Identification de gènes candidats impliqués dans la régulation de la teneur en acide ascorbique chez la tomate : impacts sur le potentiel antioxydant et la qualité post-récolte du fruit

Celine Bournonville

Année 2015

Thèse n°

THÈSE

pour le

DOCTORAT DE L'UNIVERSITÉ de BORDEAUX

Ecole Doctorale des Sciences de la Vie et de la Santé

Spécialité: Biologie Végétale

Présentée et soutenue publiquement

Le 3 Mars 2015

Par Céline Bournonville

Né(e) le20 Mars 1987 à Berlin

Identification de gènes candidats impliqués dans la régulation de la teneur en acide ascorbique chez la tomate

Impacts sur le potentiel antioxydant et la qualité post-récolte du fruit

Membres du Jury

Mme Mathilde Causse, Directeur de recherche (*INRA, Avignon*).....Rapporteur
M. Miguel Botella, Professeur (*Université de Malaga, Espagne*).....Rapporteur
Mme Rebecca Stevens, Chargée de recherche (*INRA, Avignon*).....Examinateur
M. Julien Bonnet, Ingénieur (*Syngenta Seeds SA, Toulouse*).....Examinateur
M. Michel Hernould, Professeur (*Université de Bordeaux*).....Examinateur
M. Pierre Baldet, Chargé de recherche (*INRA, Bordeaux)* ....Directeur de thèse

“La connaissance, c’est savoir qu’une tomate est un fruit. La sagesse c’est ne pas la mettre dans une salade de fruit.”
Frederic Jézégou

“Knowledge is knowing that tomatoes are fruits. Wisdom is knowing not to put them in fruit salad.”
Frederic Jézégou

Au moment ou l'on décide de réaliser une thèse, on nous met en garde sur les choix relatifs au sujet, au directeur de thèse mais également au laboratoire d'accueil. Ainsi, je peux aisément affirmer que je ne regrette aucun de mes choix. Que de personnes rencontrées pendant cette thèse, que de bons moments partagés dont je ne pourrais retranscrire ici toute l'intensité mais que je garde en moi soyez en sûr, et dont je me souviendrais avec bonheur. Merci à toutes les personnes qui m'ont entourées au cours de ces trois ans pour m'avoir autant fait rire mais aussi encourager notamment dans les moments difficiles de ma thèse. De la même façon, d'avoir pris le temps de répondre a mes nombreuses questions. Eh oui, une des qualité qui m'a le plus impressionnée chez vous est le temps que vous accordez aux doctorants malgré les emplois du temps chargés. Surtout ne changez rien !

Je tiens tout d'abord a remercier les membres de mon jury, Michel Hernould, Rebecca Stevens, Mathilde Causse, Julien Bonnet et Miguel Botella pour avoir acceptés de juger ce travail.

Ensuite je tiens à remercier mon directeur de thèse, Pierre BALDET ou « Gwan chef » qui part ses encouragements et sa bonne humeur a rendu ses trois ans de thèse vraiment fabuleux. Dès le début vous avez eu confiance en moi, vous m'avez laissé faire mes propres choix et je vous en remercie. Cette autonomie tout en restant disponible si le besoin s'en faisait sentir, m'a vraiment permis de m'épanouir scientifiquement. J'ai adoré partager les moments « forts» de ma thèse tel que la découverte du premier mutant AsA+, comme les moment « difficiles » notamment lors du screening de la collection qui a constitué une des parties les plus fastidieuse de cette épopée. Votre naturel m'a beaucoup fait rire !

Ensuite, je souhaiterai remercier mon équipe, GFDF. Votre soutien mais surtout les différentes discussions scientifiques qui ont pu avoir lieu m'ont beaucoup apportées. Merci Cécile et Lucie d'avoir été là lors de mon premier congrès, vous étiez tellement sereines et enthousiastes que je suis montée sur l'estrade rassurée et j'avoue un peu amusée en voyant vos sourires dans l'assemblée. J'aimerai également remercier mon directeur d'équipe Christophe ROTHAN, qui m'a offert de très belles opportunités au cours des ces trois ans avec notamment la possibilité de partir m'expérimenter dans des laboratoires étrangers. J'ai conscience que mon « énergie scientifique » (vous seul comprenez de quoi je parle !) n'a pas toujours était facile a canaliser et a donné lieu a des discussions dynamiques, mais fondamentales au développement d'un « bon » chercheur !

Inévitablement remercier l'équipe OrFe, qui m'a fait découvrir les joies de la recherche ! Ce passage a été vraiment décisif dans mon envie de faire de la recherche. Fred, ta passion pour la recherche ... ou plus précisément du moindre fait scientifique pouvant

aller jusqu'à l'observation d'une feuille d'Arabidopsis m'a énormément amusé mais aussi, montré cet aspect fascinant de la science. Michel, votre (et toujours pas de tutoiement !) modestie n'est pas à l'échelle de l'impact que vous avez pu avoir au cours de ma thèse. Les discussions scientifiques ou moins scientifiques (dixit les blagues « à la Michel ») m'ont de nombreuses fois amenées a la réflexion ! Merci a vous tous d'avoir continués à me soutenir lors de ma thèse et surtout de m'avoir fait tant rire pendant toutes ces années !

Sans oublier les personnes qui ont eu une part importante dans l'avancé de mon projet, Joana, J.P, les jeunes padawans mais également Isabelle notre « maman tomate ». On en a passé des heures en serre !

Je remercie également le laboratoire ARO du Volcani center (ISRAEL) de m'avoir accueilli. Noam, Sonia, Badia, Shalom, Adi, Tanmayee, Shivan et Raja, merci de m'avoir fait découvrir la beauté de votre pays mais aussi la richesse des personnes qui y vivent. J'ai beaucoup aimé participer à vos séances test/dégustation ! Raja, la cuisine indienne n'a plus de secret pour moi... merci pour tes cours de cuisine, seb t'en remercie !

Je voudrai également remercier l'INRA d'Avignon, Rebecca Stevens et Helene Gautier, mais également le BIOSS de l'Université de Freiburg pour leur participation passé et à venir dans le développement de mon projet de recherche.

J'ai également une grosse pensée pour les doctorants qui vivent actuellement cette incroyable expérience : NoNo, Tracey, Charlotte, Cecile, Ruie, Clemence, Isma..... Allez ca va le faire !

Il n'est pas d'épanouissement total sans le soutiens de ses amis, alors merci Emilie et Manu, Orelhy, les jums, Cath, Chemsouille et Alban, Dubdub, Fox, Pacaa, Pauline, Tracey, Soph, Aymeric, J.P et Ade, Lamia et Benoit, NoNo et Fanny, Maud, et Miguelito. Mais également mon équipe de basket du CDC pour qui l'étude de la « Tomatoes » a pu faire l'objet de nombreux questionnements comiques !

Je pense également a ma famille et à ma belle famille..... Je sais que ca n'a pas toujours été facile de me suivre mais j'espère que cette journée vous a permis de mieux comprendre le monde dans lequel j'évolue.

Je terminerai par celui qui a été là à chaque seconde, ma moitié Sebastien. Merci pour ta patience et ton énorme soutien, mais surtout de m'avoir laisser vivre ma thèse.... Je revois encore ton sourire quand je revenais toute excitée par de nouveaux résultats, tu ne comprenais pas toujours mais tu faisais tout de même mine de t'intéresser ! C'était vraiment drôle, et en réalité très touchant. Donc merci d'avoir était mon réconfort dans les moments de doute et, dans les moments importants de t'être autant impliqué...

MERCI a tous !

Content

Abbreviation list

AsA	Ascorbate or Ascorbic acid
AMR1	Ascorbic acid Mannose pathway Regulator 1
2ODD	2-Oxoacid-Dependant Dioxygenase
^{3}Chl	Chlorophyll triplet
ABA	ABscisic Acid
AO	Ascorbate Oxidase
APX	Ascorbate PeroXidase
ARF	Auxine Response Factor
ATP	Adenosine TriPhosphate
AZ	Abscission Zone
BC	BackCross
BSA	Bulk Segregant Analysis
bZIP	basic Leucine Zipper Domain
C2H2	Cysteine2 Hystidine2
CAT	CATalase
cDNA	complementary DNA
Ch	Chorophyll
CI	Chilling Injury
COP	COnstitutive Photomorphogenic
cOxT	cyclic-Oxalyl-threonate
CRY	CRYptochrome
CSN	Signalosome
CTRL	ConTRoL
DCF	DihydrodiChloroFluorescin
DHA	Dehydroascorbic acid
DHAR	Dehydroascorbat reductase
DKG	2,3-DiKetoGulonate
DNA	DeoxyriboNucleic Acid
DTT	Dithiothreitol
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligating enzyme
eiF3	Eukaryotic initiation Factor 3
EMS	Ethyl MethaneSulfonate
ERF	Ethylene Response Factor
ERO or ROSS	Reactive Oxygen Species
FD	Federoxine
FKF	Flavin-binding Kelch repeat F-box

FNR	Ferredoxine NADP Reductase
FW	Fresh Weight
GA	Gibberellic Acid
GalDH	L-Galctose DeHydrogenase
GalUR	D-GaLactUraunate Reductase
GDP	Guanosine DiPhosphate
GGP	GDP-L-Galactose Phosphorylase
GLDH	L-Galactono-1,4-Lactone DeHydrogenase
GME	GDP-D-Mannose-3',5'-Epimerase
GMP	GDP-D-Mannose Pyrophosphorylase
GR	Glutathione Reductase
GSH	Glutathione reduced
GSSG	Glutathione oxidized
H_2O_2	Hydrogen peroxide
HL	High Light
HR	Hypersensitive Response
InsP3	Inositol (1,4,5)-triPhosphate
IVIS	In Vivo Imaging System
JA	Jasmonic Acid
LKP2	LOV Kelch protein 2
LL	Low Light
LOV	Light Oxygen Voltage
MDA	MalonylDiAldehyde
MDHA	MonoDeHydroascorbate
MeJA	Methyl Jasmonate
MIOX	Myo-Inositol OXygenase
MMDB	Micro-Tom Mutant DataBase
MyB	MYeloblastome
NAD	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NAT	Nucleobase-Ascorbate Transporteur
NEDD8	Neural precusor cell Expressed Developmentally Down-regulated 8
NEM	N-Ethylmaleimide
NES	Nucleus Excision Sequence
NGS	Next Generation Sequencing
NLS	Nucleus Localization Sequence

O_2	Singlet Oxygen
O_2^-	Superoxide anion
OH	Hydroxyl-radical
Or	Orange
OxT	Oxalyl-Threonate isomers
PAS	Per-ARNT-Sim
PC	Plastocyanin
PCD or MCP	Programme of Cell Death
PGI	Glucose-6-Phosphate Isomerase
PHOT	PHOTotropine
PLP	PAS/LOV protein
PMI	PhosphoMannose Isomerase
PQ	PlastoQuinone
PR	Pathogenesis-Related protein
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
S1	Self 1
SA	Salicylic Acid
SAG	Scenescence-Associated Genes
SAR	Systemic Acquired Resistance
SCF	Skp, Cullin, F-box protein
SDG	Senescence-Down-regulated Genes
Ser/Thr	Serine/Threonine
SNP	Single Nucleotide Polymorphism
SOD	SuperOxide Dismutase
SVCT	Sodium-dependent Vitamin C Transporters
TCA	TrichloroAcetic Acid
ThrO	Threonate
TILLING	Targeted Induced Local Lesions IN Genome
Unk	Unknown
UT	Universal Time
VDE	Violaxanthin de-epoxidase
ViT C	Vitamin C
ViT E	Vitamin E
WGS	Whole Genome Sequencing
WT	Wild Type
YFP	Yellow Fluoresent Protein
ZTL	F-box ZEITLUPE

Introduction

Figure 1: A. Couverture du livre de Piétro Andréa Matthioli qui décrit pour la première fois la tomate (édité en 1554); **B.** Première représentation d'un plan de tomate par Piétro Andréa Matthioli (1550)

Figure 2: Exemples de fruits appartenant à la famille des Solanacées. **A.** Tomate (*Solanum lycopersicum*); **B.** Mandragore (*Mandragora officinarum*); **C.** Belladone (*Atropa belladona*)

I- La tomate

1.1 Origine

Originaire des Andes, la tomate sauvage était cultivée par les Aztèques en Amérique du Sud. Les indigènes lui donnèrent le nom de « *tomalt* » ou « *tomalti* » d'où provient son appellation actuelle « *tomate* ». La description réalisée par Matthiolus botaniste et médecin italien dans « Petri Andrea Matthioli medici Commentari » prouve qu'il existait déjà une grande variété de forme et de couleur (libros sex Pedacii Dioscoridis Anazarbei, 1544) (*figure 1*). Elle fut ensuite introduite en Europe au début du XVIe siècle par les conquistadors Espagnols. A cause de sa ressemblance avec des espèces endémiques telle que la mandragore aux vertus aphrodisiaques et la belladone, toxique, elle fut d'abord cultivée comme plante ornementale, et parfois pour la médecine (*figure 2*). Ce n'est que trois siècles plus tard que l'on découvre ses vertus en tant que légume-fruit.

1.2 Botanique

En 1753, le naturaliste suédois Carl Von Linné, fondateur du système moderne de la nomenclature binomiale, rebaptise la tomate *Solanum lycopersicum* pour *lycos* (loup) et *persicum* (pêche) d'après l'étymologie grecque. Ce nom sera ensuite adopté définitivement par la communauté scientifique en 2006. Ainsi, la tomate « *Solanum lycopersicum* » est une plante herbacée appartenant à la famille des Solanacées. Cette vaste famille de dicotylédones comprend près de 98 genres et 2700 espèces occupant une grande diversité de morphologie, d'écologie et d'habitat. On y retrouve aussi bien des espèces alimentaires telles que la pomme de terre (*Solanum tuberosum*), l'aubergine (*Solanum melongena*) et les piments (*Capsicum*) que des espèces ornementales comme le *Petunia*, *Schizanthus*, *Salpiglossis* et *Datura*. Certaines de ces espèces sont toxiques car elles peuvent contenir des alcaloïdes, de l'atropine, de l'hyoscyamine, de la scopolamine voir même de la nicotine (belladone, datura, mandragore, tabac ...). De la même façon, les espèces alimentaires contiennent aussi des alcaloïdes dans les feuilles, les tiges et les racines mais les parties comestibles en sont dépourvues.

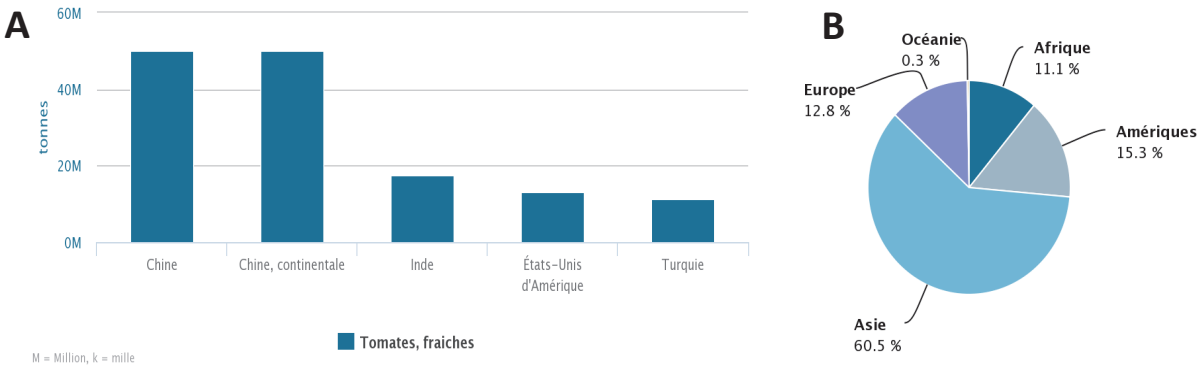


Figure 3: A. Classement mondial des principaux pays producteurs de tomate en 2012, exprimé en millions de tonnes; **B.** Part de la production mondiale par continent, exprimée en pourcentage. Les données sont issues de la section statistique de la Food and Agriculture Organization of the United Nations (FAOSTAT: http://faostat3.fao.org/faostat-gateway/go/to/home/F)

L'espèce *Solanum lycopersicum* compte plusieurs milliers de variétés botaniques dont la tomate cultivée à gros fruits, *Solanum lycopersicum* var. *esculentum,* de laquelle découlent presque tous les cultivars présents sur le marché. Mais également *Solanum lycopersicum* var. *cerasiform*, la tomate cerise, qui est la seule variété sauvage trouvée en dehors de l'Amérique du Sud (Rick, 1986). Les différentes variétés de tomate sont caractérisées selon le mode de croissance des plantes qui peut être déterminée ou indéterminée, mais également selon le fruit en fonction de la couleur, la taille ou la forme. Des collections de cultivars sont entretenues dans divers pays pour préserver les ressources génétiques (Californie: TGRC; Taïwan: AVRDC, France: INRA…). Cependant, bien qu'elle soit souvent considérée comme un légume en raison de ses nombreuses utilisations culinaires, la tomate est en réalité un fruit. En effet, selon les botanistes « un fruit est le produit végétal succédant à la fleur et qui renferme les graines de la plante ». D'autres légumes-fruits se trouvent également dans le même cas comme la courgette, le piment et l'avocat.

1.3 Importance économique

La tomate est cultivée dans de nombreux pays (170 selon la FAO) et sous des latitudes et des climats très divers. L'amélioration des connaissances tant sur le plan de la sélection variétale que des méthodes de cultures permet de produire des tomates tout au long de l'année. Les fruits produits sont destinés à la consommation en frais ou à la transformation (concentré, jus, poudres …). La demande augmente et se diversifie, créant un marché particulièrement dynamique, très ouvert aux innovations variétales. De ce fait, la production mondiale de tomate progresse régulièrement passant de 64 millions de tonnes en 1988 à plus de 100 millions aujourd'hui, dont 30 millions sont destinés à la transformation. Ce qui place la tomate au premier rang mondial en termes de production fruitière devant la banane et la pastèque, mais également au troisième rang mondial en terme de production potagère après la patate et la patate douce. Les principaux pays producteurs sont la Chine (50 millions de tonnes), l'Inde (17,5 millions de tonnes), les Etats-Unis (13,2 millions de tonnes) puis la Turquie (11,35 millions de tonnes) (*figure 3*). La France se situe seulement au 32ème rang mondial avec une production annuelle de près de 600 milles tonnes en 2012.

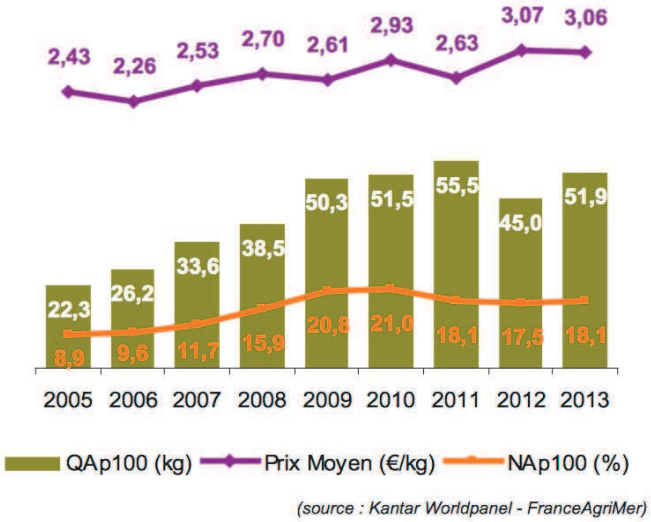


Figure 4: Evolution des achats annuels de tomate biologique en France. Données issues des campagnes réalisées par AgriMer.

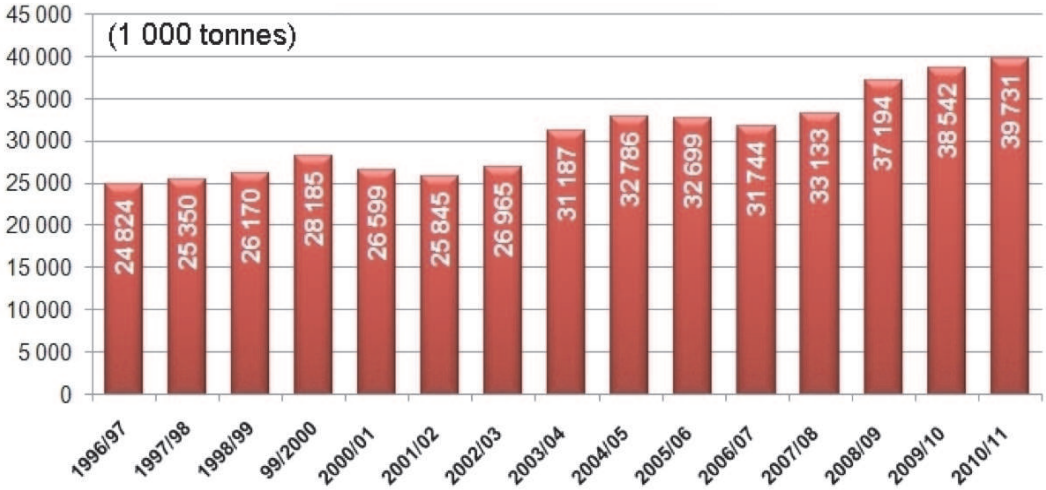


Figure 5: Evolution de la consommation mondiale de tomate depuis les années 1996/97 jusqu'aux années 2010/11, exprimée en tonnes (équivalent frais). Données issues d'une étude réalisée par France AgriMer/Ministère de l'agriculture, de l'agro-alimentaire et de la forêt.

Il semble que les producteurs français s'adaptent à la concurrence en développant des filières à hautes valeurs ajoutées tel que l'agriculture biologique, les variétés anciennes ou la filière de proximité (*figure 4*). En termes de consommation, celle-ci augmente régulièrement à l'échelle mondiale avec les pays méditerranéens comme principaux consommateurs (*figure 5*). Selon le Graphagri 2013 publié par le ministère de l'Agriculture, la tomate est le « légume » le plus consommé par les français. Ainsi, la consommation de tomate fraiche est de l'ordre de 13kg par personne et par an alors qu'elle est de 15.8kg pour la consommation de tomate transformée.

1.4 Importance nutritionnelle

La plupart des enquêtes épidémiologiques qui ont analysé les facteurs alimentaires impliqués dans la prévention de nombreuses maladies chez l'homme, ont mis en évidence le rôle important des fruits et des légumes. Ainsi le Programme National Nutrition Santé (PNNS) recommande de consommer au moins 5 portions de fruits et légumes par jour. En réalité, il faut faire attention avec ce slogan car certains fruits ou légumes ne sont pas conseillés tel que les fruits riches en fructose, sucre mal métabolisé par l'organisme, qui provoque des pics de glycémie et l'accumulation de graisse dans les cellules. L'une des particularités de la tomate est d'être riche sur le plan nutritionnel mais très peu calorique (15 kcals pour 100 g MF). Elle contient peu de glucides (moins de 20 kcals pour 100 g MF), elle est donc particulièrement recommandée en cas de surpoids ou de diabète. La tomate possède également une quantité importante d'antioxydants (carotènes, vitamine C, vitamine E, lycopène et polyphénol) impliqués dans la prévention des cancers et des maladies cardio-vasculaire. De la même façon, riche en minéraux (potassium, magnésium et phosphore), en fibres et en eau (95% de la MF), elle contribue au bon équilibre de l'organisme.

1.5 La tomate: une espèce modèle pour l'étude des fruits charnus

Au-delà de son aspect nutritionnel, la tomate (*Solanum lycopersicum*) est une plante modèle pour l'étude des processus associés au développement des fruits charnus.

Elle présente de nombreux avantages biologiques (cycle court, croisements aisés et culture tout au long de l'année, matériels biologiques disponibles importants, facilité de transformation, etc…) mais également au niveau des bases données « omics » avec de nombreuses ressources génétiques, moléculaires et biochimiques disponibles (environ 75 000 accessions) (Larry and Joanne 2007; Rodriguez *et al.,* 2011). Récemment, le génome de *Solanum lycopersicum* cv. « Heinz 1706 » a été séquencé et annoté (Tomato Consortium 2012, Aoki *et al.,* 2013). Ce génome présente une taille d'environ 900 Mb, réparti sur 12 chromosomes, dont plus de 75% est de l'hétérochromatine largement dépourvu de gènes. Approximativement 33 000 gènes ont été identifiés avec plus de 500 gènes préférentiellement exprimés dans le fruit (Tikunov *et al.,* 2013). L'évolution des technologies d'analyses a également permis de développer la cartographie de population ainsi que de marqueurs, qui ont mené à l'identification de loci impliqués dans les qualités organoleptiques du fruit tels que le locus FRUIT WEIGHT (taille du fruit) ou *locule number* (nombre de loge). De la même façon, de nombreux mutants de tomate affectés dans ces propriétés organoleptiques ont été isolés.

1.6 La tomate, structure et développement:

La tomate est une plante herbacée sensible au froid, mais vivace sous climat chaud. Généralement cultivée comme annuelle, c'est une plante à croissance en jour neutre dont la floraison est indifférente au photopériodisme, ce qui a permis son adaptation sous diverses latitudes. Elle peut mesurer de 40cm à plus de 2m de haut. La tomate est à l'origine une liane. Ce type de croissance est dit « indéterminée » c'est à dire que la tige peut se développer indéfiniment par empilement de sympodes (3 feuilles suivies d'un bouquet floral). Une mutation génétique apparue dans ce processus de croissance a induit un autre type de développement dit « déterminé » où l'extrémité du rameau se termine par un bouquet floral et la plante arrête sa croissance. Ces plantes présentent une apparence de type buisson et possèdent une version mutée du gène récessif SP (Self Pruning). Les variétés utilisées pour la consommation sont de type « indéterminée », à l'inverse, les variétés destinées à l'industrie présentent un port de type « déterminée » ce qui permet une maturation groupée des fruits et donc une récolte mécanique.

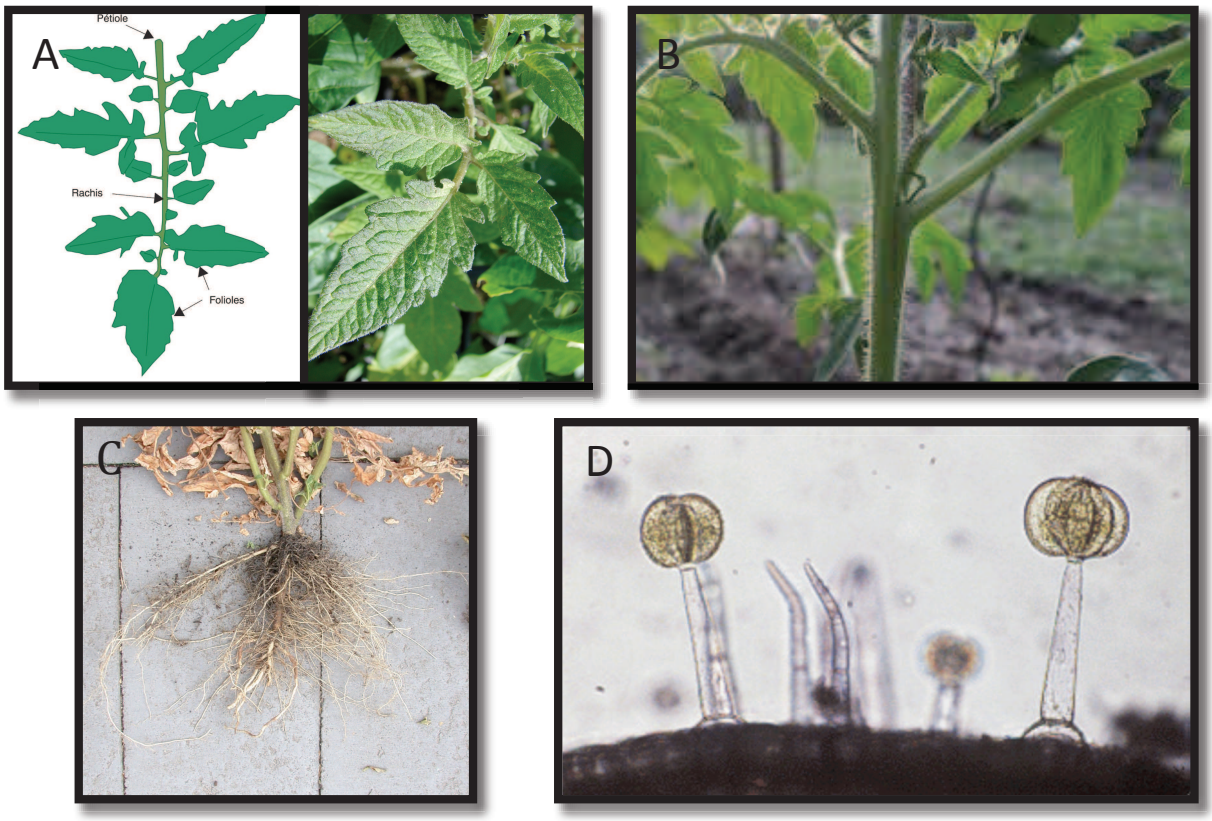


Figure 6: Présentation de l'appareil végétatif de la tomate**. A**. morphologie de la feuille; **B.** portion de tige mettant en évidence la présence de poils; **C.** système racinaire de type pivotant; **D.** Trichomes présents sur la surface des organes constituant l'appareil végétatif, de type glanduleux ou non.

