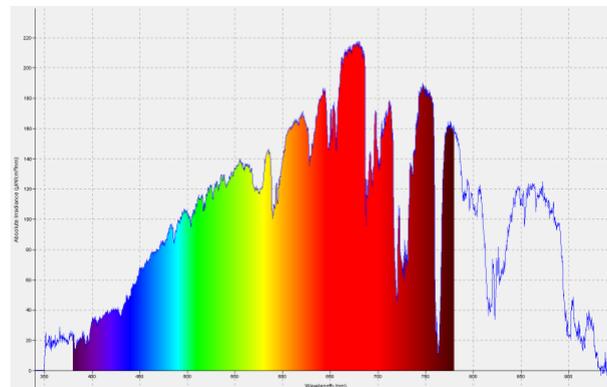
On the morning (clear sky)



Mid Day (clear sky)



Evening (clear sky)



July 2016 (cloudy sky)

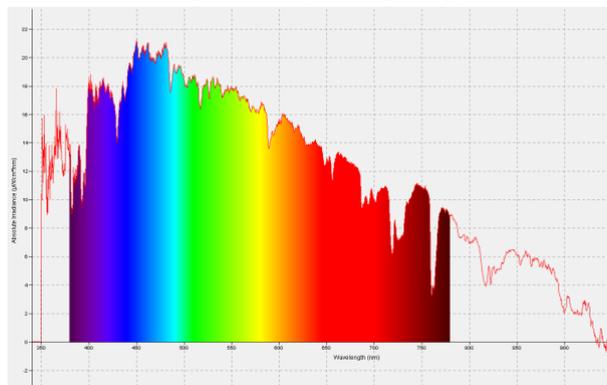


Figure 16: Variations of the solar spectrum

carbohydrates from assimilated CO<sub>2</sub>, through the Calvin cycle. Under some conditions, plants can be subjected to intense light which can be deleterious and somehow lethal. The photosynthetic activity is limited by the rate of assimilation reaction of CO<sub>2</sub> catalysed by the key master enzyme of the Calvin cycle, namely the Ribulose-1,5-bisphosphate Carboxylase Oxygenase RubisCO. When the light is too intense, the electron transport chain is saturated. In this case, the photosystems can no longer evacuate the electronic charges resulting from this excess of photons, this excess of electrons is transferred to molecular oxygen molecules resulting in the production of ROS.

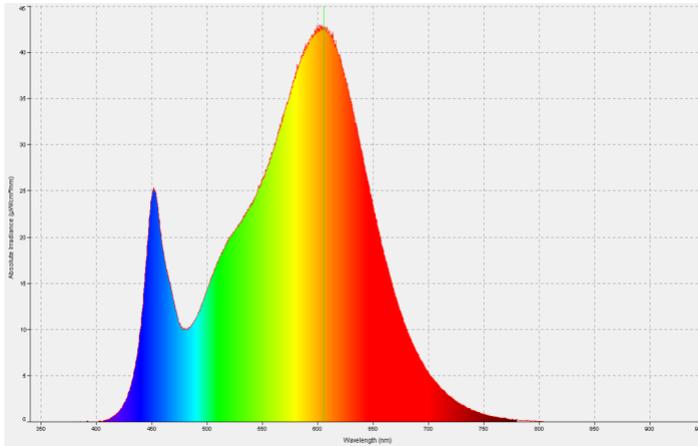
In addition to be an active radiation for photosynthesis, light also has a signalling role for the plant that can perceive environmental conditions around it and adapt. That is photomorphogenesis. The morphogenetic responses of plants to light imply numerous mechanisms corresponding to quantitative changes, the differentiation of new organs, and the modification of metabolic pathways. These responses can be i) phototropism which is a directional modification of the growth in response to a light stimulus, ii) when this movement is reversible and depends on variations in lighting conditions that is photonasty, iii) photoperiodism allows developmental responses to the variation of the circadian cycle, and finally iv) strict photomorphogenesis, which is a non-directional developmental response related to a non-directional, non-periodic and non-photosynthetic light stimulus. (Mohr, 1972, Smith, 1975, Heller, 1977).

## **i. Light and ascorbate**

Light has a very close relationship with the metabolism of ascorbic acid. However, no direct regulation mechanisms have been yet deciphered. As previously described, the availability of the reduced ascorbate pool is of great importance in the antioxidant response to the accumulation of ROS under stress. In this case, light is largely responsible for this accumulation, as it has been showed regarding Photosynthesis. In previous results, effects of plant illumination directly or indirectly affecting the genes of the ascorbic acid biosynthetic pathway have been observed. For example, prolonged exposure to a light source increases the expression of the key GGP (VTC2) gene. But again, there is no evidence that light directly induces ascorbate synthesis or whether this over-expression of GGP is a consequence of oxidative stress related to exposure.

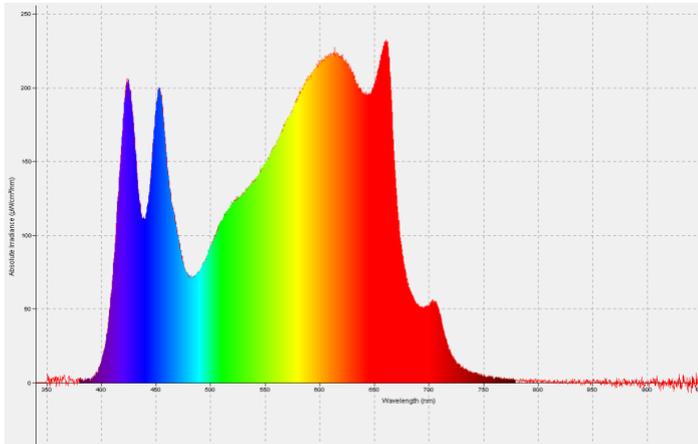
Other potential regulators have thus been identified. Screening for ozone-sensitive *Arabidopsis thaliana vtc* mutant led to the identification of AMR1 (Ascorbic Acid Mannose pathway Regulator 1), whose overexpression induces a decrease in ascorbate content

Samsung 100 W  
(HPE Green house)  
IRC warm white – 3000°K



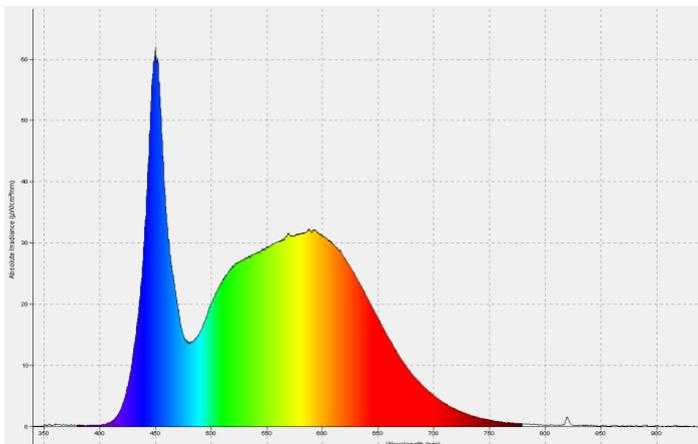
JAZ 50 ms  
Slot 10 µm  
Mesure at 105 cm  
15 sept 2014  
Li-Cor = 60 µmol/m²/s

LED Samsung SMD 28-35 100W  
(BF Green house)



2015 July  
Aspect pinky  
Int. Time = 50 ms  
Distance 30 cm

Tube LedPower 30 W  
(Room 3128)  
IRC cold white – 6500°K



JAZ 100 ms  
Mesure à 30 cm  
14 June 2013  
Li-Cor = 60 µmol/m²/s  
  
Test Lusignan  
50 µmol/m²/s

**Figure 17:** LED spectrum used in the greenhouses and the growth chamber.

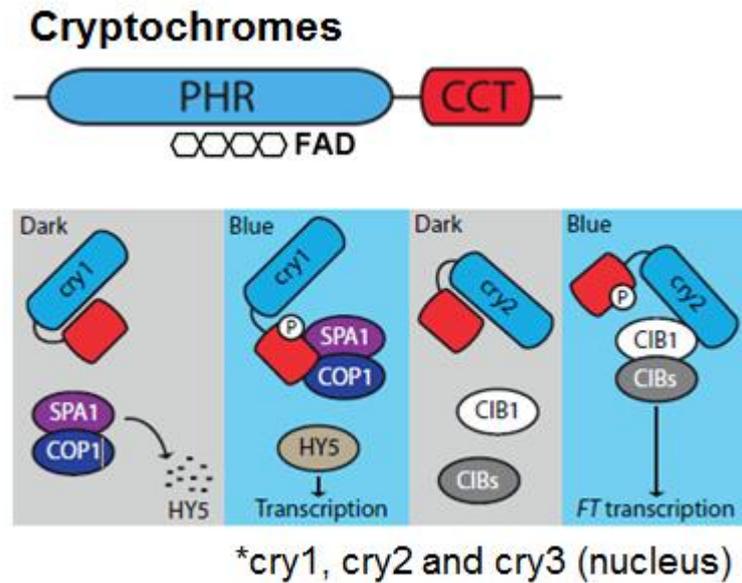
(Zhang *et al.*, 2009). In the *amr1* mutants, the majority of the genes involved in the main ascorbate biosynthetic pathway including GMP, GME, GGP, GPP, GalDH and GLDH, are overexpressed. This led to the conclusion that AMR1 acts as a repressor of the main pathway of ascorbate biosynthesis. This protein appears to be related to proteasome degradation complexes. Interestingly, the expression of the AMR1 gene would be controlled by environmental factors. It is positively correlated with foliar development and negatively with light intensity.

Still related to protein degradation, an interaction of another enzyme of the L-galactose pathway, namely the GDP-D-mannose pyrophosphorylase (GMP or VTC1 by analogy to the *Atvtc1* Arabidopsis mutant) with the CSN5B subunit of the COP9 signalosome has been shown to induce a decrease in ascorbate in the dark (Mach, 2013). Conversely, the loss of function of CSN5B causes an increase in the ascorbate pool. However, all the mechanisms linked to the CSN complex are known to act in the nucleus, and such a localization of VTC1 has not been yet demonstrated (Wang *et al.*, 2013).

Finally, InsP3 is a metabolite derived from the phospho-inositol pathway, which is involved in the response to a wide variety of stresses such as light, drought, cold or salinity (Khodakovskaya *et al.*, 2010; Alimohammadi *et al.*, 2012). Decreasing the amount of InsP3 in transgenic tomato lines alters light signalling, affecting many pathways responsible for the synthesis of secondary metabolites such as increasing the ascorbate pool. This suggests that InsP3 plays a negative regulatory role in photomorphogenic responses. Nevertheless, the specific pathways related to the involvement of InsP3 in the transduction of the light signal remain to be clarified.

## **i. Photo-Sensor Systems**

As described above, light provides energy for plant growth, but it is also one of the most important informational signal for plants. Indeed, plants collect information carried in the absorption of a photon to drive biological activity. The study of the molecular basis involved in this processes has grown a lot since the cloning of the CRY1 locus (Ahmad and Cashmore, 1993). Nowadays, advances in fundamental understanding of the mechanisms have led to develop new technic as optogenetics. This approach allows to control gene expression or pattern using light signals. Thus, plant have developed specialized photoreceptors that can mediate a response to changes in light intensity, quality, direction and duration. To date, five photosensory systems have been



**Figure 18:** Cryptochromes structure and function.

Domain structures: PHR, photolyase homology region; CCT, cryptochrome C-terminus.

Cryptochrome photo-activation and indirect regulation of transcription by cry1 (left). In darkness, HY5 is degraded by the action of the COP1–SPA1 complex. In blue light, cry1 alters its structure to bind to and sequester COP1–SPA1, resulting in the accumulation of HY5 and the promotion of gene expression. Direct regulation of transcription by cry2 (right). Upon photoactivation, cry2 binds to CIB1 and other CIB proteins via its PHR domain to promote flowering by inducing gene expression including FT (Adapted from Christie *et al.*, 2015).

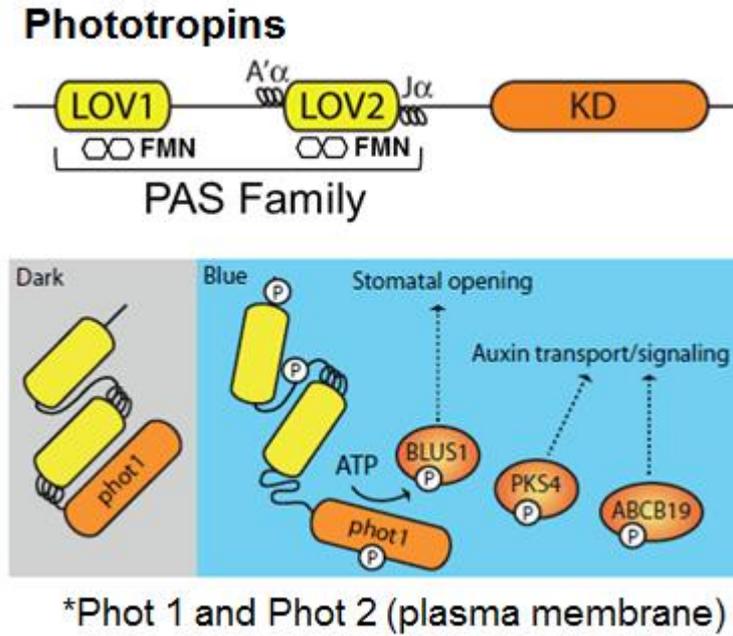
identified in higher plants. The phytochromes (phys) respond largely to red (600-700 nm) and far-red (700-750 nm) regions of the solar spectrum (Chen and Chory, 2012), whereas UV Resistance locus 8 (UVR8) monitors ultra-violet B (UV-B) wavelengths (280-315 nm) to regulate both developmental and UV-protective processes (Jenkins, 2014). Plant response to blue light (390-500 nm) is extensive and mediated by three different classes of photoreceptors: the cryptochromes (crys), the phototropins (phot) and the members of the Zeitlupe family (ztl, fkf1 and lkp2) (Chaves *et al.*, 2011; Christie, 2007; Suetsugu and Wada, 2013)

## **Cryptochromes**

Cryptochromes were the first plant blue-light receptors to be characterized at the molecular level (Ahmad and Cashmore, 1993). In addition to have a function in the photoperiodic control of flowering, they participate in the photomorphogenic development of plants, and they have a role in setting up the circadian clock (liu *et al.*, 2011). Three cryptochromes have been identified in Arabidopsis (cry1–cry3). Arabidopsis cry1 and cry2 are localized predominantly in the nucleus (Cashmore *et al.*, 1999; Kleiner *et al.*, 1999). Like other blue light-sensitive proteins, Arabidopsis cryptochromes capture the light signal by non-covalently binding a flavin chromophore, in this case FAD, within the photolyase homology region (PHR) (Hoang *et al.*, 2008). CRY proteins also contain a distinctive cryptochrome C-terminus (CCT) which triggers signalling. The sensing of the light signal by the PHR-FAD bond causes a conformational change in the CCT and thus allows the interaction with other signal components (Chaves *et al.*, 2011; Li *et al.*, 2011). By being located in the nucleus, cry1 and cry2 participate in the regulation of plant development, mainly by modulating transcription factor abundance and gene expression. Two mechanisms of transcriptional control have been well described for plant cryptochromes. The first mechanism consists in the inactivation of the Constitutive Photomorphogenic 1 (COP1)/Suppressor of phyA1 (SPA1) complex, which is responsible for the degradation of the bZIP Long Hypocotyl transcription factor 5 (HY5) in the dark (Osterlund *et al.*, 2000). This COP1–SPA1–cry1 interaction leads to an accumulation of HY5 and other factors, which, in turn, regulate the transcription of genes required for de-etiolation. A second similar mechanism involves cry 2 as a repressor of the degradation of Constans (CO), which is an essential transcriptional regulator of flowering (Zuo *et al.*, 2011).

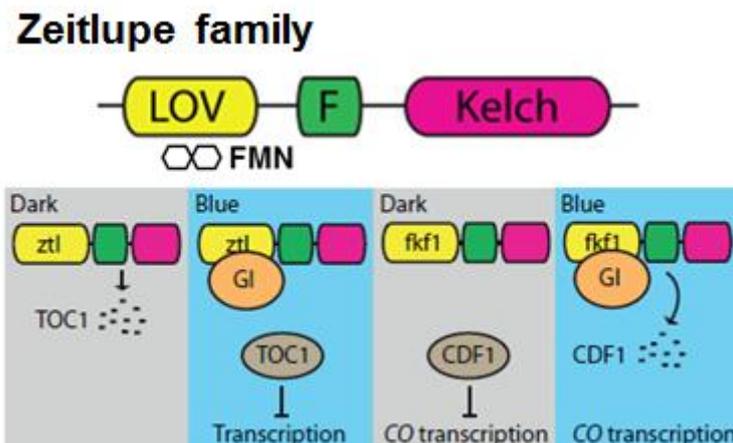
## **Phototropins**

Phototropin blue-light receptors were so named after the characterization of their role in mediating higher plant phototropism (Christie *et al.*, 1999). Both PHOT1 and PHOT2 are predominantly localized to the plasma membrane (Sakamoto and Briggs, 2002; Kong



**Figure 19:** Phototropins structure and function.

Illustration of the two LOV domains and the kinase domain (KD). Light induces autophosphorylation and substrate phosphorylation by KD domain. In the dark state, phot1 kinase activity is inhibited. Blue light drives a conformational change in the protein that results in autophosphorylation within the kinase domain, the linker region between the LOV domains and sequences upstream of LOV1. Concomitantly, phot1 can phosphorylate substrate targets, here are represented BLUS1, PKS4 and ABCB19. (Adapted from Christie *et al.*, 2015).



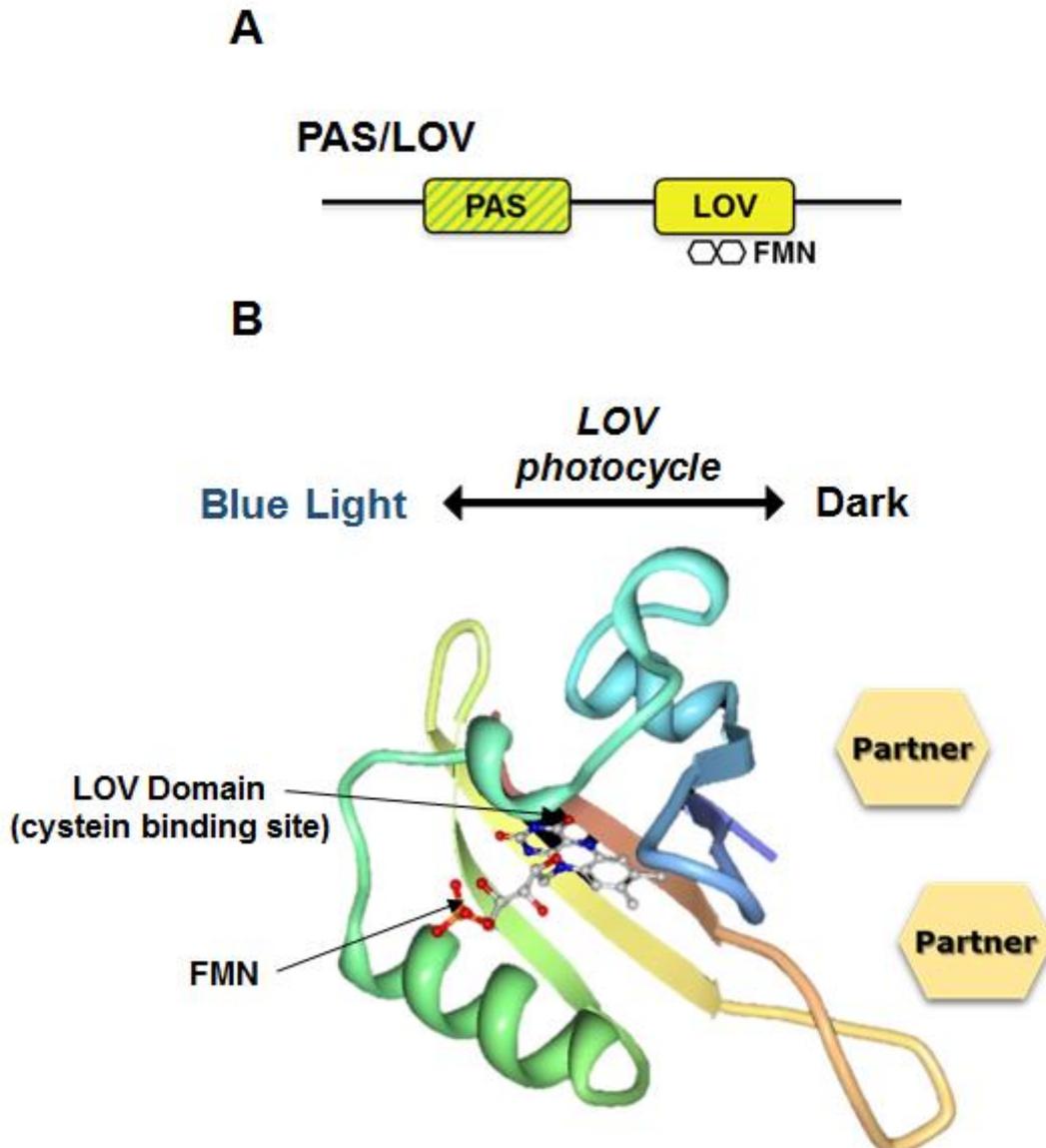
**Figure 20:** Zeitlupe family structure and functions

Domain structures: LOV, light voltage oxygen; F, F-box; Kelch, kelch repeats. Photo-activation of ztl and stabilization of TOC1 (left). In darkness, ztl functions to degrade TOC1. GI binding to ztl in blue light results in TOC1 accumulation and the transcriptional repression of circadian genes by inhibiting SCFztl activity. Blue-light-induced binding of GI to fkf1 (right) results in the degradation of the transcriptional repressor CDF1 to promote CO expression and flowering under long days. (Adapted from Christie *et al.*, 2015).

*et al.*, 2006), but are not integral membrane proteins. PHOT1 and PHOT2 display three specialized domains from N to C-terminus: a serine/threonine kinase domain and two LOV domains. Phototropins are the only blue-light receptor identified to date that contain two LOV domains. These LOV domains belong to the Per-ARNT-SIM (PAS) superfamily which is involved in the sensing of a wide range of environmental signals. Similarly to PHR domain of the cryptochromes, under illumination LOV domains can bind covalently a flavin mono nucleotide (FMN) as chromophore to a conserved cysteine residue in order to activate the signal (Salomon *et al.*, 2000; Swartz *et al.*, 2001). This photochemical reaction is fully reversible in darkness and is called the LOV photocycle. Phototropins are also light-activated serine/threonine kinases that undergo autophosphorylation in response to blue light irradiation.

### **Other Related Blue-Light Receptors**

The numerous studies of the blue-light receptor families led to the definition of several specific sub-families related to their function. Among them, there is the zeitlupe (ztl) family that, like cryptochromes, contributes to the regulation of the circadian cycle and the control of flowering (Takase *et al.*, 2011). In a similar manner to CRY1/2, the ZTL proteins act by interacting with different partners, controlling the degradation and stability of the compounds for which they are responsible. ZTL proteins localize to the cytosol or the nucleus (Takase *et al.*, 2011) and comprise three members: Zeitlupe (ztl), Flavin-binding kelch repeat F-box 1 (fkl1) and LOV kelch protein 2 (lkp2) (Ito *et al.*, 2012; Suetsugu and Wada, 2013). Each protein contains three domains: (i), a LOV domain which is able to bind oxidized FMN as chromophore in response to blue light (Imaizumi *et al.*, 2003); (ii), an F-box domain, involved in targeting proteins for degradation *via* the ubiquitin-proteasome system (Ito *et al.*, 2012); (iii), six kelch repeats at the C-terminus utilized to mediate protein-protein interactions and hetero-dimerization.



**Figure 21:** Illustration of the PAS/LOV protein and the LOV cycle.

**A** PAS/LOV protein sequence and the Flavin FMN co-factor binding at the LOV domain

**B** 3D-view of the LOV domain displaying the bound between the cysteine residue and the FMN. Blue light induces a conformational change leading to the recruitment of partners, darkness reverses the process.

## PASLOV Proteins

PASLOV proteins (PLP) have been described for a while but their function remained unknown. They are unique LOV-containing proteins and they are unrelated to the phototropins or the members of the ztl family (Kasahara *et al.*, 2010). The Arabidopsis *AtPLP* homolog has 3 isoforms (PLPA, PLPB, and PLPC) and was first described by Crosson *et al.* (2003) who named them PLP while in several studies authors defined them as PASLOV proteins according to their PAS and LOV domains. The LOV domains are members of the large and diverse superfamily of PAS domains (Christie *et al.*, 1999), so their peptide sequence are very homologous. At first glance, one may consider that the PASLOV proteins contain two LOV domains and thus could assimilate it to belong to the phototropin family. However, the PAS domains of Arabidopsis PLPs are distinguishable from a LOV domain because of the absence of the conserved cysteine residue involved in the covalent binding of the Flavin chromophore FMN inducing a conformational change of the protein and *in fine* its activity in the photochemical reaction. Like in other flavoproteins, in the case of the PASLOV proteins, blue light may trigger a change in the conformation of the unique LOV domain affecting the interaction of PLPs with PLP-interacting proteins. So far, no precise mechanism has been defined for the PAS domain. Because these PAVLOV proteins have never been associated with any physiological process, they were poorly studied in the recent years. Using a yeast two hybrid system on a bank of Arabidopsis cDNA, Ogura and co-workers (2008) described an interaction between AtVTC2, AtVTC5 and BLH10 (BEL1-like homeodomain 10 A and B proteins) with AtPLPA and AtPLPB. Additionally, it has been shown that these molecular interactions were significantly diminished under blue light but not under dark, green, red and far-red (Ogura *et al.*, 2008). Besides, in relation with light signalling studies, the activity of the promoter of the Soybean *GmPLP1* fused with GUS protein has been analysed under different light conditions (Luo *et al.*, 2013). This work showed that the GUS activity driven by the *GmPLP1* promoter was up-regulated under dark and blue light (Luo *et al.*, 2013). Still in soybean, *GmPLP1* has recently been presented as a negative regulator of hypocotyl elongation under circadian clock control and *GmPLP1* gene expression was shown to be induced in the dark (Li *et al.*, 2018). In addition, overexpression of *GmPLP1* gene affected the expression of other genes related to the CRY1 signalling pathway. Future advances in the characterization of PASLOV will be necessary to identify the molecular basis underlying these phenotypic observations.



## OBJECTIVES OF THE THESIS WORK

Since a decade, it is no longer possible to count the number of publications presenting ascorbic acid (vitamin C, AsA) as essential in human health and plant development. Undeniably, plant-derived ascorbic acid has a dual function both as a major source of vitamin C in the human diet, and as a central element in the development and protection of plants. Therefore, the understanding of the molecular mechanisms involved in the metabolism of ascorbate and its regulation, is an economic and agronomic issue in the improvement of the nutritional and post-harvest quality traits of the plant products, especially the fruits. Although ascorbate is well documented for its antioxidant capacities and its central role in the redox status of the plants, the regulation of the main actors of its biosynthesis pathway is relatively unknown. The characterization of the major pathway of ascorbate synthesis, namely L-galactose pathway (Smirnoff, 1996; Wheeler *et al.*, 1998), has obviously been a necessary basis for all researches aiming to deepen the mechanism of its homeostasis in higher plants. To our knowledge, all eukaryote organisms need ascorbic acid for living. To demonstrate this fundamental role of ascorbic acid in plants, in the double *Atvtc2/vtc5* Arabidopsis mutant, the seedlings rapidly stopped to develop after germination and needed to be rescued by addition of exogenous ascorbate in the germination medium (Dowdle *et al.*, 2007).

In plants, ascorbic acid has a particular role in response to change of environmental stimuli (light, temperature, drought, etc.). In that sense, plants need to be able to rapidly manage the ascorbate pool for an effective antioxidant response. Many studies have been interested in the relationship between stress response and ascorbic acid metabolism to identify the regulatory mechanisms involved. Several works showed that environmental stresses could trigger a modulation of ascorbate content through transcriptional or post-transcriptional regulations of genes of the L-galactose pathway. As an example, drought or salt stress highlighted two transcription factors, EsWax1 and ERF98, which regulate the expression of two ascorbate-related genes, namely *VTC1* and *VTC2* genes by interacting with their promoters (Zhu *et al.*, 2014; Zhang *et al.*, 2012). Among the environmental factors that regulate the accumulation of ascorbate in plants, light has a particular place as it has been the subject of many studies. Thus, in *Arabidopsis thaliana* leaves, high-light conditions induced an increase in ascorbate concentration by enhancing the *VTC2* expression and activity (Dowdle *et al.*, 2007).

Despite all these works on the identification of regulatory mechanisms, it is still puzzling to clearly establish whether ascorbate variations are the origin or the result of observable physiological events.

The main objective of my PhD work was to continue the study of the regulatory processes that modulate AsA content in tomato, in order to get closer to a description of the physiological role of ascorbate in plants, especially in fruits.

Based on the knowledge of the enzymes involved, and the corresponding genes, many studies have attempted to elucidate the link between ascorbate and the environmental and physiological stimuli that the plants face. These events can be either external signals (or assimilated to stress) or signal integrated into molecular processes related to the plant development, hence emphasizing the multifaceted physiological roles of ascorbic acid. It is within this framework that my thesis work is inserted in the Fruit Biology and Pathology laboratory (UMR 1332 INRA-Bordeaux) whose research project deals with the study of the development and composition of fleshy fruit, and the functional analysis of genes controlling these traits. To assess this question of the physiological role of ascorbic acid associated with these traits, more than a decade ago a reverse genetic strategy was set up in order to generate ascorbate-deficient tomato mutants. Initially, an RNA interference (RNAi) transgenic approach targeting key genes of ascorbate metabolism allowed to generate several RNAi lines of L-galactose pathway genes (*GMP*, *GME* and *GLDH*) and the recycling (*AO*, *DHAR*). The study of the galactono-1,4-lactone dehydrogenase (*GLDH*) revealed the link between the AsA biosynthesis and the mitochondrial function as well as its impact on plant and fruits development (Alhaghdow *et al.*, 2007). In addition, this reverse approach permitted to enrich the knowledge on the function of the GDP-D-mannose-3,5-epimerase (*GME*), a metabolic crossroad between AsA and cell wall biosynthesis pathway, both essential for the plant development and fruit quality (Gilbert *et al.*, 2009 and 2016). In a second time, a TILLING strategy was applied on targeted AsA-related genes to rapidly identify allelic series in tomato. This was possible thanks to the use of a highly mutagenized EMS MicroTom collection (Just *et al.*, 2013) created at the INRA laboratory and also in collaboration with colleagues in Japan (Pr Ezura group at Tsukuba University). Several AsA-deficient mutants were identified for *GMP*, *GME*, and *GGP* (Baldet *et al.*, 2013 and unpublished data). At that time, the tomato genome was released and allowed to identify all the genes of the L-galactose existing in Tomato. This study revealed that the two *GGP* proteins are not totally and functionally redundant since the KO of *SIGGP1* was not fully complemented by *SIGGP2*, which is about 100 fold less expressed than its counterpart in Tomato wild-type (Massot *et al.*, 2012). The development of the EMS mutant collection, finally allowed to undertake a forward genetic approach, in the

quest of new regulatory proteins controlling fruit quality traits. Indeed, the screening of this mutant population for a trait of interest, combined with a mapping by sequencing approach have made possible to reveal new genes underlying phenotypic variations (Garcia *et al.*, 2016). Prior to my PhD, this forward genetic strategy has been carried out in the frame of the study of the regulation of the ascorbate metabolism in tomato fruits. A tremendous phenotyping work was set up to screen for high fruit AsA content in 500 families of the EMS Micro-Tom tomato mutant population. Five mutant families which display a significant ascorbate content 2 to 5 times higher than the WT fruits were identified, providing a precious material for further analyses (Celine Bournonville, thesis 2015). The identification of the causal mutation was a success story for one of them, named P21H6 mutant.