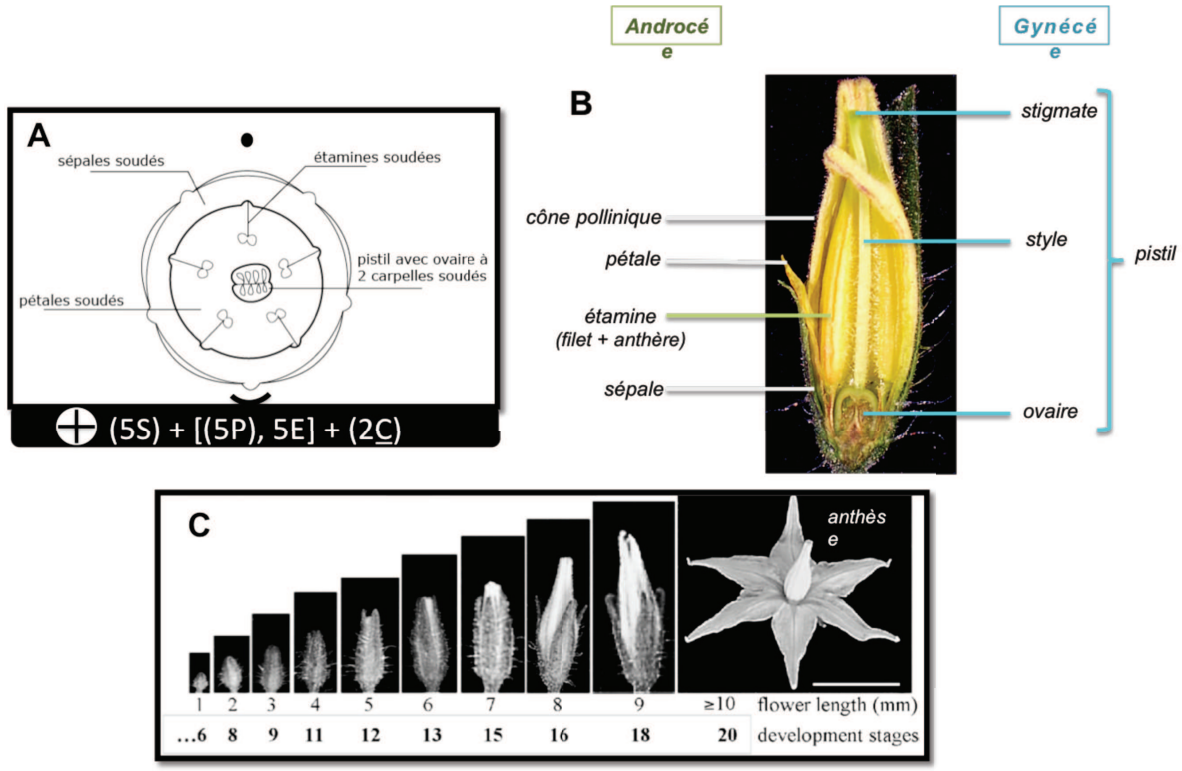


Figure 7: Structure et développement de la fleur de tomate *Solanum lycopersicum*. A. Diagramme floral: S (sépale), P (pétale), E (étamine), C (carpelle supère), «(organes soudés)», symétrie axiale B. Coupe longitudinale montrant les différents organes de la fleur. **C.** Diagramme illustrant les différents stades de développement floral depuis le stade 6 (1mm de longueur) jusqu'au stade 20 (≥ 10mm).

Appareil végétatif:

La croissance de la tige, monopodiale au début devient sympodiale après 4 ou 5 feuilles, c'est à dire que les bourgeons axillaires donnent naissance à des ramifications successives, tandis que les bourgeons terminaux produisent des fleurs ou bien avortent. De la même façon, les rameaux issus des bourgeons axillaires produisent des feuilles à chaque nœud et se terminent aussi par une inflorescence. Les feuilles, alternes, longues de 10 à 25 cm, sont composées et imparipennés. Elles comprennent de 5 à 7 folioles aux lobes découpés dont le bord du limbe est denté (*figure 6A*). La tige et les feuilles portent deux types de poils: simple ou glanduleux (*figure 6B et D*). Ces derniers contiennent une huile essentielle qui donne à la tomate son odeur caractéristique. Le système racinaire est de type pivotant à tendance fasciculée et peut mesurer jusqu'à 1m (*figure 6C*).

Fleur:

Les fleurs de tomates présentent la caractéristique d'être hermaphrodite et autoféconde, c'est-à-dire que le pollen d'une fleur peut féconder la même fleur. Ces fleurs sont actinomorphes à symétrie pentamérie (*figure 7A*). Le calice compte cinq sépales verts qui persistent après la fécondation au sommet du fruit, la corolle compte cinq pétales jaune vif soudés à la base, et l'androcée cinq étamines dont les anthères allongées forment un cône resserré autour du pistil. Les étamines possèdent une déhiscence introrse, ce qui permet au pollen d'être libéré vers l'intérieur de la fleur. Le gynécée, est constitué d'un pistil formé de deux carpelles soudés, structurant un ovaire supère généralement biloculaire à placentation centrale, certaines variétés pouvant être pluriloculaires (*figure 7B*). Lorsque la fleur est au stade anthèse, c'est à dire le moment où la fleur est complètement ouverte et où l'anthère s'épanouit pour libérer le pollen, la fécondation peut avoir lieu (*figure 7C*). Un jour avant l'ouverture de la fleur le stigmate rentre dans une période de « réceptivité ». Durant cette période de 1 à 7 jours, le pollen pourra venir se déposer sur le stigmate du pistil et féconder les ovules contenus dans l'ovaire. Après la fécondation, l'ovaire va évoluer pour donner le fruit. Le cycle de développement de la graine à la graine varie entre 90 à 120 jours en conditions optimales. Dans certains cas, le fruit se développe comme si la fleur avait été fécondée mais il ne possède pas de graines, on parle de parthénocarpie (du grec « graine vierge »).

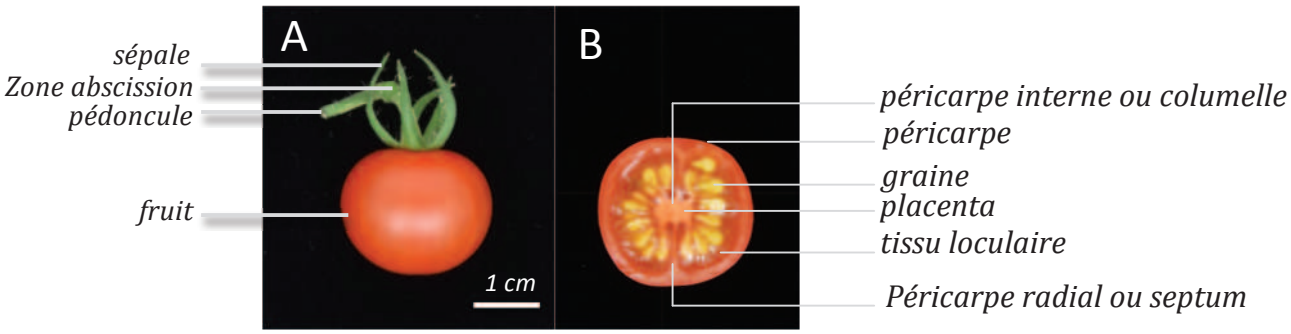


Figure 8: Fruit de tomate *Solanum lycopersicum*. **A.** Fruit de tomate à maturité. **B.** coupe transversale montrant les différentes structures.

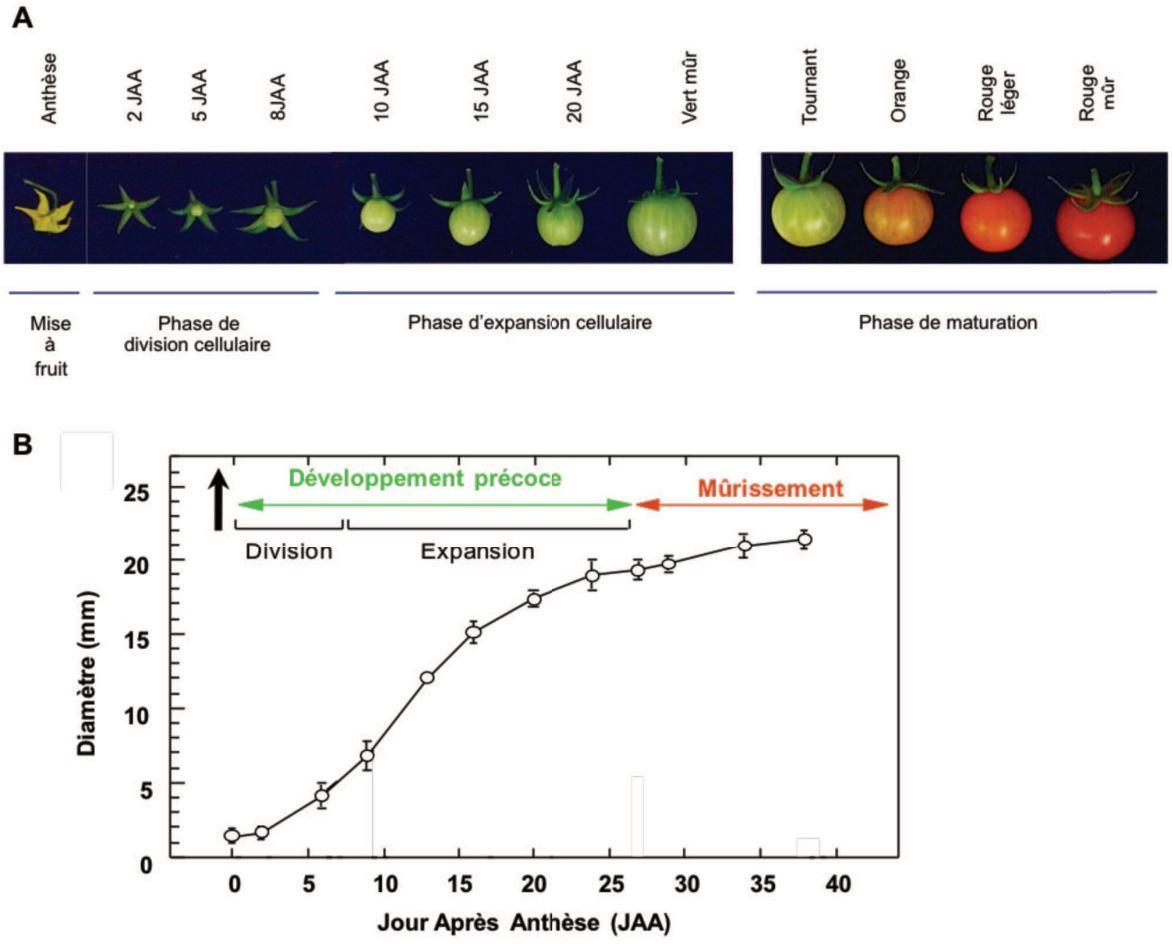


Figure 9 : Le développement du fruit de tomate *Solanum lycopersicum.* A. Fleurs et fruits de tomate à différents stades de développement. JAA : jours après anthèse **B.** Diamètre du fruit au cours des 3 phases de développement. La flèche verticale symbolise la mise à fruit.

Fruit:

La couleur des fruits diffère selon les variétés, il existe ainsi une gamme importante de coloration allant du rouge, blanc, jaune, orange, noir-violacées, au fruit vert. Le fruit de tomate est composé d'un péricarpe: peau et partie charnue, et de la pulpe: placenta et graines. C'est un fruit charnu de type baie, indéhiscent (*figure 8*).

Après la fécondation, la paroi de l'ovaire se transforme en péricarpe, lequel est structuré en trois couches cellulaires: l'exocarpe, le mésocarpe et l'endocarpe. La partie la plus externe, l'exocarpe, est formée de l'épiderme externe « la peau » qui consiste en une couche de cellules épidermiques puis d'un tissu collenchymateux où s'accumule l'amidon ainsi que quelques plastes. L'exocarpe est recouvert d'un dépôt de cuticule qui s'épaissit avec l'âge. Le mésocarpe, couche intermédiaire, est un parenchyme formé de grandes cellules comportant des vacuoles de grande taille. Les cellules du mésocarpe sont le siège d'un processus particulier appelé l'endoréduplication, c'est-à-dire qui permet la duplication du matériel génétique sans division cellulaire (Bourdon *et al.,* 2010). Ces cellules possèdent également de nombreux chloroplastes qui permettent au fruit de produire environ 20% de la photosynthèse; néanmoins, la source principale des photoassimilats reste les feuilles (Hetherington *et al.,* 1998, Massot *et al.,* 2012). Enfin l'endocarpe, qui est la couche la plus interne, est formé d'une assise cellulaire qui tapisse l'intérieur de la loge où se trouvent les graines. Chez certains fruits, il peut prendre une consistance cartilagineuse (pomme, poire….) ou encore ligneuse (cerise, pêche…). A l'intérieur du fruit, les ovules fécondés vont évoluer pour donner naissance aux graines. Elles sont entourées d'un mucilage provenant de la gélification de l'enveloppe de la graine. Le phénomène de double fécondation permet la formation de zygote diploïde ainsi que de la ploïdie de l'endosperme.

Le développement du fruit comprend plusieurs phases de développement (*figure 9*). La première étape, juste après la fécondation, est caractérisée par une activité de division cellulaire intense qui permet l'augmentation progressivement de l'épaisseur du péricarpe. Lorsque le fruit atteint un diamètre de 0,8 à 1 cm, le taux de division diminue, le fruit entre alors dans la deuxième étape de son développement. Durant cette seconde étape, les cellules entament une phase d'expansion cellulaire où le fruit va pouvoir grossir en diamètre. Cette augmentation est possible grâce au processus d'endoréduplication (Bergervoet *et al.,* 1996; Bertin *et al.,* 2005). La troisième étape correspond à la phase de maturation du fruit qui est liée à la maturation des graines

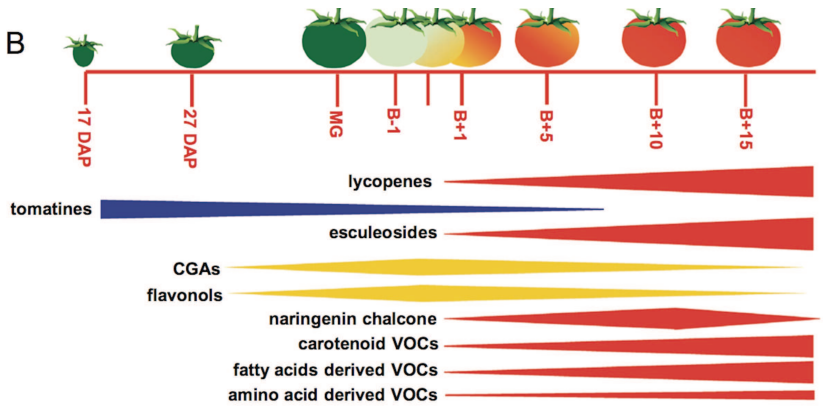


Figure 10: Représentation schématique des changements ayant lieu au niveau des métabolites secondaires durant le développement du fruit de tomate (Tohge *et al.,* 2014)

elles-mêmes. C'est un processus complexe dont la finalité est de rendre le fruit attractif et de promouvoir ainsi la consommation par des organismes qui vont faciliter la libération et la dissémination des graines. C'est un phénomène génétiquement programmé, impliquant des bouleversements biochimiques, physiologiques et structuraux dans l'ensemble des tissus du fruit. Ceux-ci se traduisent par des changements au niveau de la couleur, de la fermeté, du contenu nutritionnel, et de la production d'arômes. La tomate est un fruit climactérique dont le murissement va se dérouler en deux phases. Durant la première phase le fruit entre dans le stade «Mature Green » jusqu'à ce qu'il atteigne sa taille finale, laquelle varie selon les cultivars mais aussi sous l'influence de l'environnement (Giovannoni *et al.,* 2004; Chevalier *et al.,* 2007). Cette phase aboutit à l 'acquisition de la compétence à mûrir. En revanche, la seconde phase est une phase climactérique dépendante de l'éthylène qui une fois enclenchée peut se poursuivre même si le fruit est détaché de la plante. La phase climactérique est caractérisée par une augmentation de la vitesse de respiration, associée à l'apparition du pic transitoire de production d'éthylène. Cette transition développementale va induire un premier changement de couleur du fruit (jaune) où ont lieu la conversion progressive des chloroplastes en chromoplastes mais également l'accumulation de caroténoïdes (β-carotène et xanthophylle). Ce stade est appelé stade « Breaker ». Puis au fur et à mesure de la maturation du fruit, d'autres changements métaboliques ont lieu, le fruit débute un changement de coloration en passant par des stades successifs de maturation: tournant, orange, rouge léger et rouge mûre (*figure 10*; Tohge *et al.,* 2014). Parmi ces changements, l'accumulation du lycopène pigment responsable de la couleur rouge, de sucres, la synthèse d'arômes ainsi que l'oxydation des acides contenus dans les fruits verts, entraine une diminution de l'acidité des fruits. Ces acides sont utilisés comme combustibles cellulaires, transformés en sucres ou précurseurs de protéines. Pour l'ensemble des fruits charnus, c'est au cours de ce processus de maturation que s'élaborent les qualités organoleptiques du fruit.

Enfin, une zone d'abscission (AZ) va se former au niveau d'une position prédéterminée du pédicelle, pour permettre au fruit de tomber une fois mature. Cette zone AZ contient un groupe de petites cellules sans vacuoles. Chez la tomate, la différenciation de cette zone est contrôlée par une famille de facteurs de transcription MADS-box appelés JOINTLESS (Mao *et al.,* 2000). Le contrôle et la coordination de tous ces évènements sont donc dépendants d'une régulation spatio-temporelle fine.

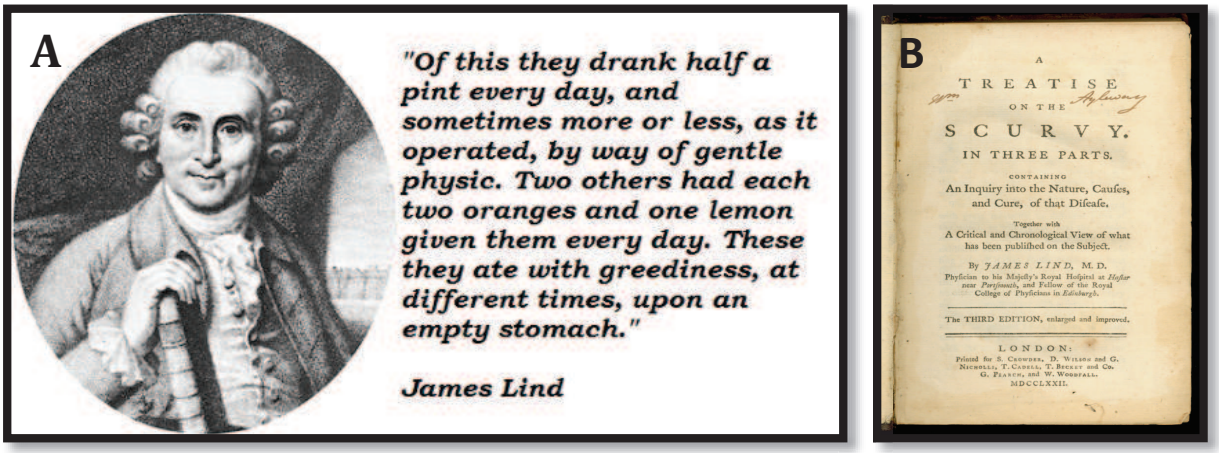


Figure 11: Portrait de James Lind (1716-1794), médecin écossais dans la Marine Royale Britannique. Il est le premier à avoir développé un essai clinique en montrant que les agrumes pouvaient guérir du scorbut. **B.** Illustration de la troisième édition de « A Treatise on the Scurvy ». Livre rédigé par Lind en trois parties. Contenant une enquête sur la nature, les causes et le traitement du scorbut. Il y développe un aspect critique et chronologique sur le sujet. Cette édition a été imprimée à Londres par S. Crowder en 1772.

Figure 12: Albert Szent Györgyi (1893-1986) scientifique hongrois qui a reçut le prix Nobel de Médecine et Physiologie pour avoir découvert la vitamine C et les flavonoïdes.

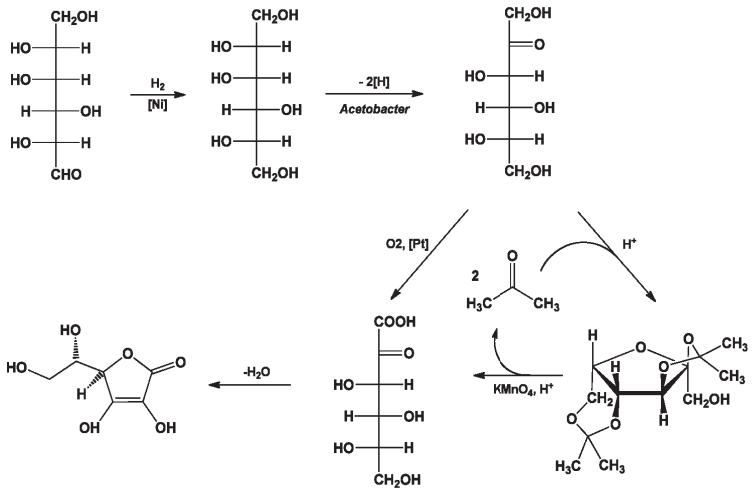


Figure 13: Procédé de Reichstein découvert par Tadeusz Reichstein (1897-1996) et ses collègues en 1933. C'est le procédé chimique couramment utilisé par l'industrie pharmaceutique et qui permet la production d'acide ascorbique à partir de D-glucose.

II- La vitamine C ou Acide ascorbique

2-1 Histoire

En 1747, un jeune médecin du nom de James Lind embarque sur les navires de la flotte britannique pour surveiller l'état de santé des marins (*figure 11a*). Après quelques mois en mer, de nombreux marins tombèrent malades présentant des symptômes graves tels que les gencives qui saignent, les dents avariés, la présence de bleues sur le corps et la fragilité des os. Ils étaient atteints d'une maladie bien connue appelée le scorbut. Lind soupçonnait un rôle du régime alimentaire et décida de changer le mode d'alimentation pour certains d'entre eux. Il choisit alors six types de régimes à base de vinaigre, de cidre, d'ail, de jus de citron associé à de l'orange, d'acide sulfurique ou d'eau de mer. Sept jours plus tard, les marins ayant mangés des agrumes montrèrent des signes de guérison. Cette première expérience établissait pour la première fois un lien entre la vitamine C et la prévention de certaines maladies. Lind publia ces résultats dans son ouvrage « *A treatise of the scurvy* » publié en 1753 (*figure 11b*). En 1907, la molécule « anti-scorbut » fut classée par Casimir Funk parmi les vitamines, et appelée vitamine C par Drummond. Ce n'est qu'en 1928 qu'un biochimiste hongrois, Albert Szent Györgyi (Prix Nobel de médecine et physiologie en 1937), isole la vitamine C à partir des glandes surrénales puis plus tard à partir de citron, produit qui fut nommé en 1932 « acide ascorbique » en référence à ses effets bénéfiques anti-scorbut (*figure 12*). Puis en 1933, grâce aux travaux de Edmond Hirst sur la structure de la vitamine C, Walter Norman Haworth et Tadeus Reichstein furent les premiers à la synthétiser artificiellement (*figure 13*). En 1934, Hoffmann-La Roche devient la première entreprise pharmaceutique à produire massivement et commercialiser la vitamine C, sous la marque Redexon. En 1957, l'américain J.J Burns a prouvé que la raison de la susceptibilité de quelques mammifères au scorbut était lié à l'incapacité de leur foie à produire une L-gulono-γ-lactone oxidase active, enzyme qui catalyse la dernière étape de la voie de biosynthèse. Au cours des différentes études, l'incapacité de la majorité des primates (dont l'homme), du cochon d'inde, de certains oiseaux et poissons à synthétiser la vitamine C été ainsi démontrée. Dans les années 1970, Linus Pauling (prix Nobel de chimie en 1954) effectue d'importantes recherches et découvre les bienfaits de la

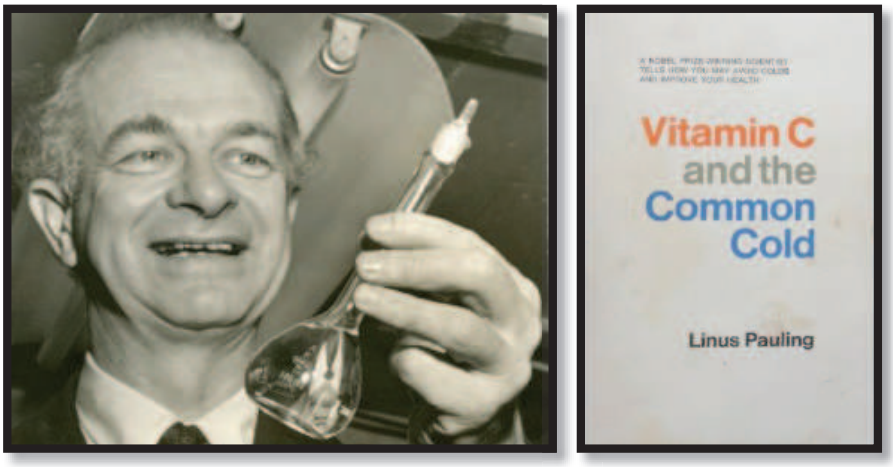


Figure 14: A. Linus Pauling (1901—1994) prix Nobel de chimie en 1954, est le fondateur de la médecine ortho moléculaire en popularisant l'utilisation de vitamine C. **B.** Livre rédigé par Linus Pauling « Vitamin C and the Common Cold » publié en 1970. Dans ce livre, il propose l'idée que la consommation de grande quantité de vitamine C peut permettre de soigner les rhumes et certains types de cancers.

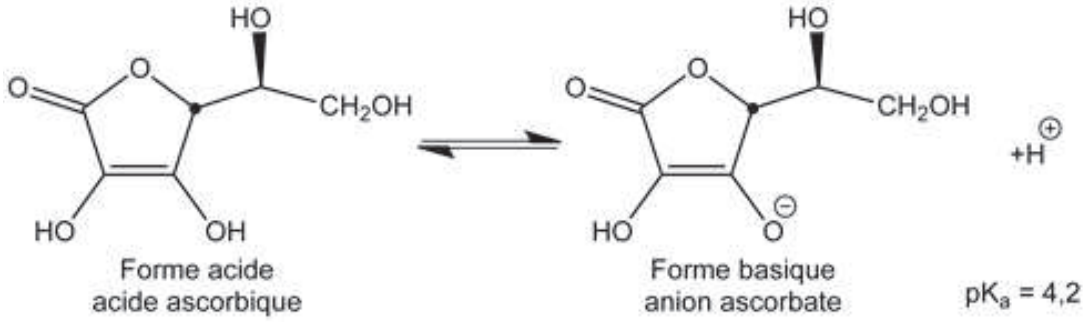


Figure 15: Différentes formes de la molécule de vitamine C suivant son caractère acido-basique: L-acide ascorbique (forme acide); ascorbate (forme basique)

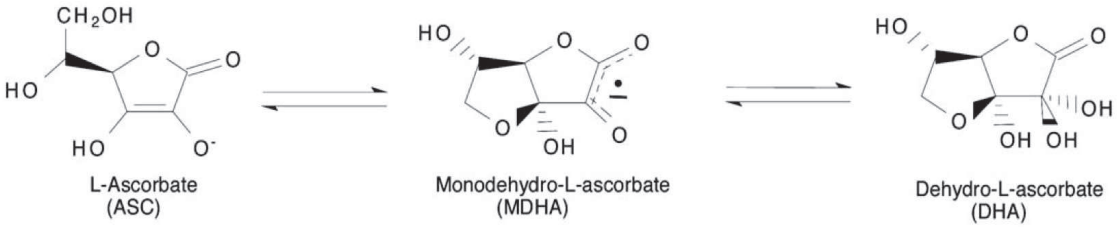


Figure 16: Différentes formes de l'acide ascorbique selon son état d'oxydo-réduction. L-ascorbate (AsA), Monodéhydro L-ascorbate (MDHA) et Déhydro-L-ascorbate (DHA). D'après Potter *et al.,* 2002

vitamine C sur le rhume (*figure 14a*). Il fait alors paraître « *Vitamin C and the Common Cold* », ouvrage contesté par la communauté médicale (*figure 14b*). En 1971, il débute une longue collaboration avec le cancérologue écossais Ewan Cameron pour démontrer un rôle bénéfique de la vitamine C dans le cadre de certaines formes de cancer. En 2008, des chercheurs de l'université de Montpellier découvrent que les hématies présentes chez les humains et les primates, ont développées un mécanisme pour utiliser de manière efficace la vitamine C présente dans le corps. Ce mécanisme n'est pas retrouvé chez les organismes capables de synthétiser leur propre vitamine C. Finalement, tout aux longs des siècles, les études scientifiques ont montré l'importance de cette vitamine dans le règne animal. Elles donnent ainsi un rôle fondamental aux végétaux dans le régime alimentaire.

2-2 Description

La vitamine C peut être également appelée « acide ascorbique » ou « ascorbate ». Le terme « vitamine C » est utilisé lorsqu'on fait référence à ses propriétés nutritionnelles. Les termes « acide ascorbique » ou « ascorbate » sont assez similaires car ils désignent la molécule purifiée mais dépend du caractère acido-basique. Ainsi, lorsque la molécule est sous une forme acide on parle d'« acide ascorbique » en revanche lorsqu'elle est sous une forme basique on parle d'« ascorbate » (*figure 15*).

La vitamine C est un acide organique hydrosoluble. Il s'agit d'un composé simple constitué de 6 carbones (formule chimique $C_6H_8O_6$), dérivé du glucose. Elle existe sous trois degrés d'oxydo-réduction différents: la forme réduite (L- acide ascorbique), la forme semi-réduite ou mono-oxydée (MDHA), et la forme oxydée (DHA) (*figure 16*). L'ensemble de ces formes constitue le « pool » de vitamine C, qui peut être plus ou moins oxydé selon l'état de la cellule. Néanmoins, seule la forme réduite possède une activité antioxydante grâce à sa capacité à libérer un proton au niveau du groupement hydroxyle du carbone 3.