My PhD thesis presented here is divided in the three chapters. The first chapter is about the functional characterization of the gene (*Solyc05g007020*) underlying the AsA-enriched phenotype identified in the P21H6 mutant family. This part is presented as a project for publication that we wish to submit shortly. It presents the identification of the mutated gene and the beginning of its functional characterization in connection with the metabolism of AsA regulation. The protein responsible of the AsA-enriched fruit phenotype is defined as a negative regulator. In this case, the protein is a PASLOV protein (PLP) that belongs to a new class of photoreceptor poorly studied. This *S/PLP* contains one LOV domain which is known to be involved in the blue light signaling. The role of PLP as negative regulator of AsA accumulation in tomato plant was thereafter confirmed using a CRISPR directed mutagenesis strategy. As suggested by previous works, we showed that *S/PLP* interact with *S/GGP* (GDP-L-galactose phosphorylase), the well-admitted key enzyme of the L-galactose pathway. This interaction is regulated by blue light and takes place in the cytoplasm and the nucleus. Our results strengthen the central role of GGP in the AsA biosynthesis and suggest a new regulation mechanism by blue light of the GGP function in addition to its metabolic activity.

The second part of the PhD is also presented as a project of publication but in a less advanced manner. This chapter presents the identification and the preliminary characterization of the second AsA-enriched Tomato mutant, named P17C5. This mutant displayed the highest increase of fruit AsA level (5 to 10 times the WT). Besides this remarkable AsA phenotype, the original P17C5 mutant appeared difficult to cultivate and study as it displayed a strong parthenocarpic-like phenotype (seedless fruits) making difficult the generation of a segregant population necessary for further mapping. Luckily, the mutated locus was dominant making possible the transfer to another Tomato cultivar, namely M82, and thanks to this crossing, the causal mutation was identified. It corresponded to a mutation in a cis-acting open reading frame (uORF) upstream of the

*S/GGP1* gene. This result emphasizes the role played by GGP in the regulation of the L-galactose pathway. Preliminary results related to the parthenocarpic-like phenotype suggest a problem of male sterility associated with pollen development processes. The regulation of the L-galactose pathway dealing with a higher AsA level, and its possible link with the processes involved in seed and pollen development, opens a new path in the understanding of the various roles of ascorbate in the cell metabolism.

At last, the third chapter of the manuscript deals with preliminary results in the study of impact of ascorbate on the post-harvest fruit quality. In this framework, chilling stress experiments were carried out using the fruits from the P21H6 mutant. Several parameters of the fruit quality were analysed, they tend to demonstrate that an increase of the fruit AsA content improve the fruit shelf life and its maturation capacity.

Taken as a whole, our results confirmed definitively the master position of the GGP protein in the L-galactose biosynthesis pathway. In addition, the two AsA-enriched mutants characterized in this PhD work are precious plant material for deciphering the role of GGP in the regulation of this vital antioxidant existing in all living organisms (except in bacteria) and in an agronomic point of view for studying the impact of ascorbate on the establishment of the fruit Quality especially related to post-harvest traits.



# RESULTS

# **PART 1: A BLIND REGULATOR**

## **THE MASTER ENZYME GGP REGULATES ASCORBIC ACID CONTENT THROUGH ITS INTERACTION WITH A NEW CLASS OF PHOTORECEPTOR IN TOMATO.**

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### **Introduction**

Ascorbic acid (AsA) is one of the most important biochemical in living organisms. It has a leading role in antioxidant defences, in response to biotic and abiotic stresses, by participating in the elimination of ROS (Reactive Oxygen Species) produced in such conditions. Ascorbic acid plays also a crucial role in the control of the ROS level produced during a part of cell metabolism like photosynthesis in plants. Due to its high antioxidant potential, ascorbic acid is one of the most important trait for the nutritional quality of the fruits and vegetables. Indeed, in humans and few animals evolution has led to the loss of the L-gulonolactone oxidase activity which catalyses the last steps of the biosynthetic pathway (Burns JJ, 1957). Consequently, humans are unable to synthesize ascorbic acid, thus defined as vitamin C, and must have a daily intake through the consumption of fruits and vegetables. However, ascorbic acid content is a critical fruit quality trait that has been lost or somehow lowered in many cultivated varieties of fleshy fruits. Understanding the regulation of ascorbic acid accumulation and its metabolism in order to improve crop species of agronomical interest is an important issue in plant breeding for many fleshy fruit species including tomato. Tomato stands for the model in fleshy fruit development and quality, it is also a major crop plant species in which vitamin C makes a major contribution to its nutritional quality.

Ascorbic acid metabolism in plants was widely described since the establishment of the Smirnoff-Wheeler pathway (also called L-galactose pathway), but very little is known about the regulatory mechanisms (Wheeler *et al.*, 1998) involved. The GGP protein, also known as VTC2 by analogy in *Arabidopsis*, corresponds to a GDP-L-galactose phosphorylase (Linster and Clarke, 2008), and is so far considered as the controlling enzyme of the L-galactose pathway (Bulley *et al.*, 2009; Li *et al.*, 2013 and 2014). The GGP enzyme supports the first committed step in ascorbate biosynthesis in plants, converting GDP-L-galactose to L-galactose-1-phosphosphate. The *Arabidopsis* knock-out *vtc2* mutants display a drastic decrease in ascorbic acid though a residual amount of ascorbic acid is still produced (Dowdle *et al.*, 2007). That makes sense with the presence in *Arabidopsis* of another gene encoding a GDP-L-galactose phosphorylase, namely *AtVTC5*. The *AtVTC5* gene has a high sequence homology (~66% identical) with its counterpart *AtVTC2* but it is 100 to 1000 less expressed. Other studies have hypothesized the existence of additional alternative pathways (Wheeler *et al.*, 2015). Among them, only the galacturonate and myo-inositol pathways are relevant. However, these alternative routes have not been completely demonstrated and some results tend to invalidate these assumptions. Indeed, the *Arabidopsis vtc2/vtc5* double mutant is unable to grow without addition of exogenous ascorbate (Dowdle *et al.*, 2007). This suggests that there would be no other way than the L-Galactose pathway to complement the ascorbate deficiency. The VTC2 enzyme is the only one to have a significant effect on AsA accumulation when it is overexpressed, although GME acts synergistically with it to increase ascorbate in leaves (Bulley *et al.*, 2009 and 2012). Nothing is really known about the precise regulation of VTC2, with exception of the recent works of Laing and co-workers that described the activity of a uORF (upstream Open Reading Frame) in the 5'-UTR of the *VTC2* gene. These authors demonstrated that this uORF could regulate the AsA biosynthesis by controlling the level of translation of the VTC2 protein. Hence, this work showed that in the presence of high ascorbate concentration the VTC2 protein was downregulated (Laing *et al.*, 2015). At the cellular level, a fluorescent fusion protein approach has emphasized that the *AtVTC2* protein was localized in both cytoplasmic and nuclear compartment (Müller-Moulé, 2008). This unexpected nuclear localization for a metabolic enzyme suggests that GGP might also act as a dual-function protein: a regulatory factor as well as a catalytic enzyme.

The accumulation of ascorbic acid is highly dependent on environmental conditions, like stresses induced by salt, drought, and particularly by high light conditions. Despite this, the knowledge of the regulation still remain limited. The existence of such regulators has been recently demonstrated with the discovery of few proteins acting at transcriptional or post-transcriptional level on the regulation of some genes and enzyme of the L-galactose pathway. These studies mainly carried out in the *Arabidopsis* leaf led to the identification of AMR1, ERF98 and CNS5B proteins as positive or negative regulators. However, for some

of these effectors the underlying mechanisms remain to be determined (Zhang and Huang, 2010; Zhang *et al.*, 2012; Alimohammadi *et al.*, 2012). Regarding the effect of light on plant development, previous works showed that light might affect directly or indirectly the expression of AsA-related genes of the L-galactose pathway. For example in Arabidopsis, darkness conditions induced the degradation of the GDP-D-mannose pyrophosphorylase (VTC1) by the CSN5B-Cop9 complex associated with the proteasome (Wang *et al.*, 2013). Likewise, the AMR1/SCF complex has been shown to modulate the expression of the whole AsA-related genes of the L-galactose pathway through an unknown mechanism (Zhang *et al.*, 2009). Interestingly, prolonged exposure to light increased the expression of *GGP* the key gene of the biosynthetic pathway (Dowdle *et al.*, 2007). Additionally, some studies have shown that *GGP* is clearly regulated by light, thus suggesting a circadian regulation (Tabata *et al.*, 2002; Dowdle *et al.*, 2007; Müller-Moulé, 2008). All these studies demonstrate the obvious link between AsA metabolism and light signalling, but up to date there is still no clue that the light directly induces ascorbate biosynthesis or whether the over-expression or activation of *GGP* should be a consequence of an oxidative stress related to light exposure.

Thus, accumulation of ascorbic acid is closely associated with light quality and quantity along the day and night. Indeed, it is well-established that there is a positive correlation between the light intensity and the accumulation of AsA in photosynthetic tissues (Córdoba-Pedregosa *et al.*, 2003; Gautier *et al.*, 2008; Bartoli *et al.*, 2009). In that sense, light seems to affect AsA content in tomato more in the leaves than in the fruits (Massot *et al.*, 2012). Furthermore, Gautier *et al.*, (2009) demonstrated that the fruit AsA content was not limited by leaf photosynthesis but was dependent on direct fruit irradiance. As previously described, the availability of the reduced ascorbate pool is of great importance in the antioxidant response to the accumulation of ROS under stress. Light is largely responsible for this accumulation, as it is the case during photosynthesis. Surprisingly, the literature is poor regarding the molecular mechanisms relating ascorbic acid and photo-sensory systems. The photoreceptor proteins which collect the light signal during the absorption of a photon to drive and govern biological activity are classified according to their wavelengths detection, and the physiological processes involved. Phytochromes (red and far-red) and UVR8 (UVB) play a crucial role in plant development as well as in UV protection. The largest family includes the blue light photoreceptors, which are involved in the circadian rhythm and the phototropism through protein and gene expression modifications. These blue light sensors are all flavoproteins, requiring a flavin (FMN or FAD) as co-factor, to be activated in addition to harbor an effector domain. Most of them are well characterized such as the phototropins as well as the proteins involved in the circadian clock (Christie *et al.*, 2015; Crosson *et al.*, 2003; Briggs, 2001). Recent reviews mentioned another type of photoreceptor proteins containing a unique LOV (Light

Oxygen Voltage) domain, named PLP (PAS-LOV Protein), and whose biological function remains to be established. It has been demonstrated that the LOV domain of the photoreceptor can change its conformation when exposed to blue light and this modification is reversible in the absence of blue light signal (Kasahara *et al.*, 2010). However, Li and co-workers (2018) showed that the GmPLP1 expression can be triggered by darkness and red light. Interestingly, a yeast two hybrid screening performed with a cDNA bank from Arabidopsis leaf allowed to the identification of AtGGP1 (VTC2) and AtGGP2 (VTC5) as being potential PLP-interacting proteins (Ogura *et al.*, 2008). In their study carried out in soybean Li and co-workers observed some phenotypic alterations in the *Gmplp* mutants, particularly in term of the growth of the hypocotyls. Nevertheless, no hypothesis was proposed to explain a putative function of the GmPLP protein in relation with such developmental processes (Li *et al.*, 2018).

In our laboratory, an EMS population of mutants have been created in the MicroTom tomato cultivar. This artificially generated mutants exhibited a genetic and phenotypic variability far beyond the natural variation found in domesticated species (Just *et al.*, 2013; Garcia *et al.*, 2016). Hence, it represented an original plant material to go further in the identification of allelic series of already known genes. In a forward genetic strategy, the screening of this population for a trait of interest, combined with a mapping by sequencing approach has succeeded to identify new genes underlying phenotypic variations related to fruit colour (Garcia *et al.*, 2016). In the present study related to ascorbic acid metabolism, 500 tomato mutant families from this EMS Micro-Tom tomato mutant collection were screened for high ascorbate fruit content. This tremendous phenotyping work on more than 6000 plants, resulted in the finding of five mutant families displaying a significant increase in fruit ascorbate content from 2 to 5 times higher than the WT fruits. Here is presented the strategy, the identification of the causal mutation and the characterization for one of the five mutant families and the functional characterisation of the corresponding protein. This P21H6 mutant family displayed an enrichment of the ascorbate content in the fruits of 3 to 4 times that of the WT fruits. Besides, the P21H6 mutant family displayed no obvious phenotypic alteration at the level of the vegetative and reproductive parts of the plant. The identification of the mutation responsible for the ascorbate enriched phenotype let to identify a new regulator of the L-Galactose pathway. The functional characterization of the protein reveals a direct interaction with the key enzyme of the L-galactose pathway, namely the GDP-L-galactose phosphorylase (SIGGP) to modulate the ascorbate content in the whole plant. These results confirm the central role played by the GGP, both at the metabolic level and in regulatory processes.

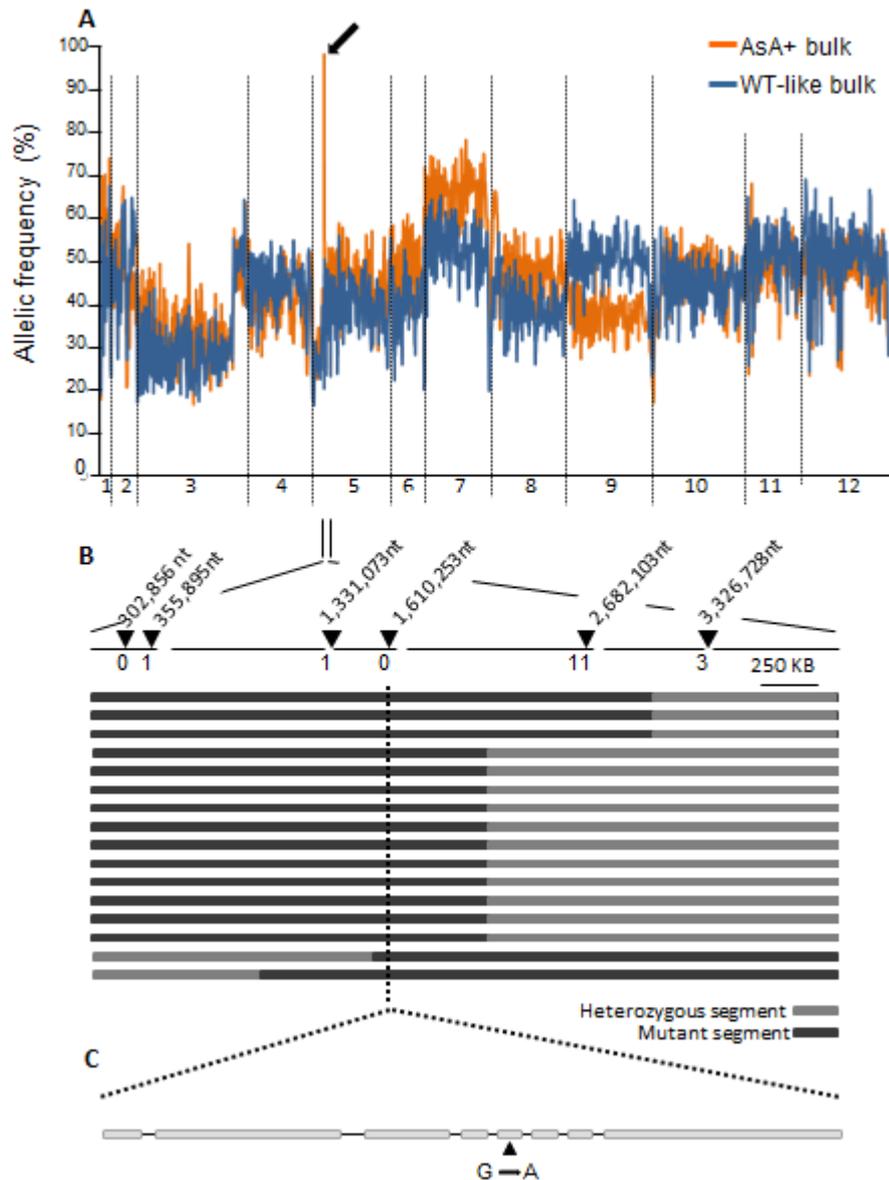
## Results

### **Detection of AsA-enriched mutant in an EMS MicroTom Tomato population.**

A forward genetic approach was performed to discover new regulator of the ascorbate metabolism in plants, more precisely regarding the level of vitamin C content in fruits. In that aim, the EMS (Ethyl Methane Sulfonate) mutant collection generated in the MicroTom Tomato cultivar at INRA Bordeaux (Just *et al.*, 2013) was screened to find mutants producing AsA-enriched (also called AsA+) fruits.

To discover mutants displaying a modification of AsA content within fruits, 500 M2 and M3 mutant families were cultured in green house. In order to minimize any environmental effects, the plants were grown by batch of 100 families (total 1200 plants) at the same place in the green house leading to successive cultivation for the whole primary screening that lasted 9 months. For each family, 12 plants were sown to get the chance to observe expressed recessive traits. The whole culture represented more than 6000 plants including the WT control plants. On each plant, at least 4 fruits at the red ripe stage were pooled and assayed for AsA content. The range of AsA content in the mutants screened varied from 0.5 to 4  $\mu\text{moles.g}^{-1}$  FW (Supplemental Fig.1A). The WT mean value is about 1 to 1.3  $\mu\text{moles.g}^{-1}$  FW depending on the period of the year when fruits were harvested, e.g. summer and autumn when growth conditions were favourable or winter when conditions were less adequate. Such variations in AsA content were expected as it is well known that plant AsA content is regulated by environmental factors, like light irradiance (Gatzek *et al.*, 2002; Gautier *et al.*, 2008; Bartoli *et al.*, 2009).

An ascorbate threshold value for selecting AsA+ mutants was set at 2  $\mu\text{moles.g}^{-1}$  FW *i.e.* twice the value of the WT resulting in the selection of 93 families (193 plants in total). These selected plants were cut to allow a new growth and they were used in a second screening for confirmation of the "AsA+" phenotyping. Following this second screening process, 5 families with increased fruit AsA contents comprised between 3 to 5 times that of the WT fruits were selected (Supplemental Fig 1B). We present here the characterization of one of these mutants, named P21H6. For this family, only one plant over 12 sown displayed AsA+ phenotype. Interestingly, AsA content was 2.5  $\mu\text{moles.g}^{-1}$  FW at the first screening at the end of the autumn period, whereas after the confirmation performed during the following spring season, the AsA content increased up to only 4  $\mu\text{moles.g}^{-1}$  FW, hence demonstrating the impact of light environment changes in the green



**Figure 1. Identification of the *plp* mutation responsible for the Asa+ enriched fruit phenotype by Mapping-by-sequencing.**

**A. Identification of the chromosome associated with the Asa+ phenotype.** Pattern of the mutation allelic frequencies obtained in the mutant and WT-like bulks are represented along tomato chromosomes by black and grey lines, respectively. The plot represents allelic frequencies (y axis) against genome positions (x axis). A sliding window of 5 SNPs. The x axis displays the 12 tomato chromosomes, the black arrow indicate the peak of allelic frequency (AF) of the chromosome 5 region carrying the putative causal mutations, since it displayed an AF > 0.95 (orange line) in the AsA+ bulk and an AF < 0.4 (grey line) in the wild-type-like bulk.

**B. Fine mapping of the causal mutation using the BC1F2 population.** Recombinant analysis of 44 BC1F2 individuals displaying the Asa+ phenotype allowed us to locate the causal mutation at position 1,610,253 nucleotides. Marker positions are indicated by black triangles. Number of recombinants are shown below the position of the markers. Chromosomal constitution of the recombinants are represented by black and grey bars, for mutant and heterozygous segment respectively.

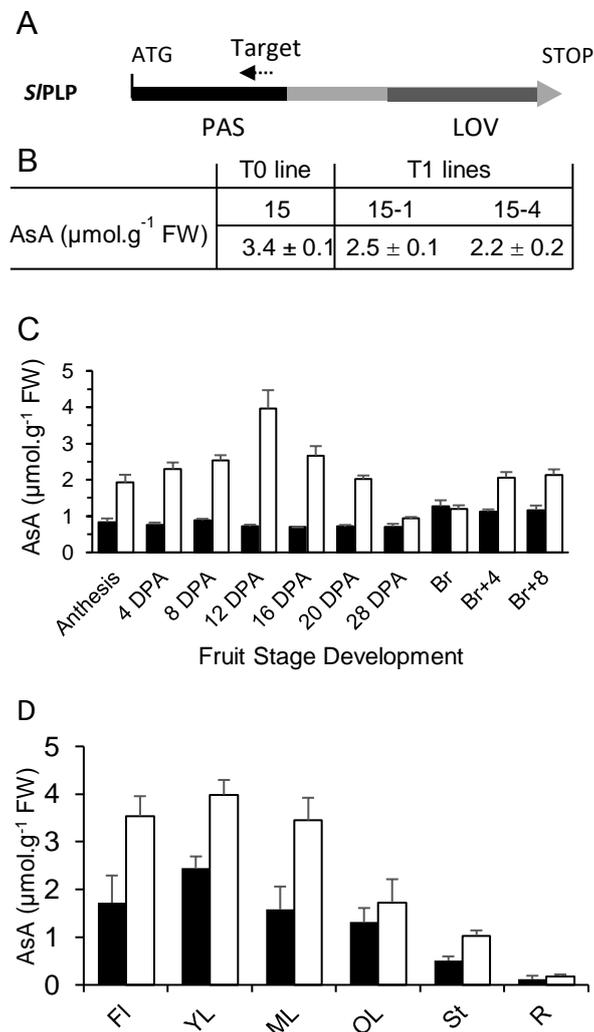
**C.** A single nucleotide transversion, G to A at position 1,610,253 in the Solyc05g007020 fifth exon sequence led to a STOP codon.

house. The P21H6 mutant was chosen as there was no alteration in the phenotype of the vegetative and reproductive organs (Supplemental Fig.1C).

### **Identification of the causal mutation of the AsA-enriched P21H6 mutant.**

In order to identify the mutated locus responsible for the AsA+ phenotype, the strategy was (i) the determination of the genetic inheritance features of the mutation using classical Mendelian genetic; and (ii) the identification of the causal mutation using mapping by sequencing strategy as described by Petit *et al.* (2016). In the P21H6-3 mutant, the AsA+ phenotype was analysed in the progeny after self-pollination (S1). For 12 S1 plants assayed, all produced AsA enriched fruits whereas fruits from plants of the back-cross (BC1F1) displayed a WT-like AsA content phenotype (Supplemental Fig.2A). The resulting BC1F2 segregant population consisting of 441 was analysed for the AsA-enriched phenotype (Supplemental Fig.2B). This analysis showed that 115 plants were defined as "AsA+" and 326 as "WT-like", thus confirming a Mendelian 1:2:1 segregation and involving a single recessive mutation.

From the BC1F2 population, two bulked pools of plants displaying either AsA+ or WT-like fruit phenotypes were generated, each of which contained 44 individuals (Supplemental Fig.2A). Pooled genomic DNA from each bulk was then sequenced to a tomato genome coverage depth of 39X, the trimmed sequences were mapped onto the tomato reference genome, and EMS mutation variants were filtered to exclude natural polymorphisms found in cv Micro-Tom (Kobayashi *et al.*, 2014) compared with the cv Heinz 1706 reference genome (Supplemental Table 1 and 2). Analysis of the allelic variant frequencies in the two bulks led to the identification of chromosome 5 as the genome region carrying the causal mutation, since it displayed high mutant AFs ( $AF > 0.95$ ) in the AsA+ bulk and much lower frequencies ( $AF < 0.4$ ) in the wild-type-like bulk (Fig.1A). Analysis of the putative effects of the mutations on protein functionality highlighted 2 genes carrying mutations in exons. Among these, one affected an "unknown protein", while the second affected a predicted PLP (*SIPLP*; *Solyc05g007020*) that was located at the top of the SNPs plot according to AF analysis. Given the previous results, underlying the link between PLP and the key enzyme GGP1, we considered the latter one to be the most likely candidate. To unequivocally associate the *p/p* mutation with the AsA+ trait, and to exclude any other mutated locus recombinant plants selected from the BC1F2 progeny were used (Fig.1B). To this end, we used the EMS-induced SNPs surrounding the *SIPLP* gene as genetic markers. The analysis clearly indicated that a single G to A nucleotide transversion occurred in the fifth exon corresponding to the LOV domain of the *SIPLP* (Fig.1C). This



**Figure 2: AsA content in developing fruits and several tomato plant organs from *S/PLP* mutant and WT plants.**

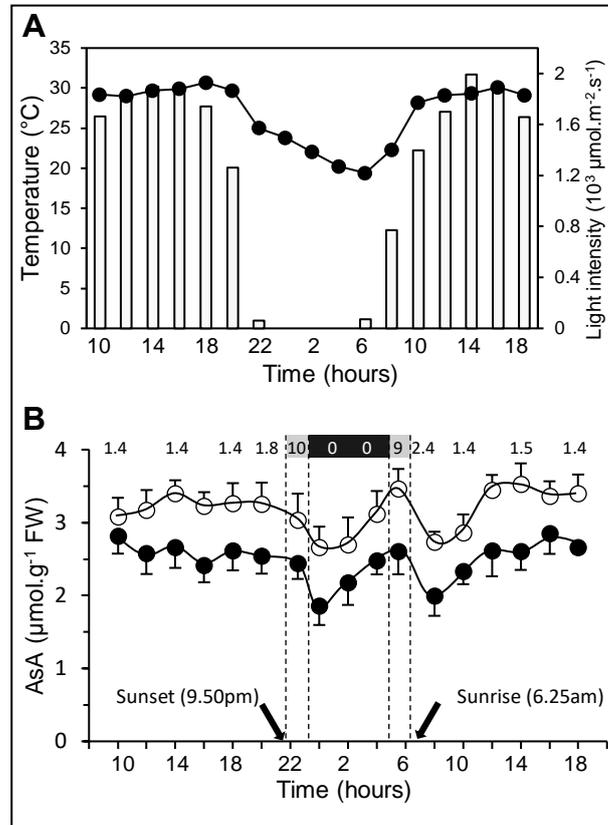
**A.** *S/PLP* is illustrated by the PAS and LOV domains. The sequence (dashed arrow) in the PAS domain was the target sequence for the CRISPR-Cas9 construct. **B.** AsA was assayed in red ripe fruits ( $n=4$ ) from T0 line 15 and the progeny T1 lines 15-1 and 15-4. **C.** In the course of fruit development from anthesis up to ripening from breaker (Br) to red ripe stage (Br+8), **D.** Fully-opened flowers (FI), and several vegetative organs including leaves at 3 stage of development, young leaf (YL), mature leaf (ML), old leaf (OL), stem (St) and roots (R) from one month-old plants were assayed for the AsA content. Data are the means  $\pm$  SD of a total three individual plants per organs and three organs per plant, except for anthesis ( $n=100$ ) and 4DPA ( $n=20$ ) for which between 10-20 plants were used to get enough material from 15-5 line and control.

nucleotide change resulted to the knocking-out of the protein with the appearance of a STOP codon instead of glutamine codon and was responsible for the AsA<sup>+</sup> fruit phenotype.

The functional validation of the SIPLP as being a negative regulator of the AsA biosynthesis in tomato plants was performed through a CRISPR-Cas9 strategy to generate *plp* mutant lines in the MicroTom Tomato. Within the large family of photoreceptor proteins harbouring a LOV domain, this domain displays a very high level of sequence homology. Consequently, the choice for the CRISPR construct was to target instead the PAS domain to produce KO mutations in the 5' terminal region of the *SIPLP* gene (Fig.2A). Several T0 lines were generated and mature fruits were analysed for the AsA content. Five T0 lines displaying at least a 2-fold increase of AsA were selected to continue to the next T1 generation (Supplemental Table 3). The sequencing of the *SIPLP* gene for several T1 plants of lines 6, 15, 17 and 21 revealed that the CRISPR-Cas9 had induced various deletions which can be from 1 to 8 nucleotides (Supplemental Fig.3). All of these deletions resulted to a shift of the open reading frame and the appearance of a STOP codon in the downstream coding sequence of the gene. For further studies, homozygous T2 plants of the lines 15-1 and 15-5 were used indifferently as they harboured the same mutation (Fig. 2B; Supplemental Fig. 3).