Table 1 Role of ascorbate in plant growth and development and abiotic stress tolerance

Enzyme/protein	Target plant	Gene	Gene source	Type of genetic manipulation	Ascorbate content	Phenotypic changes	Reference
GDP-mannose pyrophosphorylase	Tobacco	*GMPase*	Tomato	Overexpression	2.0–4.0-fold increase	Increased tolerance to temperature stress	Wang et al. 2011
Phosphomannose Isomerase	*Arabidopsis*	*PMI1*	*Arabidopsis*	RNAi	0.47–0.65-fold decrease	No phenotypic changes under normal growth conditions in both mutants	Maruta et al. 2008
		PMI2	–	T-DNA knockout	No change		
Phosphomannomutase	Tobacco	*NbPMM*	Tobacco	VIGS	Up to 3.0-fold decrease	–	Qian et al. 2007
	Arabidopsis	*NbPMM*	Tobacco	VVMEE	0.2–0.5-fold increase	–	
		AtPMM	*Arabidopsis*	Overexpression	0.25–0.33-fold increase	Increased tolerance to MV stress	
	Tobacco	*PMM*	Acerola	Overexpression	2.0 fold increase	–	Badejo et al. 2009
VTC4/Myoinositol monophosphatase (IMP)	*Arabidopsis*	*VTC4*	–	T-DNA knockout	0.61–0.75-fold decrease	22.4% –34% decreases in myoinositol content	Torabinejad et al. 2009
						Slow seed germination under control conditions	
						Slightly hypersensitive to ABA and NaCl during seed germination	
GDP-L-galactose phosphorylase	*Arabidopsis*	*vtc5-1* and *vtc5-2*	*Arabidopsis*	T-DNA knockout	0.2-fold decrease	Plant growth retardation and bleaching of the cotyledons	Dowdle et al. 2007
L-Galactose dehydrogenase	Tobacco (BY–2 cells)	*L–GalLDH*	Tobacco	Overexpression	1.5–2.0-fold increase	Higher mitotic index in cells	Tokunaga et al. 2005
						Reduced browning and cells death of cultures	
						Increased tolerance to MV	
L-galactono-1,4-lactone dehydrogenase	Tobacco (BY–2 cells)	*GLDH*	Tobacco	Antisense downregulation	0.30-fold decrease	Adversely effected plant cell division, growth and structure of plant cell	Tabata et al. 2001
	Tobacco	*RrGalLDH*	*Rosa roxburghii*	Overexpression	2.1-fold increase	Enhanced tolerance to salt stress	Liu et al. 2013a
Monodehydroascorbate reductase	Tobacco	*AtMDAR1*	*Arabidopsis*	Overexpression	Up to 2.2-fold increase	Enhanced tolerance to ozone, salt and PEG stresses	Eltayeb et al. 2007
	Tobacco	*Am-MDAR*	*Avicennia marina*	Overexpression	Up to 2.0-fold increase	Increased tolerance to salt stress	Kavitha et al. 2010
	Tobacco	*MDAR-OX*	*Arabidopsis*	Overexpression	Up to 1.1-fold increase	No change in Aluminium tolerance	Yin et al. 2010
Dehydroascorbate reductase	Tobacco	*DHAR-OX*	*Arabidopsis*	Overexpression	Up to 1.3-fold increase	Increased tolerance to Al stress	Yin et al. 2010
	Tobacco	*DHAR*	*Arabidopsis*	Overexpression	1.9–2.1-fold increase	Enhanced tolerance to ozone, drought and salinity	Eltayeb et al. 2006
	Tobacco	*DHAR*	Wheat	Overexpression	2.1-fold increase	Increased ozone tolerance and NPR	Chen and Gallie 2005
			Tobacco	Antisense downregulation	0. 29-fold decrease	Substantially reduced stomatal area and low NPR	
	Tobacco	*DHAR*	Human	Overexpression	No significant change	Enhanced tolerance to low temperature and NaCl	Kwon et al. 2003
Ascorbate peroxidase	Tobacco	*tAPx*	Tobacco	Overexpression	No change	Increased tolerance to *MV and chilling stresses* under light conditions	Yabuta et al. 2002
			Tobacco/ Spinach	Antisense downregulation	–	Plants failed to grow	
	Arabidopsis	*HvAPX1*	Barley	Overexpression	–	Increased tolerance to salt stress	Xu et al. 2008
	Arabidopsis	*OsAPXa and OsAPXb*	Rice	Overexpression	–	Increased tolerance to salt stress	Lu et al. 2007
	Tobacco	*CAPCA1*	Pepper	Overexpression	–	Increased plant growth	Sarowar et al. 2005
						Increased tolerance to MV stress	
	Tobacco BY-2 cells	*cAPX*	*Arabidopsis*	Antisense downregulation	No change	Increased tolerance against heat and salt stresses	Ishikawa et al. 2005
	Tobacco	*StAPX*	Tomato	Overexpression	–	Improved seed germination	Sun et al. 2010a
						Increased tolerance to salt and osmotic stresses	
	Rice	*Apx1/ Apx2*	Rice	RNAi (Apx1+ Apx2)	Up to 1.5-fold decrease	No change in plant growth and development	Rosa et al. 2010
						Increased tolerance to aluminium	
				RNAi (Apx1 or Apx2)	–	Produced semi-dwarf phenotype	
	Rice	*OsAPx-R*	Rice	RNAi	–	Delayed plant development	Lazzarotto et al. 2011
	Rice	*OsAPXa*	Rice	Overexpression	–	Increased spikelet fertility under cold stress	Sato et al. 2011
	Rice	*Osapx2*	Rice	Overexpression	–	Enhanced stress tolerance	Zhang et al. 2013
						Sensitive to abiotic stresses	
			–	T-DNA knockout	–	Semi-dwarf seedlings, yellow-green leaves, leaf lesion-mimic and seed sterility	
	Alfalfa	*Osapx2*	Rice	Overexpression	–	Increased salt resistance	Guan et al. 2012
	Tomato	*cAPX*	Pea	Overexpression	–	Enhanced tolerance to UV-B and heat stresses	Wang et al. 2006
	Tomato	*cAPX*	Pea	Overexpression	–	Enhanced tolerance to chilling and salt stresses	Wang et al. 2005
	Tomato	*LetAPX*	Tomato	Antisense downregulation	No significant change	Transgenic plants photosynthetically less efficient and sensitive to chilling stress	Duan et al. 2012b
Ascorbate oxidase	Tobacco	*AAO*	Cucumber	Overexpression	No change	Plants become susceptible to ozone	Sanmartin et al. 2003
	Tobacco	*AAO*	Cucumber	Overexpression	No change	Increased drought tolerance due to reduced stomatal conductance	Fotopoulos et al. 2008
	Tobacco	*AAO*	Pumpkin	Overexpression	2.0-fold increase in apoplastic ASA	Number of smaller flowers significantly increased 6% to 14% reduction of in seed weight	Pignocchi et al. 2003
			Tobacco	Antisense downregulation	2.0-fold increase in apoplastic ASA	No significant changes	
	Tobacco	*AAO*	Tobacco	Overexpression	–	Severe inhibition of germination and seed yield under high salinity	Yamamoto et al. 2005
	Tobacco	*AAO*	Tobacco	Antisense downregulation	–	Increased tolerance to salt stress	Yamamoto et al. 2005
					–	Increased seed yield under salt stress	
	Arabidopsis	*AAO*	–	T-DNA knockout		Increased tolerance to salt stress	
						Increased seed yield under salt stress	
Myoinositol oxygenase	Rice	*OsMIOX*	Rice	Overexpression	No change	Increased drought tolerance	Duan et al. 2012a
ASA mannose pathway regulator 1	*Arabidopsis*	*AMR1*	–	T-DNA knockout	2.0–3.0-fold increase	Increased ozone tolerance	Zhang et al. 2009

Table 1: Tableau récapitulatif des différentes études menées chez les plantes sur des mutants et/ou des transformants présentant une modification de la teneur en ascorbate. Les travaux réalisés montrent un rôle essentiel de l'ascorbate dans la croissance, le développement et la défense contre les stress biotiques et abiotiques. (Venkatesch 2014)

2-3 Rôle biologique chez les plantes

L'ascorbate a de nombreuses fonctions bénéfiques chez les plantes. Au-delà de son caractère antioxydant, la modification de l'expression de gènes impliqués dans les voies de biosynthèse et de recyclage, a permis d'identifier de nombreux rôles essentiels tels que la régulation des défenses de la plante, la survie, mais aussi par l'intermédiaire des phytohormones, la modulation de la croissance des plantes (*table 1*).

Régulation de la floraison:

La transition entre phase végétative et la phase reproductive sont des étapes cruciales pour la survie des plantes à fleurs. Ces processus sont contrôlés par de nombreux paramètres endogènes mais aussi exogènes, tels que le stade de développement de la plante et les signaux environnementaux (photopériode, vernalisation, intensité de la lumière, eau et nutriments) (Bernie *et al.,* 1993; Bernie 1998). Les études menées sur les mutants déficients en ascorbate *vct1, vtc2, vtc3 et vtc4* chez la plante modèle *Arabidopsis thaliana*, montrent que dans des conditions de culture de type « jour long » (16h de photopériode), ces mutants présentent une floraison précoce par rapport au WT (Conklin et Barth 2004). De la même façon, l'augmentation de la teneur en ascorbate grâce à l'apport de L-galactono-1,4-lactone, précurseur direct de l'ascorbate dans la voie de biosynthèse, induit un retard d'environ cinq jours. Ces observations suggèrent un effet inhibiteur dans le déclenchement de l'induction florale. Par ailleurs, les données moléculaires montrent qu'au niveau de l'apex végétatif d'*Arabidopsis thaliana,* l'ascorbate aurait un effet antagoniste aux gibbérellines, lesquelles activent l'expression de gènes du développement floral tel que *LEAFY* (Blasquez *et al.,* 1998). L'analyse de la régulation induite par l'ascorbate montre un niveau d'expression important de ces gènes, dans les mutants déficients. Ainsi, l'ascorbate agit comme un signal endogène qui influence l'induction florale en agissant sur l'expression de gènes de floraisons et affectant ainsi les processus de signalisation des phytohormones (Kotchoni *et al.,* 2009).

Enzyme	*Metal ion centre*	*Change in activity*	*Physiological role*	*Enzymatic activity*
4-Hydroxylphenylpyruvate dioxygenase EC 1.13.11.27	Iron	Increase	Tyrosine metabolism	Decarboxylation and hydroxylation of 4-hydroxyphenyl pyruvic acid to homogentisic acid
gamma Butyrobetaine 2-oxoglutarate 4-dioxygenase EC 1.14.11.1	Iron	Increase	Carnitine biosynthesis	Hydroxylation of butyrobetaine to carnitine
Proline hydroxylase EC 1.14.11.2	Iron	Increase	Procollagen synthesis (animals)	Hydroxylation of proline (4-hydroxylating)
Lysine hydroxylase EC 1.14.11.4	Iron	Increase	Collagen biosynthesis (animals) Extensin biosynthesis (plants)	Hydroxylation of lysine
Procollagen proline 2-oxoglutarate 3 dioxygenase EC 1.14.11.7	Iron	Increase	Procollagen biosynthesis (animals) Exensin biosynthesis (plants)	Hydroxylation of proline (3-hydroxylating)
Trimethyllysine 2-oxoglutarate dioxygenase EC 1.14.11.8	Iron	Increase	Carnitine biosynthesis	Hydroxylation of trimethyl lysine
Cholesterol 7-alpha monooxygenase EC 1.14.13.17		Increase	Cholesterol catabolism; bile acid synthesis (animals)	Hydroxylation of cholesterol
Dopamine beta monooxygenase EC 1.14.17.1	Copper	Increase	Noradrenaline (norepinephrine) synthesis	β-hydroxylation of dopamine
Peptidyl glycine alpha amidating monooxygenase EC 1.14.17.3	Copper	Increase	Peptide amidation in peptide hormone metabolism	C-terminal glycine amidation
Mitochondrial glycerol-3-phosphate dehydrogenase EC 1.1.99.5	Iron	Increase	NAD (P) H and ATP production; aid in insulin release	Dehydrogenation of triose phosphate
Catechol-*O*-methyl transferase EC 2.1.1.6		Decrease	Adrenaline (epinephrine) inactivation (animals)	Increased bioavailability of adrenaline (epinephrine)
Thymine dioxygenase EC 1.14.11.6	Iron	Increase	Pyrimidine metabolism (fungi)	7-Hydroxylation of thymine
Pyrimidine deoxynucleoside 2′ dioxygenase EC 1.14.11.3	Iron	Increase	Pyrimidine metabolism (fungi)	Deoxyuridine to uridine
Deacetoxycephalosporin C synthetase	Iron	Increase	Antibiotic metabolism (fungi)	Penicillin N to deacetylcephalosporin
1-Aminocyclopropane-1-carboxylate oxidase	Iron	Increase	Ethylene (plant hormone) biosynthesis	Oxidation of 1-aminocyclopropane to ethylene and cyanoformic acid
Violaxanthin de-epoxidase		Increase	Zeaxanthin biosynthesis and the xanthophyll cycle (plants)	De-epoxidation of violaxanthin and antheroxanthin
Gibberellin 3-β-dioxygenase EC 1.14.11.15	Iron	Increase	Gibberin (plant hormone) biosynthesis	C_{20} oxidative decarboxylation and activation of gibberellins
Thioglucoside glucohydrolase EC 3.2.3.1		Increase	Catabolism of glucosinolates (plants)	Hydrolysis of *S*-glucosides

Table 2: Liste des enzymes affectées par des modifications de la teneur en ascorbate. Ces enzymes sont des mono- ou dioxygénases qui contiennent du cuivre ou du fer dans leur site catalytique. (Davey *et al.,* 2000)

Cofacteur:

Une des fonctions principales de l'ascorbate dans le métabolisme, est d'agir sur un nombre important de réactions enzymatiques, en tant que cofacteur (*table 2*) (De Tullio *et al.,* 1999; Arrigoni and De Tullio 2000). La plupart des enzymes sont des mono- ou dioxygénase qui contiennent du fer ou du cuivre dans leur site actif. Le rôle de l'ascorbate est de maintenir l'ion métallique du site actif sous forme réduite, et d'augmenter ainsi l'activité de l'enzyme (Davey *et al.,* 2000). Chez les plantes par exemple, l'ascorbate est utilisé comme coenzyme d'une Fer dioxygénase, qui participe dans la modification post-traductionnelle des protéines de la paroi cellulaire. L'ascorbate peut également moduler l'absorption et le relargage de fer par la ferritine (Fry SC 1986). Dans un aspect plus développemental, l'AsA est un cofacteur des enzymes 1-aminocyclopropane-1-carboxylate oxydase (ACC oxydase) et GA2-oxydase, lesquelles sont impliquées dans la synthèse de phytohormones tels que l'éthylène et les GA (Smirnoff N 1996). De même, il sert de cofacteur enzymatique pour la violaxanthine de-époxidase (VDE) qui catalyse la conversion de la violaxanthine en zéaxanthine. Le cycle xanthophylle est important pour la protection des centres réactionnels de la photosynthèse (*cf chapître Photosynthèse*). L'ascorbate permet aussi la régénération de l'α-tocophérol (vitamine E) à partir radical tocophéroxyl (Asada *et al.,* 1994).

Antioxydant:

Au cours de l'évolution, l'enrichissement en O_2 de l'atmosphère a induit l'apparition de la vie en conditions "aérobie" ce qui a permis la diversification des métabolismes avec plus 1 000 réactions utilisant l'oxygène (Falkowski 2006; Halliwell 2006). Ce mode de vie a également favorisé le développement d'organismes multicellulaires plus complexes. Néanmoins, la nature chimique de la molécule d'oxygène signifie que la vie en présence de celle-ci comporte un risque. La configuration de spin de ses électrons met en évidence que quelques molécules seulement sont capables de réduire l'oxygène, donnant lieu à des molécules ou des radicaux très réactifs mais instables, qui sont appelés des « espèces réactives de l'oxygène » (EROs). Parmi les EROs on trouve l'anion superoxyde (O_2-), le peroxyde d'hydrogène (H_2O_2), radical hydroxyle (OH) et l'oxygène singuler (O^2). Les EROs sont générées par le métabolisme aérobie dans les chloroplastes, les mitochondries ou les peroxysomes. Lors de stress biotique ou abiotique leur quantité augmente et devient

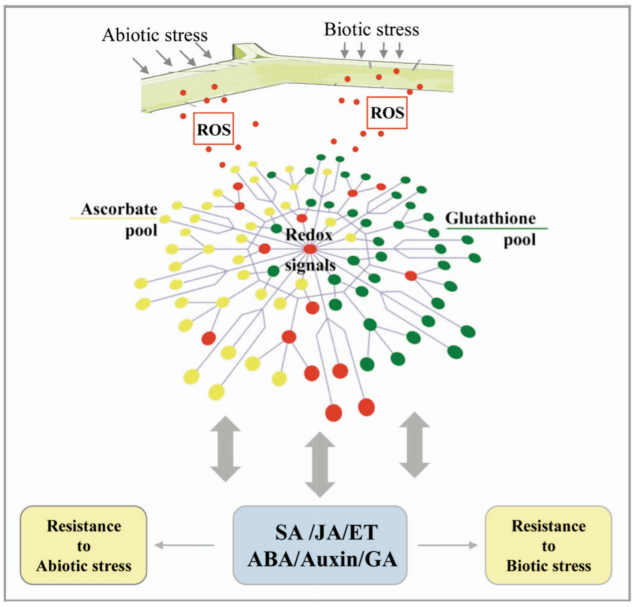


Figure 17: Modélisation du rôle du signal rédox dans l'intégration des informations provenant de l'environnement et du métabolisme. (Foyer *et al.,* 2012)

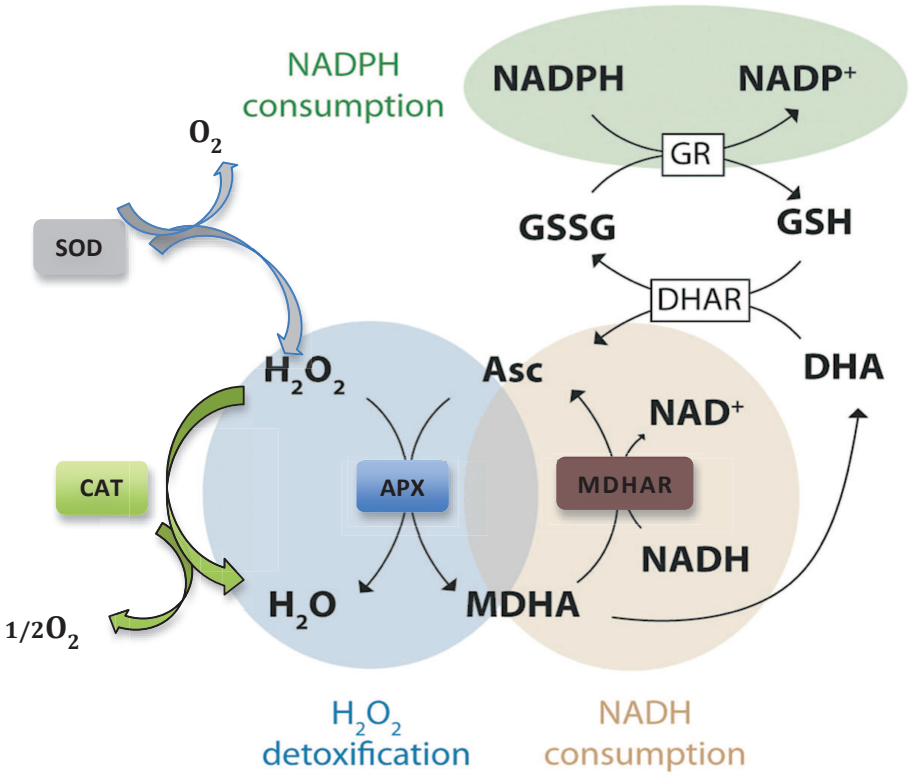


Figure 18: Détoxification des espèces réactives de l'oxygène (ERO). Plusieurs couples de donneur d'électrons sont utilisés: ASA/MDHA-DHA; GSH/GSSG; NADH/NAD+; NADPH/NADP+. (Gest *et al.,* 2012)

nocive pour la cellule et donc la plante, où elles induisent des dommages au niveau des protéines, des lipides et des acides nucléiques. On parle alors de stress oxydatif. Pour pallier à ce stress, il existe des mécanismes capables de les neutraliser. La détoxification des EROs est réalisée par l'intermédiaire d'antioxydants tels que l'ascorbate (AsA) ou le glutathion (GSH) (*figure 17*), directement ou dans des réactions catalysées par la superoxyde dismutase (SOD), l'ascorbate peroxydase (APX) et la catalase (CAT). L'ascorbate interagit de manière enzymatique et non enzymatique avec les EROs (Noctor et Foyer 1998 and 2011). Le modèle d'homéostasie rédox est basé sur cette interaction EROs-ascorbate, laquelle va agir comme un signal qui va réguler les processus sous-jacents telle que la mort cellulaire programmée. L'ascorbate peut également réparer des molécules organiques oxydées, dans certains cas de façon plus efficace que le glutathion, et ainsi générer des intermédiaires moins toxiques (Sturgeon *et al.,* 1998). L'ascorbate sous sa forme réduite (L- acide ascorbique) va être capable de donner un proton, il sera alors sous une forme oxydée (*figure 18*). L'APX est une enzyme importante qui va utiliser l'ascorbate réduit pour convertir H_2O_2 en eau (Batini *et al.,* 1995). Le premier produit issu de l'oxydation de l'acide ascorbique est le monodéhydroascorbate (MDHA), relativement stable (Smirnoff 2000). Cette stabilité résulte de la façon dont le MDHA peut délocaliser les électrons de rechange autour d'un anneau central de carbone et de ses trois groupes carbonyles (Bielski 1982). Le MDHA peut se coupler à un autre radical MDHA pour donner deux autres molécules, l'ascorbate et le déhydroascorbate (DHA) qui n'est pas un radical (Smirnoff 2000). Le DHA et le MDHA peuvent être directement réduits en ascorbate grâce aux enzymes DHAR et MDHAR (Foyer and Noctor 2011). De la même façon, le cytochrome c transmembranaire est également impliqué dans la réduction du MDHA à l'intérieur du compartiment apoplastique. Ainsi, dans ce compartiment, l'ascorbate est la première ligne de défense contre les EROs générés. L'ascorbate peut ainsi limiter la peroxydation des lipides membranaires ce qui protège les cellules et retarde la mort cellulaire. Néanmoins, la manière dont l'ascorbate est impliqué dans l'initiation et le contrôle de la transduction du signal rédox et comment ces derniers déclenchent d'autres réponses annexes, tels que l'expression des gènes afin d'optimiser les stratégies de survie, sont encore très peu décrits.

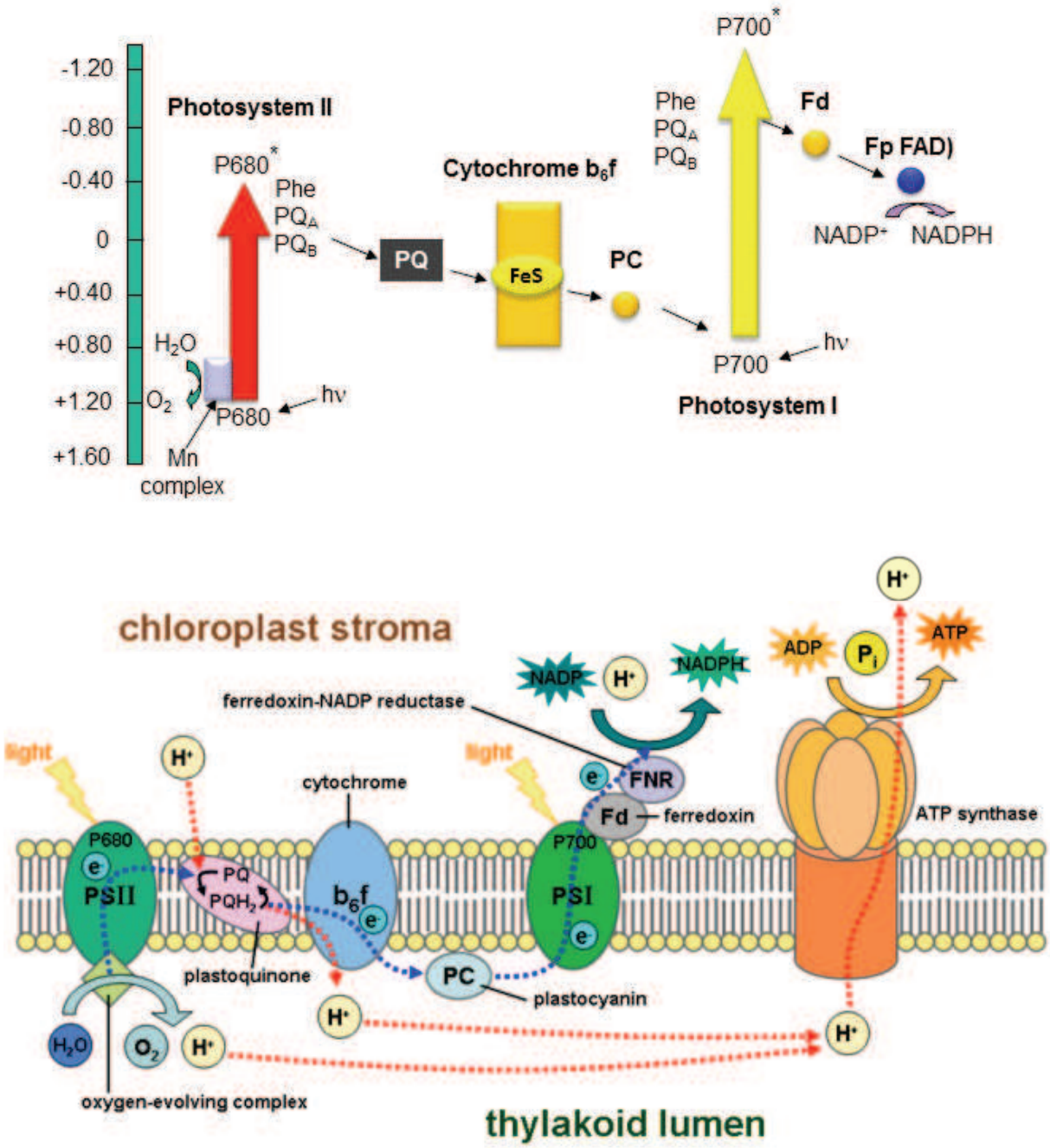


Figure 19: A. Schéma en Z des photosystèmes I et II (PSI et PSII). Les deux photosystèmes fonctionnent en série. Un photon active d'abord un électron, qu'il éjecte du PSII. Cet électron sert à pomper un proton à travers la membrane, contribuant ainsi à la production d'une molécule d'ATP par chimio-osmose. L'électron passe alors dans la chaine de cytochrome qui l'amène au PSI. Lorsque ce dernier absorbe un photon, il éjecte un électron énergétique qui servira à la production de NADPH. **B.** Processus de photosynthèse au niveau de la membrane des thylakoïdes. Lorsqu'un photon frappe une molécule de pigment du PSII, il excite un de ses électrons. Cet électron se lie à un proton arraché à l'eau et passe dans la chaine des cytochromes membranaires. Lors du clivage de l'eau, l'oxygène quitte la cellule tandis que les ions hydrogènes restent dans l'espace interne des thylakoïdes. Au niveau de la pompe à protons (complexe B6f), l'énergie fournie par le photon sert à l'importation d'un proton dans l'espace interne du thylakoïdes à travers la membrane. Lorsque le PSI absorbe un photon, son pigment transfert un second électron énergétique à un complexe réducteur (Fd), générant ainsi du NADPH. De l'ATP est produit à la face externe de la membrane (stroma), pendant que les protons diffusent au travers de l'ATP synthase.
(cf figure at http://employees.csbsju.edu/hjakubowski/classes/ch331/oxphos/olphotsynthesis.html)

Photosynthèse:

La photosynthèse est le processus responsable de la transformation de l'énergie lumineuse en énergie chimique, c'est à dire la synthèse de matière organique. Lors de la photosynthèse, l'énergie lumineuse est absorbée par une série de réactions d'oxydo-réduction pour être transmise aux centres réactionnels des photosystèmes (PSI et PSII) et ainsi permettre la production d'ATP et de NADPH. Chaque photosystème est constitué d'un réseau de molécules de chlorophylle « a », de pigments accessoires et de protéines associées maintenues dans une matrice protéique à la surface des membranes photosynthétiques. Ces éléments sont disposés en deux composantes associées: (1) une antenne collectrice formée d'une centaine de pigments photosensibles comme la chlorophylle a et b, le carotène et les xanthophylles, qui captent les photons issus de l'énergie lumineuse; et (2) le centre réactionnel, constitué d'une ou deux molécules de chlorophylle « a » associées à une matrice protéique qui exporte l'énergie du photosystème. Le processus de photosynthèse est réalisé au niveau des chloroplastes où elle se déroule en deux phases: la phase claire et la phase sombre (*figure 19*).

La phase claire correspond à un ensemble de réactions photochimiques, qui dépend de la lumière. L'énergie lumineuse est absorbée par des pigments, transformée en pouvoir réducteur (NADPH), en énergie (ATP). La phase sombre correspond à la phase d'assimilation du dioxyde de carbone (CO_2) qui utilise les molécules organiques produites lors de la phase claire (NADPH et ATP), pour la synthèse de molécule organique (sucres). L'assimilation du CO_2 est réalisée en quatre étapes dont trois se déroulent au sein du cycle de Calvin.