### **PLP is a repressor of the ascorbate accumulation in tomato plants**

As mentioned before, no obvious morphological and physiological change was observed at the whole plant level in the mutant compared to the WT. To characterize the consequences of the knocking out of the *SIPLP* gene on the AsA metabolism, AsA assays were carried out in the time course of fruit development as well as in all the tomato plant organs. As shown in Fig. 2C, all along its growth, fruits of the *PLP* mutant accumulates more AsA than the WT, with a peak at 12DPA corresponding to the end of the active cell division phase and the transition to the cell expansion phase. Interestingly, the difference of AsA content between mutant and WT declined to become negligible at the beginning of the maturation phase (Breaker stage) and to raise onwards during the maturation. In the same manner, all vegetative and reproductive organs of the mutant accumulate more AsA than the WT (Fig. 2D). As being a photosensing regulator and in order to assess the question of the temporality of the action of the SIPLP protein, the daily variations of AsA content were investigated. For that experience, one month-old tomato plants cultivated in the green house during the most favourable light conditions (between May and August 2018) were placed outside at night fall and maintained accordingly for about 32h. Every two hours, physical parameters (PAR, light spectrum, temperature, hygrometry) were



**Figure 3. AsA change in *Slpp* mutant and WT plants.**

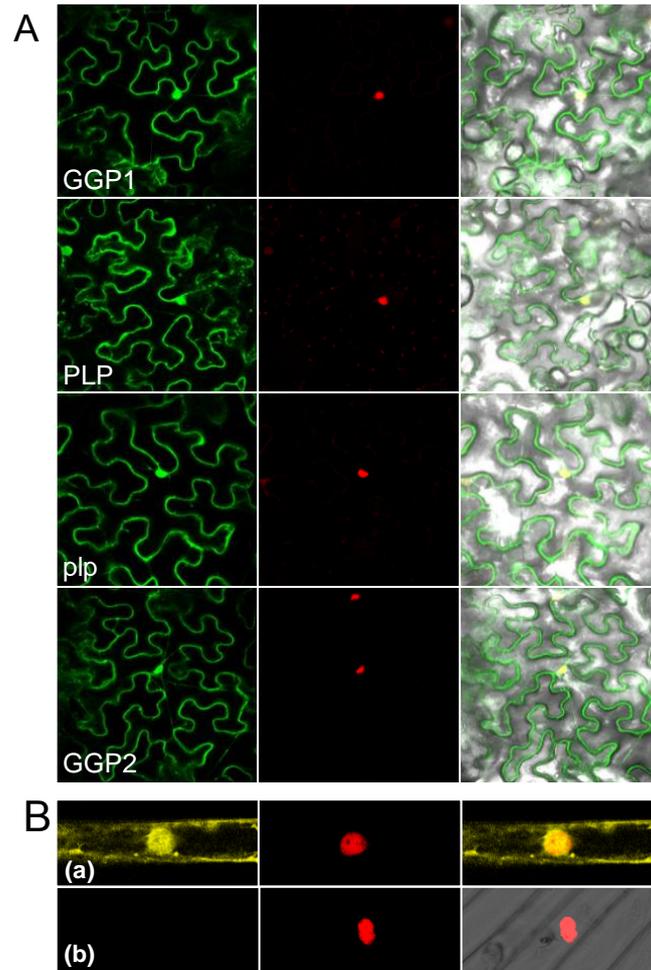
*Slpp* mutant (T2 line 15-5) and WT plants (n=51 per type) have been cultured in the green house for 1 month in June 2018. During July 9<sup>th</sup> night, all the plants were removed outside of the green house and maintained under natural light condition during 32 hours. In the time course of the day and night, physical measurements were recorded (**A**) and mature leaves were harvested for AsA assay (**B**). Data are the means  $\pm$  SD of a total three leaves from three individual plants from 15-5 line (empty circles) and control (full circles). Above the figure are illustrated the two blue-hour periods before sunrise and after sunset (grey and dashed line), the night (black) and the day (white) periods as well as the values of the blue light (430-480nm)/red light (630-680nm) ratio. Hours are expressed as local hour time.

recorded, and plant materials harvested. A special attention was paid for the period after sunset and before sunrise, known as the blue hour period, and that lasted about 1 hour around 6am and 10pm on July 10th 2018. This is consistent with the presence of a LOV domain, defined as a blue light photoreceptor domain, in the SIPLP. As illustrated in Fig. 3, while the level of AsA was always higher in the mutant, the variation in AsA content followed the same pattern in the T2 line 15-5 mutant and the WT plants.

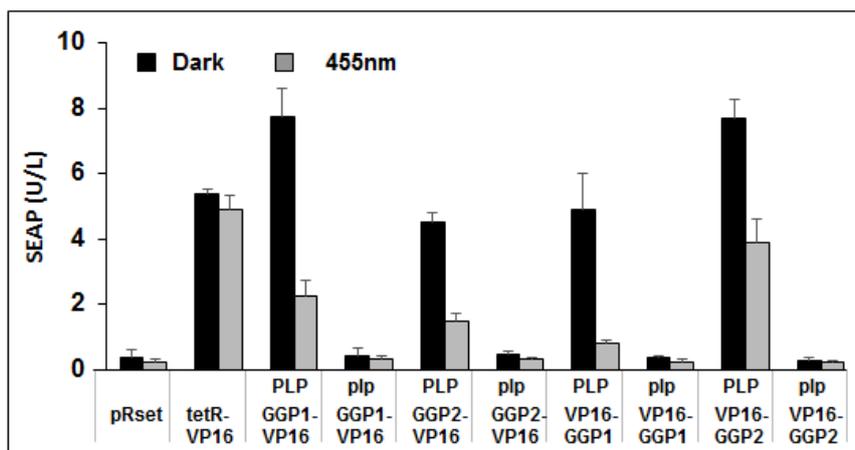
### **The SIPLP interacts with SIGGP in the cytosol and the nucleus under the dependence of light signalling**

Previous works carried have suggested a potential interaction between AtPLP and AtVTC2, which was governed by the light spectrum (Ogura *et al.*, 2008). Moreover, Müller-Moulé (2008) showed that in Arabidopsis leaf, AtVTC2 (homolog to *SIGGP1*) was detected in the cytosol and the nucleus.

To decipher the function of the SIPLP protein, we first studied its subcellular localization by transiently expressing 35S-GFP-SIPLP either under its wild-type or its truncated form as well as the two 35S-GFP-SIGGP1 and 35S-GFP-SIGGP2 in *Nicotiana benthamiana* leaves. Confocal microscopy analyses showed that the wild-type form of SIPLP localized like SIGGP1 and SIGGP2 in the nucleus and the cytoplasm (Fig. 4B). Surprisingly, the truncated SIPLP protein displayed the same sub-cellular localization. Next, we tested the physical interactions of SIPLP with SIGGP1 using BiFC experiments in epidermal onion cell. As shown in Figure 4B, this interaction between the two proteins was confirmed, but not at all when the SIPLP mutated protein was used. Interestingly, the interaction occurred both in the cytoplasm, which was expected in light of the GGP activity in the AsA biosynthesis, and into the nucleus. The nuclear localization of these two protein partners raised the question regarding the meaning of such an interaction and the regulatory process involved. With the presence of a LOV domain in the SIPLP, which is (i) defined as a blue light sensor, (ii) is truncated in our mutant PLP protein, and (iii) its impact on AsA metabolism, we hypothesized that such an interaction might be controlled through the regulation by blue light. In that frame, a heterologous system based on animal cell coupled to optogenetic approach was used in a combination of vectors for the *SIGGP1*, *SIGGP2*, *SIPLP* and *Slplp* sequences (Supplemental Table 4). In our optogenetic system two conditions were experimented, dark and blue light (455nm). It clearly appeared that the WT SIPLP interacts strongly with SIGGP1 in the dark condition whereas under blue light regime a weaker interaction with the SIGGP1 protein was observed, representing only 30% compared of that in darkness (Fig. 5). Interestingly, this experiment revealed also interaction between SIPLP and SIGGP2 which was impaired under blue light.



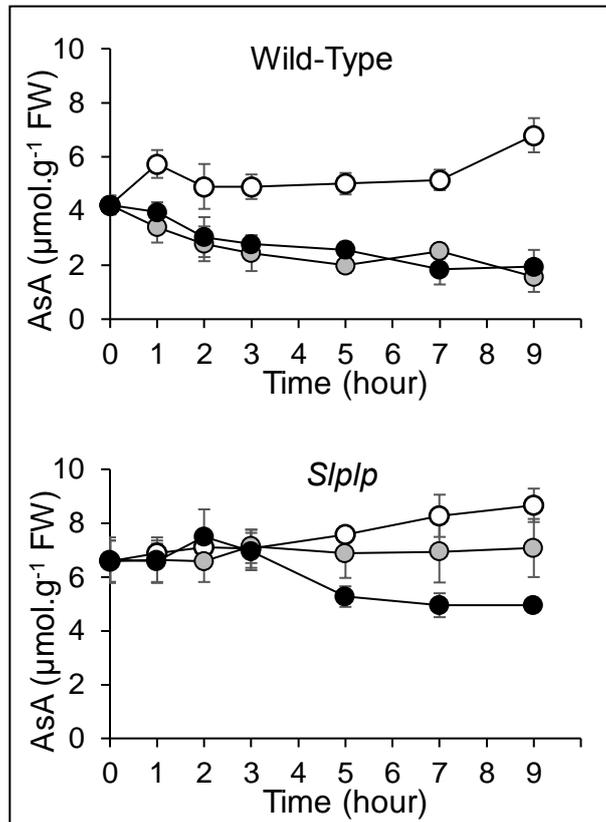
**Figure 4: Subcellular-localization of SIPLP and SIGGP.** **A.** Localization of 35S-GFP-fused proteins in *Nicotiana benthamiana* leaves, co-transformed with nuclear NLS-mcherry as nuclear marker. **B.** BiFC experiment in epidermal onion cell. Are representation of the complementation between (a) GGP1-cYFP and nYFP-PLP; (b) GGP1-cYFP and nYFP-plp.



**Figure 5: Interaction between SIPLP and SIGGP under light conditions.** All the potential partners are fused to either tetR (DNA binding domain) or VP16 (polymerase II activator), in order to test all the combinations. The rate of interaction between the fusion proteins is followed through the intensity of luciferase luminescence triggered by the reconstruction of the system in animal cell system. Dark and blue light conditions are tested independently.

On the contrary, no interaction between the *Slplp* truncated protein and SIGGP1 or SIGGP2 was observed in both conditions, hence demonstrating the loss of function of the *Slplp* mutant protein. These results confirmed the interaction between SIPLP and SIGGP, and clearly validate our hypothesis of its regulation by blue light signalling. In order to decipher the nuclear localization of the SIPLP-SIGGP complex, *in silico* analyses of the two corresponding genes (<http://nls-mapper.iab.keio.ac.jp>) revealed that SIGGP and SIPLP contained respectively 3 and 2 predicted bipartite Nuclear Localization Signal (NLS) in their peptide sequence with a score of 3.3 to 3.7, meaning that they both could be localized in the cytoplasm and the nucleus (Supplemental Table 5).

We finally examined the possible role of the blue light signalling at the physiological level by analysing the impact of various light regimes on AsA content in WT and mutant plants. One month-old plants cultured in the green house were submitted during 9 hours to three light conditions, white light, blue light and darkness in a growth chamber. Under white light, WT and the *Slplp* mutant plants displayed a similar pattern, with a 70% and 40% increase of AsA content, respectively. In the WT plants maintained under blue light and darkness, AsA content decreased gradually in a similar way to reach 40% of the initial value at T0. Under blue light, the AsA content was sustained to the initial value (around 6.5 $\mu$ mol.g<sup>-1</sup> FW) in the *Slplp* mutant plants, to remain constant for up to 2 to 3 hours and thereafter decreased suddenly to reach 70% of the T0 value. These results demonstrated that blue light is perceived in the WT plants as a signal similar to darkness, whereas in the *Slplp* mutant plants blue light did not induce such an effect.



**Figure 6: AsA change in *S/plp* mutant and WT plants under different light conditions.**

One month-old plants cultured in the green house in April 2016 were removed at night and placed in a growth chamber. After 14h of darkness, at T0 the plants were submitted to three light conditions, continuous darkness (black circles), blue light (grey circles) or white light (white circles). In the time course and up to 9h, AsA in the leaves (n=3) was assayed. Data are the means  $\pm$  SD of a total three individual plants from 15-5 line and control.

## Discussion

Ascorbic acid has been portrayed for a long time as an essential compound both for the human health and plant development. Thus, the study of the vitamin c metabolism in plants has become of crucial interest from an agronomic and economic point of view. Since the highlighting of the ascorbate main biosynthetic pathway (Wheeler et al 1998) and the characterization of the enzymes involved (Conklin *et al.*, 1999 and 2006; Linster *et al.*, 2007), many studies have tried to decipher the regulatory mechanisms involved. Our combined forward genetic and mapping by sequencing approaches using a highly mutagenized EMS Micro-Tom population (Garcia *et al.*, 2016) allowed identifying a new candidate gene related with higher AsA accumulation capacity of the tomato plants, and so the fruits (2 to 4 fold that of the WT). The AsA+ phenotype originated from a recessive mutation corresponding to a stop codon in the *Solyc05g007020* gene which encodes a photoreceptor PASLOV protein, so named SIPLP. Further genetic characterization using CRISPR Cas9 strategy and the generation of KO lines for the *SIPLP* gene confirmed the causal mutation identified. Up today, the biological function of this photoreceptor PLP sensitive to blue light remained elusive. Our work allowed (i) undoubtedly defining SIPLP as a negative regulator of AsA biosynthesis in Tomato, (ii) confirming the interaction of the PLP with the GGP protein, (iii) demonstrating that the interaction was driven by blue light signaling, and (iv) establishing the dual nuclear and cytoplasmic localization of this interaction PLP/GGP. Although the role of light on AsA metabolism was for a long time well admitted, the molecular mechanisms governing the AsA biosynthesis pathway are far to be established and must be relatively complex.

### **PLP, a new type of regulator modulating the AsA level by interacting with GGP the key enzyme of the L-galactose pathway**

Since now a decade, GDP-L-galactose pyrophosphorylase (GGP1 or VTC2) is presented as a central actor in the regulation of the AsA biosynthesis in plants (Macknight *et al.*, 2017). Several clues led to such a definition. In many plant species, including Tomato, *GGP* gene is one of the most expressed among the AsA-related genes of the L-galactose pathway, catalyzing the first committed reaction of this pathway (Dowdle *et al.*, 2007). In Arabidopsis, VTC2 has been localized in the nucleus attributing to GGP a putative regulatory role (Müller-Moulé 2008). In that sense, Laing et al. (2015) suggested a

negative feedback mechanism involving GGP that could control AsA biosynthesis. According to their model, the regulation was based on the AsA level perceived by cellular system. Hence, the translation of a small regulatory peptide encoded by a uORF located in the 5'-UTR region of the *GGP1* gene would be induced in the presence of high AsA level, and reversely when AsA level is low. This peptide could then interact with the polysomal translation machinery and inhibit the production of GGP1 protein. At last, gene expression studies showed that *VTC2* is regulated by light and suggested a circadian regulation (Tabata *et al.*, 2002; Dowdle *et al.*, 2007; Müller-Moulé 2008). However, it is quite unlikely that GGP can act alone, or through its interaction with one partner, to control the expression or translation of other actors of the AsA biosynthesis pathway.

Our results synthesize these observations of a regulatory role of GGP and the effect of light, and raise many questions regarding its physiological significance, notably the interaction of SIPLP with SIGGP1 taking place in the cytoplasmic and nuclear compartments. In the photoreceptor family harboring LOV domains, these LOV domains have the property of conformational changes dependent on blue light (Kasahara *et al.*, 2010). Light induces the covalent fixation of flavin which induces the folding of the protein and the recruitment of partner proteins. This reversible process is called the LOV photocycle (Salomon *et al.*, 2001). It is thus tempting to suggest that in this case the partner protein could be GGP1 and GGP2 and that the trigger signal is blue light (Fig. 5). This SIPLP/SIGGP interaction could play a role driving either the degradation of GGP through COP9 signalosome which is known to be involved in light-mediated regulation of AsA synthesis (Mach, 2013), either its inhibition or to act in concert directly on the expression of other genes as a regulatory complex. Moreover, other flavoproteins such as cryptochromes or phototropins, have at least a second functional domain in addition to LOV which recognizes the light signal. In the case of phototropins, whose domains are homologous to those of PLP, a serine/threonine kinase domain allows its autophosphorylation as well as the phosphorylation of its partner proteins to trigger a cascade of response (Chaves *et al.*, 2011; Christie, 2007; Suetsugu and Wada, 2013). These mechanisms would be consistent with those already described involving photoreceptors. In some cases, blue light sensors may modulate the degradation of transcription factors by their interaction with subunits of the signalosome COP9 (see Review Christie, 2015). The second possibility would be the repression of AsA biosynthesis through the inhibition of the GGP activity by PLP. This would be a very simple and rapid way to modulate or stop the biosynthesis of AsA in the cell as a function of the perception of the light signal. In the third option, PLP could alter the partitioning of GGP between the cytoplasm and the nucleus, by sequestering GGP in the nuclear compartment, and so reducing its capacity to participate to the L-galactose pathway. These assumptions are perfectly relevant given the dual compartmentation of the *S/PLP* and *S/GGP* (Fig. 4) and that was already shown for *VTC2* by Müller-Moulé (2008).

However, we cannot rule out the possibility that PLP could also participate with GGP in a multimeric complex. Up to now, the PAS domain analysis did not reveal a particular function and no additional PLP function was noted in addition to the ability to interact with GGP1. According to literature, LOV domains are able to dimerize enhancing their affinity with some targeted proteins (Heintz and Schlichting, 2016). In addition to PLP and GGP1, other partners could be recruited to form a bigger regulatory complex. One candidate could be the homolog of *AtBLH10* protein that has been identified as a potential interacting with *AtPLP* by a double-hybrid screening of an *Arabidopsis* cDNA library (Ogura *et al.*, 2008). *BLH10* belongs to the family of TALE homeodomain proteins which are involved in several developmental processes (Hackbusch *et al.*, 2005). Interestingly, in a previous integrative study carried out in Tomato (Garcia *et al.*, 2009), a positive correlation was found between AsA content and the expression level of *S/BEL1* gene, homolog of *BLH10* (Bournonville C., thesis 2016).

The intervention of a transcription factor is coherent with the localization of the two PLP and GGP proteins but this leads to the next question regarding the translocation of a putative PLP-GGP1 complex into the nucleus. According to predictive sequence analyzes (Supplemental Table 5), *SIPLP* and *SIGGP1* display at least one bipartite nuclear localization signal (NLS) in their peptide sequence, and the score so defined tolerates a dual cytoplasmic and nuclear localization. Accordingly, they could move independently into the nucleus and *vice versa*, but it is difficult to predict if this translocation is dependent or not on the interaction between PLP and GGP1. The microscopic observations in Tobacco leaf showed that like the WT *SIPLP*, the mutated *Slplp* was also localized in the nucleus (Fig. 4) even truncated of its LOV domain and unable to interact with *SIGGP* (Fig. 5). This is in favor of the existence of others partners to form a multimeric regulatory complex which would make possible the translocation of PLP independently of the interaction with *SIGGP*. Nevertheless, it remains to determine if the interaction occurs separately in each compartment and finally what is the mechanism/signal that governs the formation of such a regulatory complex and its localization in the nucleus.

### **Is blue light a signal that triggers the nuclear translocation of GGP depending on regulatory PLP complex?**

The study of the functions of photoreceptors sensitive to blue light has often reported a link with the circadian clock, by opposing the processes triggered by blue wavelengths contained in the sunlight to the nocturnal mechanisms put in place at night

(Christie, 2007). This raises the question of a possible role of the blue signal in the translocation of GGP in the nucleus in interaction with a regulatory complex, so called PLP complex. Our optogenetic experiments demonstrated first that both darkness and blue light conditions permit the interaction between PLP and GGP and second that the strength of the interaction varied depending on the perceived signal (Fig. 5). We can assume that such a situation may probably exist in plants under natural conditions. Since blue light is part of the wavelengths of the white light spectrum, one can imagine that PLP would be continuously interacting with GGP. As discussed previously, in such a case PLP could be likened to a repressor which would permanently and in a very efficient manner modulate GGP activity. This is consistent with the higher capacity to always accumulate AsA observed in the *plp* mutant at the whole plant level (Fig. 2 and 3). Likewise, this is confirmed by the absence of decline of the AsA content in the mutant contrary to what was observed in the WT under the blue light (Fig. 6). However, the true effect of blue light signalling in the regulation of GGP through its interaction with PLP must be much more complex than just this inhibition mechanism, especially when considering the issue of the nuclear localisation of these two proteins. Indeed, according to various conditions, including the hour of the day, the sky clarity which is impacted by atmospheric pollution, water vapour, cloudy periods as well as the season which influences the angle of sun rays, the solar spectrum varies regularly. These spectrum changes must be so many signals to which plants need to adapt. Among the rays of the sun, blue and red wavelengths have a very critical function since they are essential for photosynthesis and many others processes related to plant development (see review Casal, 2013). Any fluctuations of the blue over red light ratio (B/R) would be assimilated by the plants as a signal. In the present case, it is tempting to assume that this is the variation of the B/R ratio that triggers the interaction between GGP and PLP complex, and somehow may be perceived as *primo* signal in the regulation mechanism of the AsA accumulation in photosynthetic organs. Indeed, this is perfectly corroborated by the B/Rs ratios in favour of blue wavelength during the two blue-hours periods (B/R=9-10), and the effect that follows as shown by the decline of AsA content at dawn and dusk (Fig. 3). Such a B/R ratio in favour of blue wavelength exists also under cloudy sky (Just D. personal data). Considering all these features, we could propose the following scheme. First, solar spectra recorded during day/night combined with the pattern of AsA content of the tomato plant (Fig. 3) allow to define three specific light periods along the days during which the mechanism of interaction between GGP and PLP complex would operate: (1) just after sunset and before sunrise which corresponds to so-called "blue hours", (2) when the sky becomes cloudy and (3) finally during the dark night. Second, all the light transitions that happen during daytime (dawn, dusk or cloud) resulting in variations of the blue signal correspond to specific sequences which must induce distinct responses in term of light regulatory mechanisms. The sequences can be

defined as follow (1) a dark-blue signal-light sequence at the transition before dawn (2) a reverse sequence light-blue signal-dark at the transition after dusk and (3) a more or less rapid sequence light-blue signal-light when a cloud dims the sun light incidence. According to the scenario, the plants must adapt differentially, and the response must be totally specific depending on whether the time is moving towards night or day (scenario 1 or 2). In scenario n°2, the progressive increase of the B/R ratio during the decline of the light at the sunset phase would initiate the interaction between GGP and PLP complex. As the darkness grows, the interaction accentuated perhaps through phosphorylation /dephosphorylation cascades of the LOV domains by homology with the phototropins (Christie *et al.*, 2015 and ref therein), resulting in the decline of AsA content before it becomes fully dark. When the night is definitively set other molecular processes would operate leading to reverse the tendency to reactivate the AsA biosynthesis. Accordingly, the AsA content decline during sunset, and reversely its increase during night time (Fig. 3) fit perfectly this scheme. In addition, when the night was artificially maintained, it took approximatively 2 to 3h for the *plp* mutant plant to adapt and to set up these other mechanisms that trigger the decrease of the AsA pool whereas in the WT it was almost instantaneous (Fig. 6). Prolonged blue light induced the same effect than at night in the WT, but as expected the *plp* mutant plants cannot perceive this blue signal, so they continue to maintain a stable AsA level. This demonstrates clearly that another type of regulation must exist during the night, independent and in addition to that induced during the interaction between GGP and PLP complex. Indeed, other molecular processes associating AsA and light have been identified, such as those involving the AMR1 protein (Zhang *et al.*, 2009) and COP9 signalosome complex (Mach, 2013) suggesting a more complex regulation. In scenario n°1, at dawn the darkness progressively declines letting appear the blue signal. But this time the system is reversed as it is moving towards increasing light, and so lowering the B/R ratio. At first, blue signal would reverse the nocturnal regulation process as shown by the AsA content decrease that follows (Fig. 3), to as a final point reset the mechanism towards a diurnal regulation to activate the production of AsA for the rest of the daytime. For the last scenario n°3, variations in the quality of the light spectrum caused by more or less long cloudy periods could just as quickly and easily activate this mechanism. The blue light would therefore be perceived as a signal allowing the switch – to initiation or – to inhibition of the interaction between PLP complex and GGP, resulting in the decrease or increase of the AsA biosynthesis. Within this regulatory complex, PLP would therefore play a rhythmic role on a daily basis, but it could also allow the plant to quickly adapt its AsA pool according to the environmental conditions. This type of antagonist function for PAS/LOV proteins has already been described in *Neurospora* (Elvin, 2005). In this well documented study, Elvin *et co-workers* demonstrated that PLP have opposite effects either to prevent or to promote the resetting

of the circadian clock to ensure integrity and correct phase of the circadian clockwork within the complete photoperiods. Finally, PLP complex is still operational even in the *p/p* mutant plants in which the absence of PLP repression lead to higher AsA synthesis that is distributed to the whole organs (Fig. 2), including fruits which as a sink takes advantage of this in the time course of its development.

To end, the identification of potential partners in the PLP complex is an essential step to characterize in detail this regulatory mechanism, notably regarding possible process of degradation/sequestration of GGP. This would help to decipher also the issue about the translocation of the complex from the cytoplasm to the nucleus. The dependence of GGP on PLP in its localization and the influence of light are essential elements in understanding this regulation. Using a more global approach would make possible to identify these other protein partners, and even GGP as being the key enzyme of the biosynthesis pathway, is reasonably a relevant candidate in this regulation model, proteins of the AsA recycling must be considered also as interacting ones.



## Materials and Methods

### Plant material and culture conditions.

*Solanum lycopersicum* L. cv. MicroTom was used for all experiments performed in this study. Plant culture conditions in greenhouse were as described by Rothan et al. (2016). All the fruits analysed for their AsA content were produced on the plants cultured in the green house, whatever the season of the year. For the study of the light effect on AsA metabolism, the experiments were carried out in spring-summer time during the most favourable periods in term of weather. In that frame, WT and mutant tomato plants were grown in greenhouse one month prior to be used. Thereafter, the plants remained either in the greenhouse in the case of the analysis of the ascorbate change in the time course of fruit development or in the whole plant organs. In the case of the "light assay", they were moved outdoors to benefit from direct natural sunlight. The shift of the plants outside of the green house was always done after night fall to avoid any stress conditions. For the "light assay", the harvesting time of the plant materials was designed according to the light period (<https://jekophoto.eu/tools/>) which depended on the day of the year and the location (here Bordeaux). This experiment was carried out in perfect weather conditions two times, on May 19th and July 10th 2018. One month-old plants cultured in the green house were moved out the night before the beginning of the experiment, the T0 was at 10am, and every two hours 3 leaves from three plants were harvested. At the same time physical parameters were measured, temperature, hygrometry, the Photosynthetically Active Radiation (PAR, in  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ) using a Quantum photometer with a light sensor LI-190R (LI-COR Corporate, Lincoln Nebraska, USA) and the light spectrum was recorded with a JAZ Spectrometer (Ocean Optics Inc, Largo, Florida, USA). Finally, when specific light wavelengths were used, the one month-old plants were moved as previously at night fall into a growth chamber whose parameters were 16/8 h (light/dark) photoperiod under  $400 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}$  using LED light (6 Tubes LedPower EXS 30 W for a  $50 \times 120 \text{cm}$  surface) at  $25^\circ\text{C}$  and  $20^\circ\text{C}$ , respectively. After 14h of night, the plants were either kept under white light, or placed under blue light with a blue filter (Rosco supergel Nightblue#74), or left in the dark (Supplemental Figure 1). For the transient transformation experiments by agroinfiltration, wild-type *Nicotiana benthamiana* L. (tobacco) plants were cultured in soil during one month in greenhouse prior to be used. The watering was carried out three times per week, but only once with a Liquoplant fertilizing solution ( $1.85 \text{ g L}^{-1}$ , Plantin SARL Courthezon France) and twice using tap water at pH 6. For the BiFC (Bimolecular Fluorescence complementation) analyses by confocal microscopy, freshly harvested onion *Allium cepa* was purchased from the local market.

## Mapping by sequencing

The mapping by sequencing approach was performed as described in Petit *et al.* (2016). A mapping BC1F2 population of 440 plants was created by crossing the P21H6 cv Micro-Tom ascorbate-enriched mutant line with a wild-type cv Micro-Tom parental line. Two bulks were then constituted by pooling 44 plants displaying either a mutant fruit phenotype (AsA+ bulk) or 44 plants with a wild-type phenotype (wild-type-like bulk). To this end, five leaf discs (5 mm diameter each) were collected from each BC1F2 plant (approximately 600 mg fresh weight) and pooled into the AsA+ bulk and in the wild-type-like bulk. The same amount of plant material also was collected from the wild-type parental line. Genomic DNA was extracted from each bulk and the parental line using a cetyl-trimethyl-ammonium bromide method as described by Petit *et al.* (2014). DNA was suspended in 200 mL of distilled water and quantified by fluorometric measurement with a Quant-it dsDNA assay kit (Invitrogen). Illumina paired-end shotgun-indexed libraries were prepared using the TruSeq DNA PCR-Free LT Sample Preparation Kit according to the manufacturer's instructions (Illumina). The libraries were validated using an Agilent High Sensitivity DNA chip (Agilent Technologies) and sequenced using an Illumina HiSeq 2000 at the "Institut National de la Recherche Agronomique" EPGV facility, operating in a 100-bp paired-end run mode. Raw fastq files were mapped to the tomato reference genome sequence *S. lycopersicum* build release SL3.0 (<ftp://ftp.solgenomics.net>) using BWA version 0.7.12 (Li *et al.*, 2009; <http://bio-bwa.sourceforge.net/>). Variant calling (SNPs and INDELS) was performed using SAMtools version 1.2 (Li, Handsaker, *et al.*, 2009; <http://htslib.org>). As the tomato reference genome (cv Heinz 1706) used to map the reads is distinct from that of cv Micro-Tom, the variants identified would include both cv Heinz 1706/cv Micro-Tom natural polymorphisms in addition to EMS mutations. In this context, additional sequencing to a minimum depth of 203 of the cv Micro-Tom line was performed to consider and further remove the cv Heinz 1706/cv Micro-Tom natural polymorphism. The output file included various quality parameters relevant to sequencing and mapping that were subsequently used to filter the variants. The cv Micro-Tom line output file (.vcf) included all variants (SNPs plus INDELS) corresponding to natural polymorphisms between cv Micro-Tom and cv Heinz 1706. The two .vcf output files obtained from the AsA+ and wild-type-like bulks included variants (SNPs plus INDELS) corresponding to natural polymorphisms between cv Micro-Tom and cv Heinz 1706 and also to EMS mutations. The .vcf files were annotated using SnpEff version 4.1 (<http://snpeff.sourceforge.net/SnpEff.html>; Cingolani *et al.*, 2012) using ITAG2.40 gene models (<ftp://ftp.solgenomics.net>) SNP allelic frequencies between AsA+ and wild-type-like bulks and the cv Micro-Tom parental line were compared using a custom Python script version 2.6.5 (<https://www.python.org>). Once the putative causal mutation was detected using the mapping-by-sequencing procedure, the EMS-induced SNPs flanking the putative mutation were used as markers for genotyping the

BC1F2 individuals using a KASP assay (Smith and Maughan, 2015). Specific primer design was performed using batchprimer3 software (Smith and Maughan, 2015; <http://probes.pw.usda.gov/batchprimer>), and genotyping was done using KASP procedures (LGC Genomics).

## **CRISPR cas 9**

CRISPR/Cas9 mutagenesis was performed as described in Fauser *et al.* (2014). A construction comprising a single sgRNA alongside the Cas9 endonuclease gene, was designed to induce target deletions in SIPASLOV-coding sequence.

The sgRNA target sequence was designed using CRISPR-P 2.0 web software (<http://crispr.hzau.edu.cn/CRISPR2/>; Lei *et al.*, 2014). Since targeting-RNAs are inserted into pDECAS9 vector, the final plasmid was used to transform tomato cotyledons through *Agrobacterium* infection as described by Culiañez-Macia (2004). The T0 plants resulting from the regeneration of the cotyledons were genotyped and their fruits phenotyped for ascorbate content. T1 seeds from selected AsA-enriched T0 plants were sown for further characterization. The CRISPR/Cas9 positive lines were further genotyped for indel mutations using primers flanking the target sequence.

## **Ascorbate assay**

Mature leaves, and fruits at several stage of development were harvested at 12 am UT and thoroughly frozen in liquid nitrogen. Ascorbate content was measured according to (Stevens *et al.*, 2006) using 30 mg FW and 100 mg FW for the leaves and fruits, respectively.

## **Cloning**

All constructs used in the experiments were realized using gateway® technology (Invitrogen). The cDNA without STOP codon (NS) of GGP1, GGP2, PLP wild-type and PLP mutated were synthesized by GeneArt® Gene Synthesis (Invitrogen), and directly provided into entry vector pDONR201™ (Supplemental Table 4). The mutation of PLP mut was the same as the one identified in EMS mutant. In order to obtain fusion proteins with fluorescent tag either in C-terminal or N-terminal, specific primers (listed above) was used to add a STOP codon and the flanking AttB sequences by PCR reaction. Classical BP recombination reactions allowed to insert the new sequences into a pDONR201™ and then LR reaction permitted to transfer our sequence of interest into the different destination vectors.

## Interaction in plant cell

To assess the interaction of PLP and GGP1 in plant cell, BiFC (Bimolecular Fluorescence Complementation) (Walter *et al.*, 2004) experiment was performed in onion epidermal cells by biolistic transformation. Each cDNA of the genes of interest were inserted using GATEWAY technic in C0, C2, N0 and N2 vectors (see supplemental table 4) in order to test all possible orientations of the protein fusions. Then, 2.5 µg of plasmid DNA of each construct are mixed with 25 µl of a suspension (250 µg / µl) of gold microparticles (diameter = 0.6 µm) in 50% ethanol (v / v) then 25 µl of 2.5 M CaCl<sub>2</sub> and 10 µl of 0.1 M spermidine are added. The microparticles may sediment for 10 min before to be washed consecutively with 70% and 100% ethanol. Eight microliters of the microparticle suspension (30 µl) are used for transformation of epidermal onion cells using the PDE-1000He particle gun (Biorad). Before transformation, the onion epidermis is taken from the innermost scales of the bulb and deposited, upper face contact with MS medium. Transformation of onion epidermis cells is at a pressure of 710 mm Hg at a helium pressure of 1100 psi and at a distance of 6 cm.

## Optogenetic assay

In this animal cell system well described by Muller *et al.* (2014), similarly to the yeast two hybrid method, the *S1PLP* (WT or mutated) and *S1GGP1* as well as *S1GGP2* coding sequences were fused to either tetR (a DNA binding domain) or VP16 (an activator of polymerase II). When an interaction between the potential partners occurs, the system is completely reconstructed, leading to the activation of a polymerase by VP16 and the expression of the luciferase whose activity is assayed by the light produced.

## Tobacco transient transformation

The *Agrobacterium tumefaciens* electro-competent strain GV3101, harboring binary vector, was used for transient transformation studies. The competent strains were transformed with fluorescent fusion constructs previously obtained by gateway cloning. Transformed agrobacteria were selected on LB medium supplemented with suitable antibiotic and conserved at - 80°C. Before the agroinfiltration, inoculated LB cultures incubate overnight at 28°C until 0.6 to 0.8 OD<sub>600nm</sub>. For subsequent infiltration, the culture were centrifuged and the pellet suspended in water to reach 0.2 OD<sub>600nm</sub> in the case of sub-cellular localization. Then, 50-100µL of this bacterial solution was infiltrated in the leaf epidermis at the level of a wounding by needle using a 1mL syringe to improve infiltration. The plants were maintained in normal culture conditions (light, temperature) for 48h and the observation was carried out on the underside of the leaf epidermis.

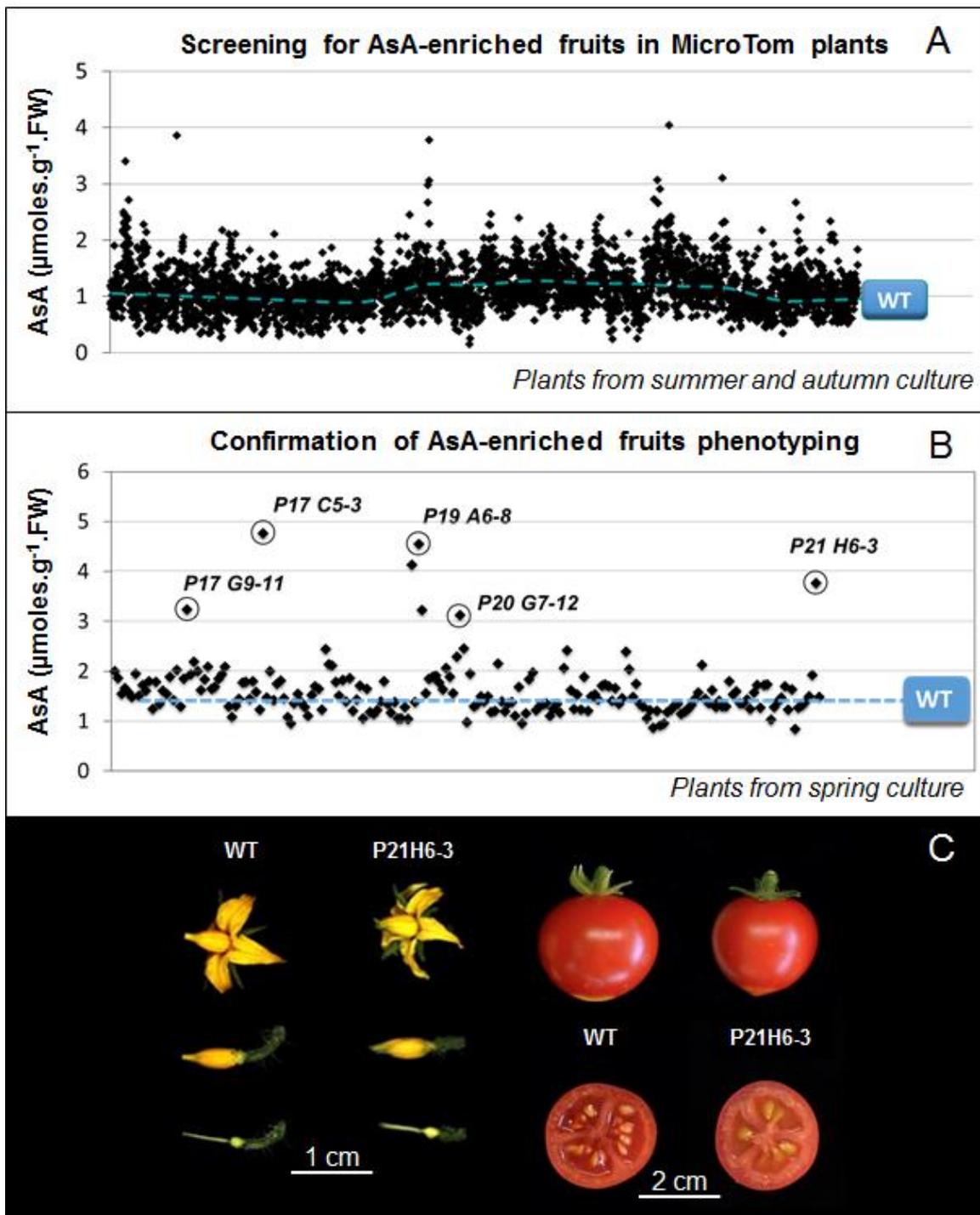
## Imaging

Imaging was performed at the **Bordeaux Imaging Center, member of the national infrastructure France BioImaging**, using a ZEISS LSM 880 confocal laser scanning microscopy system equipped with 40x objectives. The excitation wavelengths used for the eGFP (or YFP) and mCherry are 514nm and 543nm, respectively. The emission windows defined for their observation are respectively between 525 and 600 nm for the eGFP (or YFP) and between 580 and 650 nm for the mCherry.



## Supplemental

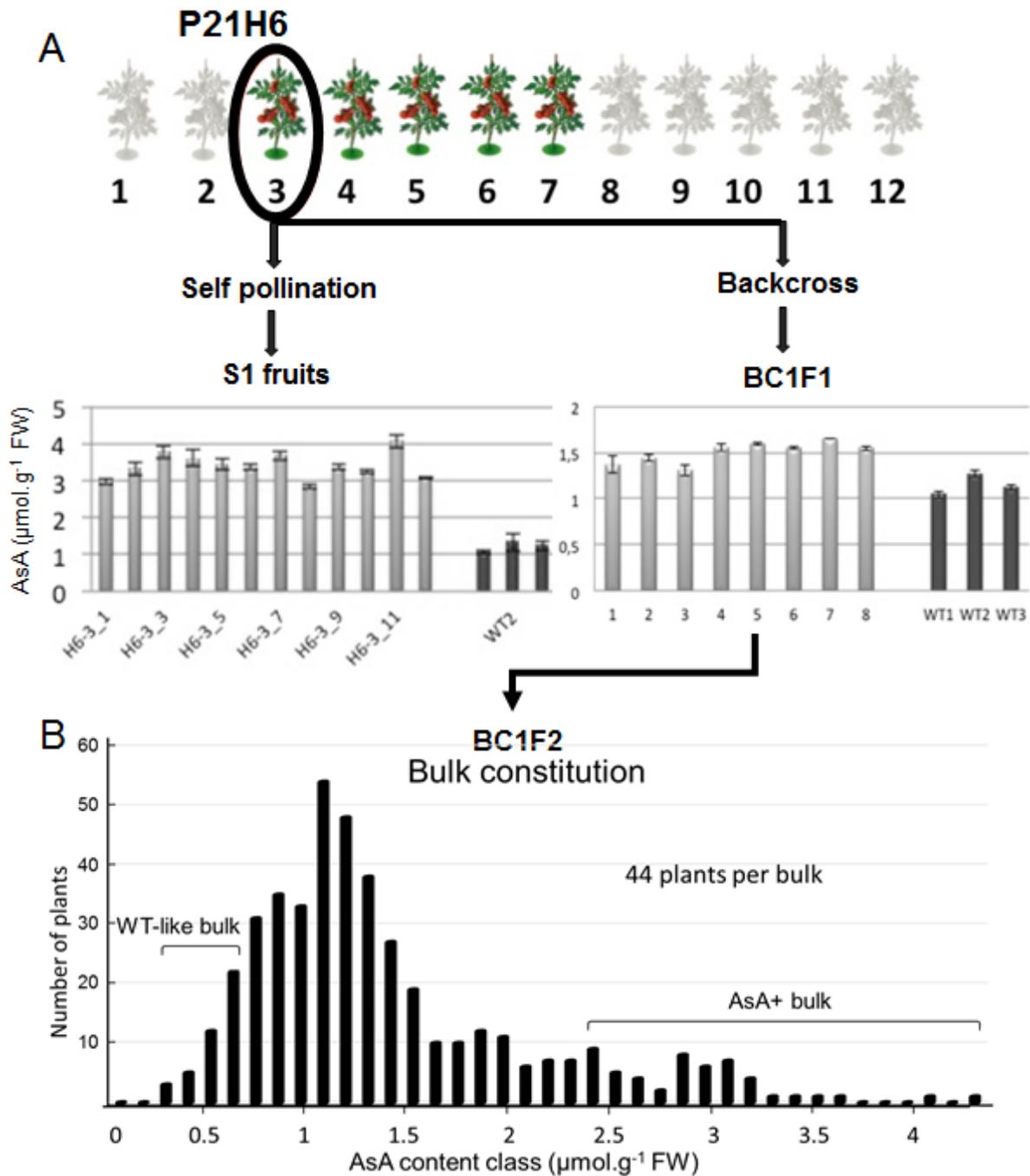
**Supplemental Figure 1: Screening of the EMS MicroTom population. A.** The screening consisted in the culture of 500 M2/M3 EMS MicroTom families each represented by 12 plants (Total 6000 plants including the WT plants). Red ripe fruits ( $n=4$ ) were assayed for their AsA content. **B.** Plants producing fruits with at least 2 times the value of the WT fruits ( $1$  to  $1.3 \mu\text{mol.g}^{-1}$  FW) were cut to allow a new growth and used for a second confirmation screening ( $n=193$  plants – 93 families). In circles are the 5 plants/family selected as AsA-enriched mutants. **C.** Illustration of the intact flower at anthesis, the anther cone, the style-ovary, the red ripe fruit and a cross section showing the pericarp and the locular tissue embedding the seeds of the P21H6-3 mutant compared to the WT.



**Supplemental Figure 2: Identification of the *plp* mutation responsible for the AsA-enriched fruit phenotype by Mapping-by-sequencing.**

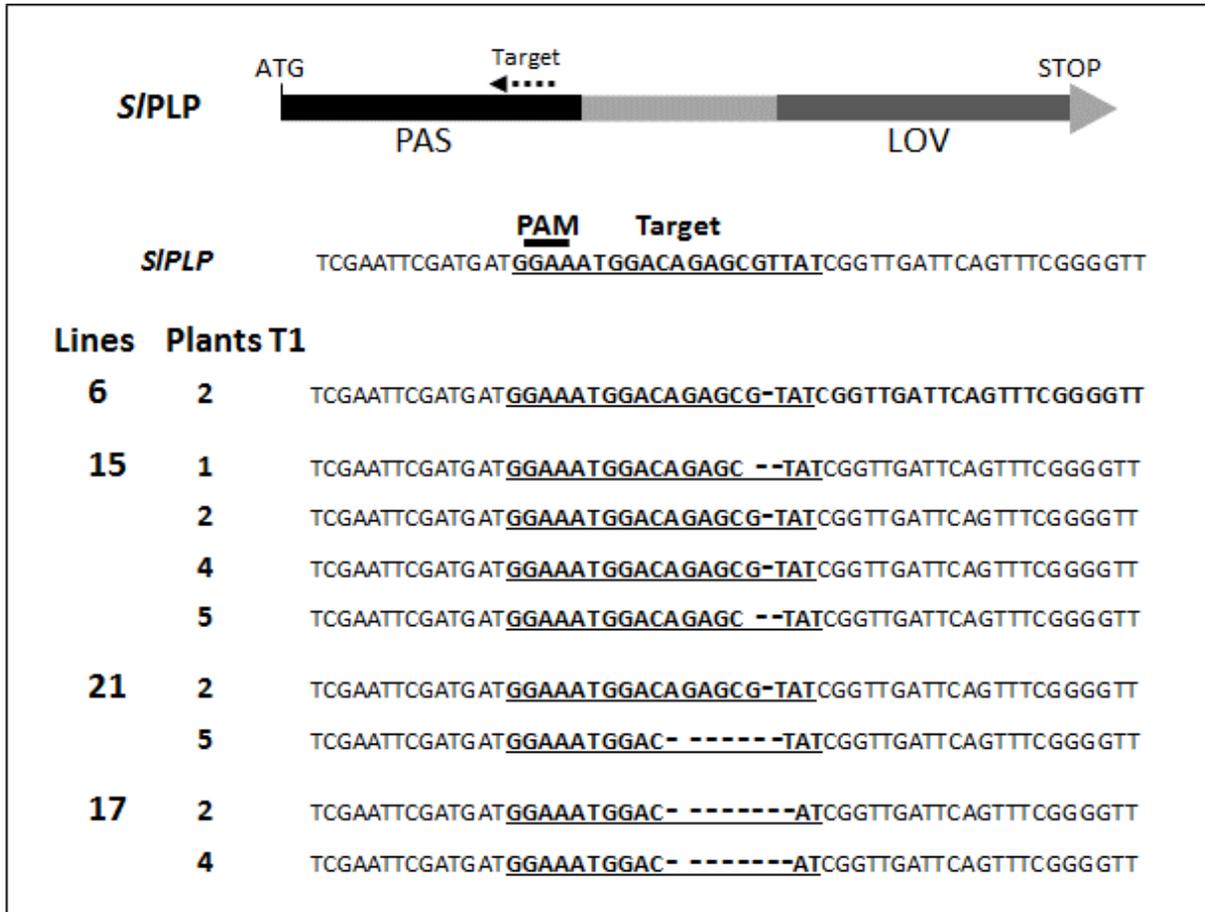
**A.** 12 M2/M3 seeds were sown for the P21H6 mutant family. Among them only 5 plants were studied (colored). Among 12 plants, those displaying an absence of fruit or a delayed maturation were not analyzed (grey). Mutant plant n°3 displaying a strong AsA+ phenotype ( $2.5 \mu\text{mol}\cdot\text{g}^{-1}$  FW) was preserved, 12 progenies of the P21H6-3 were analyzed after self-pollination (S1) for fruit AsA content. The P21H6-3 mutant was back-crossed with the WT parent, all the BC1F1 plants showed a WT-like phenotype.

**B.** The BC1F2 progeny (440 plants) was screened for AsA+ fruits (approximately 25% of the plants) and WT-like fruits (approximately 75% of the plants) to constitute the wild-type-like and the AsA+ bulks (44 individuals each).



**Supplemental Figure 3. CRISPR Cas9 strategy for the *S/PLP* gene.**

*S/PLP* is illustrated by the PAS and LOV domains. The sequence (dashed arrow or underlined) in the PAS domain was the target sequence for the CRISPR-Cas9 construct. Among the T0 transformants generated, only those producing AsA-enriched fruits ([AsA] >2 fold the WT) were selected. Thereafter the corresponding T1 lines were cultured (12 plants per T1 line) for confirmation. Among those displaying the AsA+ phenotype, a selection of them were sequenced to determine the type of mutation induced. Here is shown for some T1 lines examples of deletions detected in the *S/PLP* gene at the targeted region.



**Supplemental Figure 4: Result of the different combinations tested for the BiFC experiments between SIGGP1 and SIPLP or Slplp.** nYFP and cYFP are the two parts of the fluorochrome while Ct and Nt indicate on which side of the protein the tag is fused. (+) corresponds to an efficient complementation in both nucleus and cytoplasm; (-) means no complementation.

|     |                | GGP1     |         |         |         |
|-----|----------------|----------|---------|---------|---------|
|     |                | cYFP -Ct | nYFP-Ct | cYFP-Nt | nYFP-Nt |
| PLP | <b>cYFP-Nt</b> |          | +       | -       | -       |
|     | <b>nYFP-Nt</b> | +        |         | -       | -       |
|     | <b>cYFP-Ct</b> | +        | +       |         | -       |
|     | <b>nYFP-Ct</b> | +        | +       | -       |         |
| plp | <b>cYFP-Nt</b> |          | -       | -       | -       |
|     | <b>nYFP-Nt</b> | -        |         | -       | -       |
|     | <b>cYFP-Ct</b> | -        | -       |         | -       |
|     | <b>nYFP-Ct</b> | -        | -       | -       |         |

**Supplemental Table 1:** Illumina sequencing of BC<sub>1</sub>F<sub>2</sub> bulk individuals displaying an AsA+ mutant fruit or a WT-like fruit.

|  | <b>P21H6</b>       | <b>P21H6 WT-like</b> |
|--|--------------------|----------------------|
| Scored phenotype                                     | Asa+ fruit         | WT like fruit        |
| Tomato reference genome                              | Heinz 1706 (SL3.0) | Heinz 1706 (SL3.0)   |
| Nb of BC <sub>1</sub> F <sub>2</sub> plants per bulk | 44                 | 44                   |
| Nb of paired reads                                   | 338.529.621        | 328.853.978          |
| % mapped reads                                       | 88.23%             | 92.23%               |
| Insert size  | 352 bp             | 343 bp               |
| Sequence length                                      | 100 bp             | 100 bp               |
| Coverage   | 36X                | 35X                  |

The sequence of the reference genome represents the tomato whole-genome shotgun chromosomes from build release SL3.0, available on the SGN website (<http://solgenomics.net>).

**Supplemental Table 2:** Number of SNPs in the mutant and the WT-like bulks for the *asa* mutant.

| <b>Chromosome</b> | <b>Total variants<sup>a</sup></b> | <b>EMS mutations<sup>b</sup></b> |
|-------------------|-----------------------------------|----------------------------------|
| 1                 | 94,297                            | 1,438                            |
| 2                 | 231,487                           | 932                              |
| 3                 | 131,392                           | 1,072                            |
| 4                 | 175,353                           | 9,470                            |
| 5                 | 586,553                           | 1,058                            |
| 6                 | 51,968                            | 728                              |
| 7                 | 109,043                           | 891                              |
| 8                 | 31,317                            | 701                              |
| 9                 | 48,388                            | 773                              |
| 10                | 27,557                            | 717                              |
| 11                | 249,496                           | 919                              |
| 12                | 68,554                            | 842                              |

Only variants with a read depth between 10 and 100 reads were considered to remove false-positive variants due to erratic read mapping. Number of total variants (natural polymorphisms between the Heinz 1706/Micro-Tom and EMS mutations) and only EMS mutations per chromosome are reported in the second and third column, respectively

<sup>a</sup>Total variants (natural polymorphisms and EMS mutations) obtained in the mutant and WT-like bulks (10 < read depth < 100). <sup>b</sup>EMS mutations obtained after removing natural polymorphisms between Micro-Tom and Heinz 1706 (10 < read depth < 100).

**Supplemental Table 3:** AsA content in rep ripe fruits of T0 and T1 CRISPR PASLOV plants. AsA was assayed in red ripe fruits of T0 and the corresponding T1 lines and compared to the WT plants. Data are the means  $\pm$  SD of four fruits. The differences between the AsA values in the WT fruits as well as for the CRISPR fruits be influenced by the season of the year of the cultivation.

|                 |               | <b>AsA</b>                                    |             |               |             |               |             |               |
|-----------------|---------------|---|-------------|---------------|-------------|---------------|-------------|---------------|
| <b>Plants</b>   |               | <i>(<math>\mu\text{mol.g}^{-1}</math> FW)</i> |             |               |             |               |             |               |
| <b>WT</b>       | 1.3 $\pm$ 0.2 |   |             |               |             |               |             |               |
| <b>T0 Lines</b> | <b>6</b>      | 3.2 $\pm$ 0.3                                 | <b>15</b>   | 3.4 $\pm$ 0.1 | <b>17</b>   | 4.7 $\pm$ 0.3 | <b>21</b>   | 3.1 $\pm$ 0.1 |
| <b>WT</b>       | 0.9 $\pm$ 0.1 |   |             |               |             |               |             |               |
| <b>T1 Lines</b> | <b>6-1</b>    | 2.5 $\pm$ 0.2                                 | <b>15-1</b> | 2.5 $\pm$ 0.1 | <b>17-3</b> | 2.2 $\pm$ 0.1 | <b>21-1</b> | 2.7 $\pm$ 0.1 |
|                 | <b>6-2</b>    | 2.6 $\pm$ 0.1                                 | <b>15-2</b> | 2.4 $\pm$ 0.1 | <b>17-4</b> | 2.0 $\pm$ 0.1 | <b>21-2</b> | 2.7 $\pm$ 0.2 |
|                 | <b>6-3</b>    | 1.6 $\pm$ 0.1                                 | <b>15-3</b> | 2.4 $\pm$ 0.2 | <b>17-5</b> | 1.1 $\pm$ 0.1 | <b>21-3</b> | 2.3 $\pm$ 0.1 |
|                 | <b>6-4</b>    | 1.7 $\pm$ 0.2                                 | <b>15-4</b> | 2.2 $\pm$ 0.2 | <b>17-6</b> | 2.2 $\pm$ 0.1 | <b>21-4</b> | 2.2 $\pm$ 0.1 |
|                 | <b>6-5</b>    | 1.5 $\pm$ 0.1                                 | <b>15-5</b> | 2.0 $\pm$ 0.2 | <b>17-7</b> | 1.7 $\pm$ 0.1 | <b>21-5</b> | 2.2 $\pm$ 0.1 |
|                 | <b>6-6</b>    | 2.51 $\pm$ 0.2                                | <b>15-6</b> | 3.0 $\pm$ 0.1 |             |               | <b>21-6</b> | 1.3 $\pm$ 0.1 |
|                 | <b>6-7</b>    | 2.1 $\pm$ 0.1                                 | <b>15-7</b> | 2.1 $\pm$ 0.1 |             |               | <b>21-7</b> | 1.7 $\pm$ 0.1 |

**Supplemental Table 4:** Constructs and set of primers used for cytological analysis.

| Name                    | Primers                   | Sequence (5' to 3')  |
|-------------------------|---------------------------|--|
| attB1_SIGGP1_attB2      | SIGGP1_attB1FW            | GGGACAAAGTTTGACAAAAAAGCAGGCTTCAIGCTCAAAAATAAGAGGGTTCC            |
|                         | SIGGP1_Stop_attB2REV      | GGGACACCACCTTTGTACAAGAAAAGCTGGGTGCTAGGGCGGGAACCATGGTACTGTGG      |
| attB1_SIGGP2_attB2      | SIGGP2_attB1FW            | GGGACAAAGTTTGACAAAAAAGCAGGCTTCAIGTTGACTGTAAAAGAGGGTGC            |
|                         | SIGGP2_Stop_attB2REV      | GGGACACCACCTTTGTACAAGAAAAGCTGGGTGCTAGCTGCAACACCAAAACAATCTGG      |
| attB1_SIPASLOVwt_attB2  | SIPASLOVwt_attB1FW        | GGGACAAAGTTTGACAAAAAAGCAGGCTTCAIGGAAATCACAACTGG                  |
|                         | SIPASLOVwt_Stop_attB2REV  | GGGACACCACCTTTGTACAAGAAAAGCTGGGTGCTAGGGAGGTACTTGCACCCATTGAC      |
| attB1_SIPASLOVmut_attB2 | SIPASLOVmut_attB1FW       | GGGACAAAGTTTGACAAAAAAGCAGGCTTCAIGGAAATCACAACTGG                  |
|                         | SIPASLOVmut_Stop_attB2REV | GGGACACCACCTTTGTACAAGAAAAGCTGGGTGCTAGGAAATTGTGTGCCTCTCTGTATCTTCC |

| Use                                  | Usual Name | Name         | Description  |
|--------------------------------------|------------|--------------|--------------|
| Gateway Cloning                      | -          | pDONR201     | Entry vector |
| Localization / Stable transformation | GFP Ct     | pK7FWG2      | 35S_CDS_eGFP |
|                                      | GFP Nt     | pK7WGF2      | 35S_eGFP_CDS |
| BiFC                                 | N2         | NeYFP/pJUGW2 | 35S_CDS_nYFP |
|                                      | N0         | NeYFP/pJUGW0 | 35S_nYFP_CDS |
|                                      | C2         | CeYFP/pJUGW2 | 35S_CDS_cYFP |
|                                      | C0         | CeYFP/pJUGW0 | 35S_cYFP_CDS |

**Supplemental Table 5:** Predicted Nuclear Localization Sequences (NLS) in the *S/PLP* and *S/GGP*. *In silico* analyses to define putative NLS of the proteins *S/PLP* and *S/GGP* using the following website <http://nls-mapper.iab.keio.ac.jp>. Score classes for sub-cellular localization: 1 - 2 exclusively cytoplasm; 3 - 5 nucleus and the cytoplasm; 7 - 8 partially nucleus; 10-8 means exclusively nucleus

|              | Position | Sequence                         | Score |
|--------------|----------|----------------------------------|-------|
| <i>S/PLP</i> | 48       | RGFLKVFYGYSKNEVLGKNGRVFQGPKTNRR  | 3.6   |
|              | 230      | RLVIDRRCCQSGTSLLSASLNISFGRIKQSFV | 3.3   |
| <i>S/GGP</i> | 2        | MLKIKRVPTLVSNFQKDEADEIAARGAGC    | 3.3   |
|              | 114      | RHLKKRPTEFRVDKVLQPFDGSKFNFTKVGQE | 3.4   |
|              | 167      | IDPEKSPSVVAINVSPIEYGHVLLIPKVLEC  | 3.7   |

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## PART 2: STERILE DEREGULATION

### KNOCKING-OUT SIGGP NEGATIVE FEEDBACK LEADS TO HIGH ASCORBATE ACCUMULATION RESULTING IN A DEFECT OF THE REPRODUCTIVE SYSTEM IN TOMATO.

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### Introduction

Ascorbic acid (Vitamin C) is one of the only molecules that is necessary for all animal and plant kingdom. Ascorbic acid exists as two chemical forms, reduced (Ascorbate or AsA) and oxidized (monodehydroascorbate MDHA and dehydroascorbate DHA), AsA being the only active form of the molecule. In both kingdoms, AsA acts as the major antioxidant of the cell. Moreover, with exception of humans and few other mammals, all animals and plants produce their own ascorbic acid. Because humans are unable to synthesize ascorbic acid, this molecule is defined as a vitamin and so plants are products of great nutritional value. Indeed, fruits and vegetables are the only sources of ascorbic acid in human diet and in term of health they can help to reach the recommended daily intake of about 120 mg of vitamin C daily in average for an adult. In higher plants, especially in some fruits produced by native plants of the southern hemisphere in South America and Australia, ascorbic acid concentration can thus represent more than 2% and up to 5% of the fresh weight of the fruits, respectively in Camu camu (*Myrciaria dubia*) or Kakadu plum (*Terminalia ferdinandiana*). As a powerful antioxidant, its role in plants is as much related to the response to stress as to physiological processes by scavenging reactive oxygen species (ROS), mainly produced by oxidative metabolism and photosynthesis. In plants, the amount of AsA can therefore vary very rapidly in response to environmental changes, like for example during abiotic stresses.

In plants, several ascorbic acid biosynthetic pathways have been described so far (Ishikawa *et al.*, 2006). However, the only pathway that has been fully demonstrated is known as the L-galactose pathway, it is present in all higher plants, and is now well admitted to be the main route for synthesizing ascorbic acid (Wheeler *et al.*, 1998; Linster *et al.*, 2008). The pathway starts with the use of D-glucose-6P, and *via* the synthesis of several hexose phosphate leads to GDP-L-galactose which is then converted in L-galactose-1P by the GDP-L-galactose phosphorylase (GGP). In *Arabidopsis thaliana*, this enzyme is encoded by two highly conserved genes which have been firstly identified as *AtVTC2* and *AtVTC5* genes through the characterisation of several *vtc* (vitamin c defective) mutants (Conklin *et al.*, 2000; Dowdle *et al.*, 2007). Although the corresponding proteins VTC2 and VTC5 display the same enzymatic activity, the *Atvtc2* mutant plants showed a strong decrease of AsA level, thus demonstrating no effective complementation by the *AtVTC5* protein. The double *Atvtc2-vtc5* mutant cannot produce AsA as the seedlings were unable to develop after germination and consequently needed to be rescued by addition of exogenous ascorbate in the germination medium (Dowdle *et al.*, 2007). GGP was the last enzyme of the L-galactose pathway that has been characterized (Linster *et al.*, 2007; Laing *et al.*, 2007), and it is now described as the key step of the pathway (For review see Macknight *et al.*, 2017). Indeed, many studies have emphasized its role as a control point in the pathway, and several clues led to such a definition. In many plant species, including Tomato, GGP gene is one of the most expressed among the AsA-related genes of the L-galactose pathway (GAO *et al.*, 2011). In *Arabidopsis* like in Tomato, VTC2 has been localized in the nucleus attributing to GGP a putative regulatory role (Müller-Moulé 2008, Deslous *et al.*, unpublished data). At last, gene expression studies showed that GGP is regulated by light and suggested a circadian regulation (Tabata *et al.*, 2002; Dowdle *et al.*, 2007; Müller-Moulé 2008). Recently, Laing and co-workers (2015) characterized a new regulatory protein of the L-galactose pathway controlling the GGP level. This protein encoded by a *cis*-acting uORF located in the 5'UTR region of the GGP1 gene, has been shown to act as a negative feedback by reducing the amount of GGP at the translational level and *in fine* the level of ascorbate biosynthesis. These authors hypothesized that this regulation was driven by the AsA level in the cell, high AsA resulting to inhibition and *vice versa*.