Dans certaines conditions, les plantes peuvent être soumises à un éclairement intense, durant l'été par exemple, dans ce cas l'excès de lumière peut s'avérer dangereux. L'activité photosynthétique est limitée par la vitesse de réaction d'assimilation du CO_2, et ainsi lorsque l'éclairement est trop important, la chaine de transport des électrons est saturée. Dans ce cas, les photosystèmes ne peuvent plus évacuer les charges électroniques provenant de cet excès de photons, entrainant la production d'EROs par transfert de ces électrons vers les molécules d'oxygènes moléculaires. Dans ce contexte, l'ascorbate est essentiel (*figure 20*). Le pool d'ascorbate dans les chloroplastes est important (20-300mM), et augmente avec l'intensité lumineuse. Dans le stroma, au niveau du site accepteur du PSI, la ferrédoxine (Fd) plutôt que de réduire le pool de NADP+ peut réduire l'oxygène moléculaire en anion

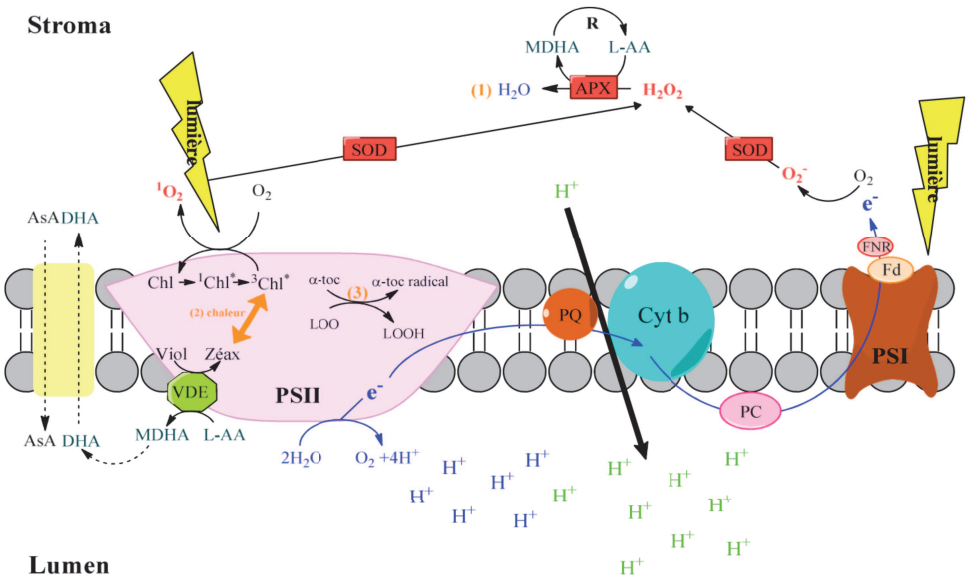


Figure 20: Rôle de l'ascorbate (L-AsA) au cours d'un stress lumineux. (1) Cycle eau-eau (2) dissipation thermique par formation de zéaxanthine (3) réduction des lipides par le L-tocophérol. Ascorbate peroxydase (APX); superoxyde dismutase (SOD); chlorophylle (Chl); cytochrome b (Cyt b); déhydroascorbate (DHA); monodéhydroascorbate (MDHA); ferredoxine (Fd); ferredoxine NADP réductase (FNR); peroxyde d'hydrogène (H_2O_2); radical lipide peroxyl (LOO); lipide hydroperoxyde (LOOH); anion superoxyde (O_2^-); Oxygène singulet (½ O2); plastoquinone (PQ); L-tocopherol (L-toc); violaxanthine (Viol); violaxanthine dé-époxydase (VDE). (Thèse Capucine MASSOT 2010)

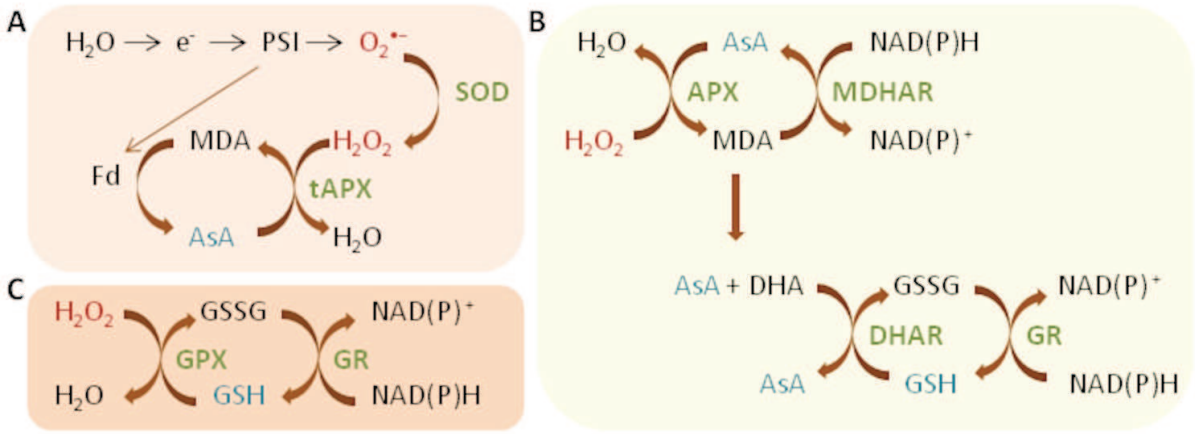


Figure 21: Différentes voies impliquées dans la détoxification des EROs chez les plantes. **A.** Cycle eau-eau (réaction de Mehler); **B.** Cycle ascorbate/glutathion; **C.** Cycle de la glutathion peroxydase. Les EROs sont indiqués en rouge, les enzymes en vert et les antioxydants en bleu. Abréviations: Ascorbate (ASA); Glutathion (GSH); Superoxyde dismutase (SOD); Glutathion peroxydase (GPX); Déhydroascorbate (DHA); DHA réductase (DHAR); Monodéhydroascorbate (MDA); MDA réductase (MDAR); Ferrédoxine (Fd); Glutathion réductase (GR); Glutathion oxydé (GSSG); Photosystème I (PSI); APX liée aux thylakoïdes (tAPX). (Wituszynska and Karpinsky 2013)

superoxyde. Cet anion superoxyde est ensuite réduit en peroxyde d'hydrogène par la SOD, puis en H_2O grâce à l'APX par oxydation de l'ascorbate. On appelle ce cycle eau-eau, la réaction de Mehler (*figure 21*) (Wituszynska and Karpinsky 2013). Le MDHA produit peut être directement réduit en ascorbate par le PSI et/ou le cycle ascorbate/GSH (Ivanov 2000). La MDHAR liée au niveau des thylakoïdes, utilise la Fd réduite comme donneur d'électron pour régénérer l'ascorbate (Foyer CH 1997; Appel and Hirt 2004). Dans l'espace interne des thylakoïdes (PSII), la dissipation de l'énergie d'excitation excédentaire par un quenching non photochimique (QNP) de la fluorescence de la chlorophylle a. Cette dissipation sera sous la forme d'émission de chaleur, permettant de prévenir la photoinhibition et les dommages causés à l'appareil photosynthétique. Au cours de ce processus, la VDE, enzyme responsable de la conversion de la violaxanthine en antheraxanthine et zeaxanthine, se lie à la membrane des thylakoïdes où elle va pouvoir utiliser l'ascorbate comme source de pouvoir réducteur pour sa réaction (Hager and Holocher 1994). L'ascorbate a donc un rôle important dans la formation du QNP. Ainsi, dans le mutant *vtc2* déficient en AsA la quantité de zéaxanthine est plus basse que chez le WT (Müller-Moulé *et al.,* 2002). De même, l'étude de mutants présentant une teneur en ascorbate plus importante comme par exemple le mutant *miox4*, révèle le rôle de l'ascorbate comme facteur limitant de l'activité de la VDE (Toth *et al.,* 2011). Au niveau du PSII, on constate également que les molécules de chlorophylles des centres réactionnels ne peuvent plus se désactiver par photochimie. Les chlorophylles à l'état de triplet (^{3}Chl) réagissent alors avec l'oxygène, pour donner naissance à l'oxygène singulet ($^1O_2$). L'oxygène singulet est une forme active de l'oxygène susceptible de conduire à la dégradation des structures plastidiales. L'ascorbate est donc un acteur primordial pour le maintien de l'intégrité des photosystèmes PSI et PSII.

Croissance:

La croissance chez les plantes supérieures est le résultat de deux types de processus, la prolifération et l'expansion cellulaires. Dans ce contexte, l'ascorbate est un élément essentiel. L'étude de mutants et de plantes transgéniques montre une altération de la croissance et/ou du développement. Dowdle *et al.,* (2007) ont montré que le double mutant *vtc2/vtc5,* incapable de synthétiser l'ascorbate, ne pouvait se développer au-delà de la germination (Dowdle *et al.,* 2007). De même, des lignées de transformant *GME*, présentant une diminution de l'ascorbate d'environ 50%, montrent une retard sévère de

la croissance (Gilbert *et al.,* 2009). Le pool ascorbate réduit/oxydé (MDHA-DHA) semble jouer un rôle important dans le control du cycle cellulaire en influençant la proportion de cellules entrant dans la phase S. Lorsqu'on utilise la lycorine, un inhibiteur de la synthèse d'ascorbate, on bloque ainsi la division et l'expansion cellulaire (Citterio *et al.,* 1994). Pour expliquer ce phénomène, l'hypothèse serait liée au cycle rédox qui existe au travers de la membrane plasmique (cf chapitre anitoxydant). Le transfert des électrons au travers de cette membrane stimule une H^+-ATPase, laquelle est capable de stimuler la croissance cellulaire. Mais également le rôle de cofacteur pour des enzymes impliquées dans la synthèse des parois cellulaires peut expliquer l'effet stimulateur de l'ascorbate (Cooper *et al.,* 1994; De Tullio *et al.,* 1999).

L'ascorbate semble donc être directement et indirectement impliqué dans la régulation de la croissance de la plante. Il va servir de signal au niveau du plasmalemme, interface dynamique qui perçoit et transmet les informations de l'environnement extérieur au noyau pour induire une réponse adaptée. Ainsi, le ratio entre le pool d'ascorbate réduit et oxydé dans l'apoplasme, permet de moduler la croissance et la division cellulaire.

- *Expansion cellulaire:*

L'ascorbate et ses formes oxydées influencent l'expansion cellulaire par de nombreux mécanismes, souvent liés à la paroi (Smirnoff N 1996). Tout d'abord, l'ascorbate est capable de réguler les processus cellulaires en liant sa biosynthèse avec d'autres métabolismes. La L-galactose 1-P phosphatase (GPP) est une enzyme qui intervient à la fois sur la biosynthèse de l'ascorbate mais aussi celle du myo-inositol. La biosynthèse et le catabolisme du myo-inositol sont essentiels pour la production de phosphatidyl-inositol, de polysaccharides pariétaux, et l'ancrage membranaire de type glycérophosphoinositide. L'enzyme L-galactono-lactone déshydrogénase (GalLDH), intervient sur les processus liés à la croissance cellulaire. Chez les transformants de tomate RNAi *SlGalLDH* l'expansion cellulaire diminue ce qui se traduit par une taille des feuilles et des fruits moins importante que chez le WT (Alhagdow *et al.,* 2007). Ensuite, l'ascorbate intervient dans l'élimination des radicaux libres impliqués dans la synthèse de xylogène, régulant ainsi la lignification des parois cellulaires (Kvaratskhelia *et al.,* 1997; Davey *et al.,* 2000). Pour finir, l'ascorbate peut servir de substrat pour la biosynthèse d'acide oxalique (Loewus *et al.,* 1988). La concentration apoplastique

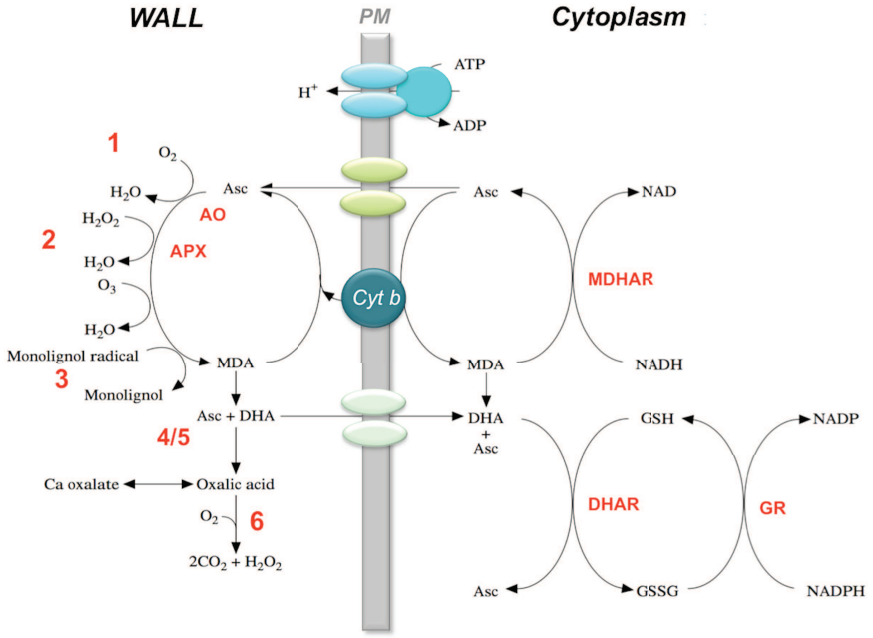


Figure 22: Rôle de l'ascorbate (Asc) dans le cytoplasme et la paroi durant l'expansion cellulaire. **(1)** L'ascorbate oxydase (AO) catalyse l'oxydation de l'ascorbate en monodéhydroascorbate (MDHA). Le MDHA accepte les électrons provenant du cytochrome *b* (*Cyt b*) ce qui induit une dépolarisation de la membrane. Cette dépolarisation active une H+-ATPase qui permet le relâchement de la paroi. **(2)** L'ascorbate peroxydase (APX) en neutralisant le peroxyde d'hydrogène, maintient la plasticité membranaire. **(3)** L'ascorbate inhibe la réticulation par oxydation des polysaccharides pariétaux mais également la polymérisation de la lignine par piégeage de peroxyde d'hydrogène et des radicaux de monolignol. **(4**) Le déhydroascorbate (DHA) peut réagir avec les chaines latérales d'acides aminées des polypeptides pariétaux et ainsi prévenir la réticulation de la matrice de polysaccharide. **(5)** L'ascorbate et le DHA permettent la formation d'oxalate, lequel est à l'origine du relâchement de la paroi *via* la fixation de calcium. **(6)** La dégradation de l'oxalate par l'oxalate oxydase entraine la production de peroxyde d'hydrogène et de calcium, favorisant ainsi le resserrement de la paroi. (Smirnoff 1996) Abréviations: glutathion réductase (GR); DHA réductase (DHAR); MDA réductase (MDAR)

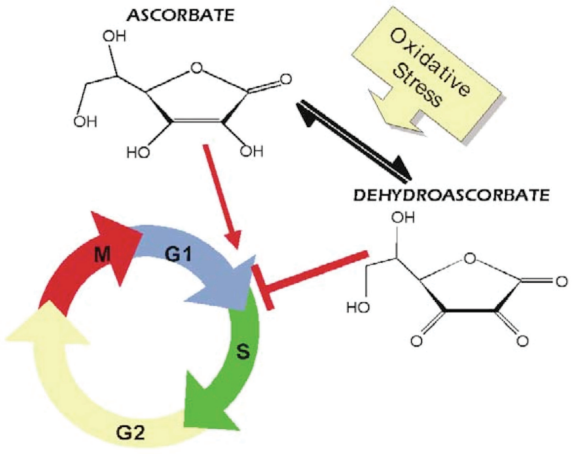


Figure 23: Influence de l'ascorbate dans la régulation du cycle cellulaire. Le cycle cellulaire comprend 4 phases distinctes: G1 (croissance cellulaire et préparation à la réplication); S (réplication de l'ADN); G2 (croissance cellulaire et préparation à la mitose); M (mitose ou division cellulaire). Une quantité suffisante d'ascorbate est nécessaire pour l'apparition de la division cellulaire (passage de la phase G1 à la phase S) alors qu'un excès de DHA aura un effet inhibiteur. Le changement dans le rapport ascorbate réduit/oxydé est aussi important pour la cellule afin d'induire la réponse adaptée lors d'un stress. (Potter *et al.,* 2002).

d'oxalate permet de réguler la teneur en ion calcium par piégeage au niveau de la paroi et formation de cristaux d'oxalate de calcium. Cette diminution du calcium augmente l'extensibilité de la paroi en diminuant les liaisons croisées entre les chaines de polygalacturonate (Lin and Varner 1991)

Lorsqu'on s'intéresse aux enzymes qui interviennent dans le cycle rédox de l'ascorbate (*figure 22*), il apparaît évident que l'APX au niveau de la paroi, aide au maintien de la plasticité en neutralisant le peroxyde d'hydrogène (Hidalgo *et al.,* 1989; Takahama U 1994). De même, l'AO qui catalyse l'oxydation de l'ascorbate, induit la formation des formes oxydées (MDHA et DHA), éléments qui affectent directement l'expansion cellulaire (Smirnoff 1996). En effet, Hidalgo *et al.,* (1989) montrent que le MDHA stimule la croissance et l'expansion cellulaire des racines d'oignon (Hidalgo *et al.,* 1989; Gonzales-Reyes 1995). Il semble qu'au moment où le MDHA est réduit, il joue le rôle d'accepteur d'électron *via* le cytochrome b, permettant alors l'augmentation du transport électrons au travers de la membrane. Cette dépolarisation de la membrane va activer l'H^+-ATPase (transport de protons vers l'apoplasme) ce qui stimule l'expansion cellulaire (Rayle and Cleland 1992). Le DHA peut réagir avec les chaines latérales des résidus de lysine et d'arginine, empêchant les liaisons des protéines structurales avec l'hémicellulose et le polygalacturonate (Lin and Varner, 1991). L'activité de l'ascorbate oxydase est très faible dans les axes embryonnaires de semis de pois pendant les étapes précoces de la germination, néanmoins une augmentation marquée de l'activité précède juste le début de la croissance de l'axe embryonnaire (Pallanca and Smirnoff 1999).

Ces données mettent en évidence que l 'ascorbate et les enzymes associées, peuvent intervenir dans la plasticité pariétale et ainsi jouer un rôle dans l'expansion cellulaire.

- *Division cellulaire:*

L'ascorbate serait capable de réguler le cycle cellulaire (*figure 23*). La teneur en ascorbate dans les méristèmes est plus forte que dans les tissus où les divisions cellulaires sont faibles, tel que le centre quiescent (CQ). L'augmentation du pool d'ascorbate, induit une augmentation du taux de division cellulaire (Liso *et al.,* 1988; Davey *et al.,* 1999). Dans des primordia de racines *d'Allium, Pisum* et *Luminus,* l'ajout d'ascorbate exogène a permis de montrer que ce phénomène serait induit par une augmentation du nombre de cellules progressant de la phase G1 (cellule compétente) à

la phase S (synthèse ADN) du cycle cellulaire (Citterio *et al.,* 1994; De Cabo *et al.,* 1996; Arrigoni *et al.,* 1997). Les études réalisées sur le CQ dans des racines de maïs, lequel est constitué de cellules bloquées en phase G1, montrent qu'il contient une quantité importante d'AO. Ce qui corrèle avec des niveaux faibles d'ascorbate réduit dans cette zone. Cette hypothèse a été confirmée dans le maïs et l'*Allium*, où l'ajout d'ascorbate au niveau du CQ a permis aux cellules de reprendre leur cycle cellulaire (Liso *et al.,* 1988; Kerk *et al.,* 1995). De la même façon, la répression de la GalDH, une enzyme de la voie de biosynthèse, dans des cellules BY-2 de tabac, réduit de près de 30% la teneur en ascorbate (Kato *et al.,* 1999). Cette modification de la teneur induit une diminution du taux de division cellulaire ainsi que de la croissance. Les cellules de BY-2 sont alors bloquées en phase G1 du cycle cellulaire, et parallèlement, la quantité d'AO et de DHA diminue. L'augmentation de la quantité de DHA a un effet inhibiteur seulement durant la phase G1 et non la phase G2 (Potters *et al.,* 2000). L'ascorbate a donc un rôle important durant la phase G1 du cycle cellulaire. Il semble que l'ascorbate soit capable de réguler la croissance des plantes en agissant sur la biosynthèse de protéines riches en hydroxyproline nécessaires à la progression des phases G1 et G2 du cycle cellulaire (Ito *et al.,* 1998; De Tullio *et al.,* 1999).

Régulation du processus de senescence: exemple de la feuille:

Il existe différents types de senescence selon l'organe: feuille, fleur, fruit ainsi que la senescence après la récolte. La senescence naturelle observée durant la dernière étape du développement foliaire, est un processus dégénératif programmé. La sénescence est un type de mort cellulaire programmé (MCP) mais elle présente la caractéristique d'être réversible (Thomas *et al.,* 2003). Elle est contrôlée par de nombreux facteurs développementaux tels que l'âge et le stade de développement des organes reproducteurs; mais également environnementaux comme le froid, la sécheresse ou les attaques par les pathogènes. Elle est caractérisée par une expression modifiée de certains gènes (senescence-down-regulated genes ou *SDG;* scenescence-associated genes ou *SAG*), une dégénérescence active de macromolécules, et le recyclage de nutriments (azote, carbone et nutriment) (Quirino *et al.,* 2000; Lim *et al.,* 2003). Ainsi, les nutriments sont transportés depuis les tissus sénescents jusqu'au tissus jeunes et les organes reproducteurs (fleurs, graines et fruits). On constate donc que le nombre de feuilles sénescentes est plus important quand les organes reproducteurs sont

présents. En revanche, les tissus vasculaires sont maintenus jusqu'à des stades très tardifs du processus de sénescence (Gan *et al.,* 1997). Les protéines sont dégradées en acides aminés, les ARN sont décomposés et les lipides membranaires sont métabolisés en sucres (Buchanan-Wollaston 1997). La fragmentation de l'ADN a lieu seulement dans les dernières étapes. Dans ces stades tardifs du processus, les cellules sont donc peroxydées, l'ADN est dégradé et certains organites sont détruits. Le jaunissement des feuilles dues à la dégradation de la chlorophylle est souvent considéré comme le principal marqueur de la senescence.

L'ascorbate régule l'expression des gènes *SAG*. Une accumulation faible d'ascorbate accélère la sénescence alors qu'une teneur en ascorbate élevée retarde le processus. Chez le mutant d'Arabidopsis déficient en ascorbate *Atvtc1*, des feuilles détachés et conditionnées dans le noir présentent une dégradation de la chlorophylle plus rapide, induisant une sénescence foliaire plus précoce que chez le WT. Parmi ces gènes, *SAG13* et *SAG15* montrent une expression prématurée dans *vtc1* (Barth *et al.,* 2004). De la même façon, les gènes *PR-1, PR-2* et *PR-5* (Pathogenesis-Related protein) sont plus exprimés dans les mutants *vtc1* et *vtc2* que chez le WT. Cette modification de l'expression des gènes *SAG* serait la conséquence d'une augmentation importante de la quantité d'EROs (Thompson *et al.,* 1987). Chez les plantes supérieures, le cycle Ascorbate-Glutathion (AsA-GSH) est un système antioxydant important pour la protection (cf chapitre antioxydant). Dans le processus de senescence, la mélatonine (N-acetyl-5-méthoxytryptamine) est un antioxydant qui permet le retard de la dégénérescence cellulaire par le maintien d'un niveau élevé de l'activité du cycle AsA-GSH (Wang *et al.,* 2012). En conclusion, l'ascorbate est capable de réguler le processus de sénescence en régulant la quantité de EROs et/ou l'expression des gènes SAG.

2-4 Métabolisme, catabolisme et recyclage de la vitamine C

2-4i Biosysnthèse

La voie de biosynthèse de la vitamine C a été élucidée très tôt chez les animaux. En 1954, Isherwood *et al.,* ayant constaté que les plantules de cresson (*Lepidium sativum L.*) étaient capables de réaliser l'oxydation de la galactono-lactone (GalL) en ascorbate, proposèrent un schéma analogue entre la voie décrite chez les animaux et celle présente chez les plantes.

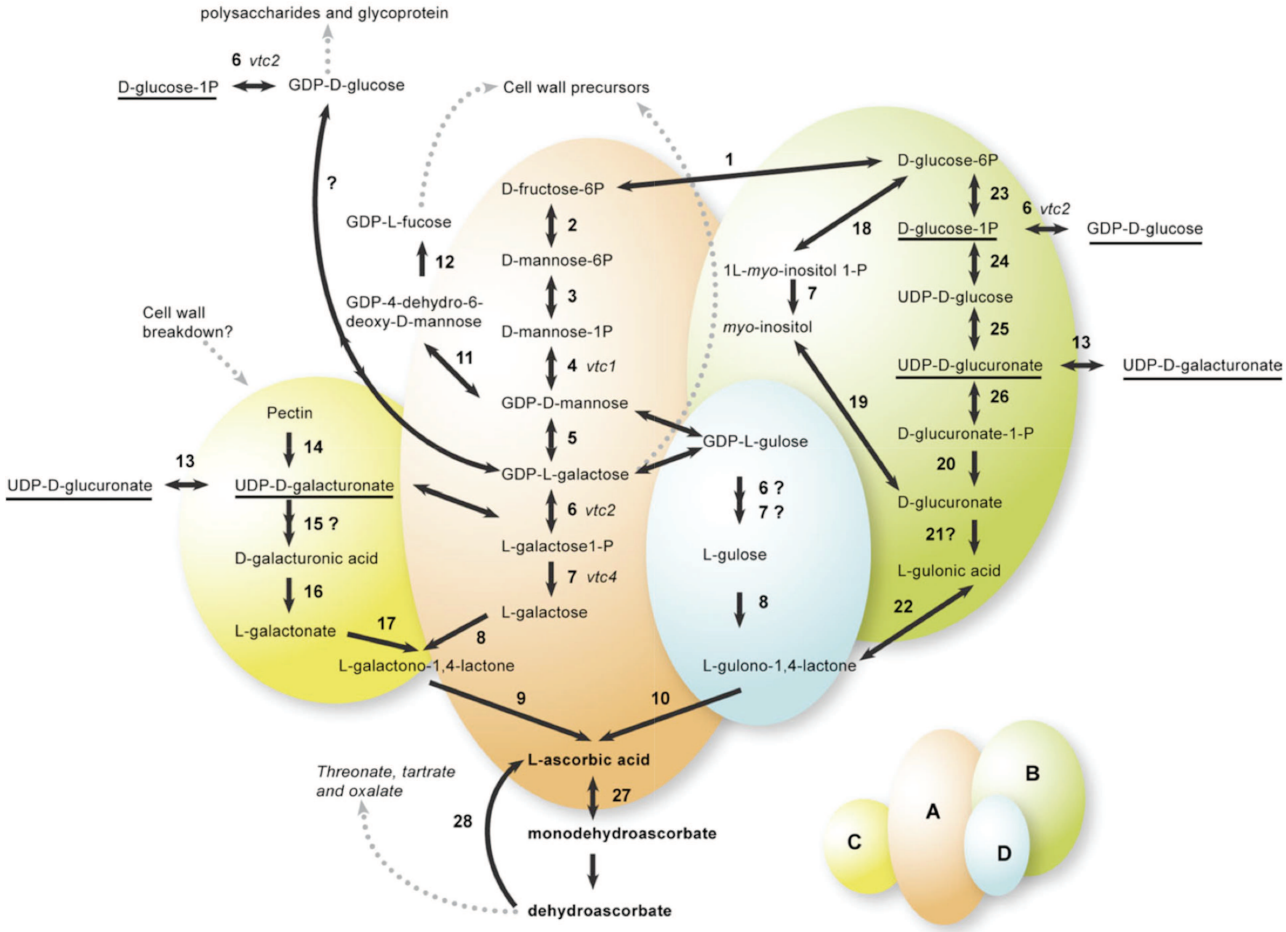


Figure 24: Voies impliquées dans la biosynthèse de l'ascorbate chez les plantes. **(A)** Voie du L-Galactose (2-9); **(B)** Voie "animale" du myo-inositol (7, 18-26); **(C)** Voie du D-Galacturonate (14-17); **(D)** voie du L-Gulose (5-8, 10). Les enzymes identifiées ont été numérotées. **1:** glucose-6-phosphate isomérase (PGI); **2:** mannose-6-phosphate isomérase (PMI); **3:** phosphomannose mutase (PMM); **4:** GDP-mannose pyrophosphorylase (GMP); **5:** GDP-mannose-3#,5#-épimérase (GME); **6**: GDP-L-galactose phosphorylase (GGP); **7:** L-galactose-1-phosphate phosphatase (GPP); **8:** L-galactose déshydrogénase (GDH); **9:** L-galactono-1,4-lactone déshydrogénase (GLDH); **10**: L-gulono-1,4- lactone oxidase; **11:** GDP-D-mannose-4,6-déshydratase; **12:** GDP-L-fucose synthase; **13:** UDP-galacturonate épimérase; **14:** polygalacturonate 4-a-galacturonosyltransférase; **15:** galacturonate-1-phosphate uridyltransférase and galacturonate-1-phosphate phosphatase (hypothétique); **16:** D-galacturonic acide réductase; **17:** aldonolactonase; **18:** L-myo-inositol 1-phosphate synthase; **19:** myo- inositol oxygénase (MIOX4); **20:** D-glucurono-1-phosphate phosphatase; **21:** glucuronate réductase; **22:** gulonolactonase; **23:** phosphoglucomutase; **24:** UDP-glucose-pyrophosphorylase; **25:** UDP-glucose déshydrogénase; **26:** glucuronate-1-phosphate uridyltransférase; **27:** monodéhydroascorbate réductase; **28:** déhydroascorbate réductase.(Bulley *et al.,* 2009)

Avec cependant, une différence au niveau de la transition D-galacto/L-galacto. Ce n'est qu'en 1990, que Smirnoff met en évidence les voies de biosynthèse chez les plantes. Puis l'étude de mutants d'*Arabidopsis thaliana* déficient en ascorbate a permis à Wheeler *et al.,* en 1998 d'identifier la voie majeure de biosynthèse chez les plantes (voie du D-mannose/L-galactose). Quelques années plus tard, d'autres voies alternatives ont été décrites: voie du galacturonate, voie du gulose, mais aussi la voie du myo-inositol (*figure 24*) et plusieurs hypothèses ont émergé quant à la présence d'un lien entre les voies de biosynthèse de l'ascorbate avec les polysaccharides pariétaux et les cires. La résolution de la voie de biosynthèse a ouvert la voie à l'identification des facteurs génétiques et biochimiques contrôlant l'accumulation d'AsA dans les plantes.

Voie principale de biosynthèse chez les plantes: D-mannose/L-galactose

La voie principale de biosynthèse de l'ascorbate appelée voie du L-galactose ou voie de Smirnoff-Wheeler, est différente de celle impliquée chez l'Homme (ul-Hassan and Lehninger 1956; Linster *et al.,* 2008). Elle a été caractérisée pour la première fois sur la plante modèle *Arabidopsis thaliana,* où l'ensemble des gènes impliqués ont été identifiés (Ishikawa *et al.,* 2006a). La voie du L-galactose est localisée au niveau du cytosol, à l'exception de la dernière étape où l'enzyme GLDH est localisée au niveau des mitochondries (Siendones *et al.,* 1999; Bartoli *et al.,* 2000). L'intégration de la GLDH dans la chaine de transport des électrons *via* le cytochrome c, semble être importante pour coordonner le métabolisme de l'ascorbate avec le métabolisme énergétique intervenant dans le statut rédox de la cellule. L'incapacité des autres voies alternatives à compenser la perte d'ascorbate dans les doubles mutants déficients *vtc2/vct5*, ainsi que des mutants knock-out *vtc2 et vtc5* correspondant aux gènes codant pour l'enzyme GGP pour GDP-L-galactose phosphorylase met en évidence la position essentielle de cette voie dans la constitution du pool d'ascorbate chez les plantes (Conklin *et al.,* 1999; Dowdle *et al.,* 2007). Cette voie de biosynthèse implique 10 étapes enzymatiques avec comme précurseur le D-glucose. L'UDP-D-glucose dérivé de l'amidon, est considéré comme le substrat principal pour la synthèse *de novo* d'ascorbate. Elle combine deux éléments importants: la non-inversion du squelette carboné, et la L-galactono-1,4-lactone comme précurseur immédiat de l'acide L-ascorbique. Les six premières étapes sont responsables de la biosynthèse de sucres nucléotidiques, lesquels servent également des précurseurs pour la formation des polysaccharides de la paroi cellulaire

et des glycoprotéines. Les quatre étapes qui suivent sont spécifiques de la synthèse de l'ascorbate. Ainsi, l'enzyme GGP est la première enzyme de la voie spécifique de la synthèse. Suivant l'espèce considérée, plusieurs des enzymes jouent un rôle de régulateur clef dans la détermination de la concentration en ascorbate finale comme par exemple l'enzyme GGP chez le kiwi et la pomme, et les enzymes GME et GPP chez la tomate (Stevens *et al.,* 2007; Li *et al.,* 2008; Ioannidi *et al.,* 2009; Bulley *et al.,* 2009; Mingjung *et al.,* 2010).

Voie du D-galacturonate:

En 1969, Isherwood *et al.,* décrivent une voie analogue à la voie de biosynthèse caractérisée chez les animaux, voie qui propose la conversion du D-galactose par un mécanisme ‘‘d'inversion du squelette carboné". Lors de cette étude, ils montrent ainsi que le méthyl ester d'acide galacturonique pouvait être directement convertie en acide L-ascorbique, sans impliquer les réactions intervenant dans la voie des hexoses monophosphates. Plus tard, Agius *et al.,* identifient le gène codant une D-galacturonate reductase NADPH dépendante (GalUR) chez la fraise, et montrent ainsi l'existence d'une voie alternative appelée la voie du D-Galacturonate (Agius *et al.,* 2003). Badejo *et al.,* (2011) montrent que l'incubation d'un extrait de fruits de tomate à maturité en présence de D-galacturonate permet l'augmentation de la teneur en ascorbate, ce qui n'est pas le cas pour l'extrait issu de fruits vert. L'activité des deux enzymes associées à cette voie (GalUR et aldonolactonase), est détectable dans les fractions insolubles de fruits rouges suggérant que cette voie est dépendante du stade de développement du fruit. Le D-galacturonate est un composant abondant de la paroi cellulaire et provient du turnover des pectines durant le processus de senescence. Des données métaboliques récentes, montrent une forte accumulation en D-galacturonate durant la maturation (Carrari *et al.,* 2006). Un argument fort en faveur de l'existence de cette voie repose donc d'une part sur les travaux réalisés chez la fraise (Loewus *et al.,* 1956; Agius *et al.,* 2003), et d'autre part sur l'observation que la teneur en ascorbate est 2 à 3 fois supérieure à celle du témoin, chez des plantes d'Arabidopsis surexprimant *GalUR*. Il n'y a donc pas de confirmation directe mais de forte présomption quant à la participation de cette voie dans l'accumulation de l'ascorbate durant la maturation du fruit. Si on prend le cas des protistes capables de réaliser la photosynthèse, ils utilisent la voie du D-galacturonate

pour la synthèse d'ascorbate (Ishikawa *et al.,* 2008). Il est possible que le manque de connaissance concernant l'aldonolactonase, une enzyme essentielle dans ce schéma de synthèse de l'AsA, explique que cette voie ne soit pas encore caractérisée chez les plantes supérieures.

Voie du L-gulose:

Dans la voie du L-galactose, l'enzyme GME catalyse la conversion du GDP-D-mannose en GDP-L-galactose. Les études à la fois de l'enzyme recombinante et de l'enzyme native, ont révélé que le GDP-L-galactose n'était pas le seul produit issu de cette épimérisation (Wolucka and Montagu 2002). En effet, l'activité de la GME produit également du GDP-L-gulose (*figure 24*). Ainsi, l'enzyme GME catalyse deux types distincts d'épimérisation produisant dans un cas le GDP-L-galactose (voie L-galactose) et dans l'autre cas le GDP-L-gulose (voie L-gulose) (Wolucka *et al.,* 2003; Botella *et al.,* 2004). Les réactions qui suivent la formation du GDP-L-gulose sont très peu décrites chez les plantes, néanmoins l'intermédiaire L-acide gulonique (Wolucka and Montagu 2003) ainsi que l'activité de la L-gulono-1,4-lactone déshydrogénase (Wagner *et al.,* 2003) ont été caractérisées.

Voie du Myo-inositol:

La possibilité qu'une voie de biosynthèse animale de l'AsA soit présente chez les plantes n'a jamais été écartée. La surexpression chez la laitue et le tabac du gène codant pour la L-gulono-1,4-lactone oxydase de rat, induit une augmentation de la teneur en ascorbate d'environ sept fois (Jain and Nessler 2000). Plus récemment, l'expression ectopique de ce gène dans des feuilles de mutants d'Arabidopsis déficients (*vtc*), restaure les teneurs en AsA (Radzio *et al.,* 2003). Chez les animaux le substrat utilisé pour cette enzyme est la L-gulono-1,4-lactone, il est intéressant de constater que le mutant *vtc1* d'Arabidopsis produit très peu de cet intermédiaire. La voie du *myo*-inositol utilise comme substrat le *myo*-inositol, osmolite accumulé en condition de stress (Zhu *et al.,* 2014), qui est converti en acide D-glucuronique par la myo-inositol oxygénase (Brown *et al.,* 2006). Ainsi, dans certaines conditions, ces données suggèrent la présence d'une voie alternative qui peut compenser la voie du L-galactose.

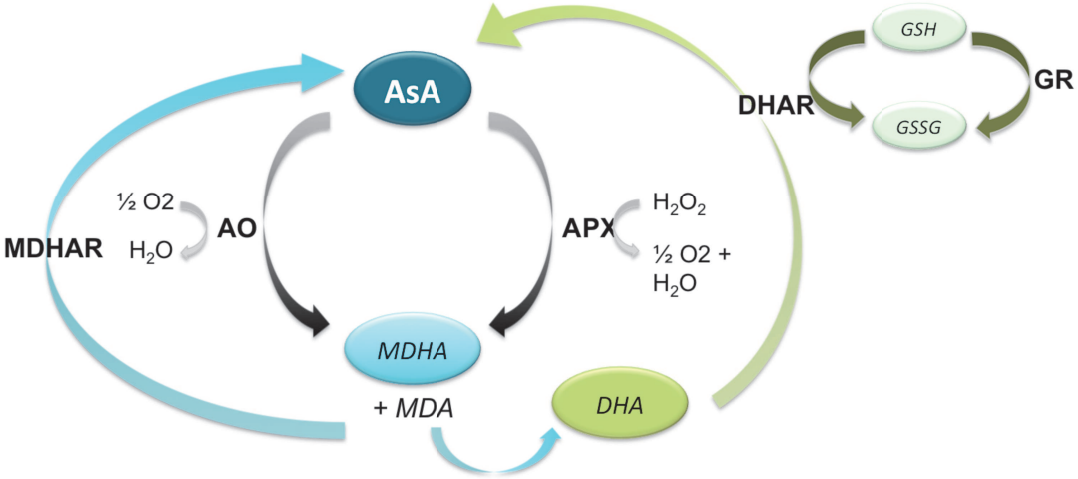


Figure 25: Voie du recyclage de l'ascorbate (AsA) chez les plantes. Ascorbate oxydase (AO); ascorbate (APX); Monodéhydroascorbate (MDHA); déhydroascorbate (DHA); MDHA réductase (MDHAR); DHA réductase (DHAR); glutathion réduit (GSH); glutathion oxydé (GSSG); glutathion réductase (GR)

2-4ii Recyclage

Le recyclage de l'ascorbate est très important notamment durant les mécanismes de réponse à des stress biotique et abiotique (*figure 25*). Dans ce contexte, la capacité de l'AsA à neutraliser les radicaux libres par des réactions enzymatiques impliquant la SOD, l'AO, l'APX et la catalase, ou non enzymatiques utilisant directement l'AsA ou le GSH nécessite la mise en place d'une voie de recyclage (*cf chapitre antioxydant*). L'APX va utiliser la capacité de donneur d'électron de l'ascorbate pour réduire les radicaux libres de type H_2O_2 en H_2O (Noctor and Foyer 1998). Une des particularités de cette enzyme est qu'elle est instable en absence d'ascorbate (Ishikawa and Shigeoka 2008). A l'issue de cette détoxification, l'ascorbate est oxydé en un radical le MDHA, qui peut à son tour être réduit par une réductase, la MDHAR, pour former à nouveau de l'ascorbate "actif". Cette réaction de réduction nécessite un apport d'électron, lequel est généralement fourni par l'oxydation de NADH. Cependant, le NADPH, la ferrédoxine et le cytochrome b peuvent également être utilisés. Lorsque l'activité de la MDHAR n'est pas suffisante, deux molécules de MDHA interagissent pour former une molécule d'ascorbate et une molécule de DHA. Cette forme di-oxydée DHA qui n'est pas un radical peut être de la même façon réduite par une réductase, la DHAR qui utilise le glutathion réduit comme cofacteur (GSH), sinon le DHA est dégradé en 2,3-diketo-L-gulonic acid (*cf chapitre catabolisme*). La dernière étape du recyclage consiste à la régénération du GSSG oxydé par une glutathion réductase (GR). Cette voie de recyclage est appelée le cycle ascorbate-glutathion. De nombreuses publications montrent que la surexpression des enzymes MDHAR et DHAR permet d'augmenter le pool d'ascorbate réduit dans les cellules (Chen *et al.,* 2003; Eltayeb *et al.,* 2007). L'utilisation de ^{14}C-ascorbate a permis d'estimer un taux de renouvellement ou turnover d'environ 2 à 3% du pool par heure dans les feuilles d'Arabidopsis (Conklin *et al.,* 1997). Une étude réalisée au niveau de l'axe embryonnaire du Pois en germination, montre que le turnover dans ce cas est d'environ 13% (Pallanca and Smirnoff 2000). Suivant l'organe et le stade de développement, le renouvellement de l'ascorbate est plus ou moins important. La voie de recyclage permet donc de maintenir un statut redox essentiel pour la fonction antioxydant de l'ascorbate dans la plante.

2-4iii Catabolisme

La dégradation de l'ascorbate semble avoir lieu au niveau de l'apoplasme des cellules, à partir de DHA (Green and Fry 2005). Chez la plupart des plantes, la dégradation du DHA induit la formation de deux produits: l'oxalate (OxA) et le L-thréonate (ThrO). Dans certains cas comme chez le raisin, l'ascorbate peut également être dégradé *via* le L-idonate en L-thréonate puis en L-tartrate (Green and Fry 2005). Cette dégradation du DHA peut être réalisée de façon enzymatique ou non enzymatique conduisant à la formation irréversible de 2,3-diketogulonate (DKG). Ainsi, le DHA peut être oxydé en présence de radicaux H_2O_2 de façon non enzymatique en mono-anion (cyclic-oxalyl-threonate; cOxT) ou en di-anion (oxalyl-threonate isomers; OxT) (Parsons *et al.,* 2011). En absence de H_2O_2, le DHA qui est instable à pH physiologique, va être spontanément délactonisé en DKG par réaction enzymatique. Le DKG à l'inverse est relativement stable; néanmoins au cours du temps il sera oxydé pour donner de nombreux produits, parmi lesquels le 2-carboxy-1-xylonolactone, le 2-carboxy-1-lyxonolactone et le 2-carboxy-1-threo-pentonate. Les intermédiaires produits en présence ou non de radicaux libres, vont être ensuite hydrolysés en oxalate, L-threonate et L-tartrate. Les produits issus du catabolisme sont susceptibles de jouer un rôle métabolique. Présent chez les plantes supérieures, l'oxalate est impliqué dans l'osmorégulation et le contrôle de la concentration en calcium. De même, le tartatre est un composé très important pour l'établissement des caractéristiques organoleptiques de la baie de raisin. Bien que les intermédiaires de cette voie aient été identifiés, les enzymes impliquées ne sont pas bien caractérisées.

2-5 Transport

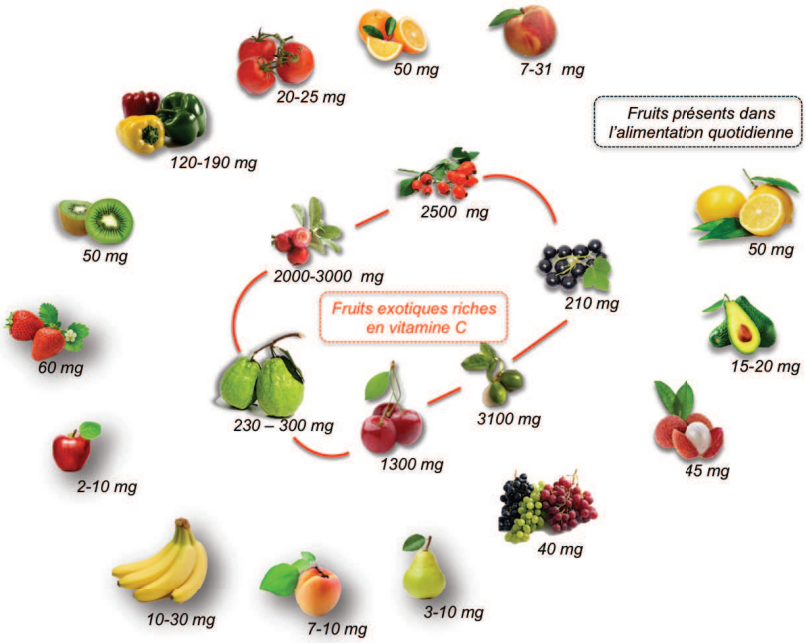
L'ascorbate est une petite molécule mobile dont le transport intra- et intercellulaire a un impact sur la taille du pool d'ascorbate.

- Transport intracellulaire

Une fois l'ascorbate synthétisé à la surface externe de la membrane interne des mitochondries, il peut être transporté dans les différents compartiments cellulaires incluant l'apoplasme. Le plasmalemme, membrane plasmique qui délimite la cellule, est

à la fois un pont et une barrière entre le cytoplasme et le milieu extérieur. C'est une interface dynamique qui perçoit et transmet les informations provenant de l'environnement. La présence de système de transport va permettre une allocation de l'ascorbate en réponse à des demandes physiologique, développementale et métabolique de la plante. Le transport à travers les membranes peut être réalisé grâce à un système passif (diffusion) ou actif (transporteur). Dans le cas de l'ascorbate, celui-ci ne peut pas diffuser simplement au travers de la bicouche lipidique principalement à cause de sa charge négative à pH physiologique. En revanche, le DHA qui est une molécule neutre avec une hydrophobicité plus importante peut diffuser aisément. Néanmoins, le transport de l'ascorbate et du DHA est médié essentiellement grâce à un système de diffusion facilité et/ou de transport actif (cf figure chapitre 6). Ces systèmes de transporteurs ne sont pas clairement identifiés chez les plantes. Les études menées sur des organelles isolées, et des protoplastes, mais également en tenant compte des similarités avec les mammifères, permettent d'émettre des hypothèses sur les modalités de ce transport. Par exemple, Beck *et al.,* (1983) et Anderson *et al.,* (1983) montrent que l'absorption d'ascorbate dans les chloroplastes d'épinard suit une cinétique de type Michaelis-Menten dont les Km sont très élevés. De même, l'absorption d'ascorbate dans des vacuoles isolées présente une cinétique qui suggèrerait une mécanisme de saturation, indiquant l'absence d'un transporteur spécifique (Rautenkranz *et al.,* 1994). Très récemment une protéine transporteur de phosphate de la famille des PHT4 a été décrite comme un transporteur d'ascorbate localisé au niveau de l'enveloppe du chloroplaste chez la plante *Arabidopsis thaliana* (Miyaji et al., 2015). A l'inverse, les transporteurs présents chez les mammifères ont été largement caractérisés (**S**odium-dependent **V**itamin **C** **T**ransporters; SVCT1 et CVSCT2) (Daruwala *et al.,* 1999; Tsukaguchi *et al.,* 1999). Ces protéines appartiennent à une famille des nucleobase-ascorbate transporteur (NAT) présents aussi chez les bactéries et les plantes supérieures (de Koning and Diallinas 2000). Des études récentes d'homologie, ont permis d'identifier 12 NAT orthologues dans le génome d'*Arabidopsis thaliana.* Parmi ceux-ci, le gène At5g62890 possède une région conservée similaire aux transporteurs SVCTs de mammifères (Li and Schultes 2002) mais l'implication de cette protéine dans le transport de l'ascorbate reste à étudier. Lorsqu'on s'intéresse au transport du DHA chez les animaux, on constate un rôle substantiel d'un transporteur de glucose (Wilson 2004). De manière intéressante, l'ajout d'un inhibiteur du transport de glucose tel que la

A



B

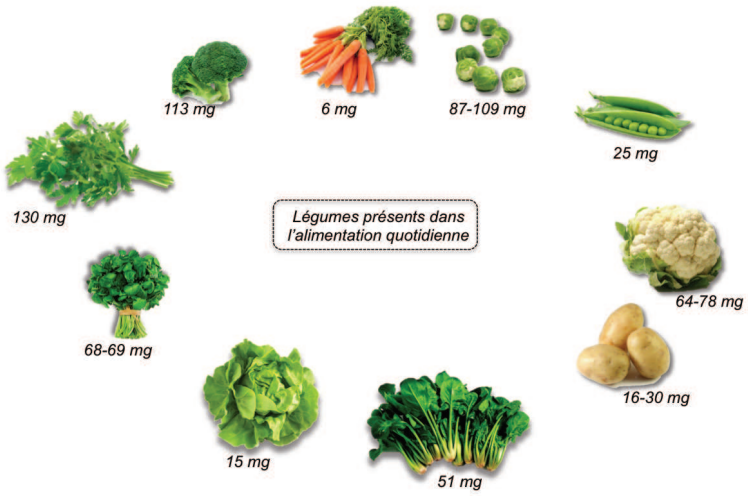


Figure 26: Teneur en vitamine C dans les fruits (A) et les légumes (B) exprimée en mg.100g^{-1} de matière fraiche. **A.** Au centre, les fruits les plus riches en vitamine C parmi lesquels la prune kakadu (3100mg), la cerise Acerola (1300mg), la goyave (200-300mg), le camu camu (2000-3000mg), la baie de Goji (2500mg) et le cassis (210mg). **B.** Légumes retrouvés dans l'alimentation quotidienne. D'après Davey *et al.,* 2000

cytochalasine B ou le génistéine, bloque l'absorption de DHA et de glucose dans des mitochondries isolées à partir de cellules BY-2 de tabac (Szarka *et al.,* 2004). Horemans *et al.,* (2000b) proposent la présence au niveau de la membrane plasmique d'un transporteur dans lequel le DHA apoplastique va être échangé avec l'ascorbate cytologique. Malheureusement, la protéine ou le gène associé à l'activité de transport n'a pas été identité. A l'exception de la récente découverte d'un transporteur d'ascorbate chloroplastique chez Arabidopsis (Miyaji *et al.,* 2015), les autres processus/protéines impliqués dans le transport de l'ascorbate entre les différents compartiments cellulaires restent donc à élucider.

- Transport à longue distance

La biosynthèse de l'ascorbate a lieu dans la majorité des tissus de la plante. Cependant, la quantité d'ascorbate est plus importante dans les organes photosynthétiques, les fruits et les méristèmes (Davey *et al.,* 2000). A l'échelle de la plante, l'ascorbate peut être localisé au niveau des cellules compagnes, dans les tubes criblés et les vaisseaux du xylème, indiquant que l'ascorbate peut être transporté dans la plante à travers les tissus vasculaires (Zechmann *et al.,* 2011). Des études d'autoradiographie utilisant le ^{14}C-AsA ont montré un mouvement de l'ascorbate radio-marqué à travers le phloème, depuis les organes sources, les feuilles, jusqu'aux organes puits, racines, organes floraux, tubercules, fruits (Franceschi and Tarlyn 2002; Tedone *et al.,* 2004). Hancock *et al.,* (2003) suggèrent que la synthèse d'ascorbate par la voie L-Galactose peut avoir lieu dans le phloème. Ainsi, le transport à longue distance de l'ascorbate peut être nécessaire afin de compléter la synthèse *in situ* réalisée par les organes puits. Cependant dans le fruit, la contribution de ce transport dans la teneur finale en AsA reste à déterminer. La combinaison entre la voie principale de biosynthèse et des voies alternatives peut en être la conséquence (*cf chapitre biosynthèse*).

2-6 Variabilité de la teneur en vitamine C chez les plantes

2-6i Variabilité selon le genre et l'espèce

Les fruits et les légumes représentent la source principale de vitamine C dans l'alimentation (*figure 26*). Ainsi, les variétés de fruits exotiques telles que le camu camu, la prune kakadu ou la baie de Goji, présentent des teneurs en vitamine C très élevées.

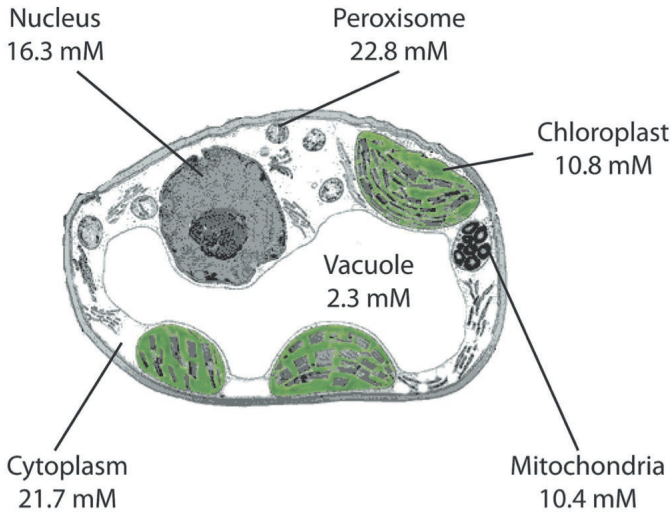


Figure 27: Distribution subcellulaire de l'ascorbate dans des cellules photosynthétique de feuilles de rosette d'*Arabidopsis thaliana (accession Col-0)*. La teneur en ascorbate a été déterminée par une méthode d'immunomarquage dans des conditions de forte intensité lumineuse (700 µmol $m^{-2}s^{-1}$) (Gest *et al.,* 2012)

	Mitochondria	Chloroplasts	Nuclei	Peroxisomes	Cytosol	Vacuoles
% change	35% (*)	104% (***)	35% (*)	-31% (**)	35% (**)	395% (***)

Table 3: Changement subcellulaire de la distribution en ascorbate dans des cellules photosynthétiques de feuilles de rosette d'*Arabidopsis thaliana (accession Col-0)* exposées à un excès de lumière (700 µmol $m^{-2}s^{-1}$) durant deux semaines, par rapport à des plantes exposées à des intensités lumineuses plus réduites (150 µmol $m^{-2}s^{-1}$). (Zeichmann *et al.,* 2011)

A l'opposé la poire, la pomme et l'abricot sont plutôt pauvres en vitamine C. On retrouve la même diversité parmi les légumes (*figure 26b*). Cependant, selon les genres et les espèces, les teneurs sont également très variables mais tendent à être plus importantes dans les accessions sauvages par rapport aux accessions domestiquées (Gest *et al.,* 2013). La diversité naturelle retrouvée chez la tomate est un bon exemple puisque les espèces sauvages telle que *Solanum pennelli,* dont les fruits sont de petite taille, possèdent jusqu'à cinq fois plus d'ascorbate que les cultivars commerciaux appartenant à l'espèce *Solanum lycopersicum* (Stevens *et al.,* 2007; George *et al.,* 2010).

2-6ii Variabilité au sein de la plante

2-6ii a. Variabilité spatiale

La distribution de l'ascorbate est variable selon l'organe considéré, le tissu mais également au sein même de la cellule.

<u>Distribution subcellulaire:</u>

La distribution de l'ascorbate dans les différents compartiments cellulaires a une grande importance dans le développement, la croissance mais aussi la protection de la plante. Pour cela, le pool d'ascorbate réduit/oxydé est impliqué dans la création d'un signal rédox qui va permettre de moduler l'expression de gènes ainsi que certaines activités enzymatiques. Des méthodes de microscopie électronique ont permis de localiser l'ascorbate dans les mitochondries, les chloroplastes, le noyau, les peroxysomes, le cytosol, les vacuoles, l'apoplasme et le long des membranes du réticulum endoplasmique (Gest *et al.,* 2012; *figure 27*). Lors d'un stress lumineux, la teneur en ascorbate va augmenter dans la plupart des compartiments cellulaires (*table 3*). De manière surprenante, l'augmentation la plus importante sera observée dans les vacuoles (+395%). Ceci met en évidence un rôle essentiel dans la détoxification directe ou indirecte des H_2O_2 produits dans les chloroplastes et transportés dans les vacuoles. De la même façon, dans les chloroplastes et les mitochondries, l'ascorbate est détecté au niveau du stroma et de la matrice, alors qu'en condition de stress lumineux on le retrouve dans les crêtes et le lumen des thylakoïdes. Ces observations concordent avec la capacité de quenching non-photochimique (QNP) de l'ascorbate, qui permet de diminuer la formation d'EROs par dissipation de chaleur.

Distribution dans la plante:

Il existe une différence de teneur en AsA entre les différents organes de la plante. Ainsi, les organes photosynthétiques autrement appelés organes sources (feuilles, tige) possèdent une capacité plus importante à synthétiser l'ascorbate que les organes puits (fruits, tubercules, racines, méristèmes, stolons…). Franceshi *et al.,* montrent que la concentration en AsA dans les feuilles d'*Arabidipsis thaliana* incubées durant 4h et 12h dans du L-Galactose entraine une augmentation de 3 et 7 fois de la synthèse d'AsA, respectivement. Au contraire, l'incubation d'organes puits parmi lesquelles les fleurs et les siliques, n'induit pas d'augmentation significative (Franceshi et Tarlyn 2002). Des résultats similaires ont été obtenus chez *M. Sativa*. Parallèlement, d'un point vu « capacité antioxydante », les organes photosynthétiques présentent une concentration en ascorbate plus importante (Loewus *et al.,* 1987; Gara *et al.,* 1997; Cordoba-Pedregosa *et al.,* 2003). Cette différence observée entre les organes puits et sources est liée à la nécessité de translocation de l'ascorbate depuis les organes sources vers les organes puits, ce qui permet la réalisation des processus de croissance.

Lorsqu'on s'intéresse aux organes eux même, on constate qu'il existe un gradient décroissant d'ascorbate entre les tissus extérieurs et intérieurs. Chez la tomate, le péricarpe possède une teneur en ascorbate plus importante que le gel et les graines (Wokes *et al.,* 1943). De la même façon, la peau et la pulpe du fruit de tomate sont les tissus les plus riches (George *et al.,* 2010; Chandra *et al.,* 2012).

2-6ii b. Variabilité temporelle

L'ascorbate participe à de nombreux processus développementaux incluant la croissance et l'expansion cellulaire, la photosynthèse, mais également la sénescence. Ainsi, au cours du développement de la plante les teneurs en ascorbate vont varier. Durant la germination, l'ascorbate s'accumule dans l'axe embryonnaire où il va permettre la première division zygotique ainsi que la formation des cotylédons (Pallanca et Smirnoff 1999; Chen *et al.,* 2012). De la même façon, la concentration en ascorbate sera plus importante dans les jeunes feuilles que dans les feuilles matures et pré-sénescentes. (Yahia *et al,.* 2001; Chen *et al.,* 2003, Bulley *et al.,* 2009). L'accumulation des transcrits de gènes impliqués dans la voie de biosynthèse est corrélée avec la croissance et la maturation du fruit, ce qui suggère un rôle important de

l'ascorbate dans ces étapes de développement (Ioannodi *et al.,* 2009). Néanmoins, suivant le genre et l'espèce considérés, l'accumulation de l'ascorbate peut avoir lieu à différentes étapes du développement du fruit. Chez le kiwi par exemple, l'accumulation a lieu rapidement après la fécondation de la fleur et diminue durant la maturation du fruit (Bulley *et al.,* 2009). En revanche, chez la tomate ou le melon, l'accumulation de l'ascorbate a lieu au cours de la maturation (Agius *et al.,* 2003; Pateraki *et al.,* 2004).

2-6ii c. Variabilité liée à l'environnement

Les facteurs environnementaux ont une forte influence sur la composition chimique des espèces horticoles (Klein and Perry, 1982). Ils peuvent être de types abiotiques, c'est à dire qui regroupe l'ensemble des facteurs physico-chimique capables d'influencer une biocénose (lumière, température, salinité, conditions de cultures…), ou biotiques lesquels correspondent à des facteurs liés aux êtres vivant (virus, bactérie, champignon, insecte…). Lors d'un stress biotique ou abiotique la production d'EROs augmente et peut alors devenir toxique pour les cellules. Les EROs sont responsables de la peroxydation des membranes, de dommages sur l'ADN, de la dénaturation de protéines mais également peuvent induire la mort des cellules (Fridovich 1998). La plante possède des mécanismes qui lui permettent de se protéger, parmi lesquels l'activation de voies de signalisation responsables de la synthèse de protéines particulières (PR, HSP, LEAP), ou la stimulation des systèmes antioxydants (Zhu *et al.,* 2001). L'ascorbate joue un rôle important dans ces deux processus. En effet, l'ascorbate n'est pas seulement un antioxydant essentiel, il sert aussi de cofacteur pour l'activité d'une 2-oxoacide-dépendant dioxygénase (2ODD). La famille des 2ODD est responsable de la synthèse d'un large éventail de métabolites secondaires parmi lesquels les hormones (Arrigoni and De Tullio 2002). Les hormones sont un élément fondamental pour la transmission du signal et par conséquent la mise en place d'une réponse adaptée. Les mutants d'Arabidopsis déficients en ascorbate (*vtc*) sont de bons modèles pour démontrer que la réponse des plantes dépend des stimuli qu'elles perçoivent. Les mutants *vtc* sont ainsi sensibles aux stress abiotiques alors qu'ils montrent une résistance aux pathogènes (Pastori *et al.,* 2003; Barth *et al.,* 2004; Colville *et al.,* 2008). Il existe donc une variation du pool d'ascorbate lorsque les conditions environnementales ne sont pas favorables.