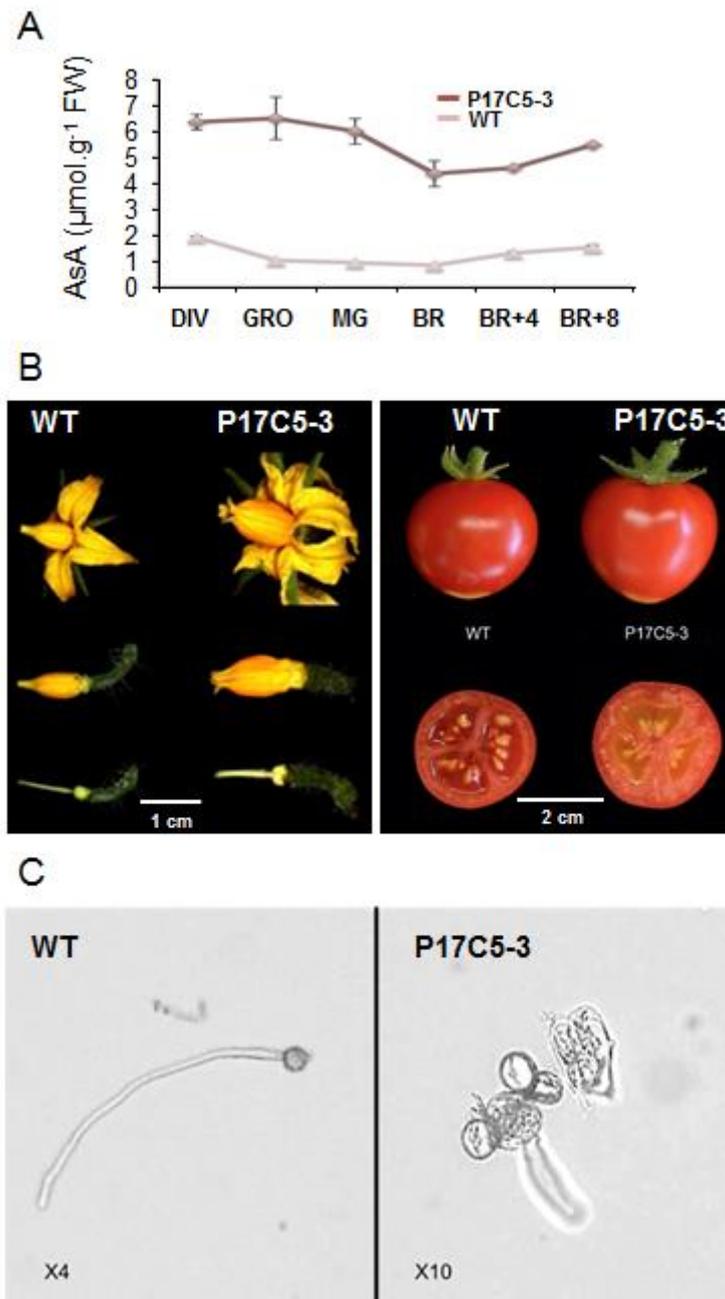
The biosynthesis of AsA is largely conditioned by the intensity of the light captured by the plants. Basically, high light means high AsA content and reversely. For instance, it has been shown that in *Arabidopsis thaliana* leaves, high-light conditions induced an increase in AsA concentration by enhancing the GGP expression and activity (Dowdle *et al.*, 2007). But in some cases, high light can give rise to stress like when the plants are growing at low temperature under high irradiance or undergo a sudden change of light intensity. In such environments, the photosynthetic machinery unable to coop with the

excess of photons induces a burst of ROS production at the level of the photosystem I and II resulting in an oxidative stress condition. Regulatory proteins such as AMR1 or the CSN5B/COP9 complex were shown to directly affect AsA-related gene expression or the activity of the corresponding enzymes in response to modulation of light regime (Zhang and Huang, 2010; Zhang *et al.*, 2012; Alimohammadi *et al.*, 2012). However, the precise mechanisms involved in this control are totally unknown.

Due to its high health potential, many studies have been conducted for enhancing the ascorbic acid pool in plants mainly by overexpressing some genes of the L-galactose pathway. Generally the gain was not what was expected. (Macknight *et al.*, 2017). The most representative increase was obtained by overexpressing the GGP gene, so this led to raise GGP as the key enzyme of the L-galactose pathway (Bulley *et al.*, 2009). Intriguingly, when this GGP overexpression strategy was carried out in Tomato, among the transgenic lines generated, those displaying the highest AsA content produced fruits having non-viable seeds, or in the extreme case parthenocarpic fruits (seedless) (Bulley *et al.*, 2012). These authors suggested that there might be a threshold limit of AsA level beyond which seed development would be impaired leading for instance to such an unexpected parthenocarpic phenotype of the fruits. Besides, GDP-D-mannose epimerase which corresponds to the enzyme that precedes GGP has been described to be also an important step of the pathway. Indeed, the silencing of the two Tomato SIGME1 and SIGME2 resulted to a 60% decrease of AsA content associated with problems of plant growth due to impairment of both cell division and cell expansion processes (Gilbert *et al.*, 2009). Alternatively, silencing of the SIGME1 only resulted in the production of small and poorly seeded fruits without changing the AsA content (Gilbert *et al.*, 2016). Such effects originated from the GDP-D-mannose-epimerase activity which is at the crossroads of AsA and cell wall biosynthesis (Gilbert *et al.*, 2009, Dumont *et al.*, 2010). Over-expression of *SIGMEs* led also to a significant effect on the ascorbate pool in tomato fruits (Zhang *et al.*, 2011), Thus, the GME and GGP double seem to play distinct and important roles in the L-galactose pathway as they can to act synergistically to stimulate AsA concentration (Bulley *et al.*, 2009 and 2012; Ioannidi *et al.*, 2009).

To investigate the regulation of the ascorbic acid biosynthesis pathway in plants, and so in fruits, a forward strategy combining the screening of an EMS mutant population in the MicroTom Tomato cv and mapping by sequencing has been developed to identify new regulators. This approach allowed identifying five mutant families producing AsA-enriched fruits whose ascorbate content was 2 to 5 times higher than the WT fruits (Deslous *et al.* unpublished data). Among these plants defined as AsA+ mutants, one family named the P17C5 family displayed the highest AsA content. Interestingly, the AsA+ phenotype was dominant. In addition, the P17C5 mutant also showed morphological alterations, like

abnormal flowers and at the fruit level a low seed number up to total absence of seeds. Such a parthenocarpic phenotype made the subsequent genetic characterization difficult. Despite this, the identification of the causal mutation was possible thanks to out-crossing the original P17C5 mutant with another tomato cultivar, namely M82, which allowed generating a segregant F2 population necessary for the mapping by sequencing approach. Here we describe the finding of the P17C5 AsA+ mutant and the characterization of the mutated protein which strengthen the role played by the GDP-L-galactose phosphorylase (SIGGP1) in the regulation of the ascorbate metabolism.



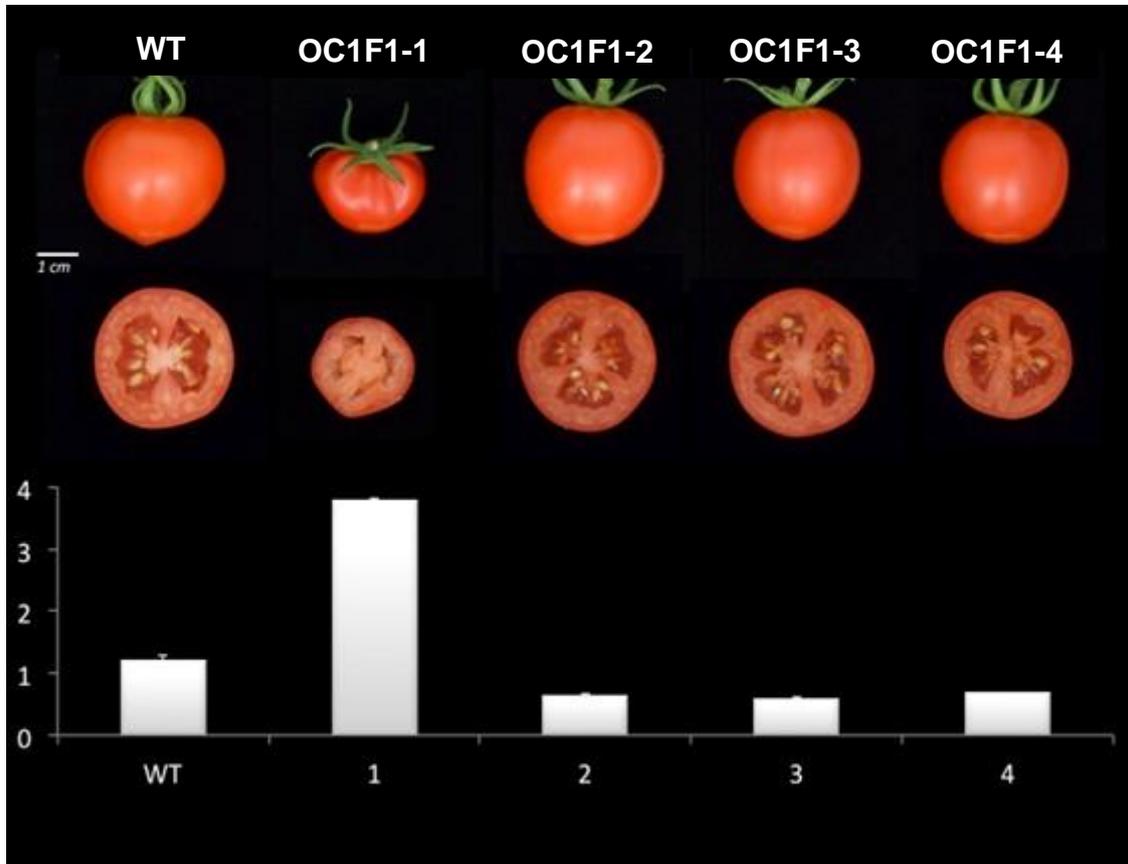
**Figure 1:** Phenotyping of P17C5-3 mutant.

**A.** AsA content during tomato fruit development in the P17C5-3 AsA-enriched mutant and WT. Fruits are analysed at different stage of development: cell division (DIV), cell growth (GRO), mature green (MG), breaker (BR), breaker +4 days or orange (BR+4), breaker +8 days or red ripe (BR+8). For each assay, three fruits of individual plants were pooled and analysed in technical triplicate. **B.** Alteration of the flowers and fruits morphology. **C.** Microscopic observation of pollen grains during in vitro germination in WT and P17C5-3 lines. Pollen was incubated 4h in germination solution. Pollen tube growth is clearly observed in the WT whereas in P17C5-3 pollen grain produced a shorter pollen tube and displayed some aggregation phenomena.

## Results

### **Characterization of a new AsA-enriched mutant from the screening of a highly mutagenized EMS MicroTom population**

In a previous work (Deslous et al., unpublished data), a direct genetic approach was set up to identify AsA-enriched fruit (also named AsA+) from 500 M2 mutant families of a MicroTom EMS population generated at INRA-Bordeaux. This screening allowed isolating five families producing fruits whose AsA content ranged from 2 to 5 times that of the WT. We will describe here the family named P17C5 that showed the more remarkable AsA+ phenotype (Supplemental Fig.1A). Among 12 M2 P17C5 mutant plants analyzed, only one plant (n°3) produced red ripe fruits that had 4 to 5 times more AsA depending on the period of the year (Supplemental Fig. 1). The analysis of the AsA accumulation in the fruits showed that it was already high at the early stages of fruit development and maintained high all along up to the ripe fruits in comparison with the WT fruits (Fig.1). In addition to the AsA+ trait, the P17C5-3 mutant had flowers bigger than the WT, for some of them the anther cones were abnormally merged together and displayed carpel fusion that could explain the significant thickness of the style (Fig. 1). As a result, most of the fruits of the P17C5-3 mutant showed an obligatory parthenocarpy-like trait (seedless). Nevertheless, few fruits contained small seeds (Fig.1) but their germination capacity was significantly low compared to other AsA+ families. Besides, the vegetative parts of the P17C5-3 mutant were indistinguishable from the WT plants. In order to characterize the nature of the P17C5-3 mutation, the analysis of the segregation of the AsA+ trait in the progeny was performed as well as the back-cross with the WT MicroTom parent (Supplemental Fig. 1). In this unique P17C5-3 mutant, to favour the production of seeded fruits, the trusses were vibrated manually to improve self-pollination (S1), which finally made possible to obtain only 5 M3 seeds. Among the five S1 plants, only one showed the fruit AsA+ phenotype. The backcrossing of P17C5-3 resulted in just under 50% of BC1F1 plants (5/12) displaying an "AsA+" fruit phenotype, but those fruits still exhibited the parthenocarpic phenotype. At this stage, we could conclude that (1) the mutation is dominant as the phenotype was observed in BC1F1 plants, and (2) that the P17C5-3 parent was heterozygous for the mutation since only half of the F1 presented the AsA+ phenotype. At last, no change in fruit AsA content was observed in the BC1F2 population obtained from the BC1F1 plants that displayed the WT-like trait (Supplemental Fig.1B). The absence of seeds in the AsA+ fruits of the BC1F1 made impossible the generation of the BC1F2 population that was necessary to identify the causal mutation using a mapping by sequencing strategy. Theoretically, the association between the



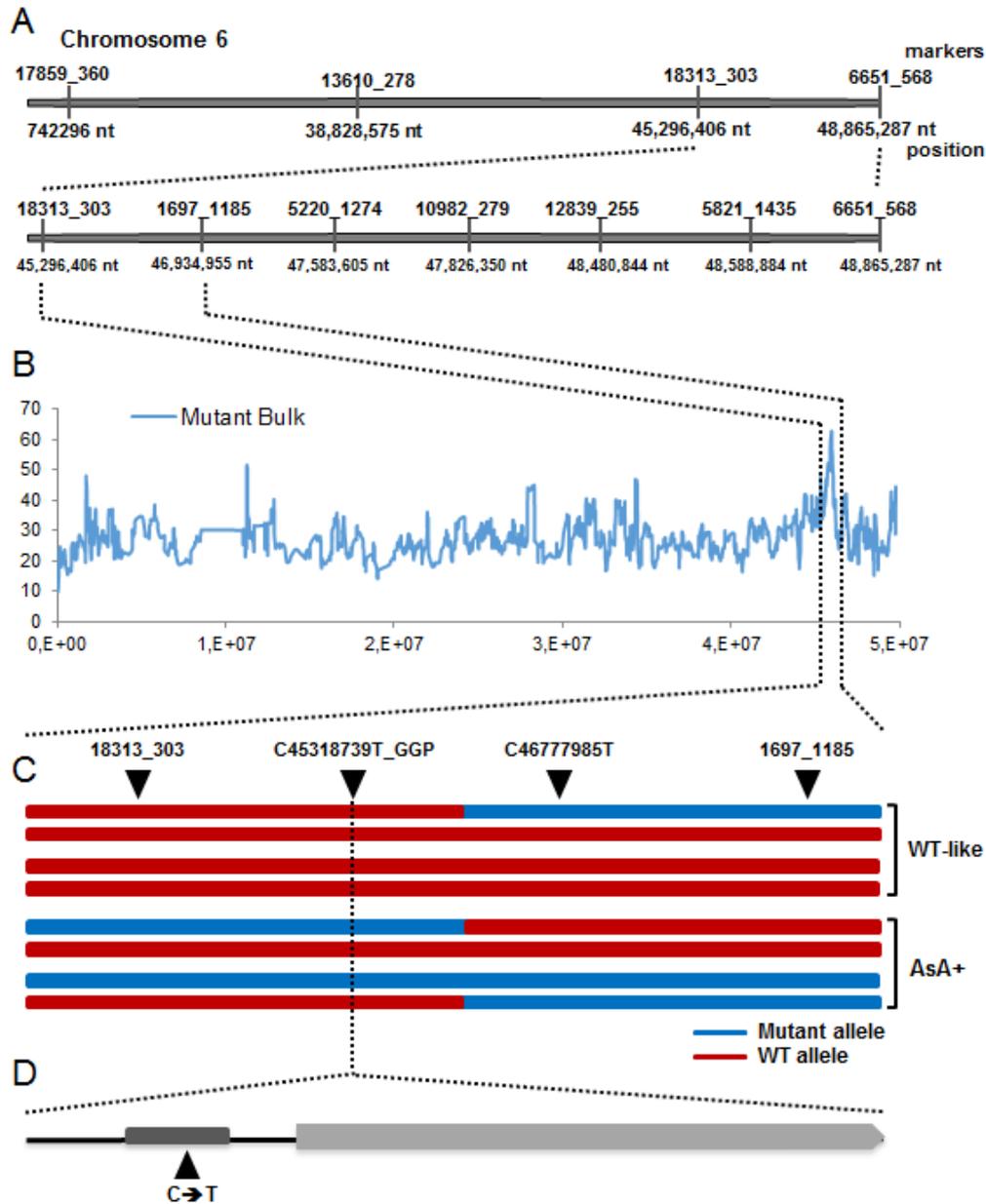
**Figure 2:** AsA content in red ripe fruits from OC1F1 plants (♀ P17C5-3 x ♂ M82). Among four BC1F1 plants (P17C5-3xM82) cultivated in the green house, only one produced smaller and parthenocarpic fruits (n°1) whereas the other three produced fruits displayed a WT-like phenotype. Three fruits at the red ripe stage were pooled and three technical triplicates (±SD) were analysed. AsA content is expressed in μmoles.g<sup>-1</sup> FW.

parthenocarpic and AsA+ phenotypes could result of either two linked loci or one single locus, thus leading to an interesting question about the relationship between these two phenotypes.

In parallel, we decided to check the possible transfer of the beneficial P17C5-3 dominant mutation to another genetic background of commercial interest. The selected tomato cultivar to carry out the interspecies cross was the M82 cultivar, a well-known and well-studied cultivar that displays morphological and genetic characteristics very different from that of Micro-Tom. The present out-cross consisted in several flower pollinations using the P17C5-3 gynoecium as female part and the M82 androecium as male part. The fruits of the OC1 contained few seeds (in average 4 seeds/fruit), and among the first four OC1F1 seeds sown, only one plant displayed the fruit AsA+ phenotype (Fig.2). Such an AsA+ trait in the F1 generation is consistent with the previous observations and the dominant nature of the P17C5-3 mutation. Unfortunately and despite a lot of attention regarding intensive manual self-pollination, the OC1F1 plant n°1 produced small and seed-less fruits (Fig.2) compared to the WT M82 or the other BC1F1-2 to 4 WT-like plants. This very precious plant n°1 was finally cut to allow developing new shoots. Intriguingly, that time the intensive manual self-pollination on the new inflorescences resulted in the production of several bigger fruits containing F2 seeds. Thanks to that, 144 seeds were sown to obtain the OC1F2 segregant population, hence allowing the mapping by sequencing. Analysis of the AsA content of the red ripe fruits showed a range of AsA level up to 20 times that of the WT fruit (Supplemental Fig. 1C). Moreover, this OC1F2 population revealed a large variety of morphological traits in comparison to the MicroTom and M82 parents, like plant height, leaf shape, flower morphology, truss number, fruit number per truss, fruit diameter and shape, and finally the seed content of the fruits. Statistical analyses allowed discarding any significant links between the AsA+ phenotype and all these traits, with exception of the parthenocarpy (Supplemental Fig. 2).

### **Identification of the causal mutation of the AsA-enriched P17C5 mutant.**

As mentioned before, the characterization of the causal mutation of the P17C5-3 plant was quite complicated. This originated from the link of the AsA + phenotype with the strong alteration of seed development, thus resulting to parthenocarpy-like fruits. Luckily, out-crossing the P17C5-3 mutant with M82 cultivar and the dominant character of the responsible mutation allowed the mapping by sequencing strategy to succeed and the identification of the mutation. Each individual of the 144 OC1F2 population plants was phenotyped for their AsA content to construct two bulked pools, AsA+ and WT-like. Plants



**Figure 3:** Identification of the *uorf* mutation responsible for the AsA-enriched fruit phenotype

**A.** Identification of the chromosome associated with the Asa+ phenotype. Localization of the loci associated to AsA+ phenotype in OC1F1 mutant population using bulk segregant analysis (BSA), in the sixth chromosome of Tomato genome.

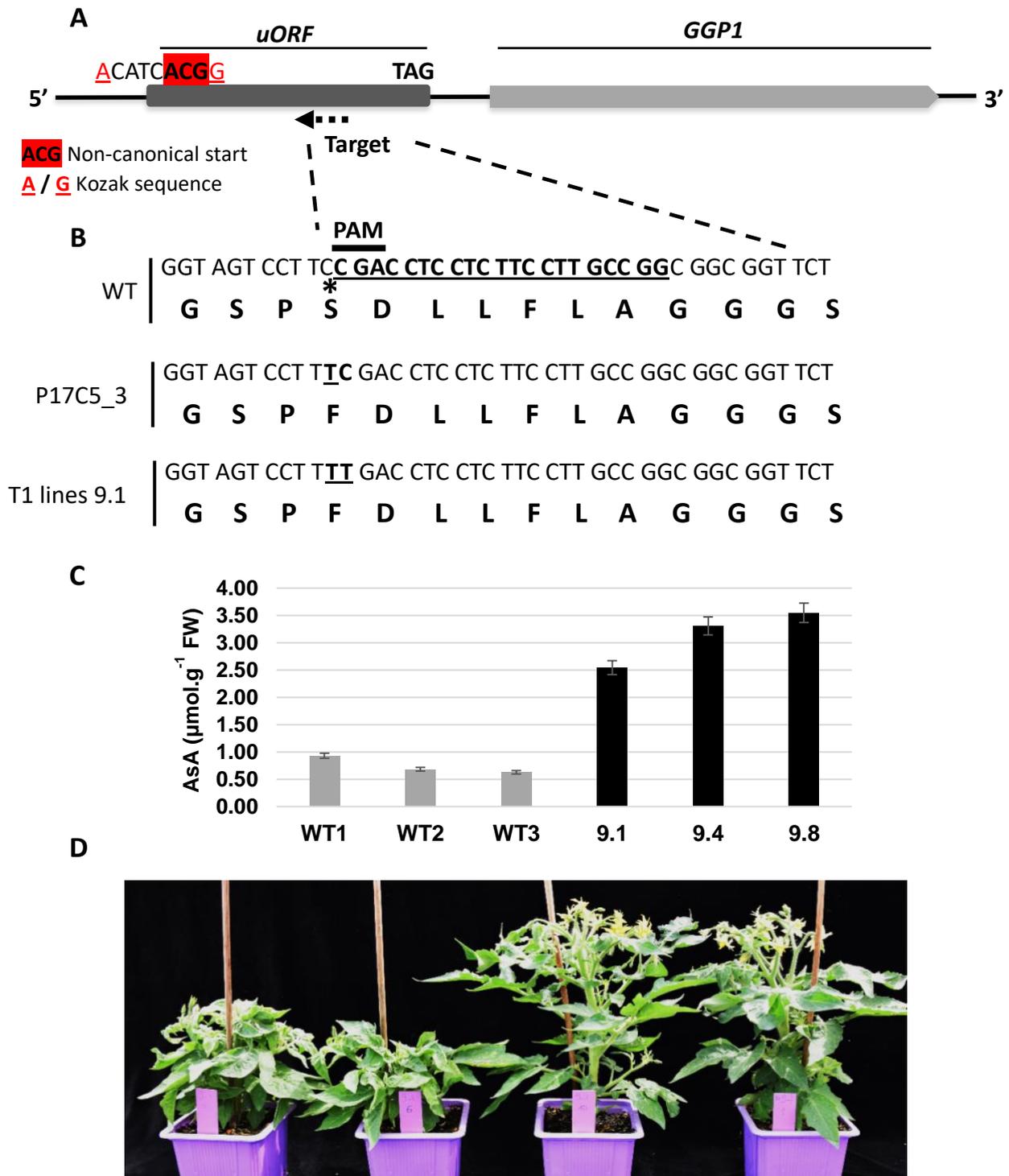
**B.** Pattern of the mutation allelic frequencies (AF) obtained in the mutant bulk is represented along tomato sixth chromosome. In the graph was not shown the AF of WT-like since individuals are all homozygous WT at the locus and the polymorphism from M82 genome tend to blur the analysis. The plot represents allelic frequencies (y axis) against genome positions (x axis). The dashed lines indicate the peak of allelic frequency of the chromosome 6 region carrying the putative causal mutations, since it displayed an AF ~66% in the AsA+ bulk and an AF = 0 (not shown) in the WT-like bulk.

**C.** Fine mapping of the causal mutation using the OC1F2 population. Recombinant analysis of 144 OC1F2 individuals displaying the WT-like and Asa+ phenotype allowed us to locate the causal mutation. Marker positions are indicated by black triangles.

**D.** A single nucleotide transversion, C to T in 5'UTR of the *Solyc06g073320* sequence led to a change from Serine to Phenylalanine.

displaying the AsA<sup>+</sup> and WT-like fruit phenotypes were selected to generate the corresponding bulks, each containing 33 and 30 individuals, respectively (Supplemental Fig. 1C). Because of the combined P17C5 and M82 genomes, we first undertook a bulk segregant analysis (BSA) with known genetic markers widely distributed along the 12 tomato chromosomes (Shirasawa *et al.*, 2013). This resulted to the identification of the locus on the q arm of chromosome six (Fig. 3A). Then, the investigation of the mutated locus responsible for the AsA<sup>+</sup> phenotype was performed using the mapping by sequencing strategy described by Petit *et al.* (2016). The two pooled genomic DNA from each bulk described above were then sequenced before the sequences were mapped onto the tomato reference genome. EMS mutation variants were filtered to exclude natural polymorphisms found in cv Micro-Tom (Kobayashi *et al.*, 2014) compared with the sixth chromosome sequence from cv Heinz 1706 reference genome. Next, analysis of allelic variant frequencies (AF) in the two bulks allowed the identification of the responsible loci (Fig. 3B). Because of the mutation was defined as dominant, individuals of WT-like bulk are obligatory homozygous WT for the locus of interest resulting in a null AF at the location. Moreover, the AsA<sup>+</sup> bulk might contain heterozygous and homozygous mutated loci represented by an AF~66%. In addition, for the M82 genome the analysis made the AF values very noisy, and so not reliable. In the same manner, the WT-like AF was not useful for this determination and consequently the putative responsible mutations were identified into non-common SNPs. Among the SNP candidates underlying the AsA<sup>+</sup> phenotype, one appeared highly relevant, being a 5'UTR variant in SIGGP1 which encodes the key enzyme of the L-galactose pathway, namely the GDP-L-galactose phosphorylase. To definitively discriminate the candidates and identify the mutation associated to the AsA<sup>+</sup> trait, a fine mapping was performed using more precise genetic markers within GGP locus (Fig. 3C). Finally, this last analysis confirmed the association of the EMS-induced SNP in SIGGP with the AsA<sup>+</sup> trait, and allowed definitively to reject the others candidate SNPs. The mutation corresponded to a single C to T transversion, resulting in a change at the second nucleotide position of a serine codon TCC to a phenylalanine codon TTC in an upstream open reading frame (uORF)(Fig 3D). Recently, in *Arabidopsis* the same uORF has been described to act as a repressor of AtGGP1 (VTC2) at the translational level, and its activity was hypothesized to be regulated by the AsA level (Laing *et al.*, 2015). Accordingly, the tomato SluORF-GGP1 was selected as the gene responsible of the AsA<sup>+</sup> phenotype of the P17C5-3 mutant.

To definitively validate this mutation, a new CRISPR-based technology that was recently released for plants was carried out using fusion of CRISPR-Cas9 and activation-induced cytidine deaminase for point mutagenesis at genomic regions specified by single guide RNAs (sgRNAs) (Shimatani *et al.*, 2017) (Fig. 4A). Such an approach had three aims: (1) to induce the mutation in the original genetic background, the MicroTom WT parent; (2) to discard all the EMS mutations existing in the P17C5-3 plant, particularly a possible



**Figure 4: AsA content in developing fruits and illustration of plant morphology from CRISPR Cas-9 cytidine deaminase mutants and WT plants.**

**A.** *SluORF-GGP1* is illustrated upstream to *SIGGP1* CDS. The sequence (dashed arrow) in the uORF domain was the target sequence for the CRISPR-Cas9 construct. **B.** Here is shown for T1 line 9-1 the induced mutation detected in the *SluORF-GGP1* gene at the targeted base, compared to WT and original P17C5-3 mutant. **C.** AsA was assayed in red ripe fruits from three T1 line 9. **D.** Illustration of plant phenotype observed in T2 homozygous (two left plants) and homozygous WT-like (two right plants) CRISPR lines 9-1.

locus related to the parthenocarpy and linked or not to the AsA+ phenotype; and (3) to reduce the complexity of the genetic characterization being dependent on the use of the OC1F2 progeny plants P17C5xM82. Indeed, at this stage nothing could refute the hypothesis that the parthenocarpy-like trait of P17C5-3 mutant fruits was caused by another SNP in a locus genetically linked to that identified in the SluORF-GGP1.