Les stress abiotiques:

En conditions de stress salin (10-50mM NaCl), la teneur en ascorbate augmente en réponse à une augmentation de la concentration en sel (Teleinsky *et al.,* 2008). Chez le sorgho, la sécheresse entraine une diminution des activités cytosolique des enzymes APX et MDHAR, et inversement induit une augmentation de l'activité de la DHAR (Zhang and Kirkham 1996). De la même façon, l'oxygène influence le métabolisme de l'ascorbate. Chez les variétés appartenant à l'espèce *Cucurbita pepo*, l'activité de l'AO est stimulée dans le noir en réponse à une augmentation de l'oxygène, alors qu'elle diminue durant la germination en condition d'hypoxie (De Tullio *et al.,* 2007). Reuther et Nauer (datas non publiées 1972; Lee *et al.,* 2000), montrent que des mandarines cultivées sous des températures élevées (30-35°C jour, 20-25°C nuit) ont des teneurs en ascorbate moins importantes que celles cultivées sous des températures plus fraiches (20-22°C jour, 11-13°C nuit).

Cependant, parmi les facteurs climatiques qui influencent de manière significative l'accumulation de l'ascorbate, la lumière tient une place particulière. Elle ne constitue pas un élément indispensable à la synthèse, néanmoins la quantité et l'intensité au cours de la croissance de la plante ont une influence certaine sur la quantité d'ascorbate produite. De nombreuses études principalement réalisées sur les feuilles, mettent en évidence un rôle de la lumière dans la régulation du pool d'ascorbate (Smirnoff and Pallanca 1996; Grace and Logan 1996; Smirnoff 2000b; Gatzek *et al.,* 2002). Ainsi, Gatzek *et al.,* montrent que dans des feuilles d'*Arabidopsis thaliana* la quantité d'ascorbate augmente lorsque les plantes sont acclimatées à une forte intensité lumineuse et, inversement diminue à l'obscurité. L'étude des gènes corrélés avec cette modification de la teneur en ascorbate met en évidence une régulation par la lumière de certaines enzymes appartenant à la voie principale de biosynthèse telles que la GMP, la GGP et la GPP mais également la PMI, l'APX et l'AO du cycle redox (Yoshimura *et al.,* 2000; Tabata *et al.,* 2002; Yabuta *et al.,* 2007; Dowdle *et al.,* 2007; Maruta *et al.,* 2008). En ce qui concerne les enzymes GME, GalDH et GLDH, les résultats restent contradictoires. Par ailleurs, Massot *et al.,* montrent que l'influence de la lumière est plus importante dans les feuilles que dans le fruit. Il existe donc une variation de la teneur à l'échelle de la journée, qui suit le rythme circadien de la plante (Dowdle *et al.,* 2007; Massot *et al.,* 2012). Le rythme circadien est une horloge interne capable de générer de façon autonome des oscillations d'environ 24H du métabolisme de la plante.

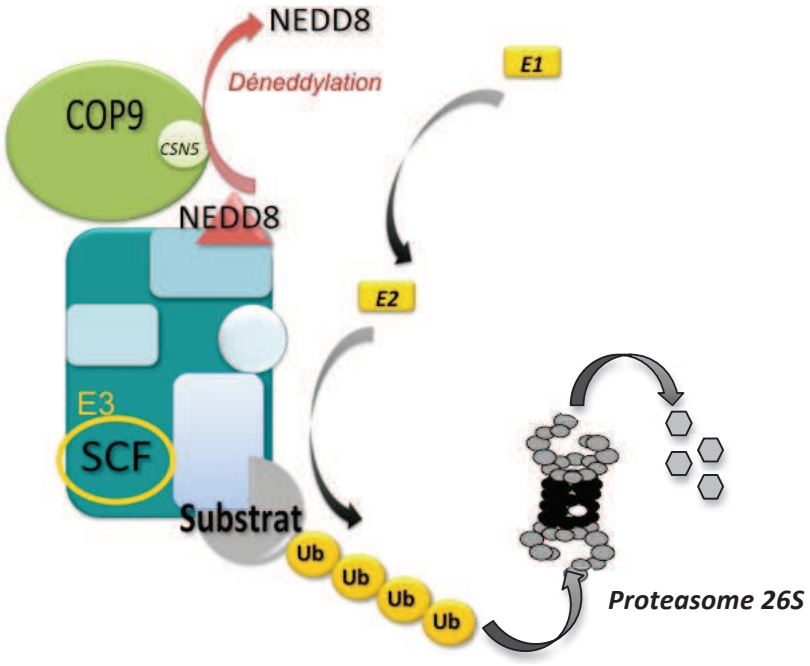


Figure 28: Schéma de fonctionnement du signalosome (CSN) chez les eucaryotes: exemple du signalosome COP9 (COnstitutive Photomorphogenic 9). Le CSN est un complexe multiproteique hautement conservé au cours de l'évolution. CSN va se lier au complexe SCF qui est une classe d'E3 ligase. SCF contient un type de protéine composée d'un domaine F-box capable de reconnaître de façon spécifique les protéines à dégrader. L'enzyme E1 va activer des molécules d'ubiquitines qui vont être transférées à E2. E2 va alors s'attacher à E3 afin que cette E3 ligase puisse lier l'ubiquitine activée sur les résidus de lysine de la protéine ciblée. Lorsque cette marque est déposée la sous unité CSN5 de CSN va hydrolyser la molécule NEDD8 pour induire la dissociation du complexe CSN/SCF. La dissociation du complexe va permettre la libération de la protéine poly-ubiquitinylée et son élimination par le protéasome 26S.

Pour cela un signal photopériodique est perçu au niveau des feuilles grâce à des photorécepteurs. Chez les plantes, il existe trois types majeurs de photorécepteurs: les phytochromes, les cryptochromes et les phototropines. Les phytochromes permettent de percevoir la durée de la période obscure, laquelle est déterminée par des signaux d'allumage et d'extinction de la lumière. Dans ce contexte, la lumière rouge (625–740nm) est efficace en tant que signal d'interruption de la période obscure. Le phytochrome est capable de percevoir la lumière rouge et d'engendrer les réponses adéquates en fonction de la longueur d'onde perçue. Ainsi, les phytochromes existent sous deux formes d'isomères inter-convertibles, un isomère actif physiologiquement qui absorbe le rouge sombre dont le maximum d'absorption se situe à 720nm (Infra Rouge proche ou rouge lointain), et l'autre isomère inactif qui absorbe le rouge clair dont le maximum d'absorption se situe à 660nm. Sous leur forme active, les phytochromes contrôlent plusieurs processus dont la photo-morphogénèse, le réglage de l'horloge interne, la germination des graines, l'induction florale et la biosynthèse de diverses substances (anthocyanes, flavones, protéines…). Bartoli *et al.,* en 2009, montrent que lorsque le ratio rouge clair/rouge lointain est bas, les concentrations en ascorbate et glutathion sont également faibles. Ce ratio fluctue au cours de la journée, mais présente des valeurs faibles au lever et au coucher du soleil. Le second type de photorécepteur, les cryptochromes, sont impliqués dans la détection de la lumière bleue (400-490nm) et ultraviolette (UV-A, 315-380nm). Ils existent sous deux formes protéiques: CRY1 et CRY2. Les cryptochromes contrôlent la photo-morphogénèse *via* l'expression de gènes cibles. Pour cela, à l'obscurité, les cryptochromes interagissent avec un complexe protéique appelé COP1 (Constitutive Photomorphogenetic 1). COP1 est un signalosome qui permet la reconnaissance spécifique de la protéine à dégrader par la mise en place d'une marque de poly-ubiquitination, laquelle sert de signal pour le protéasome (*figure 28*). COP1 induit alors la dégradation de facteurs de transcription nécessaire à l'activation de l'expression de nombreux gènes impliqués dans la photo-morphogénèse (HY5, STO, STH, HFR). Il a été démontré que le signalosome COP1 était régulé par le signalosome COP9 (Wang *et al.,* 2009). De manière intéressante, des études récentes menées chez *Arabidopsis thaliana* sur le signalosome COP9, montrent que la sous unité CSN5B, laquelle est liée à l'effet lumière, était capable de moduler la synthèse de l'ascorbate *via* une interaction avec VTC1 (GMP) (Wang *et al.,* 2013). Le troisième type de photorécepteur correspond aux phototropines.

Les phototropines détectent uniquement la lumière bleue. Elles permettent d'optimiser la photosynthèse en condition de faible éclairement, de même qu'elles protègent les cellules photosynthétiques d'un excès de lumière. Pour cela il existe deux types de phototropines , PHOT1 et PHOT2 dont les domaines LOV1 et LOV2 (Light Oxygen Voltage) sont identiques. Ces domaines appartiennent à la superfamille des domaines PAS (Per, Arnt, Sim) impliqués dans les interactions entre protéines. LOV1 et LOV2 lient le cofacteur flavine mononucléotide (FMN) pour permettre la remobilisation des chloroplastes, l'ouverture des stomates mais également le phototropisme. Ogura *et al.,* en 2008 ont montré par double hybride que chez *Arabidopsis thaliana* une phototropine appelée PAS/LOV pouvait interagir avec les protéines VTC2 (GGP) et VTC2-like. L'ensemble de ces données suggère un lien entre le métabolisme de l'ascorbate et les différents photorécepteurs présents dans les plantes.

Les conditions de cultures ont également une influence sur le pool d'ascorbate. Ce sont des facteurs intervenant avant la récolte, qui sont importants dans la qualité du fruit consommé. La demande concernant des méthodes de cultures alternatives limitant l'utilisation de fertilisants et de pesticides synthétique est de plus en plus forte. Il existe une différence notable entre les teneurs en antioxydant de produits végétaux soumis à des conditions de culture conventionnelle et ceux cultivés en conditions dites «Bio » plus respectueuses de l'environnement (Lee *et al.,* 2000). Dans le cas de la tomate, les teneurs en vitamine C semblent plus importantes chez des tomates « Bio ». Ces observations sont controversées dans le fruit mais restent significatives dans les feuilles (Caris-Veyrat *et al.,* 2004; Woese *et al.,* 1997; Worthington *et al.,* 2001). De la même façon, Augustin (1975) et Lisiewska and Kmiecik (1996) mettent en évidence que l'utilisation de fertilisants azotés entraine une diminution de la teneur en ascorbate dans de nombreux cultivars de pomme de terre et de chou. Inversement, l'utilisation de fertilisant à base de potassium induit une augmentation du pool d'ascorbate (Nagy, 1980). Plusieurs hypothèses ont été proposées pour expliquer l'impact de fertilisants azotés sur les fruits et les légumes. Il semble qu'au delà de son impact sur l'expansion foliaire, ce type de fertilisant augmente la quantité de NO_3 dans la plante (Mozafar, 1993). D'autres pratiques comme le rabattage et la diminution de la charge en fruit influencent la composition nutritionnelle et la taille du fruit. De même, les produits chimiques comme les pesticides et les régulateurs de croissance ont des effets importants.

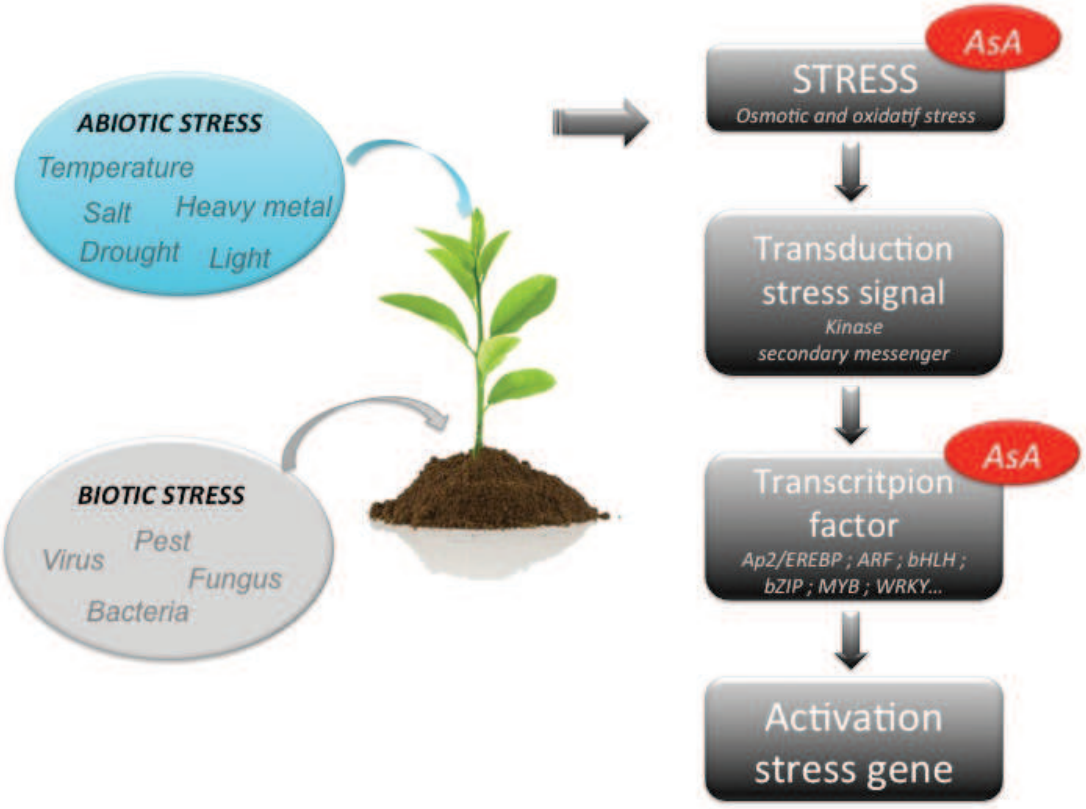


Figure 29: Schéma représentant la réponse de la plante consécutive à un stress biotique ou abiotique. L'ascorbate (AsA) joue un rôle d'antioxydant mais également dans la synthèse de messagers secondaires parmi lesquels les hormones

Ainsi,'application de gibbérellines induit une augmentation d'environ 20% de la teneur en ascorbate dans le thé vert (Liang *et al.,* 1996).

Les stress biotiques:

Les infections bactériennes induisent la production d'exo-polysaccharides qui altèrent le métabolisme de l'ascorbate en augmentant la quantité de peroxyde d'hydrogène dans les cellules de la plante. De manière générale lors d'une infection par des pathogènes, l'ascorbate régule la production de protéines de défenses appelées pathogenesis-related protein (PR). Dans le mutant déficient *vct1* les protéines PR sont activées (Pastori *et al.,* 2003). De manière intéressante, les plantes déficientes en acide salicylique (SA) montrent une adaptation aux infections par les virus à ARN, ce qui se traduit par des symptômes plus légers et une accumulation moins importante d'EROs. L'augmentation du pool d'ascorbate et de glutathion dans ces plantes semble à l'origine de la résistance observée (Wang *et al.,* 2011). De la même façon, un traitement avec des doses élevées d'ascorbate atténue la sévérité des symptômes ainsi que la réplication virale mais sans lien avec une élimination des EROs. Ceci indique que l'ascorbate permet d'augmenter la résistance des plantes aux pathogènes grâce à un mécanisme spécifique qui n'utilise pas les EROs comme signal. A l'inverse, l'abondance d'ascorbate dans des plantes infectées par des insectes herbivores, augmente leur susceptibilité. En effet, l'ascorbate fonctionne comme un phago-stimulant pour de nombreux insectes qui sont susceptible de l'utiliser pour leur métabolisme. Anthonius *et al.,* (2003) mettent en évidence que la teneur en ascorbate dans des feuilles de différents cultivars de tomate, corrèle positivement avec la survie d'une espèce de Doryphore (*Leptinotarsa decemlineata*). Néanmoins, l'impact de l'ascorbate dans ce type de réponse est complexe. Il est à la fois utilisé pour le métabolisme de l'insecte et de la plante (Goggin *et al.,* 2010). En effet dans la plante, il participe à la synthèse de métabolites secondaires tels que la tyrosinase et les tanins, impliqués dans la défense.

L'ascorbate peut donc agir efficacement comme un immuno-modulateur lorsqu'il est présent à une concentration appropriée. En plus de sa fonction antioxydante, il joue un rôle de molécule signal permettant à la plante d'avoir une réponse adaptée, favorisant alors la protection (*figure 29*).

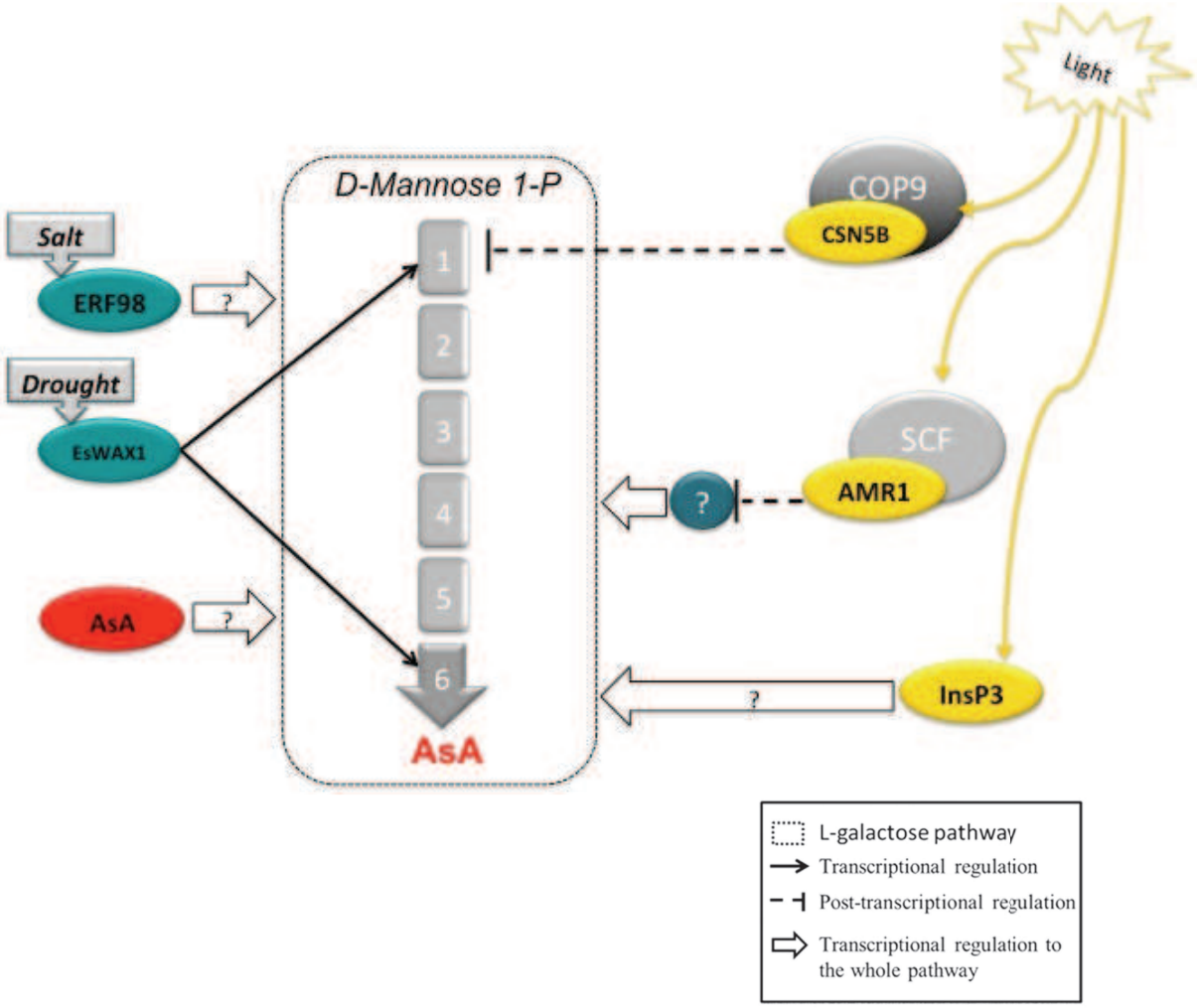


Figure 30: Vue d'ensemble des différents éléments régulateurs intervenant dans la contrôle de la voie principale de biosynthèse de l'ascorbate (AsA). Enzymes appartenant à la voie du L-galactose: (1) GDP-D-Mannose pyrophosphorylase (VTC1); (2) GDP-D-Mannose-3',5'-épimérase (GME); (3) GDP-L-Galactose phosphorylase (VTC2/VTC5); (4) L-Galactose 1-phosphate phosphatase (GPP); (5) L-Galactose déshydrogénase (GDH); (6) L-GalactonoLactone déshydrogénase (GLDH). Un stress salin induit l'expression de *ERF98* qui va à son tour stimuler l'expression de *VTC1*. Cependant l'action de *ERF98* sur les autres enzymes de la voie reste à déterminer. Lors d'une sècheresse, EsWAX1 est également capable de stimuler VTC1 et GLDH, pour induire une résistance accrue de la plante. La lumière inhibe l'expression de *AMR1*, lequel va inhiber l'expression des enzymes de la voie de biosynthèse grâce à l'action d'une protéine intermédiaire inconnue. InsP3 est un messager secondaire dans la transduction du signal lumineux capable d'induire la synthèse d'ascorbate mais les mécanismes moléculaires sont encore méconnus. A l'obscurité, CSN5B agit au niveau post-transcritptionnel où il induit la dégradation de VCT1 dans le but d'inhiber la synthèse d'ascorbate. (D'après Wang *et al.,* 2013)

2-6ii d. Variabilité liée à une régulation

Bien que les voies de biosynthèse, de recyclage et de dégradation soient relativement bien caractérisées, les mécanismes qui interviennent dans la régulation restent à ce jour méconnus. Des études récentes menées sur *Arabidopsis thaliana* ont cependant permis d'identifier certains de ces régulateurs, capables d'intervenir au niveau transcriptionnel et post-transcriptionnel sur la voie principale de biosynthèse de l'ascorbate (*figure 30*) (Zhang *et al.,* 2009; Zhang *et al.,* 2011; Alimohammadi *et al.,* 2012; Wang *et al.,* 2013; Zhu *et al.,* 2014)

AMR1 ou Ascorbic acid Mannose pathway Regulator 1:

Le criblage de mutant d'*Arabidopsis thaliana* sensible à l'ozone, a conduit à l'identification d'AMR1 (Ascorbic acid Mannose pathway Regulator 1) (Zhang *et al.,* 2009). La surexpression d'*AMR1* induit une diminution de la teneur en ascorbate dans les lignées transgéniques, et inversement chez les mutants déficients *amr1*. Dans les mutants *amr1,* la majorité des enzymes impliquées dans la voie principale de biosynthèse de l'ascorbate incluant GMP, GME, GGP, GPP, GalDH et GLDH, sont surexprimées. Ce qui a conduit à conclure que AMR1 agit comme un répresseur sur la voie principale de biosynthèse de l'ascorbate. AMR1 code une protéine contenant un motif F-box en N-terminal, lequel présente de fortes homologies avec d'autres protéines à F-box composant le complexe SCF E3 ligase (SKP1-Cullin-F-box). Ce complexe catalyse *via* la signalosome, l'ubiquitinylation de protéines destinées à être dégradée par le protéasome (Fig. 28). La reconnaissance spécifique des protéines à dégrader est réalisée grâce au domaine DUF295, domaine également présent chez AMR1. Cette forte homologie indique que les mécanismes de protéolyse contrôlés par AMR1 peuvent être responsables de la régulation de la synthèse d'ascorbate. De manière intéressante, l'expression de ce régulateur négatif semble être contrôlée durant la croissance et le développement mais également par les facteurs environnementaux. Ainsi, les niveaux de transcrit d'AMR1 augmentent lors du développement foliaire alors qu'ils diminuent lorsque l'intensité lumineuse augmente.

CSN ou COP9 signalosome:

Les études ont montrées que la lumière pouvait stimuler l'expression des enzymes impliquées dans la voie principale de biosynthèse de l'ascorbate. Néanmoins, on connaît peu de chose sur les mécanismes impliqués dans la diminution de la teneur en AsA en absence de lumière. Les travaux de Match J en 2013 montrent que la protéolyse médiée par le signalosome COP9 joue un rôle dans l'inhibition de la synthèse d'ascorbate à l'obscurité.

Ces travaux démontrent que la dégradation de VTC1 *via* l'interaction avec la sous unité CSN5B du signalosome COP9 induit une diminution de l'ascorbate à l'obscurité. De la même façon, la perte de fonction de CSN5B entraine une augmentation du pool d'ascorbate qui se traduit par une résistance accrue aux stress oxydatif et salin. Ainsi, le complexe CSN semble avoir un rôle de régulateur négatif post-transcriptionel, à l'obscurité. La découverte de l'implication de CSN dans la régulation du pool d'ascorbate amène cependant de nombreuses questions. En effet, CSN agit au niveau du noyau alors que VTC1 est localisé dans le cytosol. Pour qu'une telle hypothèse soit vérifiée, il faut encore démontrer que VTC1 subit une translocation vers le noyau, un tel mécanisme reste inconnu.

ERF98 ou Ethylen Response Facteur 98:

Un deuxième facteur de transcription de type ERF (Ethylen Response Factor) a également été identifié. Les protéines appartenant à la famille des ERF possèdent un rôle important dans la régulation transcriptionnelle au cours des processus de croissance et de développement, mais aussi dans la réponse à l'environnement (Aharoni *et al.,* 2004; Zhang *et al.,* 2004; De Boer *et al.,* 2011). ERF98 agit comme un régulateur positif sur la voie principale de biosynthèse (Zhang *et al.,* 2012). Ainsi, la surexpression de *ERF98* chez *Arabidopsis thaliana* entraine une augmentation de la teneur en ascorbate, et inversement chez les mutants déficients *erf98*. Chez les mutants *erf98*, la sensibilité au stress salin est plus élevée, indiquant alors que ERF98 peut intervenir dans la réponse au stress salin par stimulation de la synthèse d'ascorbate. Les niveaux d'expression des gènes impliqués dans la voie du D-mannose/L-galactose ainsi que de certaines enzymes intervenant dans le recyclage de l'ascorbate (APX3, APX6, ChlDHAR, CytDHAR et GR1) augmentent dans les lignées transgéniques sur-exprimant ERF98 alors qu'ils diminuent dans les mutants knock-out.

Cependant, seul *VTC1* semble être vraiment affecté dans les mutants déficients *erf98*. L'analyse des promoteurs, montre que ERF98 serait capable d'interagir avec les promoteurs de *VTC1* et *VTC2* grâce à la présence d'un motif DRE. ERF98 pourrait agir directement sur l'activité transcriptionnelle des gènes et ainsi moduler le pool d'ascorbate.

InsP3 ou Inositol (1,4,5)-triphosphate:

InsP3 est un métabolite provenant de la voie du phospho inositol, impliqué dans la réponse à une grande diversité de stress tels que la lumière, la sécheresse, le froid ou la salinité (Khodakovskaya *et al.,* 2010; Alimohammadi *et al.,* 2012). La diminution de la quantité d'InsP3 dans des lignées transgéniques de tomate modifie la signalisation lumineuse, ce qui affecte de nombreuses voies responsables de la synthèse de métabolites secondaires tel que l'augmentation du pool d'ascorbate. Ceci suggère que l'InsP3 joue un rôle de régulateur négatif dans les réponses photo-morphogéniques. Néanmoins, les voies spécifiques liées à l'implication de l'InsP3 dans la transduction du signal lumineux restent à clarifier.

MYB ou myeloblastome:

L'étude de *Eutrema salsugineum,* espèce modèle pour l'analyse des stress abiotiques, a permis l'identification d'un facteur de transcription de type R2R3-MYB appelé EsWAX1 (Zhu *et al.,* 2014). Ce facteur est impliqué dans la résistance à la sécheresse. L'expression ectopique de *EsWAX1* dans des plantes transgéniques d'*Arabidopsis thaliana* augmente significativement la quantité de cires cuticulaires mais également la teneur en ascorbate. Dans ces lignées transgéniques, l'expression de VTC1, GLDH et MIOX4 est stimulée, alors que les enzymes impliquées dans le cycle AsA-GSH ne sont pas affectées. Il semble donc que EsWAX1 soit capable de réguler la synthèse d'ascorbate en modulant l'expression des gènes VTC1, GLDH et MIOX4 dans des conditions de sécheresse. Néanmoins les mécanismes moléculaires mise en jeu restent inconnus.

Régulation feedback:

De nombreuses observations mettent en évidence que la biosynthèse de l'ascorbate pourrait être régulée par rétro-inhibition. Pallanca et Smirnoff (2000) montrent grâce à l'utilisation de D-^{14}Cglucose, que le taux de biosynthèse de l'ascorbate diminue progressivement avec l'augmentation de la taille du pool dans des plantules de pois. De même, l'activité de l'enzyme L-GalDH peut être inhibée par l'ascorbate dans les organes d'épinard (Mieda *et al.,* 2004). De plus, l'ascorbate est capable d'avoir une rétroaction sur d'autres enzymes de la voie principale de biosynthèse, puisque l'ajout d'ascorbate exogène diminue significativement l'expression de *GMP* et *GLDH* (Tabata *et al.,* 2002). Cette capacité de rétro-inhibition par l'ascorbate est aussi un mécanisme permettant de réguler le pool d'AsA dans les plantes.

De nombreuses études montrent que l'augmentation du pool d'ascorbate permet à la plante de mieux résister aux divers stress environnementaux. Ainsi, les plantes sont capables d'ajuster le pool d'ascorbate grâce à une cascade de réactions incluant l'activation et l'inhibition de l'expression de certains gènes. A ce jour, de tels éléments régulateurs ont uniquement été identifiés et caractérisés chez l'espèce modèle *Arabidopsis thaliana*. La découverte de ces systèmes de régulation dans des variétés à intérêt agronomique peut ouvrir de nouvelles perspectives dans le domaine de l'amélioration variétale.

III- Vitamine C et qualité du fruit de tomate après la récolte

3-1 Vitamine C et procédés de conservation post-récolte

Au cours des dernières années, la consommation de tomate a encore augmentée grâce à l'approvisionnement tout au long de l'année de fruits frais mais également transformés tels que les soupes, les jus de fruits, les purées et les sauces. Le développement du marché de la tomate a ainsi favorisé la production en serre et les importations, qui ont alors supplantés la production saisonnières de plein champ. Cependant, les consommateurs sont de plus en plus exigeants en ce qui concerne l'aspect extérieur, les caractéristiques nutritionnelles et organoleptiques du fruit. Les qualités sensorielles de la tomate tels que l'aspect visuel, la fermeté et le gout ont donc un impact important sur la consommation. De nombreuses études ont établi que la qualité du fruit est principalement influencée par les caractéristiques génétiques de l'espèce ou du cultivar, les procédures de cultures employées, le stade de récolte, mais aussi par les conditions de conservation des fruits après la récolte (Hodges and Forney 2000; Hodges *et al.,* 2001; Aharoni *et al.,* 2002; Wismer 2003). Les fruits et légumes frais comme les tissus vivants sont soumis à des changements continuels après la récolte. Ces changements ne peuvent pas être stoppés mais seulement contrôlés par diverses procédures post-récolte.

Le contrôle de la température et de l'humidité relative permet de ralentir le ramollissement du fruit, les pertes de gout, et le développement de la pourriture. Zepplin et Elvehjein (1944) ont montré que les pertes en vitamine C augmentent lorsque la température est élevée, néanmoins la gamme de température et le taux de perte de vitamine C sont dépendants du type de fruit considéré. Wu *et al.,* (1992) établissent que l'AsA diminue rapidement dans les haricots verts stockés à 5°C durant 3 jours, alors qu'il reste stable chez le brocoli. De la même façon, Esteve *et al.,* (1995) mettent en évidence que la concentration en AsA dans des asperges fraiches stockées à 4°C augmente 2 jours après avoir été récolté. Généralement, les fruits et les légumes stockés montrent une diminution progressive de la teneur en AsA (Adisa 1986; Pantos and Markakis 1973; Agar *et al.,* 1999).

Les traitements chimiques tels que l'utilisation de calcium, permettent de réduire les désordres physiologiques liés à la conservation et maintenir la fermeté des fruits. Bangerth en 1976 observe que le taux de vitamine C augmente dans les pommes et les tomates traitées avec du chlorure de calcium. De la même façon, Watada *et al.,* (1976) montrent que la teneur en AsA est légèrement plus élevée dans les tomates « matures green » traitées à l'éthylène par rapport à celles ayant muries sans traitement à l'éthylène. Chez le Kiwi, les fruits conservés dans une atmosphère enrichie en éthylène présentent trois fois plus d'AsA que ceux de la condition témoin (Agar *et al.,* 1999).

Les rayonnements ionisants peuvent être utilisés pour réduire la germination des tubercules, la prolifération des insectes ou même pour diminuer la vitesse de maturation de certains fruits et légumes. Mitchell *et al.,* (1992) ont étudié l'influence de faibles doses d'irradiation sur des cultures horticoles, et constatent qu'il n'y a pas d'influence sur la teneur en vitamine C. Cependant, les études menées par Graham and Stevenson (1997), ont montré que des doses d'irradiation de 2-3 kGy (unité en Gray) combinées avec une réfrigération, entrainent une augmentation de la teneur en AsA mais une diminution du pool de DHA chez la fraise.

Le contrôle des conditions atmosphériques permet également de réduire les changements physiologiques et chimiques durant le stockage des fruits et légumes. Ainsi, une réduction de l'oxygène ou du dioxyde de carbone atmosphérique permet de réduire la dégradation de l'AsA (Lee *et al.,* 2000). Des kiwi conservés sous une pression d'O_2 de 0,5, 2 ou 4 kPa à une température de 0°C présentent une diminution en AsA de 7, 12 ou 18%, respectivement, après 12 jours de stockage. De la même façon, des kiwis stockés à l'air ambiant avec 5, 10 ou 20 kPa de CO_2 présentent une diminution de la teneur en AsA de 14, 22 ou 34% respectivement. Il semble que de fortes concentrations en CO_2 stimulent la quantité d'éthylène, ce qui augmente l'activité de l'AO, laquelle est impliquée dans la dégradation de l'AsA (Mehlhorn 1990).

De manière générale, les fruits et les légumes fraîchement récoltés contiennent plus de vitamine C que ceux stockés, puisque dans des conditions défavorables de manutention et d'entreposage, la vitamine C peut être dégradée. L'ensemble de ces travaux révèle qu'il existe un lien entre le métabolisme de l'ascorbate et la qualité des fruits après la récolte.

Factors	Visible observations	Antioxidant content	Antioxidant enzymes	Oxidative products	Active O_2 species	Membrane degradation
Maturity	Scald, core and flesh browning, pitting	<LSA, <WSA, <anthocyanins, <phenols	<SOD, <CAT, >GR	>Lipid hydroperoxides,		
Storage duration	Decline in quality	>tocopherol	±SOD, ±CAT	>Lipid hydroperoxides,		Increased
Storage temperature						
Superoptimal	Yellowing	±ascorbate, <carotenoids,	>SOD, >CAT, >POD	>Lipid hydroperoxides,		
Suboptimal	Pitting, abnormal ripening, flesh browning, sweetening	<ascorbate, <GSH, <tocopherol	±SOD, ±CAT, >POD, >ASPX	>Lipid hydroperoxides, >peroxides, >MDA	>H_2O_2, >O_2^-, >·OH	Increased
Water loss	Decline in quality	<ascorbate, <carotenoids	increased			
Atmosphere						
High CO_2	Flesh browning	<LSA, <ascorbate	<ASPX, <CAT, >DHAR			Increased
Low O_2	Reduced scald	>ascorbate	>SOD, >CAT	<α-Farnesene*		Reduced
Ethylene	Yellowing, scald, russeting	>ascorbate	>ASPX, >CAT, <DHAR, <GR, <SOD	>Lipid hydroperoxides, >MDA, >α-farnesene*	>H_2O_2	
Irradiation	Browning, abnormal ripening, scald, tissue breakdown	±GSH, ±carotenoids, ±ascorbate		>DNA fragmentation	>O_3, >H_2O_2	Increased

[a]LSA = lipid soluble antioxidants, WSA = water soluble antioxidants, SOD = superoxide dismutase, CAT = catalase, GR = glutathione reductase, POD = peroxidase, GSH = reduced glutathione, MDA = malondialdehyde, O_2^- = superoxide anion, ·OH = hydroxyl radical, ASPX = ascorbate peroxidase, DHAR = dehydroascorbate reductase, α-farnesene* = oxidized α-farnesene.

Table 4: Tableau illustrant certains dommages observés après la récolte des fruits et légumes, suite à un stress oxydatif (d'après Hodges *et al.,* 2004).

Figure 31: Photographies illustrant divers symptômes pouvant apparaître lors d'un stress oxydatif chez la tomate. **A.** Infection par *Colletotrichum sp*. **B.** Brunissement au niveau des graines. **C.** Spot noir. **D.** Défaut de mûrissement. **E.** Pourriture liée à une infection par *Botrytis cinerea*. **F.** Dessiccation

3-2 Vitamine C et stress oxydatif

L'objectif des technologies mises en place pour améliorer la conservation des fruits et des légumes après leur récolte, a pour but de prolonger leur durée de vie et ainsi prolonger la période de commercialisation. Cependant, ces procédés peuvent induire de nombreux stress parmi lesquels le stress oxydatif, qui vont altérer la qualité des fruits et des légumes (Hodges *et al.,* 2004; Malacrida *et al.,* 2006). Le stress oxydatif a lieu quand la production d'EROs est supérieure à la capacité de neutralisation de la plante. Les EROs sont responsables de la dégradation des lipides, des protéines, des carbohydrates et des acides nucléiques (Hodges *et al.,* 2003) (*table 4*). Ceci se traduit par des symptômes tels que les brulures superficielles (Delong and Prange 2003), le brunissement des noyaux (Larrigaudière *et al.,* 2001), le blanchissement des pigments (Elstner and Oswald 1994), la détérioration de l'intégrité de la membrane (Biedinger *et al.,* 1990), l'inactivation de différents types d'enzymes (Casano *et al.,* 1994; Landry and Pell 1993) ainsi que des lésions et mutations au niveau du génome (*figure 31*). La tomate comme l'ensemble des fruits charnus est sensible aux basses températures en particulier durant les stades précoces de maturation du fruit. Ainsi, le stress oxydatif est impliqué dans la réponse de la tomate lors de blessures induites à des températures basses, généralement inferieur à 10°C. Les désordres physiologiques générés représentent un problème économique important puisqu'on observe une maturation anormale des fruits, une perte de fermeté lié à un phénomène de dessiccation, l'apparition de « trou » noir mais également une augmentation de la sensibilité aux pathogènes fongiques. Ces symptômes sont généralement observables lorsque les fruits sont replacés à température ambiante (Morris 1982; Sharom *et al.,* 1994). La neutralisation des EROs implique un système de réponse antioxydant dont fait partie l'AsA (*cf chapitre* « *rôle de l'ascorbate* »). Les niveaux d'AsA au moment de la récolte peuvent être considérés comme des marqueurs importants de la qualité du fruit et le reflet d'une capacité des tissus à résister à des conditions de stress (Davey and Keulemans 2004). Envisager d'augmenter le potentiel antioxydant du fruit de tomate représente donc un enjeu agronomique indispensable pour améliorer la qualité des fruits après la récolte.

3-3 La biofortification

L'enrichissement d'espèces d'intérêt agronomique par des antioxydants peut avoir lieu à deux niveaux: l'enrichissement des aliments de base avec des nutriments essentiels qui possèdent des propriétés antioxydantes (vitamine C et E), mais également, l'enrichissement des aliments de luxe présentent des bienfaits pour l'organisme par des antioxydants non essentiels tels que les flavonoïdes notamment utilisés dans le cadre de l'industrie des nutraceutiques (Zhu *et al.,* 2013). La biofortification de la tomate avec un trait de qualité tel que la vitamine C constitue une stratégie qui permettrait à la fois d'améliorer les qualités nutritionnelles mais également d'une manière générale la résistance du fruit au stress pré- et post-récolte. L'utilisation de la variabilité génétique naturelle constitue une première stratégie pour identifier certains traits de qualité grâce à l'utilisation de marqueurs moléculaires déjà développés pour la vitamine C et ceci sur de nombreuses espèces dont la tomate. La teneur en ascorbate dans les accessions sauvages varie de 11 mg pour 100 g de MF à plus de 500 mg pour 100 g de MF dans les accessions les plus riches. De nos jours, plus de 75 000 accessions sont disponibles dans plus de 120 pays. La plupart de ces ressources ont été utilisées au cours des 20 dernières années par les sélectionneurs afin d'améliorer des caractères d'intérêts qualitatif (gènes majeurs) ou quantitatif (QTL ou Quantitative Trait Loci). Plusieurs QTL contrôlant la teneur en vitamine C ont ainsi été localisés par cartographie génétique, sur des lignées d'introgressions (Rousseaux *et al.,* 2005; Stevens *et al.,* 2007). Alors que la teneur en caroténoïdes et tocochromanols (Vit E) provient d'une synthèse *de novo* est réalisée pour chacun par une seule voie métabolique, pour la teneur en AsA, il faut tenir compte de la synthèse *de novo* qui peut être réalisée *via* plusieurs voies de biosynthèse mais également de la voie de recyclage à partir des formes oxydées, ce qui offre de nombreuses stratégies de bio-ingénierie (Ishikawa *et al.,* 2006). L'avancée des connaissances dans le domaine du métabolisme de l'ascorbate a montré qu'il était possible d'augmenter la teneur dans différentes lignées transgéniques de tomate sur-exprimant ou sous-exprimant des gènes impliqués dans les voies de biosynthèses, de recyclage ou de dégradation (*table 1*). La biofortification des espèces d'intérêt agronomique est donc envisageable notamment grâce à l'identification d'éléments capables d'influencer et de réguler l'accumulation de l'ascorbate. Afin de surmonter les limitations imposées par l'utilisation de la transformation génétique stable (Organisme

Génétiquement Modifié ou OGM), l'utilisation de collection de mutants de tomate représente une stratégie alternative intéressante qui est utilisée pour modifier des traits mais également pour en créer de nouveaux plus intéressants dans les variétés cultivées. La variabilité génétique élevée qui est induite, peut permettre l'identification de génotype présentant une augmentation du pool d'ascorbate (Stevens *et al.,* 2006).

Objectives of the PhD work

The vitamin C is an essential antioxidant for the human health. Biochemical evidence links AsA to molecular events associated with oxygen sensing, redox homeostasis and carcinogenesis (Valko *et al.,* 2006). The human and some other mammalians cannot synthesize this secondary metabolite due to the mutation on the last enzyme of the biosynthesis pathway (Linster and Schaftingen 2007). Hence, plant-derived Vitamin C, mostly found in fruits and vegetables, is the major source in the human diet. Beyond this nutritional function, vitamin C plays a prominent role in plant development and protection against biotic and abiotic stresses (Smirnoff N 1996; De Tullio *et al.,* 1999; Gatzek *et al.,* 2002; Anthonius *et al.,* 2003; Pastori *et al.,* 2003; Conklin and Barth 2004; Halliwell 2006; Badejo *et al.,* 2009). Harvested fruits and vegetables are subjected to oxidative stress which can induce physiological disorders like failure to ripening process, pitting, water soaking and increased postharvest decay (Malacrida *et al.,* 2006; Lee *et al.,* 2000). These postharvest injuries are economically important problems that reduce the overall quality and marketability of many harvested fruits and vegetables.

The main objective of my PhD study was to elucidate the regulation processes that control the AsA content in tomato fruits in the aim to improve the postharvest fruit quality. For that, two strategies were developed, on the one hand using a forward genetic approach, and in the other hand a reverse genetic approach.

The first part of my work based on a forward genetic approach describes the screening of a Micro-Tom EMS (Ethyl Methane Sulfonate) tomato population in order to discover AsA+ mutants in fruits. The EMS mutant collection generated at INRA Bordeaux contained about 8000 families whose 3500 were phenotyped and grouped in 150 phenotype categories. This type of collection is a perfect tool to find mutants producing AsA-enriched fruits. In addition, thanks to the recent development of new technologies of mapping by NGS sequencing (Next Generation Sequencing) it was possible to consider identifying the causal mutation responsible for the AsA+ phenotype (Hartwig *et al.,* 2012; Abe *et al.,* 2012). The screening of 500 EMS Micro-Tom families allowed finding five AsA-enriched mutants in the fruits. Classical genetic approaches demonstrated that one of the mutants corresponded to a dominant mutation whereas the other four were recessive ones. Moreover, these five mutants

originated from distinct loci. Additionally, the two higher AsA-enriched mutants displayed an associated parthenocarpic phenotype, leading to a very intriguing question about the link between parthenocarpy and AsA metabolism. At last, the NGS-mapping strategy has been successful for one on the five mutants selected for this strategy. Indeed, in a segregate population F2 from a backcrossing with WT, combining Bulk Segregante Analysis (BSA) and Whole Genome Sequencing (WGS) strategies allowed identifying the causal mutation. The nature of the protein mutated is very original as the protein participates to the light signalisation and probably can act at the post-transcriptional level on the AsA biosynthesis pathway.

The second part is based on a reverse genetic approach in order to identify new regulator elements related to AsA. In a previous collaborative project called (VTC-Fruit) four transgenic tomato lines silenced for key enzymes of biosynthesis pathway (GME, GalDH) and recycling pathway (AO, MDHAR) have been analysed for AsA and transcriptome (micorarrays), proteome and metabolome (CPG-MS) of fruits at the orange stage. Correlation analysis revealed links between expression level of some genes and the AsA content in these transgenic lines. In this PhD work, the bio-analysis of the database VTC ToolBox allowed selecting 15 candidate genes. Most of them are transcription factors (TF). Interestingly, *in silico* analysis showed that five of these genes co-localize with AsA content QTLs previously isolated (Stevens *et al*., 2007). Several strategies to confirm the relationship of these genes and AsA metabolism have been carried out in the leaf and fruit organs. Taken as a whole, the data confirmed some possible links between these candidate TF genes and the AsA metabolism, but did not permit to conclude about the regulatory function of any of them regarding AsA. This work is still in progress notably to validate the function of the TF genes using transient transformation with protoplasts and TILLING strategy.

The last part consisted in the more agronomic aspect of this project regarding the effect of the antioxidant potential of the mutants related to the postharvest quality of the tomato fruits. For this purpose we benefited of two opposite AsA-related genotypes. First was the AsA+ mutants identified in the first part of the PhD work and displaying an increase of fruit AsA level comprised between 3 to 5 times that of the WT, and second was the AsA- mutants identified by TILLING displaying a lessening of AsA content by about 50 to 80%. These two genotypes constitute very original and unique tools for understanding the importance of AsA regarding postharvest quality, but also address

the question of the biofortification of tomato fruit. This part of my PhD gave me the chance to visit Noam Alkan laboratory at the Volcani Center (Israël), a group having a strong expertise in the postharvest domain related to abiotic and biotic stress responses in fleshy fruits. The results obtained in the frame of this short term mission are very promising as they confirmed that AsA+ mutants are more resistant to chilling stress. On the other side, the biotic part led to observations that are not conclusive and need to be reconsidered and further renewed.

Results

Part I

Identification of Ascorbic Acid Regulators using Forward Genetic screening in tomato.

Part I: Identification of Ascorbic Acid Regulators using Forward Genetic screening in tomato

The ascorbic acid metabolism is currently well described in plants (Wheeler *et al.,* 1998; Smirnoff 2000; Agius *et al.,* 2003; Wolucka and van Montagu 2003; Lorence *et al.,* 2004; Chen *et al.,* 2003; Green and Fry 2005). However, the knowledge of regulatory mechanisms still remains limited. The existence of such regulators has been recently demonstrated with the discovery of some elements of transcriptional and post-transcriptional regulation. The study carried out in the leaf of *Arabidopsis thaliana* led to the characterization of AMR1, ERF98 and CNS5B as positive or negative regulators of the main biosynthesis pathway (Zhang *et al.,* 2010; Zhang *et al.,* 2012; Alimohammadi *et al.,* 2012). Recently, other studies carried out in tomato fruit showed the implication of InsP3 and esWAX1 in the regulation of AsA content (Match J 2013; Zhu *et al.,* 2014). Fruits represent an interesting model to unravel this AsA regulation aspect. The range of AsA content in fruits is large according to the species or the genus considered. AsA can accumulate to very high levels in fruits like acerola (1300mg.100g FW^{-1}) or blackcurrant (210 mg.100gFW^{-1}). As well fruits within the same genus can display a large range of AsA content such as in tomato, where values range from 10 to 500 mg.100g FW^{-1} (Galiana-Balaguer *et al.,* 2006). Tomato fruits harvested at a mature green stage and then ripened off the vine can maintain their reduced AsA content, suggesting that the fruit contains the complete enzymatic machinery related to AsA metabolism (Jimenez *et al,.* 2002; Ahn *et al.,* 2002). Thus, the transcriptome analyses of the enzymes related to the biosynthesis pathway revealed that tomato fruits are able to synthesize their own AsA. It was also the case in apple or blackcurrant (Razavi *et al.,* 2005; Hancock *et al.,* 2007; Ioannidi *et al.,* 2009; Massot *et al.,* 2012). As an example, *GMP* gene is highly expressed in fruits. This gene encodes a GDP-D-mannose pyrophosphorylase which catalyses the synthesis of GDP-D-mannose, a precursor for both the biosynthesis of AsA as well as cell wall polysaccharides. Both classes of compounds have a crucial role in fruit ripening (Gilbert *et al.,* 2009; Handa *et al.,* 1985). In addition, it has previously been demonstrate that AsA is transported between leaves, which are source organs, and fruits, which are sink organs. In some plants, such as blackcurrant and tomato, the *de-novo* synthesis of AsA in fruit seems to be more important than its transport from source

organs (Hancock *et al.,* 2007; Gautier *et al.,* 2009). Furthermore, Massot *et al.,* (2012) demonstrated that according to light irradiance, the key enzymes of the ascorbate biosynthesis are differentially expressed in leaf and fruit. The control of ascorbate accumulation in leaf and fruit are apparently different, as leaf AsA content is usually higher in than in fruit. This suggests that the regulation of AsA synthesis also differs between leaf and fruit (Badejo *et al.,* 2008; Li *et al.,* 2009).

Ascorbic acid content is a critical fruit quality trait that has been lost or lowered in many cultivated varieties of fleshy fruits. 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 including tomato. Tomato stands for the model in fleshy fruit development and quality and, is also a major crop plant species in which vitamin C makes a major contribution to fruit nutritional quality. Strategies for genetically improving AsA content in the fruit rely on the use of genetic variability affecting fruit AsA content. This variability can be found, for example, in other tomato cultivars or in related wild species (natural genetic diversity) or has been generated by artificial means e.g. by physical (X-ray, fast-neutrons) or by treatments with chemical mutagens (artificially-induced genetic diversity). Once the chromosomal region harbouring genes/loci influencing treaits of interest has been identified, as was done for ascorbate in tomato by studying populations issued from crosses between cultivated tomato and related species (Stevens *et al.,* 2007), it can be introgressed into elite varieties by marker assisted selection (Causse *et al.,* 2001).

However, the best possible markers correspond to polymorphism specifically responsible for the ascorbate trait variation itself. Variant alleles responsible for increase in fruit ascorbate can be identified from genetic resources through either reverse genetic or forward genetic approaches. In reverse genetics approaches, genetic resources can be screened for variants in target gene, for example in a candidate gene possibly controlling ascorbate biosynthesis in the fruit. This can be done using several strategies e.g. TILLING (Targeting Induced Local Lesions IN Genomes). The function of the target gene in AsA accumulation, if any, can be further determined by analysing fruit AsA content in the genotype(s) carrying the gene variant. In forward (or direct) genetic approaches, genetic resources are first screened for the phenotype of interest and the gene underlying the phenotypic variation is then identified, classically by map-based cloning.

In EMS (Ethyl MethaneSulfonate) mutant collections, a very large genetic variability far beyond the natural variation found in domesticated species has been artificially generated. This therefore represents a very useful source of genetic diversity that can be harnessed for investigating the function of newly discovered genes/alleles and/or for breeding varieties with high AsA content in the fruit. This forward genetic approach is based on the fact that a phenotypic variation results from a genetic variation or mutation in a gene that is, in the simplest case related to the studied process corresponding to the trait of interest. An EMS-induced mutant library in the dwarf tomato cultivar Micro-Tom was developed at INRA Bordeaux (National Institute for Agronomical Research). This collection consists in 8500 families among which 3500 were phenotyped for visual traits such as fruit colour, shape and epidermis aspect, etc…. The whole phenotypic data were integrated in a web-searchable database called "Micro-Tom Mutant Database (MMDB) (Just *et al.,* 2013).

In the present work, 500 tomato mutant families of the INRA EMS Micro-Tom tomato mutant collection were screened for high fruit ascorbate content. Among them, 5 mutants displaying a significant fruit AsA content were further selected. These mutants will be named "AsA+" mutants all along the manuscript. Fruits of these mutants contained 3 to 5 times more AsA than the WT fruits. The mutations of these AsA+ mutants were characterised. Allelic tests further confirmed that AsA+ phenotypes in these mutants were induced by independent mutations. A strategy of mapping-by-sequencing was next developed in order to identify the causal mutation in one selected AsA+ mutant. The combination of classical bulked-segregant analysis (BSA) with next-generation sequencing (NGS) allowed the rapid identification of the causal mutation responsible for the AsA+ phenotype. An additional interesting outcome of the study of AsA+ mutants was the demonstration of a possible link between AsA level in the fruit and the incidence of fruit parthenocarpy.

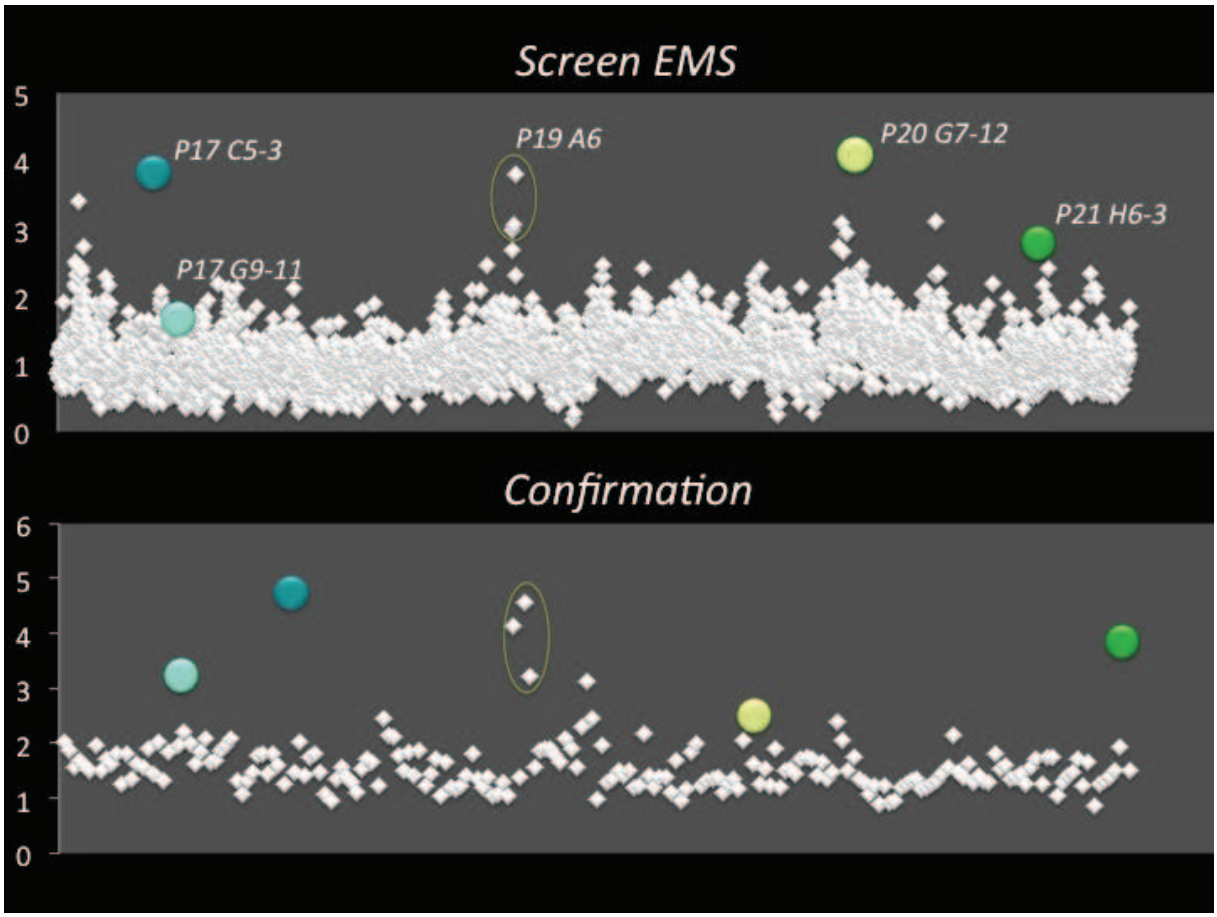


Figure 32: Screening of EMS Micro-Tom mutant population. A. First screen of 500 families realised during Autumn-Winter 2012. Each point represents AsA content of fruits from a single plant. Mutant plants with AsA content around and above 2nmoles.mg FW^{-1} were selected for the screen of confirmation performed during spring 2013 (B). For each mutant, three fruits at the red ripe stage have been pooled, and the assay have been realised in technical triplicate. The colourful circles are the selected mutants to carry on the study. AsA content is expressed in nmoles.mg FW^{-1}.

I. Screening of Micro-Tom EMS mutant collection

In order to discover mutants displaying a modification of AsA content within fruits, 500 M2 and M3 mutant families were screened. For each family, 12 plants were sown in order 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 3-4 fruits at the red ripe (RP) stage were pooled and assayed for AsA content. As shown in *figure 32*, the range of AsA content in the mutants screened varied from 0,5 to 4 nmoles.mg FW^{-1}. The WT mean value is about 1 to 1,5 nmoles.mg FW^{-1} depending on the period of the year when fruits were harvested, e.g. 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).

At the end of this screening process, no mutant line displaying low AsA content in the fruit (AsA-) could be recovered. One main reason is that mutant screening was done on tomato cultures in which the plant density was high, close to 230 plants per m2. These cultures were prone to fungal attacks and the AsA- mutants appeared more susceptible to pathogen infections, which resulted in most cases in the death of the AsA- mutant plants. In contrast, an ascorbate threshold value for selecting AsA+ mutants was set at 2 nmoles.mg FW^{-1} i.e. twice the WT value. Results obtained on the first set of plants selected as AsA-enriched fruits were further confirmed by performing new AsA measurements on the same plants. Following this screening process, 5 families with increased fruit AsA contents comprised between 3 to 5 times that of the WT fruits were selected for further characterization. To get some insights upon the possible inheritance of the AsA+ trait, selected mutants were further selfed and fruits were analysed for AsA content in the S1 (self or autopolination) plants. Noteworthy, mutant lines displaying the highest fruit AsA contents also showed a parthenocarpic fruit phenotype potentially associated with male or female sterility.

In order to identify the mutated locus responsible for the AsA+ phenotypes, the strategy for the 5 mutants selected 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 BSA/NGS-mapping.

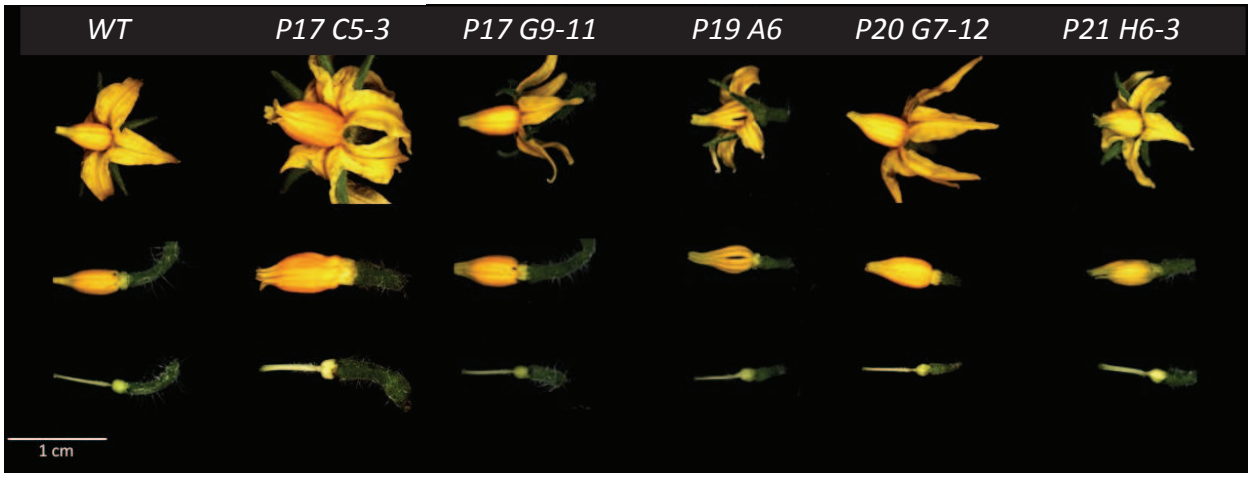


Figure 33: Comparison of floral organs from six AsA enriched EMS Micro-Tom mutants. Entire fully opened flowers (up), after the removal of the sepals and petals (middle), and after the removal of the anther cone to reveal the pistil including the stigma, the style and the ovary (low).