About 20 T0 lines were generated, and after discarding those showing ploidy disorders, 10 were sequenced. According to Shimatani and co-workers (2017), the use of this new CRISPR technology in tomato resulted in a low DNA substitution efficiency (~10%). Consequently, only three T0 lines numbered 4, 5 and 9 were kept (Supplemental table 1). Besides, the plant morphology of those T0 lines was similar to the WT. The genotyping for the SluORF showed that only line 9 carried a clear heterozygous mutation. For lines 4 and 5 the sequencing data did not allow us to conclude with certitude. This was essentially due to the low efficiency of the system CRISPR as the mutation clearly appeared to be chimeric. The induced mutation resulted in a double nucleotide C conversion to T at position 2 and 3 of the serine codon TCC to phenylalanine TTT codon, whereas in the original P17C5-3 mutation was TTC (Fig. 4B). Then, 12 T1 plants for each were cultured and analysed for their fruit AsA content (Fig. 4C; Supplemental Table 1). Plants T1 displaying a four-fold increase of AsA content compared to the WT, lines 4-10, 9-1, 9-4 and 9-8 were confirmed as heterozygous and we decided to continue to the next T2 generation only with the line 9. For line 9-1, 12 T2 were cultured, and we observed that about 50% (5/12) displayed dwarf and bushy phenotype, severe alteration of the truss, additionally malformation of the flowers, and consequently no fruit developed. The others 9-1 T2 plants developed normally and produced seeded fruits. The same phenotypes and in the same percentages were observed in lines 9-4 and 9-8. The genotyping of the most affected 9-1 T2 plants revealed that they were homozygous mutants for the SluORF, whereas the others plants were either heterozygous (2/12) or homozygous WT (5/12). The absence of fruits in these homozygous T2 plants from line 9-1 seemed to demonstrate that the CRISPR-based mutation induced in the SluORF-GGP1 gene resulted as in the P17C5-3 mutant, in the alteration of the flower development and so the fruits. Additionally, these homozygous T2 plants displayed a bushy phenotype of the vegetative part (Fig. 4D). However, at this stage we cannot rule out any off-target mutation even this is very unlikely knowing that the frequency of such an events was determined by Shimatani et al. (2017) to be between 0.14 to 0.38% in Tomato.

**Higher AsA accumulation disturbs the reproductive system of the plant**

In parallel, since the first P17C5-3 mutant, we paid attention about the question of the origin of the parthenocarpy-like phenotype. To assess the question of the link between AsA and this trait observed in both CRISPR and EMS mutated lines, all the plants from the OC1F2 population were phenotyped and the AsA content was compared to absence/presence of seeds (Supplemental Fig. 2). The results confirmed that tomato fruits tend to be incompetent to produce seeds when they accumulate high AsA level. In addition, it appeared that in these AsA+ fruits the few seeds contained were unable to germinate implying a problem of fertility related to ascorbate. All along the characterisation of this mutation, the different crosses carried out brought clues about the type of sterility. From the parental P17C5-3 mutant flowers, the fertilization with pollen from the wild-type parent MicroTom succeeded to generate the BC1F1 population. Likewise, interspecies crossing using WT M82 pollen resulted in the generation of OC1F1 seeds. Conversely, all the attempts in the pollination of M82 and MicroTom WT flowers with Sluorf-GGP1 mutant pollen never made possible to generate offspring. These observations suggest that the seedless fruit phenotype originated from male sterility problems. By definition, parthenocarpy consists in the development of seedless fruits from flower that has not been fertilized. To test this option, flowers at the pre-anthesis stage from several OC1F2 AsA+ mutant plants were emasculated. Since no fruits developed from those flowers, we could predict that the present phenotype observed is not real parthenocarpy. Analysis of the expression of a selection of known parthenocarpy-related genes during flower development in two homozygous OC1F2 P17C5xM82 mutants showed no significant difference in comparison with the M82 WT flowers (Supplemental Fig. 3), and thus confirming our hypothesis. On the contrary, genes related to the pollen development displayed more significant changes as a function of developmental stage of the flowers (Supplemental Fig 3). Preliminary analyses showed that pollen from flowers of the original P17C5-3 mutant (Fig. 1) as well as from homozygous OC1F2 P17C5xM82 mutants displayed a low viability and germination rate. Taken as a whole, these data suggested that the parthenocarpy-like phenotype observed in the fruits of the P17C5 mutant plants was definitively not parthenocarpy but more likely related to a wider problem of male sterility which was probably caused by an alteration of pollen development. At this stage of the work, histological and cytological analyses of the flowers as well as of the pollen grain development are necessary to decipher precisely the origin of the male sterility and its possible relation with the high AsA level in the plants.



## Discussion

Recently thanks to a forward genetic strategy, combining the screening of an EMS tomato population with mapping by sequencing allowed identifying new regulators participating in various physiological traits of the fruits, like its size, color, and antioxidant potential, namely here fruit ascorbic acid (AsA) content. About the AsA trait, 5 mutant families producing AsA-enriched fruits 2 to 5 times higher than WT fruits have been isolated. For one of them, the genetic characterization was achieved exposing the photosensor SIPLP as a negative regulator of AsA content through its interaction with the key enzyme of the L-galactose pathway, *SIGGP1* (Deslous et al., unpublished data).

The genetic characterization of a second AsA-enriched mutant that was 5 to 10 fold richer than the WT led to identify a new regulatory system in which, unexpectedly, *SIGGP1* was the target. The mutated protein responsible of this remarkable AsA+ phenotype was a uORF which has already been described by Laing et al., (2015). These authors hypothesized that this uORF located up-stream of the *GGP1* gene would control the AsA biosynthesis by acting on the translation level of the *GGP1* protein. Similar to riboswitch mechanism (Bocobza et al., 2007), the uORF is directed by a non-canonical initiation site consisting of a Kozak sequence (i.e. a purine at 23 and a G at +4) (Simpson et al., 2010). It would encode a small peptide (60-65 amino acids) whose expression would be regulated by the ascorbate level or its precursors. So, in the case of AsA deficiency, the uORF protein would aim to initiate the setting of ribosomes at the level of *GGP1* start codon, resulting in higher amount of *GGP1* and consequently of AsA. The interaction of small metabolites with the 5'UTR of a gene in order to influence the protein synthesis is uncommon in eukaryotic cells especially in plants, whereas uORFs are highly abundant in the genomes of angiosperms (Von Arnim et al., 2014). The regulation in which translation of a CDS is slowed down or inhibited by uORFs has been studied in *Drosophila*, but their mechanisms of action and function remain poorly understood (Medenbach et al., 2011; Andrews and Rothnagel, 2014). Interestingly, the peptide sequence of non-canonical uORFs in the 5'UTR of several *GGP* genes is conserved over a wide range of plant species (Laing et al., 2015). In addition, a very similar sequence was also found in the 5'UTR of *SIGGP2* gene, the homologue of *SIGGP1* in Tomato. Given the more drastic phenotypes observed in T2 homozygous CRISPR mutants (Fig. 4D), that is a total absence of fruit even when flowers develop, we could not rule out the probability for an off-target induced by the CRISPR mutagenesis and the consequences in the mechanism of regulation. Although *SIGGP2* is much less expressed than *SIGGP1* in tomato making insignificant the potential off-target, *SIGGP2* remains relatively more expressed in flowers than in others organ (Supplemental Fig. 4). Previous studies in tomato showed that in three transgenic lines overexpressing

*GGP1* the expression of this gene did not presage the GGP enzymatic activity, but on the contrary could reduce it (Bulley *et al.*, 2012). Nevertheless, the relative high expression of a gene in a specific tissue, here the flowers, remains a good indicator of its importance in this organ. In presence of an off-target, this would not affect the expression of *GGP2* but rather that of its specific uORF independently. In a trivial way, we could predict that uORF acts on the translation of the juxtaposed down-stream gene. Today, nothing is known about the mode of action of uORFs, like a possible long-distance action on homologous proteins. Thus, we cannot exclude the hypothesis that SluORF-GGP1 could also operate on the synthesis of SIGGP2 and *vice versa* that SluORF-GGP2 on SIGGP1. Accordingly, in the P17C5-3 original mutant in which only SluORF-GGP1 is mutated, the parthenocarpy-like phenotype observed could be compensated by the uORF-SIGGP2 but in a weaker way since the low expression level of SIGGP2. Thus, if the off-target mutation of uORF-SIGGP2 is confirmed, we could imagine that the severe phenotypes observed in the T2 homozygous of CRISPR line 9 plants is the result of the mutation of uORF-SIGGP2 in addition to that of uORF-SIGGP1. This would lead to an absolute absence of control of the GGP activity and explain such phenotypes in term of flower and plant development. Such an off-target of the SluORF-GGP2, is currently investigated by sequencing the up-stream region of the SIGGP2 gene.

All the observations on pollen viability and germination in the P17C5 mutant revealed a possible role of GGP1 in the reproductive system. Moreover, the expression pattern of *GGP1* in the different tomato organs highlighted its prevalence in the flowers (Supplemental Fig. 4). Interestingly, among the two tomato *SIGME* genes which encode the enzyme that precedes GGP, silencing of the *SIGME1* gene impaired the flower development (Gilbert-Mounet *et al.*, 2016). On the reverse, the tomato knock-out *Slgpp1* mutant showed no alteration in term of fertility despite a strong decrease in AsA content (Baldet *et al.*, 2013). This suggests that it would be precisely the excessive accumulation of AsA that would be the cause of sterility problems. Moreover, this intriguing feature connecting AsA to plant reproduction events was already observed into tomato lines overexpressing *SIGGP1* (Bulley 2012). Besides, in the Arabidopsis *vtc2* mutant Caviglia *et al.*, 2018 described an impact of AsA deficiency on hormonal metabolism in a general manner. In addition, auxin treatment of tomato plants during anthesis affected pollen availability and fertility, which resulted from the inactivation of enzyme involved in AsA recycling (Tsaniklidis *et al.*, 2014; Martinelli *et al.*, 2009). Furthermore, there is no evidence that these changes in AsA metabolism are just a stress response caused by changes in the hormonal balance.

At this stage of the work, it is still too early to establish a clear scheme regarding the possible link between the AsA-enriched phenotype and the hormonal metabolism. This

must be taken with caution since the MicroTom cultivar is affected in the brassinosteroid biosynthesis (Marti, 2006; Nomura *et al.*, 2005). In our study, the results of the different combinations of crossing indicate that the observed parthenocarpy-like phenotype is not actually real parthenocarpy, since emasculated mutant flowers failed to produce fruit and among the parthenocarpy-related genes studied none were differentially expressed between mutants and WT (Supplemental Fig. 3). The impact of a deregulation of AsA homeostasis seems to impair more globally all the steps prior to the germination of a new plant, from the formation of the flower marked by bigger flowers in P17C5-3 mutant, to the development of pollen tube, its fertilization and seed production (Fig. 1). Several studies have highlighted that AsA could participate in specific processes linked to meristem activity, seeds or embryo development. Thus, the treatment of seeds with high AsA concentration can inhibit germination (Ishibashi and Iwaya-Inoue, 2006). The male sterility observed in the AsA+ P17C5 mutant is probably the result of a combination of defective processes that occur at different stages of development, in specific organs, and requiring a very accurate control in term of redox state. As an example, the pollen grain is one of those tissues very sensitive to the modulation of AsA. The pollen tube, like roots hair cells, requires the action of ROS together with calcium ions ( $\text{Ca}^{2+}$ ) and pH, to support its polar growth (Mangano *et al.*, 2016). It is tempting to suggest that a sudden modification of the redox state by the over-production of AsA is damaging to the establishment of this structure.

Many ways remain to be explored to elucidate the precise mechanisms of regulation of GGP1 activity by this uORF, and to determine the physiological role of AsA and GGP in the processes related to the reproduction of plants. Nevertheless, the mutants from the CRISPR strategy and those preserved thanks to the out-crossing in the M82 cultivar represent valuable plant materials for the study of ascorbate metabolism and its regulation. From an agronomic point of view, the genotypic dominance of the *uORF-GGP1* mutation leading to a remarkable AsA-enriched phenotype in the whole plant, and particularly in fruits, and the feasibility to transfer this AsA+ trait to any variety of commercial interest emphasizes even more this AsA regulation issue.

## Materials & Methods

### Plant material and culture conditions

The tomato (*Solanum lycopersicum* L.) genotypes used were the dwarf determinate cultivar cv. MicroTom and the determinate cv. M82. Plants were cultured in greenhouse were as described by Rothan et al. (2016) at 18°C (night) to 28°C (day) and 60% to 80% of relative humidity. In autumn and winter seasons extra light was supplied to the plants with LED 100W spots (Samsung). Flowers were regularly vibrated to ensure optimal self-pollination and therefore normal fruit development.

### Cartography

A mapping F2 population of 144 plants was created by crossing the P17C5-3 Micro-Tom AsA+ mutant with WT plant from the M82 cultivar. Plants from the mapping population were grown in greenhouse in the conditions indicated above. Two bulks were then constituted by pooling 33 plants displaying either a mutant fruit phenotype (AsA+ bulk) or 30 plants with a wild-type phenotype (wild-type-like bulk). A bulk segregant analysis was first performed in order to identify the responsible locus. Primer sets of 48 SNP markers well spread along the genome and displaying polymorphism between the 'Micro-Tom' and the 'M82' (Shirasawa et al., 2010) were chosen for genotyping each plant. Genotype and phenotype data were analyzed using MapMaker/Exp v3.0 (Lander et al., 1987), MapMaker/QTL v1.1 (Paterson et al., 1988), and QTL Cartographer (Basten et al., 2002) software. Since, the locus was identified in the q arm of the sixth chromosome, a classical mapping-by-sequencing strategy was set up to define the causal mutation. Like the mutation was defined as dominant, it was identify in the non-common SNPs from AsA+ bulk. Once the putative causal mutation was detected using the mapping-by-sequencing procedure, the EMS-induced SNPs flanking the putative mutation were used as markers for genotyping the BC1F2 individuals using a KASP assay (Smith and Maughan, 2015). Specific primer design was performed using batchprimer3 software (Smith and Maughan, 2015; <http://probes.pw.usda.gov/batchprimer>), and genotyping was done using KASP procedures (LGC Genomics).

## **CRISPR Cas9-cytidine deaminase**

CRISPR/Cas9 mutagenesis and tomato cotyledons was performed as described in Bollier *et al.* (2018) except that the level 1 construct pICH47742:2x35S-5'UTR-hCas9(STOP)-NOST was replaced by pICH47742:2x35S::Cas9n which was subcloned from pDicAID\_nCas9-PmCDA\_NptII\_Della (Kondo, addgene #91694). The L1 construct was assembled into the L2 pICSL4723 (Addgene plasmid #86172).

## **Ascorbate assay**

Mature leaves, and fruits at several stage of development were harvested at 12 am UT and thoroughly frozen in liquid nitrogen. Ascorbate content was measured according to Stevens *et al.* (2006) using 30 mg FW and 100 mg FW for the leaves and fruits, respectively

## **Pollen observation**

Pollen germination was analysed according to the previously described protocol with some modifications (Hernould *et al.*, 1993). The buffer for germination is composed of 3.75 ppm boric acid, 5% sucrose and adjusted to pH 5.7 (KOH). The flowers are dissected to remove the anther cone and the anther is cut to release the pollen onto the buffer solution in 24 well plate (Thermo Fisher Scientific). The plate is kept for 4 hours at the greenhouse temperature (~25°C) in the dark to promote germination. The pollen germination rate was estimated by counting the number of grains harboring growing pollen tubes under an inverted microscope (Olympus, IM).

## **Gene expression analysis**

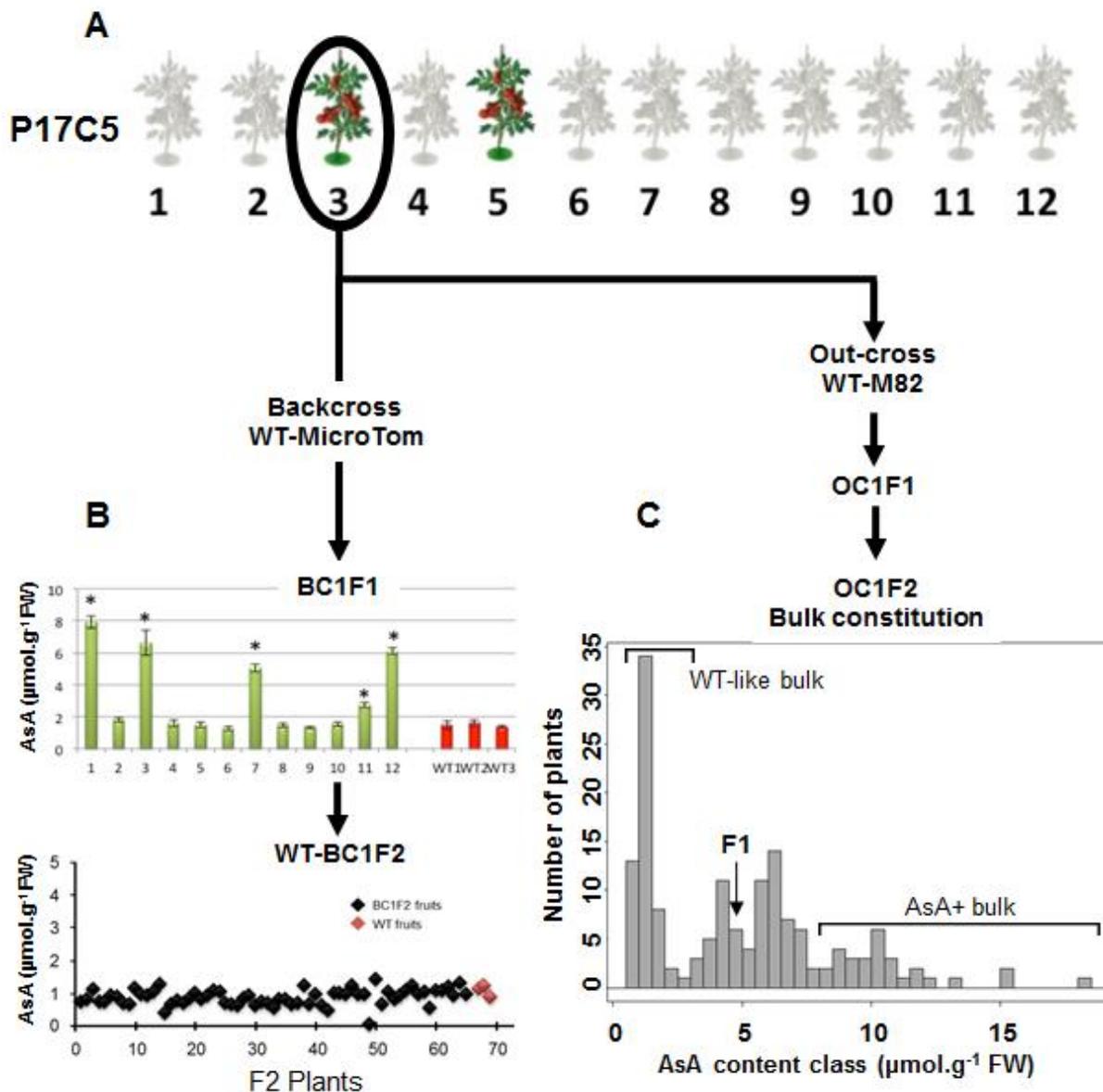
Total RNA was extracted from leaves using an RNeasy Plant Mini Kit (Qiagen). The cDNA was synthesized from 1 µg of total RNA using X. The cDNA was 10-fold-diluted with RNase-free water, and 1 µl of diluted cDNA was used as a template for quantitative and semi-quantitative PCR analysis. The qPCR was performed using a Thermal Cycler Dice Real Time System TP800 (Takara-Bio Inc.) with SYBR Premix Ex Taq II (Takara-Bio Inc.). The reaction was performed in a 20 µl volume as follows: 95°C for 10 seconds for initial denaturation, followed by 40 cycles of 5 seconds of denaturation at 95°C, and 30 seconds of annealing/extension at 55 or 60°C depending on the oligos. The expression level of each gene was normalized to the expression of reference genes (SAND, actin, α-tubulin), which were used as an internal control. The primer sequences and annealing temperatures used in the experiment are shown in Supplementary Table 3.

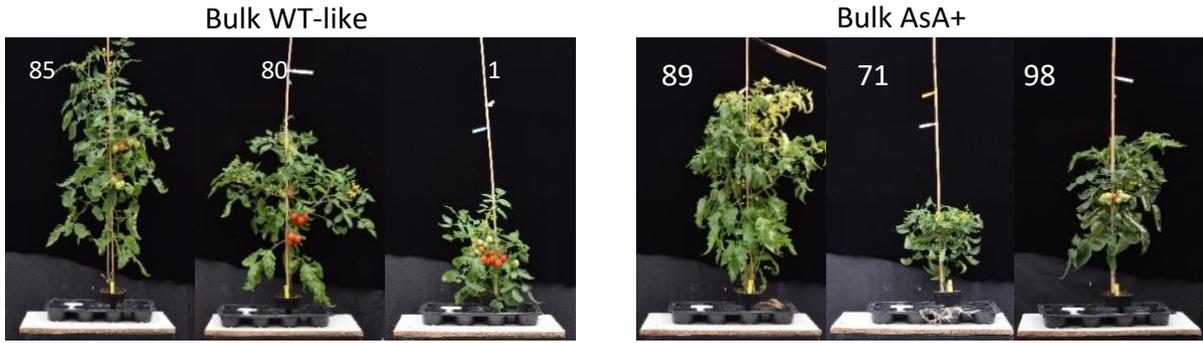


## Supplemental

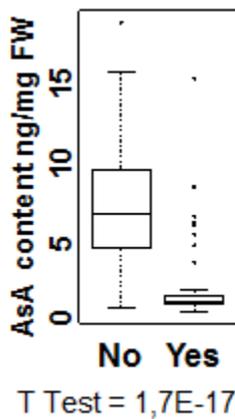
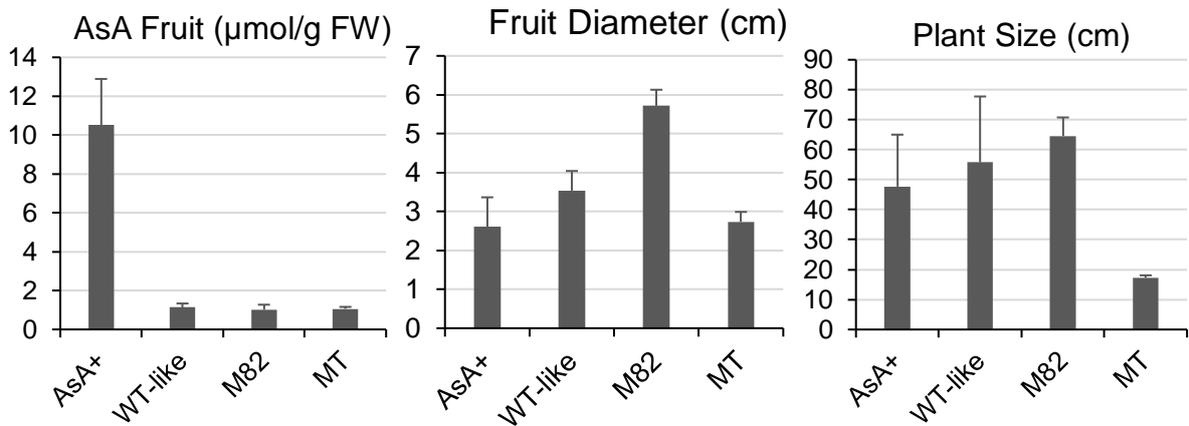
**Supplemental Figure 1:** Identification of the *uorf* mutation responsible for the AsA-enriched fruit phenotype by Mapping-by-sequencing.

**A.** 12 M2/M3 seeds were sown for the P17C5 mutant family. Among them only 2 plants were studied (colored). Among 12 plants, those displaying an absence of fruit or a delayed maturation were not analyzed (grey). Mutant plant n°3 displaying a strong AsA+ phenotype (5  $\mu\text{mol.g}^{-1}$  FW) was preserved. The P17C5-3 mutant was back-crossed with the WT parent, all the AsA+ BC1F1 plants (n=5) showed a parthenocarpic-like phenotype. Thus, the P17C5-3 mutant was out-crossed (OC) with a WT M82 cultivar. **B.** Seeds from several fruits of the BC1F1 plants that displayed a WT-like AsA phenotype were used to generate a BC1F2 segregant population. Among the fruits of 65 BC1F2 plants (Black Square) none displayed the AsA+ phenotype, their AsA level was equivalent to the WT fruits (red square). **C.** The OC1F2 progeny (144 plants) was screened for AsA+ fruits (approximately ~55% of the plants) and WT-like fruits to constitute the two bulks: the wild-type-like bulk (33 plants) and the AsA+ bulks (30 plants). For each assay, three fruits of individual plants were pooled and analyzed in technical triplicate.





Development of BC1F1 fruits from P17C5-3 x M82



**Supplemental Figure 2:** Phenotyping of the OC1F2 population P17C5-3 x M82.

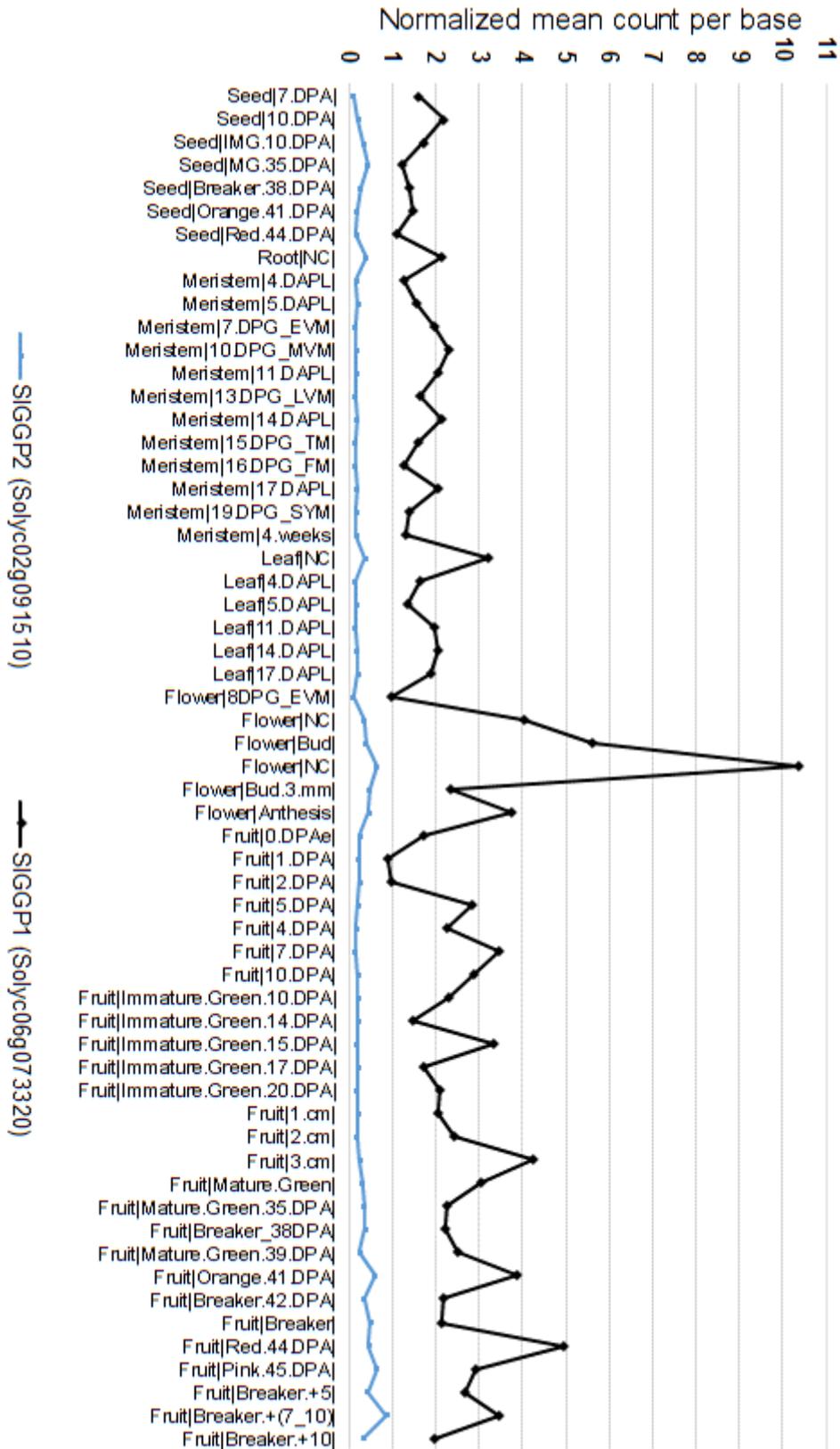
Illustration of the plant growth diversity of the two bulks from 144 OC1F2 plants.

Fruits development pattern for OC1F2 MTwtXM82wt and OC1F2 homozygous P17C5-3xM82wt.

In the OC1F2 P17C5-3xM82wt.population (144 plants), fruit diameter, plant height and AsA content were measured for each bulk AsA+ (30 plants) and WT-like (33 plants) as well as in the MT (n=4) and M82 (n=4) WT plants. In the graph are represented the mean ( $\pm$ SD) of all fruits, plants and AsA content of each bulk and control.



**Supplemental Figure 4:** Comparison of the expression patterns for *SIGGP1* and *SIGGP2* genes in tomato organs including meristems, seeds, leaf, developing flowers, and fruits at several stages of development provided by TomExpress online database at <http://tomexpress.toulouse.inra.fr/>



**Supplemental Table 1:** AsA content in rep ripe fruits of T1 CRISPR uORF plants

| T1 line 4 |                    | T1 line 5 |             | T1 line 9 |                    | WT  |             |
|-----------|--------------------|-----------|-------------|-----------|--------------------|-----|-------------|
| 4-1       | 0.88 ± 0.06        | 5-1       | 0.99 ± 0.03 | 9-1       | <b>2.54 ± 0.25</b> | WT1 | 0.63 ± 0.05 |
| 4-2       | 1.38 ± 0.25        | 5-2       | 1.50 ± 0.10 | 9-3       | 1.01 ± 0.11        | WT2 | 0.68 ± 0.12 |
| 4-3       | 0.96 ± 0.05        | 5-3       | 0.83 ± 0.16 | 9-4       | <b>3.31 ± 0.14</b> | WT3 | 0.93 ± 0.09 |
| 4-4       | 1.14 ± 0.08        | 5-4       | 1.14 ± 0.05 | 9-5       | 1.27 ± 0.00        |     |             |
| 4-5       | 1.45 ± 0.12        | 5-5       | 0.71 ± 0.07 | 9-8       | <b>3.55 ± 0.05</b> |     |             |
| 4-6       | 1.14 ± 0.22        | 5-6       | 0.84 ± 0.09 | 9-9       | 1.01 ± 0.09        |     |             |
| 4-7       | 1.49 ± 0.16        | 5-7       | 0.91 ± 0.09 | 9-10      | 1.46 ± 0.07        |     |             |
| 4-9       | 1.02 ± 0.04        | 5-8       | 0.62 ± 0.03 | 9-11      | 1.12 ± 0.16        |     |             |
| 4-10      | <b>2.60 ± 0.09</b> | 5-9       | 0.79 ± 0.10 | 9-12      | 1.46 ± 0.09        |     |             |

AsA was assayed in red ripe fruits of T1 lines and compared to the WT plants. Data are the means ± SD of four fruits. The average of the WT was 0.75 ± 0.16, and only T1 plants whose fruits displayed an AsA content 3 to 4 times (in bold) that of the WT were selected to continue to the T2 progeny.

| T2 lines | genotype   | AsA (μmol.g <sup>-1</sup> FW) |
|----------|------------|-------------------------------|
| 9-1#1    | hetero Mut | 2.47 ± 0.21                   |
| 9-1#2    | homo Mut   | nd                            |
| 9-1#3    | homo WT    | 1.01 ± 0.08                   |
| 9-1#4    | hetero Mut | 2.64 ± 0.15                   |
| 9-1#5    | homo Mut   | nd                            |
| 9-1#6    | homo Mut   | nd                            |
| 9-1#7    | homo WT    | 0.67 ± 0.01                   |
| 9-1#8    | homo Mut   | nd                            |
| 9-1#9    | homo WT    | 0.84 ± 0.09                   |
| 9-1#10   | homo WT    | 0.69 ± 0.04                   |
| 9-1#11   | homo Mut   | nd                            |
| 9-1#12   | homo WT    | 0.77 ± 0.18                   |

AsA was assayed in red ripe fruits of T2 lines and compared to the WT plants. Data are the means ± SD of four fruits. The average of the WT was 0.95 ± 0.06. nd means that no fruit was produced on the plant.

**Supplemental Table 2:** List of primers for qRT-PCR

| <b>Gene ID</b>                          | <b>Forward Primer Sequence (5'→3')</b> | <b>Reverse Primer Sequence (5'→3')</b> |
|---|--|--|
| <b>Auxine-related genes</b>             |  |  |
| ARF8                                    | AGGAAGTAATAATTCATTGAATATC              | TTAGTTGTGACTCTGTAAATTTTG               |
| ARF7                                    | GCCAAGATGTGCCAACGA                     | AAGCTGTCCCTCAATCCAAAC                  |
| ToFZY1                                  | GGATACAAAAGCAATGTGCCTTC                | TCCATTTTCCCCTTTCCAACC                  |
| SIGH3-like                              | TGTGACATAGTCCCAGTAACAATAACATCG         | TTGAAATGGAATGTAGTAAAGAGTCATGGA<br>AAGG |
| <b>GA-related genes</b>                 |  |  |
| GAST1                                   | CAACAACAGAGAAATAACCAAC                 | TTATACGATGTCTTTGAACACC                 |
| TAP3                                    | TGCAAGAGCAGCTAAGGAAGC                  | CCACATTTTCCATGAGTTCTTCC                |
| <b>Pollen development-related genes</b> |  |  |
| SISES                                   | AGCCTTTGCTTCACTCATAACAGTT              | ACTCATCATCGTTGCTTCATTCTC               |
| EMS1/EXS-like                           | TTTCGCGAACTGTTCAAGCC                   | ATTGTTGCCGAGTGAGATCCC                  |
| TAG1                                    | GCAGAAGAGGGAAGTTGATTTACAC              | GTCTAGGGTAATGGTTGTTGGTTTG              |
| SIINO                                   | TACCCCAACATGACTCACAAGC                 | TTGCTATCTCTTGGGACCATGG                 |

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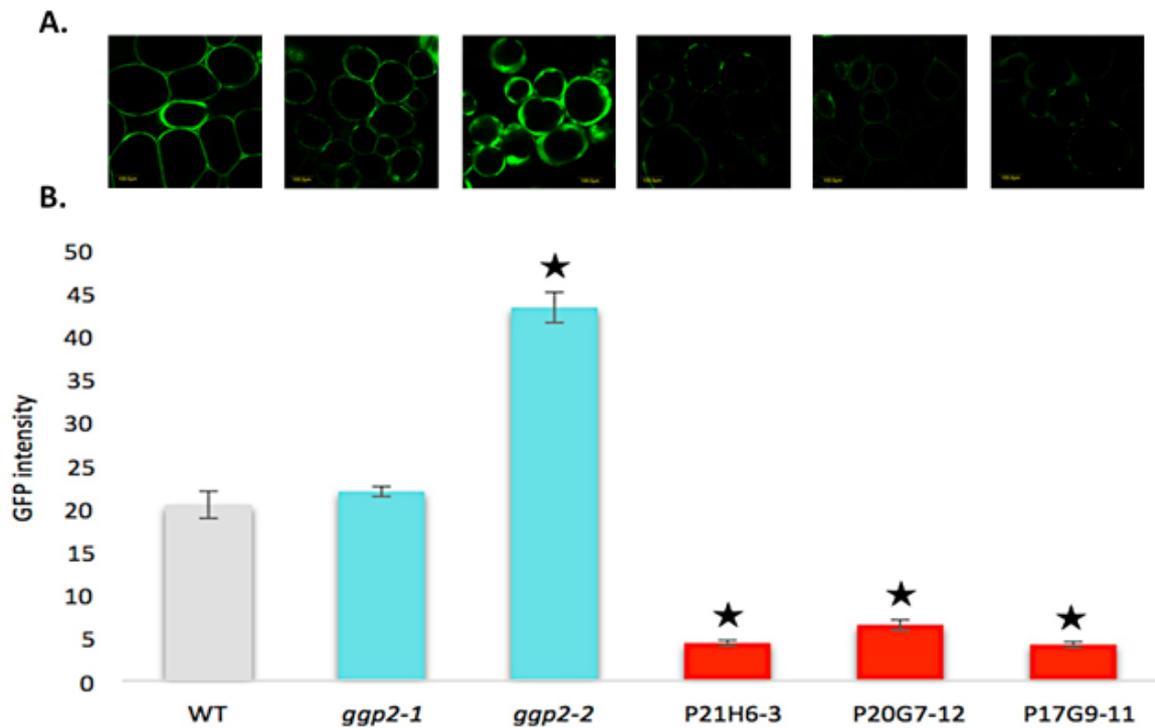
# **PART 3: CHILLING STRESS**

## **ANTIOXIDANT POTENTIAL IN POSTHARVEST FRUIT QUALITY.**

### **Introduction**

Tomato (*Solanum lycopersicum*) is one of the most important crops used as fresh vegetable as well as for a variety of processed products such as juice, ketchup, sauce, paste, etc. During the ripening process, several important changes take place in the fruit structure and its metabolic content, among which the carbohydrate accumulation, the acidity, the pigment biosynthesis, the production of flavour and aromatic compounds and also variations in the accumulation of several antioxidants. Among the antioxidants, total phenolic, flavonoid and ascorbic acid (Vitamin C; AsA) contents increase during the ripening phase (Hunt and Baker, 1980; Senter *et al.*, 1988; Buta and Spaulding, 1997; Cano *et al.*, 2003). However, many tomato fruit varieties for fresh consumption are harvested before complete ripening when fruits are still firm and turning. Therefore, extended shelf-life has been and is still an important issue in Tomato breeding program. Indeed, during postharvest period, fruits are usually stored at low temperatures and/or in high CO<sub>2</sub> atmosphere, resulting in the slowing down or the arrest of its maturation metabolism. In addition to those treatments, sometimes fruits can be exposed to exogenous ethylene to induce colour change and ripeness and this is performed usually before being delivered to the fresh food market (Kalt *et al.*, 1999). It has been shown that such low storage temperatures impair the fruit ripening and so its final quality (Javanmardi and Kubota, 2006). Today, special recommendations regarding the storage and the duration, are established for fruits and vegetables to maintain and preserve their health and nutritional traits (<http://www.fao.org/>). For example in Tomato, fruits harvested at fully mature stage also called red ripe can be stored between 7 to 10°C for 4 to 7 days. When tomato fruits have been harvested at turning stage, which corresponds at 6 to 8 days before red ripe stage depending on the cultivar and the culture season and conditions, they can be stored at 13 to 21°C for 1 to 3 weeks.

Fruit storage below a critical temperature induces the appearance of chilling injury (or stress) which corresponds to physiological disorders triggered by such exposure to low temperatures (ca. < 10°C). The severity of chilling stress depends on several intrinsic

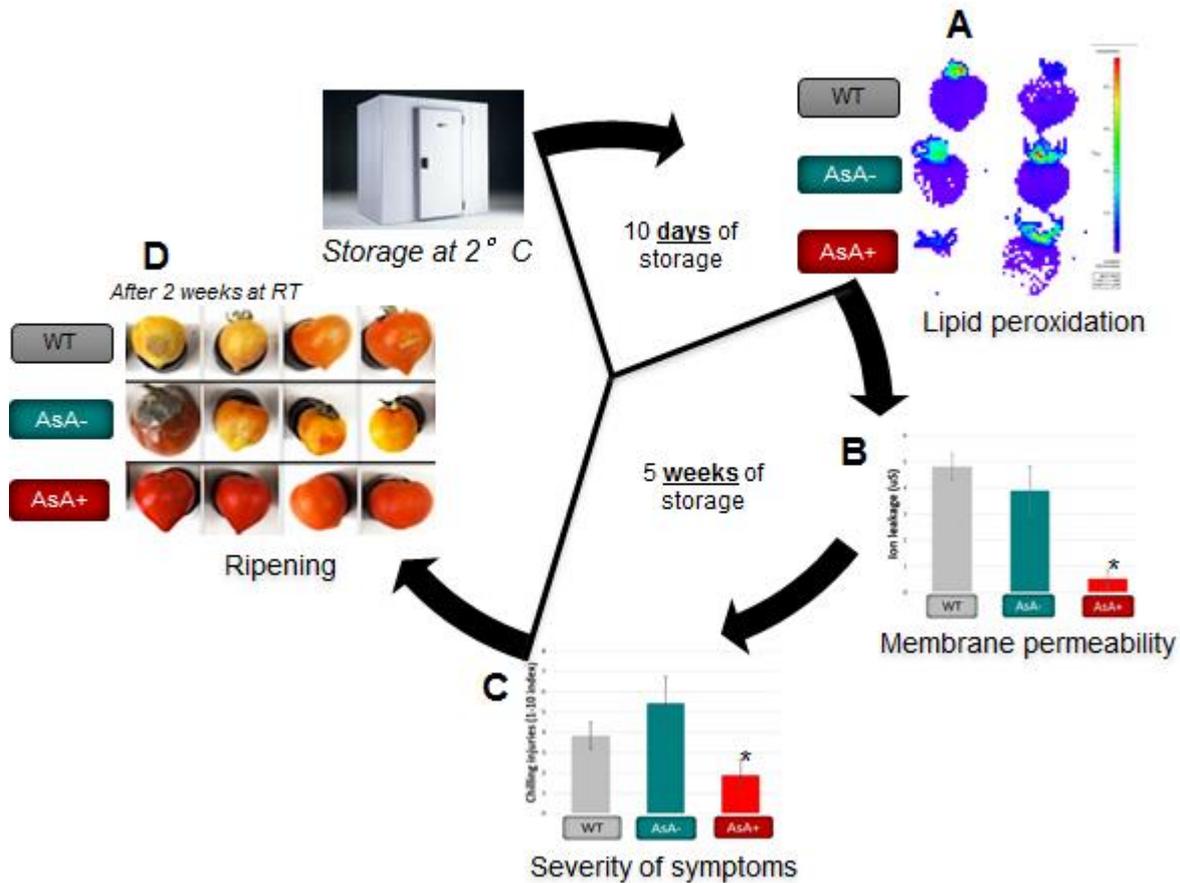


**Figure 1:** Antioxidant potential in mutant lines. A. Confocal Imaging of fruit cell pericarp after wounding stress. In green, fluorescence emission of dichlorofluorescein (DCF) exited under blue light. B. Graph showing the means of GFP intensities ( $\pm$ SD; n= 10) asterisks indicate significant differences between lines (ANOVA: Dunnett test,  $P < 0.05$ ). P21H6-3 (SIPLP mutant), P20G9 and P17G9 are three AsA+ lines from the screening of the EMS mutant collection. (From C. Bournonville thesis, 2015)

factors (e.g. cultivar, culture conditions, and exposure to stress) and extrinsic factors (e.g. temperature, duration of exposure, surrounding relative humidity, sanitation and level of mechanical injury). Chilling stress can be induced after the harvest of the fruits, during transportation, storage or once fruits are displayed at the market. Once returned to ambient temperature fruits may display various symptoms like arrest of the ripening process, pitting, water soaking, and increase of disease susceptibility (Luengwilai *et al.*, 2012). So when the generation of ROS exceeds the capacity of the tissue/cell to maintain a cellular redox homeostasis this results in oxidative stress that can be associated with the appearance of chilling damage in fruits (Hodges *et al.*, 2004). This oxidative condition and the ROS produced may have a negative impact at the level of membrane integrity, also for proteins and finally DNA. To prevent such deleterious effects, plants have developed several defence mechanisms mainly based on the setting of antioxidant compounds. Tomato fruits possess a high antioxidant potential which comprises a large variety of lipophilic compounds like its specific pigment lycopene, and also  $\beta$ -carotene, the tocochromanols (Vit E) and hydrophilic compounds like phenolic acids, flavonoids, and of course ascorbic acid (Vit C).

Currently, many cultivars have been selected by breeders for traits such as fruit yields, colour and shape uniformity, appearance, firmness, resistance to biotic and abiotic stress, and extended shelf-life (Shewfelt, 2000). However, these traits are usually not linked even opposed to nutritional and health criteria (Stevens, 1986). Moreover, this breeding strategy was encouraged by the fact that giving satisfaction to consumers appeared tricky since their requirements remained always conflicted. Hence, even taste was given high importance, fruits displaying a poor appearance but a good taste/flavour will not be chosen (Beckles, 2012).

Among the water-soluble antioxidant compounds, ascorbic acid is one of the most abundant, which is present in all the organs and cell compartments, and can accumulate to high levels in the fruits, like in some exotic fruits (see Introduction chapter). Several works have shown that changes of AsA metabolism impact the response of the tomato fruits submitted to chilling stress. Thus, the increase of MDHAR activity, an enzyme involved in the AsA recycling by reduction of its mono-oxidized form to its active and reduced ascorbate, induced a diminution of tomato firmness after chilling stress (Stevens *et al.*, 2008). In the same manner, pepper fruits (*Capsicum annuum. L*) treated with H<sub>2</sub>O<sub>2</sub> during storage displayed an increase of AsA content in addition to antioxidant activities such as DHAR and APX, and appeared significantly more resistant to postharvest damage (Bayoumi, 2008). So, fruits biofortification by increasing its antioxidant potential, and so its AsA content, can be relevant. In the case of Tomato this is all the more true with recent changes of consumer expectations for a product with healthy and nutritional values in

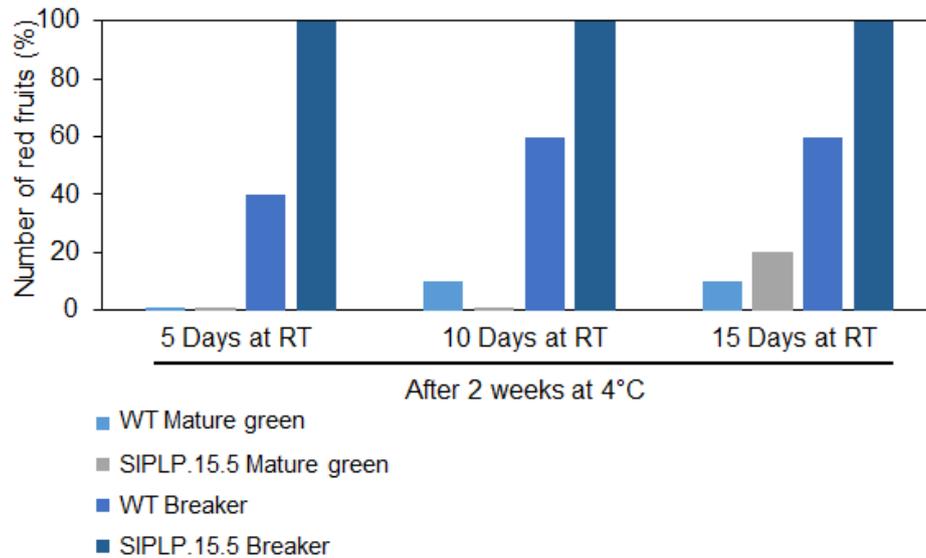


**Figure 2: Postharvest fruit quality of AsA+ mutants, resistance to chilling stress.** Susceptibility of mature green fruits stored at 2°C for five weeks. **A.** After 10 days of storage at 2°C the luminescence is quantified using IVIS (In Vitro Imaging System). Luminescence reflects intensity of lipid peroxidation. Here is shown only *p21h6-3* fruits, named as AsA+. **B.** Ion leakage of fruit after five weeks of storage at 2°C. ( $\pm$ SD; n= 15 fruits) Asterisks indicate significant differences between lines (ANOVA: Dunnett test,  $P < 0.05$ ). **C.** Chilling injury is estimated using chilling index. A number comprised from 1 to 10 was given for each fruit analysed according to the severity of the symptoms. ( $\pm$ SD; n= 15 fruits) Asterisks indicate significant differences between lines (ANOVA: Dunnett test,  $P < 0.05$ ). **D.** Photos illustrating 4 MicroTom fruits displaying altered or not a ripening stage. After five weeks at 2°C, the fruits were placed at room temperature for two weeks, in order to estimate the recovering of the ripening process that was observed in terms of appearance of red colour of the fruits (From C. Bournonville thesis, 2015)

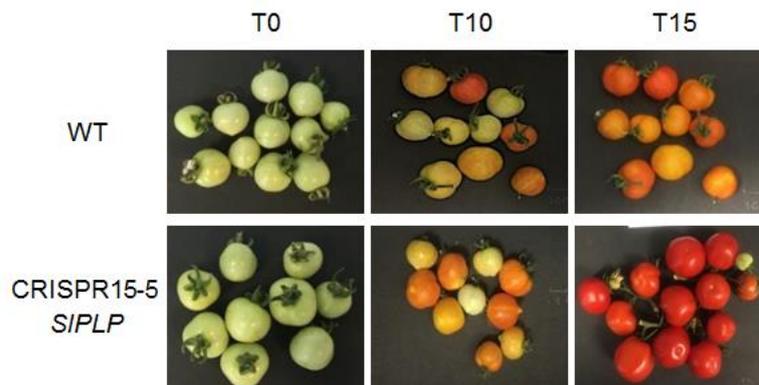
addition to taste and organoleptic traits. The biofortification of cultivated tomato with AsA represents major economic issues to improve both fruit nutritional quality and postharvest fruit resistance, which can respond to the expectations of consumers but also of the tomato producers.

As mentioned in the previous chapters, prior of my PhD work several AsA-enriched tomato mutants (AsA+) have been discovered (Céline Bournonville Thesis 2015). Besides, AsA-deficient tomato mutants (AsA-) have also been characterized (Baldet *et al.*, 2013, and unpublished data). All these mutants were used to test the hypothesis of the impact of the fruit AsA content (positive or negative) on the response to wounding and chilling stress (Céline Bournonville Thesis 2015). This preliminary study showed that AsA can play a protective role in scavenging the ROS production in the case of wounded fruits (Figure 1). Furthermore, when fruits were submitted to cold stress, the results suggested that AsA+ fruits are more resistant (or tolerant) than the AsA- and the WT fruits, as shown by the lessening of the peroxidation of membrane lipids, a better membrane permeability, and an improve capacity to ripen once the fruits returned to ambient temperature (Figure 2). However, these very promising and encouraging data needed to be confirmed as they were obtained with the original P21H6-3 mutant and using a small number of fruits.

The last part of my PhD objectives was to confirm this preliminary study. For this, we used the fruits from the CRISPR T2 line 15-5 (see chapter 1), as this line displayed only the mutation of the *PLP* gene and was at genetic level cleaner than the original EMS P21H6-3 mutant plants. Moreover, to perform this new chilling stress experiment we used a large number of fruits ( $n > 100$ ) of the AsA+ and WT plants.



**Figure 3:** Effect of chilling stress on fruit ripening. After two weeks at 4°C, mature green fruits and breaker fruits were placed at room temperature for 5 days, 10 days and 15 days. The percentage of red fruits was calculated for every series. (n=10 fruits)



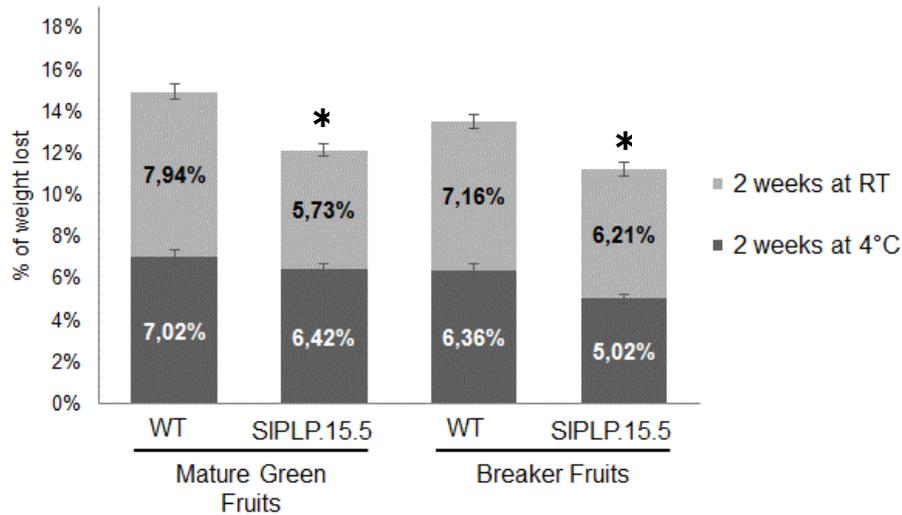
**Figure 4:** Photos illustrating the stage of maturation of fruits. After two weeks at 4°C (T0), breaker fruits were placed at room temperature for 10 days (T10) and 15 days (T15), in order to estimate the recovering of the ripening process that was observed in terms of appearance of red color of the fruits. (n=15 fruits)



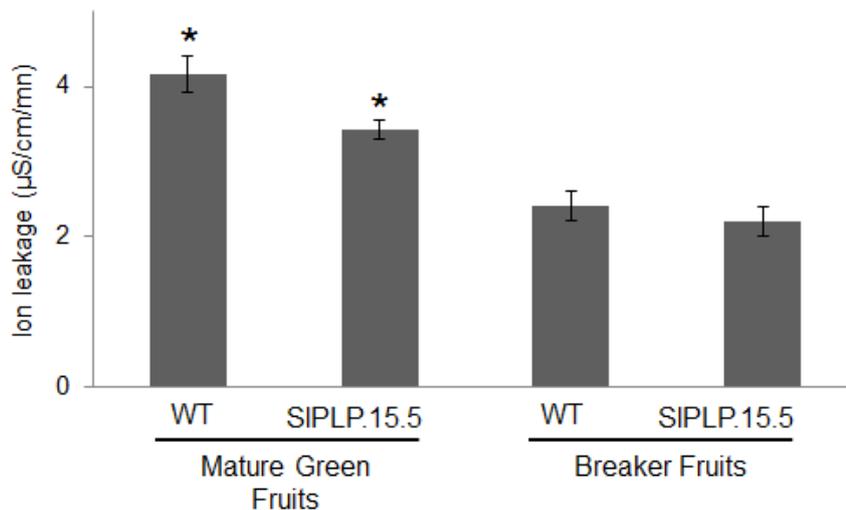
**Figure 5:** Evaluation of the fruit permeability. After two weeks at 4°C, mature green fruits were placed at room temperature for two weeks. Then they were incubated in toluidine blue 1% (W/V) for 6h at room temperature. The symbol + corresponds to the intensity of the coloring visually estimated. (n=10 fruits)

## Results

To evaluate the resistance of mutants enriched in AsA to a chilling stress, fruits at two stages of ripening, mature green and breaker stages, have been stored at 4°C during two weeks in a cold room in the dark before put back at ambient temperature (25°C) for at least 15 days. As shown in Figure 3 and 4, the capacity of the breaker fruit to restart its ripening process is better in the line 15-5 than the WT. The recovery is also quicker for the AsA+ mutant than the WT (Fig. 3). Hence, 100% of the breaker fruits of line 15-5 already reached the red ripe stage after 5 days whereas for the WT it was only 40% and reached a maximum of 60% after 15 days. For the mature green fruits the tendency is the same but to a lower extend. Damages at the epidermis level revealed by the appearance of blue spots at the fruits surface seemed to be more frequent on the WT fruits than the AsA+ fruits and must originate from alteration of the cuticle integrity (Fig. 5). In relation the membrane permeability, here at the level of fruit epidermis, the measures of water loss as well as ion leakage showed that the AsA+ fruits behave in a better after the chilling stress than the WT fruits (Fig. 6 and 7). Thus, all our results confirmed definitively previous data described (C. Bournonville Thesis) and allow to conclude that the AsA+ mutants are more resistant to chilling stress compared to WT.



**Figure 6:** Desiccation resistance. Before and after two weeks at 4°C, MG fruits and breaker fruits were weighed in order to estimate the loss in water. Then fruits were placed at room temperature for two weeks and again weighed. ( $\pm$ SD; n=10 fruits). Asterisks indicate significant differences between lines (ANOVA: Dunnett test  $P < 0.05$ )



**Figure 7:** Measure of the ion leakage in fruits after two weeks of storage at 4°C. ( $\pm$ SD; n=10 fruits). Asterisks indicate significant differences between lines (ANOVA: Dunnett test  $P < 0.05$ )

## Discussion

At the level of the global economic market the production and the commerce of plants product has massively developed these last 30 years. This has led to adapt the conditions of storage in order to supply those products almost all along the year, but also to allow long distance transportations. In that frame, cold storage conditions is very developed and adopted since the onset of the refrigerator in the life of common people. Today, cold storage recommendations have been made for almost all consumable vegetables and fruits (<http://www.fao.org/WAIRdocs/>). Since the 70's the question concerning the maintenance of the fruits quality has raised. Among the large literature on that topic, here is an example excerpted from an US Army report in 1972 (<http://www.dtic.mil/>): "Effects of pre-and post-harvest treatments such as chemical, packaging and atmosphere control on the storage of fresh fruits and vegetables have been investigated by several researchers (Salunkhe 1960, El-Mansy 1968, Watada 1964, Singh 1970). In addition, considerable research has been conducted specifically on tomato storage requirements (Magoon 1968, McCulloch 1968 Hall (1963) described the chemical and physical changes (color, firmness, placental breakdown) that take place during the storage of tomatoes."

At that time, breeding strategies started to develop to improve many traits of tomato fruits quality but that was often obtained at the expense of other traits like organoleptic and nutritional qualities. Since then, it is well documented that cold treatments induce fruits spoilage as well as loss of the nutritional qualities. The question that an improvement of the antioxidant potential of the plants, especially fruits, can have a positive impact on the post-harvest quality is relevant. Indeed, there are several works described in the literature in that sense, like for example those described for apple fruits (Davey *et al.*, 2007). In the case of actual commercial Tomato varieties, below 10-12°C corresponding to an optimum storage temperature, fruit quality can be severely impaired by chilling injury (Morris 1982). Among the physiological disorders induced by chilling temperature, disruption of cell membranes is thought to be the primary event that ultimately leads to chilling injury symptoms (Lyons, 1973; Sharom *et al.*, 1994). These damages result mainly from the peroxidation of the membrane lipids by ROS which weaken the membrane permeability. In addition, the ROS can also damage protein and nucleic acid integrity. The consequences of such damage make the fruit more sensitive to stress, including biotic and abiotic ones. Though, tomato fruit AsA content is not the most important among the others antioxidant compounds, our results tend to suggest that higher AsA level can affect positively the resistance of the fruits to chilling stress. As being a very mobile antioxidant, ascorbate present in all cell compartments could act as a first

barrier to prevent or reduce the appearance of such oxidative stress. A large number of treatments such as temperature modulation including intermittent warming, extreme atmosphere with high or low O<sub>2</sub> levels, high CO<sub>2</sub> levels, the use of chemicals like growth regulators, anti-transpiring compounds to prevent oxidative stress have been evaluated (Mari *et al.*, 1996; Toivonen, 2004; Droby *et al.*, 2009; M.A. and Mark, 2011). However, due to the variety of the mechanisms involved in the stress response, it would be unrealistic to believe that it will be easy to control especially taking into account what fruits may encounter during harvest, handling, storage and distribution.

Finally, the selection of new cultivars can be a powerful approach to improve postharvest stress resistance (Collins *et al.*, 2008). Thus, Toivonen *et al.*, (2004) showed that apple cultivars displaying high concentration of antioxidant linked to a decrease of ROS level were more resistant to browning in response to wounding. A better knowledge of fruit Biology using molecular engineering could be the key to this issue. In that aim, the biofortification of the fruits would respond to two goals, firstly by improving its postharvest physical qualities and second by the maintenance of its nutritional and healthy potential.

## Materials and Methods

### Plant material and culture conditions

*Solanum lycopersicum* L. cv. MicroTom was used for this experiment and the mutant plants corresponded to the CRISPR line 15-5 T2 (Deslous et al., in preparation). The plants (n=36) were sown on May 15<sup>th</sup> and cultured in greenhouse during spring 2018 as described by Rothan et al. (2016). The fruits used for the chilling stress were harvested in two periods in July at two stages of ripening phase, mature green and breaker. More than 100 fruits were harvested at that time in order to get data statistically valid for all the experiments planned. For each assay, a minimum of 30 mature green fruits and 30 breaker fruits from each plant WT and lines 15-5, were thoroughly stored at 4°C in the dark on trays in a cold chamber during two weeks. Then, they were placed back to room temperature (~25°C) and kept in the dark for two weeks. During and after cold storage, several parameters have been checked to evaluate the chilling susceptibility of the mutants compared to the WT.

### Evolution of the ripening

To estimate the recovery of the ripening process, the percentage of red fruits (n=15) was determined using mature green fruits (n=10 to 15) and breaker fruits (n=10 to 15) after three times T=5 days, T=10 days and T=15 days of storage at room temperature and after previously being stored two weeks at 4°C. The intensity of the red color was estimated visually by two persons independently.

### Cuticle permeability measurements

The fruit cuticle permeability was measured according to Tanaka et al. (2004) after the cold treatment and at two times T=10 days and 15 days being back to room temperature. This assay consisted to immerse the fruits (n=10) in a 1% (W/V) toluidine blue solution during 6 h. Alteration of the permeability of the cuticle was estimated by counting the appearance of blue spots at the surface of the fruits. For this experiment we only used mature green fruits since the visualization of blue spots is almost impossible once fruits are turning or ripening (Tanaka 2004)

### **Water loss measurements**

For measuring the water loss, mature green fruits (n=10) and breaker fruits (n=10) freshly collected were weighted. This corresponded to fruit fresh weight at time T=0. After the cold treatment, the same fruits were weighted as well as after two weeks of storage at room temperature. Water loss was calculated as a percentage weight loss of the fruits.

### **Ion leakage assay**

The measure of ion leakage consisted to soak each individual fruits in 100 mL of double distilled water for 1hour. The electrical conductivity was measured using a conductimeter HI 98311 (Hanna Instruments). Mature green fruits (n=10) and breaker fruits (n=10) were used after the cold treatment.



# **CONCLUSION**

The present thesis work is in line with all the projects done since more than a decade in the laboratory, on the regulation and the physiological roles associated with ascorbic acid in plants, more especially in Fruits. The plant AsA biosynthesis was established by Smirnoff and co-workers, namely the L-Galactose pathway, but little is known about regulatory mechanisms. Many environmental factors, like light and chilling stress, affect AsA levels in plants by modulating gene expressions and enzyme activities. As examples, the oxidative stress caused by light during photosynthesis and its impact for plant development, as well as at the fruits level the storage at low temperature as the most important stress related to the post-harvest phase, are the main origin of ROS production. More specifically, my PhD work was the continuation of the C. Bournonville thesis project (2012-2015), which consisted of a direct genetics approach combined with mapping by sequencing, whose purpose was to discover AsA-enriched EMS Tomato mutant lines in order to identify new regulators of AsA metabolism. Among the AsA-enriched families that emerged from the screening, two families named P21H6 and P17C5 displayed fruits with AsA-enriched phenotype that is with an accumulation 2 and 5 times higher than the WT. These two mutant families were of particular interest and therefore represent the core of the results presented here.

The mutation responsible for the phenotype observed in P21H6 was the first to be characterized. Indeed, this family was the one whose AsA content was the strongest without morphological alteration at the plant and fruit level. The mapping strategy identified a knock-out mutation in the *Solyc05g007020* gene encoding a new class of photoreceptor involved in blue light sensing named PLP (PAS/LOV Protein). The first part of my thesis work was therefore to undertake the functional characterization of this protein which has been validated as a negative regulator of AsA accumulation. Our results have demonstrated that PLP can regulate GDP-L-galactose pyrophosphorylase (GGP), the master enzyme of the L-galactose pathway, by interacting directly with GGP in both the cytoplasm and the nucleus. This interaction is modulated by light signaling, especially blue light. Blue light would act as a trigger of the processes governing the modulation of the AsA, during changes of light environment for the plant (*i.e.* clouds, canopy, sunset, sunrise etc.). Because no effector function of a molecular event has yet been identified for PLP, further experiments will have to determine the modalities and consequences of its interaction with GGP. Indeed, our assumption is that PLP would undoubtedly be part of a larger regulatory complex.

In parallel with this study, we have undertaken the genetic characterization of the second AsA-enriched P17C5-3 mutant whose fruit AsA+ phenotype was the strongest among all the EMS tomato mutants isolated. In contrast to the P21H6 line, P17C5 exhibited many morphological changes particularly related to its capacity to accumulate high AsA level, *i.e.* larger flowers, bushy growth, and above all a strong tendency not to produce fruit without seed. This fertility problem, probably related to the dominant nature of the causal mutation, necessitated the transfer of this trait by an interspecies crossing to the Tomato M82 cultivar. In addition to demonstrating the transferability of this mutation to a commercial variety, obtaining an OC1F2 MicroTomxM82 segregant population made it possible to identify the mutation responsible for the phenotypes observed in this mutant family. It corresponds to an amino acid change in an ORF upstream of the GGP gene, probably implying a decrease in function of this small regulatory peptide. Indeed, it has been shown that this uORF 5'UTR would be responsible for variation of the synthesis of the GGP protein at the translational level and this would be governed by the ascorbate level. The identified mutation was inserted in the MicroTom cultivar using a new CRISPR Cas9 deaminase strategy, and allowed to confirm the function of this ORF in the two observed phenotypes: the high AsA level and the plant developmental alterations. The high AsA biosynthesis is directly involved in the defect of pollen growth and development which meant male sterility and thus parthenocarpy trait.

Because of its strong activity as an antioxidant and as a cofactor of several reactions, AsA has often been associated with many physiological events ranging from plant growth to environmental stress responses. However, its ubiquitous functions make it very difficult to say that changes in AsA content are directly or indirectly related to these processes. Here, through the study of the regulatory mechanisms of AsA metabolism, we demonstrated that (i) light signal plays a role in the direct regulation of GGP at the molecular level, specifically blue light, (ii) the establishment of the reproductive system of plants must be dependent on a fine regulation of the AsA biosynthesis through a feedback on the GGP activity, (iii) the post-harvest quality of the tomato fruit could be easily improved by increasing its AsA content. Like other regulators such as AMR1 and EsWAX1 involved respectively in the response to light and salt stress, our results confirmed the molecular link between a signal external to the plant and the key enzyme of the AsA biosynthetic pathway, namely GGP. They allowed us to define even more precisely the essential physiological role of AsA for the correct development of the plant. Furthermore, it is interesting to observe that AsA plays a major role in response to internal signals, particularly for the establishment of the reproductive system of the plant. The homeostasis of AsA level in plants and therefore the very fine regulation of its metabolism seem all the more necessary that any deregulation has severe consequences for the plant.

For a long time, GGP has been considered a controlling enzyme, and here we confirmed its status according to the ascorbic acid biosynthesis pathway. In previous studies, to address the question of the physiological role of Ascorbate in the fruit, GGP was targeted in a targeted reverse genetic approach in order to obtain AsA-deficient mutants. The knock-out lines identified barely accumulated AsA approving the importance of GGP according to AsA synthesis. Here an exploratory forward genetic approach has been set up, so without knowing if the mutations would correspond to a protein of the ascorbate biosynthesis pathway or a new regulatory protein. Surprisingly, for the two families of AsA+ mutants characterized so far, P21H6 and P17C5, the SIGGP protein was always associated with the regulatory processes. Either because its activity was directly regulated (P17C5 mutant) or because it could act directly as a potential regulator in a larger complex (P21H6 mutant), the dysfunction of these mechanisms always led to the same phenotype, a better capacity to accumulate AsA in the plants, and so in fruits. Thus, GGP is the corner stone of the regulation of the ascorbate biosynthesis in plants, and it is certain that elucidating the functions and the regulations that surround the GGP protein would allow to go deeper in the understanding, and therefore the manipulation of the AsA metabolism in plants.

Varietal selection is an important agronomic issue, which may allow the nutritional and post-harvest improvement of species of commercial interest. In this system, ascorbate has a role to play as it seems to be beneficial for the quality of fleshy fruits. As previous studies on tomato fruit post-harvest quality suggest, decreasing the fruit AsA content has a negative effect while reversely a higher fruit AsA content improves its resistance to environmental stresses. This feature clearly raises the question about the role of AsA regarding post-harvest fruit quality and a new possible way of its improvement by modulation of AsA metabolism. Moreover, the understanding of how external signals, like light, may act to modulate directly the AsA biosynthesis at the molecular level, should allow developing new strategies to enhance AsA content in vegetables. As an example, it has been shown that exposure to light in certain conditions, allows the detached fruits to keep a stable antioxidant potential, largely represented by AsA, and thus these fruits have an extended shelf-life. Although beneficial, like any other chemical compound, AsA cannot be highly accumulated in plants. Indeed, our results showed that there must be a threshold above which an excess of AsA would be harmful for the plant. Such a positive deregulation could impaired plant sustainability in absence of fruit development. Indeed, certain physiological processes, such as pollen tube growth, require a redox status to be set up and precisely regulated. In one word: "Too much Asa kills AsA"

The forward genetics strategy applied on the EMS MicroTom population is a powerful tool that has made it possible to dig deeper into the AsA regulation mechanisms and to reveal the physiological processes with which the AsA is associated. Combined with the TILLING approach that identified ASA-deficient mutant lines, the forward approach and its AsA-enriched lines allowed collecting valuable new genetic resources for the study of AsA. Moreover, among the five AsA+ families identified during the screening of the EMS collection, 3 lines still remain to be characterized, which represent as many new tracks in the perspective of deciphering the regulation of the AsA metabolism.



# **ANNEXES**

## Primers and vectors used for GATEWAY® cloning

| • Primers used for GATEWAY® cloning        |                                  |  |  |   |                          |
|--|----------------------------------|--|--|---|--------------------------|
| Name                                       | Primers                          | Sequence (5' to 3')  |  |   |                          |
| attB1_SIGGP1_attB2                         | SIGGP1_attB1FW                   | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAIGCTCAAAAATTAAAGAGGGTTCC       |  |   |                          |
|  | SIGGP1_Stop_attB2REV             | GGGGACCACTTTGTACAAGAAAGCTGGGTGCIAGCGGGAACCATGGTACTGTGG         |  |   |                          |
| attB1_SIGGP2_attB2                         | SIGGP2_attB1FW                   | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAIGTTGACTGTAAGAGGGTGC           |  |   |                          |
|  | SIGGP2_Stop_attB2REV             | GGGGACCACTTTGTACAAGAAAGCTGGGTGICACTGCAACACCAAAACAATCTGG        |  |   |                          |
| attB1_SIPASLOVwt_attB2                     | SIPASLOVwt_attB1FW               | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAIGGAATCACAACCTGG               |  |   |                          |
|  | SIPASLOVwt_Stop_attB2REV         | GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGGAGGTACTTGCACCCATTGAC        |  |   |                          |
| attB1_SIPASLOVmut_attB2                    | SIPASLOVwt_attB1FW               | GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAIGGAATCACAACCTGG               |  |   |                          |
|  | SIPASLOVmut_Stop_attB2REV        | GGGGACCACTTTGTACAAGAAAGCTGGGTGCTGIGAAAATTGTGTGCCTCTCTGTATCTTCC |  |   |                          |
| • Vectors used in the experiments          |                                  |  |  |   |                          |
| Use  | UsualName                        | Name   | Description  | Resistance gene in bacteria                           | Resistance gene in plant |
| Gateway Cloning                            | -                                | pDONR201   | Entry vector                                       | Kanamycine  | -                        |
| Localization / Stable transformation       | GFP Ct                           | pK7FWG2  | 35S_CDS_eGFP                                       | Spectinomycine (100 µg.ml-1)                          | Kanamycine (50 µg.ml-1)  |
|  | GFP Nt                           | pK7WGF2  | 35S_eGFP_CDS                                       | Spectinomycine (100 µg.ml-1)                          | Kanamycine (50 µg.ml-1)  |
| BiFC                                       | N2                               | NeYFP/pUGW2  | 35S_CDS_nYFP                                       | Ampiciline (100 µg.ml-1)                              | -                        |
|  | N0                               | NeYFP/pUGW0  | 35S_nYFP_CDS                                       | Ampiciline (100 µg.ml-1)                              | -                        |
|  | C2                               | CeYFP/pUGW2  | 35S_CDS_cYFP                                       | Ampiciline (100 µg.ml-1)                              | -                        |
|  | C0                               | CeYFP/pUGW0  | 35S_cYFP_CDS                                       | Ampiciline (100 µg.ml-1)                              | -                        |
| • Bacterial strain used in the experiments |                                  |  |  |   |                          |
| Use  | Type                             | Name   | Medium   | Supp Antibiotic                                       | Temperature              |
| Cloning                                    | <i>E. Coli</i>                   | DH5α   |  | -   | 37°C                     |
| Transformation                             | <i>Agrobacterium Tumefaciens</i> | GV3101   | LB (Bactotryptone 1%; yeast extract 0,5%; NaCl 1%) | Gentamycine (40 µg.ml-1);<br>Rifampicine (10 µg.ml-1) | 28°C                     |
|  |                                  |  |  |   |                          |

## **Agrobacterium-mediated stable transformation of tomato**

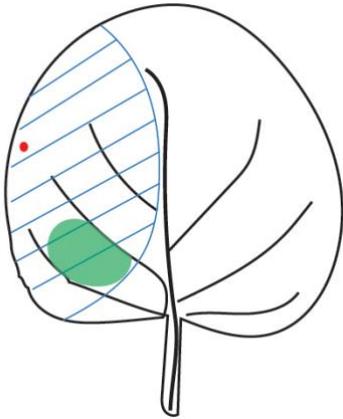
The tomato transformation is carried out through agrobacterium infection of cotyledon fragments as described in Culiañez-Macia (2004). MicroTom seedlings are grown in vitro during 7 days on MS<sub>1/2</sub> medium. Cotyledons are cut into 3 explants then laid on KCMS medium consisting in a MS medium (including vitamin) supplemented with thiamine (10mg/ml), Acetophenone (100mM), 2,4D (10 mg/ml) and kinetin (1 mg/ml) for 24 hours. The explants are then immersed for 30min in a KCMS solution containing the bacteria *A.tumefaciens* transformed with the plasmid of interest. The excess of bacterial culture is remove on sterile absorbent paper, and the explants are laid again on new solid KCMS medium for 48h in growing chamber. The explants are then cultured on 2Z selection medium (MS supplemented with Nitsch vitamins at 1x, zeatin at 2 mg/ml, timentin at 250 mg/L and kanamycin at 150 µg/mL until callus formation. The little regenerated plantlets formed from calli are transplanted into jars containing MS medium with vitamins, zeatin, a reduced concentration of timentin (75 mg/L) and kanamycin at 100 µg/mL until the appearance of roots. When roots are enough developed, plantlets are transferred in soil in mini growth chambers under controlled humidity and temperature for 2 weeks and after that acclimation phase they are normaly cultured in soil in the green house.

## Biolistic transformation of onion epidermis



To assess the interaction of *S/PASLOV* and *S/GGP1* in plant cell, BiFC (Bimolecular Fluorescence Complementation) (Walter, 2004) experiment was performed in onion epidermal cells by biolistic transformation. The cDNA of the genes of interest were inserted using GATEWAY technology in a destination vector (see table) in order to test all possible orientations of the protein fusions. Then, 2.5  $\mu\text{g}$  of plasmid DNA of each construct are mixed with 25  $\mu\text{l}$  of a suspension (250  $\mu\text{g} / \mu\text{l}$ ) of gold microparticles (diameter = 0.6  $\mu\text{m}$ ) in 50% ethanol (v/v) then 25  $\mu\text{l}$  of 2.5 M  $\text{CaCl}_2$  and 10  $\mu\text{l}$  of 0.1 M spermidine are added. The microparticles are let to sediment for 10 min before to be washed consecutively with 70% and 100% ethanol. Eight microliters of the microparticle suspension are used for transformation of epidermal onion cells using the PDE-1000He particle gun (Biorad). Before transformation, the onion epidermis is taken from the innermost scales of the bulb and deposited, upper face contact with MS medium. Transformation of onion epidermis cells is at a pressure of 710 mm Hg at a helium pressure of 1100 psi and at a distance of 6 cm. After the shooting, the epidermis was placed in the dark for 24 hours before the observation with a microscope. The onion layer is placed whole between a glass slide and a cover slip in a drop of water.

## Agroinfiltration of *Nicotiana Benthamiana* leaves



The *Agrobacterium tumefaciens* electro-competent strain GV3101, harboring binary vector, was used for transient transformation studies. The competent strains were transformed with fluorescent fusion constructs previously obtained by gateway cloning. Transformed agrobacteria were selected on LB medium supplemented with suitable antibiotic and conserved at  $-80^{\circ}\text{C}$ . Before the agroinfiltration, inoculated LB cultures were cultivated overnight at  $28^{\circ}\text{C}$  until 0.6 to 0.8  $\text{OD}_{600\text{nm}}$ . For subsequent infiltration, the cultures were centrifuged and the pellet suspended in water to reach 0.2  $\text{OD}_{600\text{nm}}$  in the case of sub-cellular localization. Then, 50-100 $\mu\text{L}$  of this bacterial solution was infiltrated at the level of a wounding by needle in the leaf epidermis using a 1mL syringe to improve infiltration. The plants were maintained in normal culture conditions (light, temperature) for 48h and the observation was carried out on the underside of the leaf epidermis.

### Observation

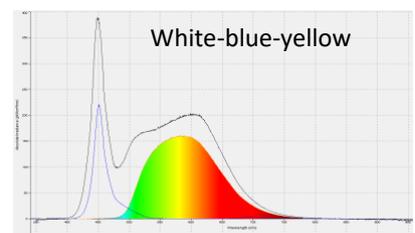
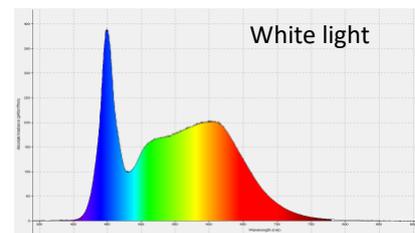
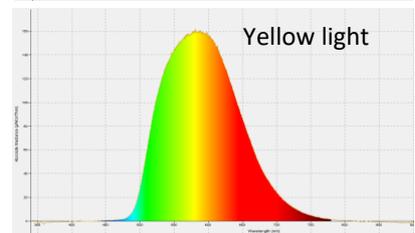
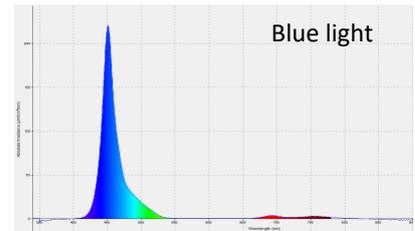
The observation is done by cutting a piece of leaf from the area between the first two ribs. *N.benthamiana* leaves samples were gently transferred between a glass slide and a cover slip in a drop of water. Live imaging was performed in the plant imaging division of the BIC platform (Bordeaux Imaging Center), using a ZEISS LSM 880 confocal laser scanning microscopy system equipped with 40x objectives. The excitation wavelengths used for the eGFP (or YFP) and mCherry are 514nm and 543nm, respectively. The emission windows defined for their observation are respectively between 525 and 600 nm for the eGFP (or YFP) and between 580 and 650 nm for the mCherry.

## Setting of the light experiment in the growth chamber.

25 plants per condition



One month-old plants cultured in the green house were placed during the nocturnal phase



in the growth chamber and let in night condition for 14h. At T<sub>0</sub>, the light was switched on. The white light was produced by 6 LED tubes (LED power 30W), the intensity measured was  $400\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at 10 cm of the source. Blue light and yellow light conditions were produced by covering the 6 LED tubes with a plastic filter Rosco supergel nightbue# 74 and Rosco supergel canary# 312, respectively. The dark condition was obtained by shading using black plastic plates (bottom shelf). The culture conditions were 16h/8h day-night at 25/20°C.

On the right part of the figure are the spectra recorded with a JAZ spectrometer at 10 cm of the light source for each light condition, the graph at the bottom represents the overlay of the three light white-blue-yellow.

## CRISPR Cas9

### Nuclease

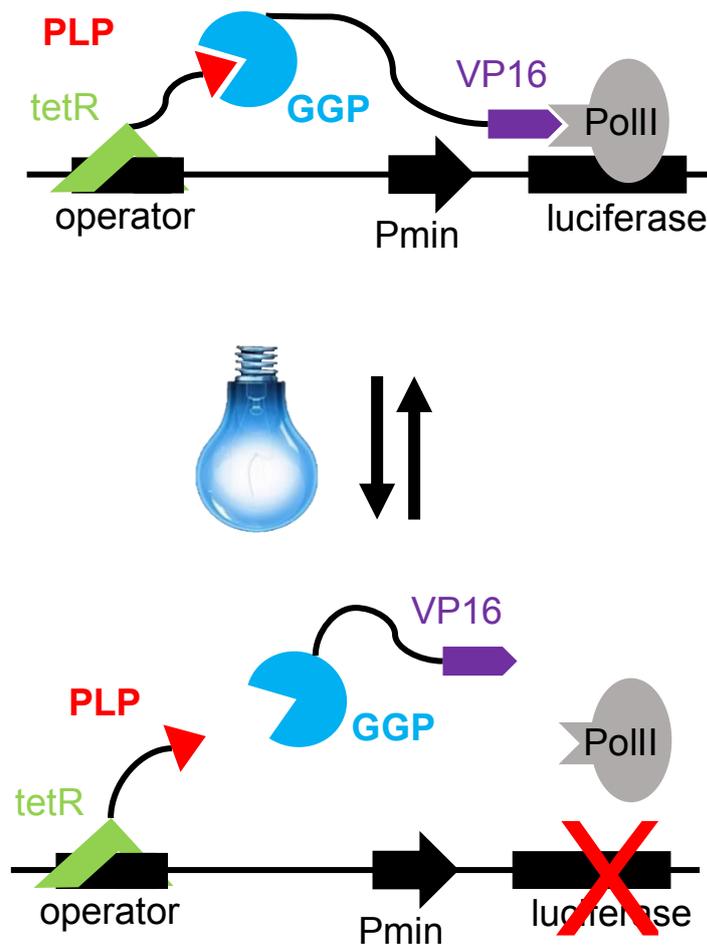
CRISPR/Cas9 mutagenesis was performed as described in Fauser et al. (2014). A construction comprising a single sgRNA alongside the Cas9 endonuclease gene, was designed to induce target deletions in *SIPASLOV*-coding sequence. The sgRNA target sequence was designed using CRISPR-P 2.0 web software (<http://crispr.hzau.edu.cn/CRISPR2/>; Lei et al., 2014). The oligonucleotide primer is annealed with its reverse complement before being inserted into pEn-Chimera by digestion-ligation. Since, targeting-RNAs are transferred into pDECAS9 vector through LR reaction, the final plasmid was used to transform tomato cotyledons through *Agrobacterium* infection. The T0 plants resulting from the regeneration of the cotyledons were genotyped and their fruits phenotyped for ascorbate content. T1 seeds from selected AsA-enriched T0 plants were sown for further characterization. The CRISPR/Cas9 positive lines were further genotyped for indel mutations using primers flanking the target sequence.

### Deaminase

For CRISPR/Cas9 mutagenesis, constructs were designed to create defined DNA substitution using a single sgRNA alongside the CRISPR/Cas9 nickase (nCas9D10A) fused to *Petromyza marinus* cytidine deaminase (PmCDA1) in order to induce point mutation in 5'UTR of *SIGGP1*-coding sequence. The sgRNA target sequence was designed using web-based tool, CRISPOR (<http://crispor.org>). All constructs were assembled using the Golden Gate cloning method (Weber et al., 2011). Level 1 constructs carrying sgRNA placed under the control of the *Arabidopsis* U6 promoter were assembled as described (Belhaj et al., 2013). Level 1 construct carrying the p2x35S::nCas9-PmCDA:TerOCS was obtained by subcloning the PmCDA from pDicAID\_nCas9-PmCDA\_NptII\_Della who was a gift from Akihiko Kondo (Addgene plasmid # 91694). The level 1 constructs were assembled into the level 2 vector pICSL4723 (Addgene plasmid #86172) as described (Weber et al., 2011).

## Optogenetic assay

In this animal cell system well described by Muller et al. (2014), similarly to the yeast two hybrid method, the *SIPLP* (WT or mutated) and *SIGGP1* as well as *SIGGP2* coding sequences were fused to either tetR (a DNA binding domain) or VP16 (an activator of polymerase II). When an interaction between the potential partners occurs, the system is completely reconstructed, leading to the activation of a polymerase by VP16 and the expression of the luciferase whose activity is assayed by the light produced.



## Ascorbic acid measurement

Mature leaves, and fruits at several stage of development were harvested and thoroughly frozen in liquid nitrogen. Ascorbate content was measured according to Stevens *et al.* (2006) using 30 mg FW and 100 mg FW for the leaves and fruits, respectively.

To evaluate total AsA content (reduced and oxidized forms), organs were ground in liquid nitrogen, the powder was then stored at  $-80^{\circ}\text{C}$ . The extractions were performed using  $500\mu\text{L}$  of trichloroacetic acid 6% (TCA). After vortexed, samples were centrifuged 25 minutes at  $12\ 000g$  at  $4^{\circ}\text{C}$ . The supernatant was recovered and ready to assay. The entire extraction step needs to perform on ice to prevent AsA degradation. In order to estimate total AsA content in the supernatant, a standard curve was realized. A concentrate solution of sodium-L-ascorbate ( $1\ \text{mg/mL}$ ) was diluted in TCA 6%. This range allow to obtain linear standard curve comprise between 0 and 1 of  $\text{DO}_{550\text{nm}}$ .

For each sample, biologic triplicates were analysed.  $20\mu\text{L}$  of supernatant (or standard) were deposited in 96 well plate ELISA and  $20\mu\text{L}$  of dithiothreitol (DTT)  $5\text{mM}$  are added to reduce the oxidized AsA. Plate was next incubated 20 minutes at  $37^{\circ}\text{C}$  under gentle stirring. Then,  $10\mu\text{L}$  of N-ethylmaleimide (NEM)  $0.5\%$  (w/v) are added to stop the reaction triggered by the DTT. After one minute at room temperature,  $80\mu\text{L}$  of colour reagent (Mix reagent A + B) was added and incubated 40 minutes at  $37^{\circ}\text{C}$ . The reduction of the  $\text{Fe}^{3+}$  contained in the reagent by AsA will lead to the formation of  $\text{Fe}^{2+}$ , which react with dipyridil that induce apparition of red colour proportional to AsA concentration. The intensity of coloration was determined by lecture of the absorbance at  $520\text{nm}$  using a Multiscan Ascent lector (ThermoFisher).

The concentration of AsA is expressed in  $\mu\text{moles.g}^{-1}\text{FW}$ . This calculation takes into account of the quantity of fresh powder, the volume of supernatant, the volume of TCA, the DO and the coefficient of the standard curve.

**Reagent A:** 31%  $\text{H}_3\text{PO}_4$  (w/v), 4.6% TCA (w/v), 0.6%  $\text{FeCl}_3$  (w/v)

Solvent: DDW

**Reagent B:** 4% 2,2-dipyridil (w/v)

Solvent: ethanol 70%



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## Abstract

Ascorbic acid (AsA, vitamin C) is one of the most important biochemical in living organisms. Due to its high antioxidant potential, AsA represents an important trait of nutritional quality in fruits and vegetables. In addition to its beneficial health value in fruit consumption, increasing fruit AsA content would likely affect postharvest quality and resistance to pathogens. Thus, understanding the regulation of AsA accumulation in order to improve crop species of agronomical interest is an important issue in plant breeding for many fleshy fruit species. To get a better understanding of the regulation of AsA level in plants and its impact on fruit quality, a highly mutagenized EMS tomato collection (cv. Micro-Tom) was screened for AsA+ fruit mutants. This forward genetic strategy combined with a mapping-by-sequencing approach, had allowed identifying new genes related to the AsA+ trait. One of the mutant line named P21H6, displayed an AsA-enrichment 2 to 4 fold that of the WT, and was the first to be genetically characterized. It allowed highlighting a new class of photoreceptor involved in blue light sensing named SIPLP as a negative regulator of AsA accumulation in tomato. We confirmed the role of the PLP in the fruit AsA+ phenotype using a directed mutagenesis strategy, undertaking its functional characterization. We demonstrate that PLP interacts with GGP (GDP-L-galactose phosphorylase), a key enzyme of the L-Galactose pathway, under blue light control and that this interaction takes place in the cytoplasm and the nucleus. Our results strengthen the central role of GGP in the AsA biosynthesis and suggest a new regulation mechanism by blue light of the GGP function in addition to its metabolic activity. Besides we started the characterization another mutant, the P17C5-3, which displayed the highest level of AsA (up to 10 times the WT). Beyond its AsA+ content, the P17C5 mutant showed strong morphological alterations including a seedless phenotype making the mapping difficult at first. Thanks to the crossing with the commercial M82 tomato cultivar, the causal mutation was identified in a cis-acting ORF, upstream of the *GGP* gene. This result confirmed the key role of GGP in the L-Galactose pathway. Preliminary studies related to the parthenocarpic phenotype suggest a problem of male sterility associated with pollen development processes. Finally, in the study of the post-harvest fruit quality, chilling stress experiments carried out with the P21H6 fruits seem to demonstrate that increasing AsA content improve the fruit shelf life and its maturation capacity. Taken as a whole, our results confirmed the key position of the GGP protein in the AsA biosynthesis pathway and they provided precious tools and plant material for deciphering the regulation of AsA and its physiological role in fruit quality and post-harvest traits.

**Keywords:** Ascorbic acid; Tomato; GDP-L-galactose phosphorylase; EMS Mutants; Light signal; Sterility

## Résumé

L'acide ascorbique (AsA, vitamine C) est l'un des composés parmi les plus importants chez les eucaryotes. En raison de son potentiel antioxydant élevé, l'AsA représente un trait important de la qualité nutritionnelle des végétaux. Au-delà de sa valeur bénéfique pour la santé, une augmentation de la teneur en AsA des fruits bénéficierait probablement à la qualité post-récolte et à la résistance aux pathogènes. Pour mieux comprendre ces régulations chez les plantes et leurs impacts sur la qualité des fruits, une collection de tomates EMS hautement mutée (cv. Micro-Tom) a été criblée pour identifier des mutants dont les fruits sont enrichis en AsA. Cette stratégie de génétique directe associant le criblage à une approche de cartographie par séquençage devait permettre d'identifier de nouveaux gènes liés au caractère AsA+. L'un des mutants, noté P21H6, présentait un enrichissement en AsA de 2 à 4 fois supérieur à celui du WT, et fut le premier à être génétiquement caractérisé. Cette étude a permis de mettre en évidence une nouvelle classe de photorécepteurs impliqués dans la détection de la lumière bleue, appelée SIPLP, en tant que régulateur négatif de l'accumulation d'AsA dans la tomate. Le rôle de PLP dans le phénotype AsA+ du fruit a été confirmé par une stratégie de mutagenèse dirigée, avant d'entreprendre sa caractérisation fonctionnelle. Nous avons démontré que SIPLP interagit avec SIGGP (GDP-L-galactose phosphorylase), une enzyme clé de la voie du L-Galactose, sous contrôle de la lumière bleue et que cette interaction a lieu dans le cytoplasme et le noyau. Nos résultats renforcent le rôle central du GGP dans la biosynthèse de l'AsA et suggèrent un nouveau mécanisme de régulation par la lumière bleue de la fonction du GGP, en plus de son activité métabolique. Parallèlement, nous avons entrepris la caractérisation d'un autre mutant, le P17C5-3, qui présentait le plus fort taux d'AsA (jusqu'à 10 fois le WT). Outre le phénotype AsA+, le mutant P17C5 présentait de fortes altérations morphologiques, notamment l'absence de graines, rendant la mise en place de la stratégie de cartographie difficile. Grâce à un croisement avec la variété commerciale M82, la mutation causale pu être identifiée dans un ORF cis-régulateur en amont de *GGP*. Ce résultat confirme le rôle clé de GGP dans la voie L-Galactose. Des études préliminaires liées au phénotype parthénocarpique suggèrent un problème de stérilité mâle associé aux processus de développement du pollen. Enfin, dans l'étude de la qualité des fruits après la récolte, des expériences de stress froid effectuées avec les fruits P21H6 semblent démontrer que l'augmentation de la teneur en AsA améliore la durée de conservation et la capacité de maturation des fruits. Dans l'ensemble, nos résultats confirment la position clé de la protéine GGP dans la voie de biosynthèse de l'AsA, et fournissent des outils et du matériel végétal précieux pour décortiquer la régulation de l'AsA et son rôle physiologique dans la qualité des fruits et les caractères post-récolte.

**Mot clefs :** Acide ascorbique ; Tomate ; GDP-L-galactose phosphorylase ; Mutants EMS ; Lumière ; Stérilité