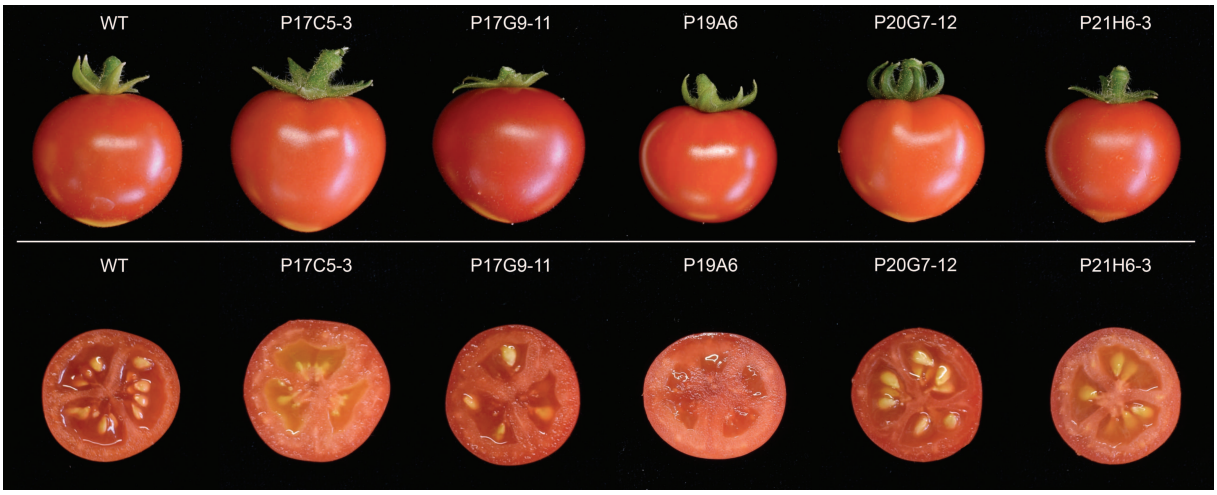


Figure 34: Photography of Fruits from six AsA enriched EMS Micro-Tom mutants. Cutting the fruit at the equatorial level allows to show the carpels containing the jelly and the seeds. Thus for some mutants like P17C5-3 and P19 16, the fruits displayed either a total or a severe absence of seeds, resulting to the parthenocarpic fruit phenotype.

II. Characterization of the 5 selected mutants

a. Genetic variability observed

o Mutant P17 C5-3

In the P17C5 family, only plant n°3 produced AsA+ fruits (*supplemental data 1*). This P17 C5-3 mutant displayed the most important ascorbate content increase in fruits among all the mutants analysed during the screen of the EMS collection. Compared to the WT, the P17 C5-3 plant had 4 to 5 times more AsA in fruits depending on the period of year. The analysis of the kinetic of AsA accumulation in the fruits showed that the AsA content was already high at the early stages of fruit development (*figure 52*). In addition, this mutant displayed an interesting phenotype at the flower and fruit levels (*figure 33 and 34*). The flowers were bigger than the WT and apparently displayed carpel fusion that could explain the significant thickness of the style. The analysis of the pollen germination showed that only few grains were able to germinate *(figure 35)*. However beyond this male sterility problem, the female organs were competent since crossing with foreign pollens resulted in the production of fertile fruits. All the fruits of the P17 C5-3 mutant showed almost obligatory parthenocarpy. Sometimes, fruits contained few seeds but plants issued from these seeds had a strongly altered phenotype making their analysis difficult.

o Mutant P17 G9-11

Among the P17G9 family, the plant n°11 displayed a slight increase just above the threshold at 2 nmol.mg FW^{-1} (*supemental data 2*). This P17G9-11 mutant was selected and the second confirmation revealed an AsA content of 3 nmoles.mg FW^{-1} that is the less important among the selected mutants. The P17 G9-11 mutant displays no obvious phenotype at the level of vegetative and reproductive parts (*figure 33 and 34*).

o Mutants P19 A6

For the screen of the P19 A6 family, 3 out of the 12 plants sown were characterized as AsA+ with a fruit AsA content of 4 to 5 nmoles.mg FW^{-1} (*supplemental data 3*). In term of AsA concentrations, these mutants are the second important ones after those of the P17 C5-3. Furthermore, as for the P17C5 family, these plants showed a strong phenotype both at the flower and fruit levels (*figure 33 and 34*).

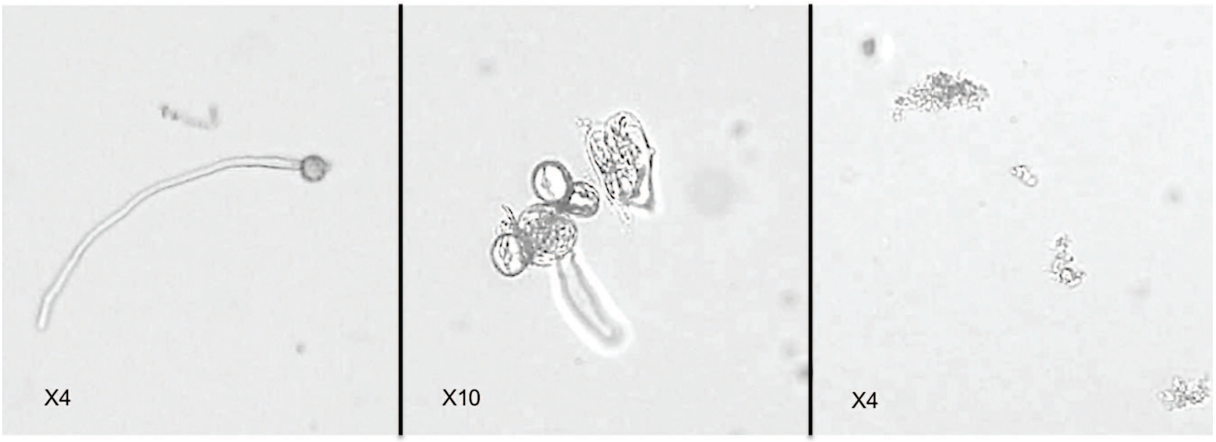


Figure 35: Microscopic observations of pollen grains during *in vitro* germination in WT, P17C5-3 and P19 A6 mutant lines. The pollen was incubating 4h in a germination solution (see Mat&Meth). Photos show that P17C5-3 displays a shorter pollen tube and some aggregation phenomena. For the P19A6 mutant, the pollen grains are almost undetectable, like if the stamens were empty.

The flowers were smaller than the WT, the anther cone had slots and the style always topped the anther cone extremity. Microscopic observation showed absence of pollen in the stamina *(figure 35)*. In addition, the AsA+ fruits of these three P19A6 mutants were smaller than the WT and had a parthenocarpy phenotype. As for P17 C5-3, this family was very remarkable but the parthenocarpic phenotype rendered difficult their characterization. At last, the tentative to backcross them with WT plants were unsuccessful, suggesting also organs female sterility.

- **Mutants P20 G7**

Among the P20 G7 family plants, two displayed the AsA+ phenotype (*supplemental data 5*). AsA content was determined to be close to 3-4 nmole.mg FW^{-1} at the first screening that was carried out in September 2012. When the confirmation was performed on the two selected AsA+ plants, in January 2013, the measured AsA concentration was reduced to 2,5 nmoles.mg FW^{-1}. At this stage, we decided to keep this family and we assumed that the observed discrepancy between the two measurements could result from environmental effects or different regulations, possibly related to light irradiance. To check this hypothesis, additional experiments under different light conditions need to be performed. This P20 G7 mutant is also a good candidate mutant because, in a general manner, no alteration in the phenotype of the vegetative and reproductive organs was observed (*figure 33 and 34*). At last, it could be noteworthy to identify the causal mutation and determine any relationship with some environmental regulation of this mutated gene.

- **Mutants P21H6-3**

For this last family selected, only one plant, mutant n°3, displayed AsA+ phenotype among 12 sown (*supplemental data 4*). AsA content is around 3nmole.mg FW^{-1} at the first screening that was carried out in September 2012. When the confirmation was performed on the two selected AsA+ plants, in January 2013, the AsA concentration increase to 3 nmoles.mg FW^{-1}, suggesting a light regulation. The P21H6-3 mutant is a good candidate as there is no alteration in the phenotype of the vegetative and reproductive organs (*figure 33 and 34*). In the frame of my work, this mutant was the first one selected for the next step with the aim to identify the causal mutation.

b. Type of Inheritance of the mutation

In order to determine the type of inheritance of the mutations responsible for the AsA+ phenotypes observed in the 5 selected mutants, the segregation analysis of the AsA+ trait was carried out using Self1 (S1) and F2 segregant populations (*supplemental 1, 2, 3, 4, 5*). All the mutants were backcrossed with WT parent. Among the five mutants selected, only P17C5-3 corresponds to a dominant mutation, while the other four carry recessive mutations. In each mutant family selected after the first screening step, one mutant plant carrying EMS mutations but displaying normal fruit AsA content was kept as control. This plant shares a similar mutagenized background as the AsA+ mutant plant and is noted as "WT +".

i. Recessive mutants

- **Mutants P17 G9-11**

To characterise the P17 G9-11 mutation, the AsA content was assayed in the progeny. First, 12 plants from S1 generation were sown. All the 12 S1 plants displayed the AsA+ phenotype, and such a phenotype was not observed at the leaf level, only in fruits. In the next step, the P17G9-11 mutant was backcrossed with a WT parent. All the 12 BC1F1 plants analysed displayed an AsA content similar to the WT. The segregant analysis revealed that among the 16 BC1F2 plants only 3 produced fruits with around 2 nmoles.mg FW^{-1} that is just twice as much AsA as the WT. Hence, this study of the P17 G9-11 mutant shows that the P17G9-11 parent carries homozygous and recessive mutation. However, for this mutant it seems that the AsA+ phenotype would be difficult to detect and follow especially in non-optimal culture conditions.

- **Mutants P19 A6**

Among 12 M2/M3 plants of the family P19A6 analysed, 3 were identified as "AsA+" suggesting a recessive mutation. It is also necessary to keep in mind that these mutants produced parthenocarpic fruits. That's why the study of the progeny is complicated. So, in the hypothesis that the corresponding "WT+" selected is heterozygous for the mutation. To check whether the "WT+" selected is heterozygous for the mutation, 18 S1 plants of the "WT+" were analysed. Two of them 3 produced AsA enriched fruits

	P17C5-3	*P17G9-11*	*P19A6*	*P20G7-12*	*P21H6-3*
AsA content (nmoles.mg FW$^{-1)}$	4 to 5	3	4 to 5	3 to 4	4
Backcrossing Effectiveness	YES	YES	NO	YES	YES
Pollen sterility	YES	NO	YES	NO	NO
Fruit parthenocarpy	YES	NO	YES	NO	NO
Dominance of the mutation	*Dominant*	*Recessive*	*Recessive*	*Recessive*	*Recessive*
NGS-mapping	NO	NO	NO	NO	YES

Table 5: Summary table of the analysis of the five AsA enriched mutants selected. Are presented the AsA content in the fruits, the backcrossing effectiveness suggesting a possible sterility of the floral female organs, the pollen sterility, the fruits with parthenocarpic phenotype, and finally the dominance of the mutation and which mutants have been analysed using NGS-mapping.

containing few seeds showing that the "WT+" was indeed heterozygous and that the AsA+ and parthenocarpy traits could be associated in dosage dependant manner. At this stage, for the P19 A6 family, we stopped the study after the first backcross.

o Mutants P20 G7

For the P20G7 family, only one of the two plants identified in the M2/M3 generation were selected. From this plant, 12 seeds corresponding to the S1 generation were sown. The AsA assay revealed that all of these S1 plants produced AsA enriched fruits. Analysis of the AsA content in leaf revealed no change compared to the WT, suggesting that the mutation can interfere exclusively with the regulation of the AsA accumulation in the fruits. The resulting BC1F2 segregant population show that 9 on the 32 plants displayed an AsA+ phenotype.

o Mutant P21H6-3

In the P21 H6-3 mutant the AsA+ phenotype was analysed in the progeny. For 12 S1 plants assayed, all produced AsA enriched fruits. Interestingly, for some plants AsA content in leaf also increased. However, this feature must be taken with care because leaf AsA content is highly influenced by environmental factors like light. The resulting BC1F2 segregant population show that 115 plants among 441 studied were defined as "AsA+" plants.

In conclusion, the *p17g9-11, p20g7-12* and *p21h6-3* mutants display a segregation of the mutation as expected in the case of a recessive mutation (*table 5*). The S1 generation appear to be homogeneous for the AsA+ phenotype. Further analysis of the F2 population confirms a mendelian 1:2:1 segregation involving a single recessive mutation. Using an allelic test that consists to cross together these three mutant families, we obtained plants producing "WT-like AsA" phenotype fruits. These results confirm that the three AsA+ phenotypes originate from mutations of independent loci. In the case of the *P19A6* mutant, the mutation seems to be also recessive because among 12 M2/M3 plants analyzed, only three displayed the AsA+ phenotype. However, it is necessary to study the corresponding F2 segregant population in order to determinate whether the parthenocarpic fruit and the AsA+ phenotypes originate from a single locus or from several loci genetically linked.

ii. Dominant mutant

As mentioned before, only one plant in the P17C5 family, named P17C5-3, showed a remarkable and very intriguing AsA+ phenotype. 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. For this unique mutant, fruits from self-pollination provided only 5 seeds. Among the five S1 plants, only one showed the AsA+ phenotype both in leaf and fruit. Interestingly, the AsA content of this S1 plant was twice higher than that of the parent P17C5-3 mutant. The backcrossing of P17C5-3 with the wild type parent allowed the characterization of the mutation. Around 50% of the 12 BC1F1 plants analysed displayed an "AsA+" phenotype associated with the parthenocarpic fruit phenotype. In the same time, the AsA concentration measured in BC1F1 fruits displaying the AsA+ trait were more important than in the parent P17C5-3. We could conclude that the mutation is firstly dominant as the phenotype is observed in BC1F1 plants, and secondly that the P17C5-3 parent was heterozygous for the mutation because only half of the F1 presented the AsA+ phenotype. As expected, no change in fruit AsA content was observed in the BC1F2 population obtained from the BC1F1 plants that did not displayed the AsA+ trait. The absence of seeds in the BC1F1 AsA+ fruits made impossible the generation of the BC1F2 population to determinate if it results from a single or several loci. Theoretically, the association of the parthenocarpic phenotype with the AsA+ phenotype can result of either two linked loci or one single locus, thus leading to an interesting question about the relationship between these two phenotypes.

Besides its crucial importance in fruit nutritional quality, vitamin C is also likely to play important roles in other fruit quality attributes such as resistance to various stresses, fruit postharvest shel-flife etc … The discovery of an AsA+ dominant mutation is really significant and attractive for breeding programs in the objective to produce fruits with better nutritional potential and especially this at the F1 hybrid level. In addition, P17C5-3 is a good resource to study the link between AsA and fruit parthenocarpy. In that aim, it was pertinent to check the possible transfer of the beneficial P17C5-3 mutation to another genetic background of commercial interest. The first tomato cultivar crossed with that AsA+ mutant was the M82 cultivar, a well-known and well-studied cultivar that displays morphological and genetic characteristics very

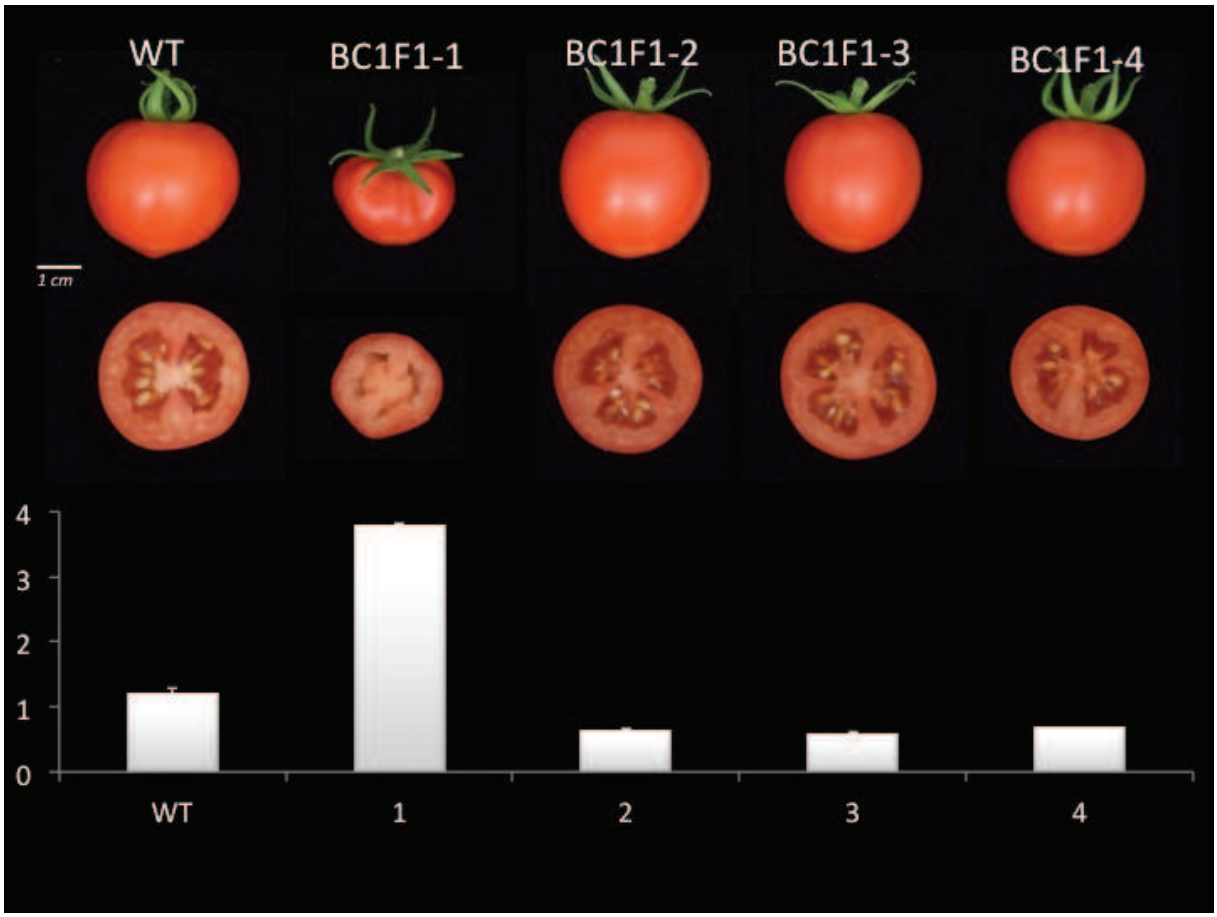


Figure 36: BC1F1 fruits between P17C5-3 Micro-Tom mutant and the M82 cultivar and AsA content**.** Among four BC1F1 plants (P17C5-3xM82) cultivated in the green house, only one produced smaller and parthenocarpic fruits (n°1) whereas the remaining three produced fruits displaying WT phenotype. Three fruits at the red ripe stage were pooled and three technical triplicates (±SD) were analysed. AsA content is expressed in nmoles.mg FW^{-1}.

different from that of the Micro-Tom cultivar. Indeed, M82 displays a bushy determinate port, the plant height varies from 1 to 2 m, and produces large fruits (diameter of 5 to 6 cm). The cross between P17C5-3 plant and M82 cultivar produced fruits with few seeds in average 4 seeds/fruit. Among four BC1F1 plants sown, only one displayed a strong AsA+ phenotype (*figure 36*). Such an AsA+ trait in the F1 generation is consistent with the previous observation carried out in Micro-Tom and the nature of the P17C5-3 mutation. This result confirms that the AsA+ phenotype of the P17C5-3 can be expressed in other genetic background. Interestingly, the AsA+ phenotype is similarly associated with fruit parthenocarpy. However, it is not possible to distinguish whether these two phenotypes are the result of a single mutation or two genetically linked mutations. To address this question it is necessary to product F2 recombinants or look for another solution.

III- Mutation NGS-mapping for the *p21h6-3* mutant

For many decades, forward genetics (or forward genetic screen) has been a powerful approach to identify genes underlying phenotypes of interest in collections of artificially-induced genetic diversity (chemical and physical mutagenesis, T-DNA insertions etc …) and determine their function. This approach is therefore at the opposite from the reverse genetic approach, which aims at determining the function of a known target gene by analysing the phenotype of genotypes carrying genetic variants for that gene. In forward genetics, searching for the genetic mutation responsible for the trait of interest is typically a multistep process that involves genetic mapping to locate the chromosomal region harbouring the causal mutation followed by a targeted search for candidate mutations within this region. It classically involves outcrossing the genotype carrying the mutation with a genotype carrying enough genetic polymorphism to design molecular markers (e.g. Single Nucleotide Polymorphism or SNP markers) that will be used to map the mutation on a genetic map and eventually identify it (this process is hence called “map-based cloning”). However, map-based cloning can be very long and tedious. In addition, while outcrossing the mutant with a distant genotype (e.g. a distant cultivated species or a related wild species) offers a high density of genetic markers that can help fine-mapping the mutation, it also produces high genetic and

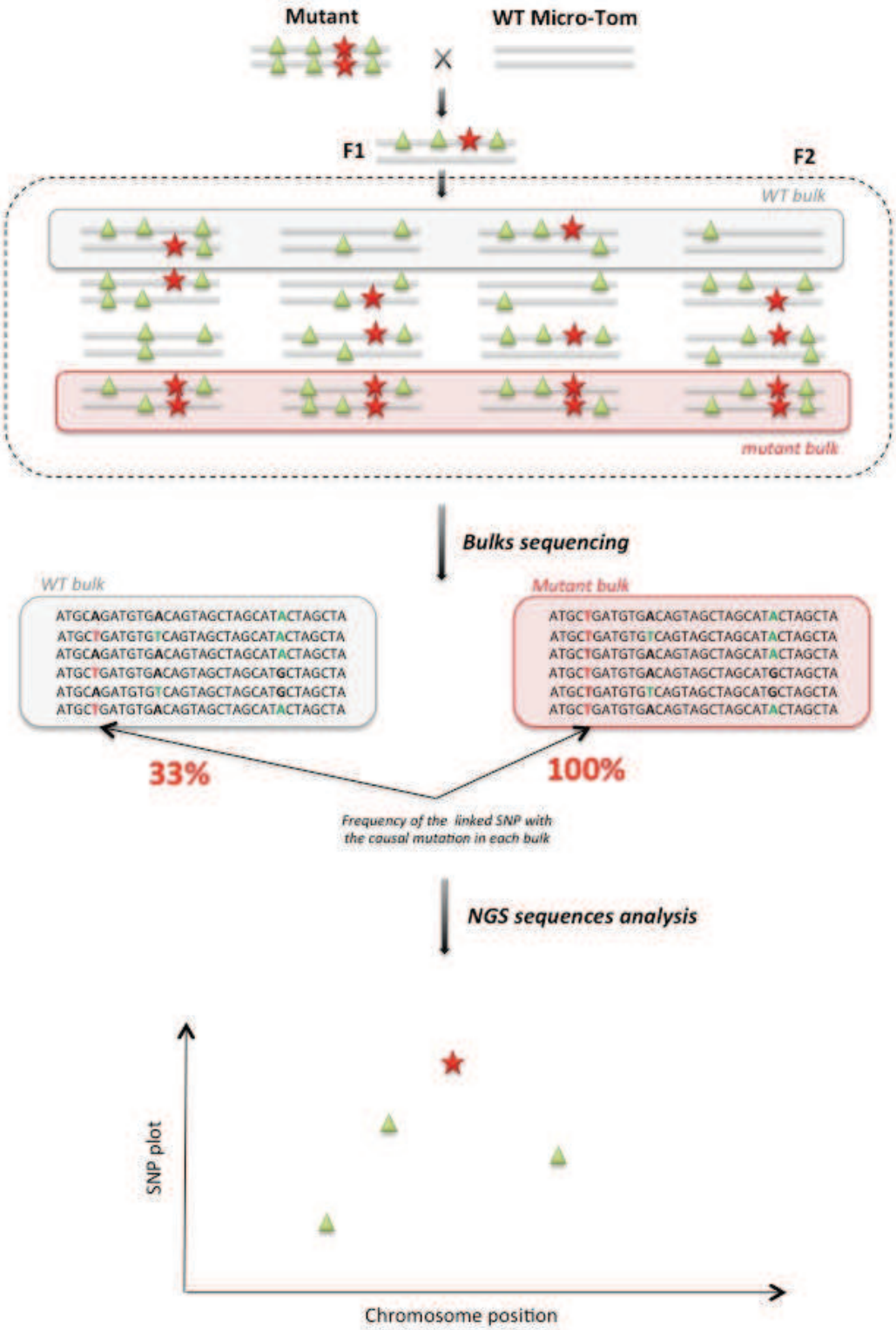


Figure 37: Overview of the NGS-mapping strategy. The M2/M3 mutant is backcrossed with the WT Micro-Tom to generate the heterozygous BC1F1, which is next, self-pollinate to generate the BC1F2 population. In the case of a recessive mutation, the AsA+ phenotype segregates as 1:3 ratio in the F2 population. Two different bulks were prepared; the WT bulk corresponded to individual plants displaying the WT phenotype and the mutant bulk corresponding to the AsA+ individual plants. Within the two bulks, leaves of each single plant of the bulk were collected and equally pooled to prepare DNA for sequencing. The analysis of NGS sequences allows to detect EMS mutations and comparison of allelic frequencies in the 2 bulks allows identifying the causal mutation. We expect that causal EMS mutation should present highest SNP index plot. (Ratio EMS frequencies in mutant/WT bulk; red start)(Allen *et al.,* 2013)

phenotypic variability that can hinder the detection of the mutant trait in the segregating population used for map-based cloning.

The recent advances in next-generation sequencing (NGS) technology now provide new ways for efficient mapping-by-sequencing strategy (Allen *et al.,* 2013; Lin *et al.,* 2014). Once a mapping population has been established, the strategy developed in this study strategy combines classical bulk-segregant analysis (BSA) with next generation sequencing (NGS) of the bulks followed by computational analysis of the phenotype and sequence data. Already demonstrated in the plant models Arabidopsis (Austin *et al.,* 2011) and rice (Abe *et al.,* 2012), this strategy allows the rapid identification of causal mutations at single-nucleotide resolution. Therefore, in the context of the huge phenotypic and genetic variability found in our EMS tomato mutant collection, forward genetic screen associated with NGS-mapping of the causal mutations of interest appears as the most straightforward approach to identify new actors involved in the regulation of AsA metabolism. Among the various AsA+ mutants mentioned in the previous section, we selected the *p21h6-3* mutant line for identifying the mutation underlying the fruit AsA+ trait by BSA-NGS mapping. The *p21h6-3* mutant offers several interesting features including (*i*) a strong fruit AsA enrichment (3–4 fold increase), (*ii*) controlled by a recessive mutation and, (iii) no additional phenotypic alteration noted for the plant.

a- Strategy developed to map the causal mutation underlying the p21h6-3 AsA+ trait by NGS

To limit phenotypic and genetic variability obtained when crossing with a distinct genetic background, the EMS mutant was backcrossed with the WT parent (*figure 37*). Outcrossing can be problematic if the mutant phenotype is difficult to recognize in the hybrid F2 progeny, or dependent on parental specific traits. The interest to achieve a non-polymorphic crossing is to reduce the introduction of polymorphisms other than the ones induced by the EMS mutations. The resulting F2 segregant population contains plants that display either the mutant trait or the WT phenotype with respect to the trait-of-interest screened. In the case of a recessive mutation responsible for the trait-of-interest, the recombinant individuals that show the mutant phenotype carry the causal

mutation. If no previous genetic mapping has been done, as is the case of the *p21h6-3* mutant, the chromosomal location of the mutation is unknown. As described above, the strategy adopted to overcome this limitation is that of the BSA-NGS mapping in which (1) DNA from bulks of BC1F2 plants displaying either the AsA+ trait or the WT-like fruit phenotype are pooled, (2) each bulk is sequenced in order to get a genome coverage of 30 to 40 X per bulk, (3) the SNP resulting from EMS mutagenesis are mapped to the reference tomato genome and their frequencies compared in each bulk.

We therefore phenotyped the BC1F2 segregant population resulting from a cross between *p21h6-3* and WT Micro-Tom to define two bulks corresponding to the "AsA+" and to the "WT-like AsA" individuals, respectively. In the next step, the pooled DNA were sequenced using a next generation Illumina sequencer 2500. Whole-genome sequencing (WGS) of the two bulks was performed by paired-end sequencing (2x 100bp) using Illumina technology platform that uses 1 lane per bulk that theoretically gives around 40 GBase /lane. According to the size of tomato genome (≅950 Mb), the expected sequencing depth is around 40X, which is sufficient to detect single nucleotide polymorphism (Austin *et al.,* 2011; Allen *et al.,* 2013; Mascher *et al.,* 2014). In our strategy, we selected 40 plants in each bulk (from a total population of 440 individuals) to maintain balance between the desired depth of sequencing and the dilution of the possible errors in the phenotyping. For each bulk, an equivalent amount of leaf was harvested on each of the 40 plants, so each individual is equally represented. A general pipeline was applied to detect variants between the two bulks. This pipeline is a bioinformatic workflow sequence that includes different steps of WGS analyses using multiple software tools (*See Materials and Methods section figure 67*). It consists in aligning the sequences obtained from the bulk to the reference tomato genome *(Heinz version 2.50*) and then to perform the detection of variants using Samtool. With this "pipeline" we were able to detect both EMS mutations and natural polymorphism between Heinz and our Micro-Tom tomato cultivar. Additionally, some WT Micro-Tom plants used to produce the EMS collection were also sequenced, to enable filtering out any non EMS SNPs resulting from natural variations within the Micro-Tom cultivar. An underlying tenet relies the frequency with the chromosomal position of the SNP in the mutant. This principle was applied as filter using allelic frequency expected in the mutant and WT bulks for each EMS mutation detected. For example, in the case of a

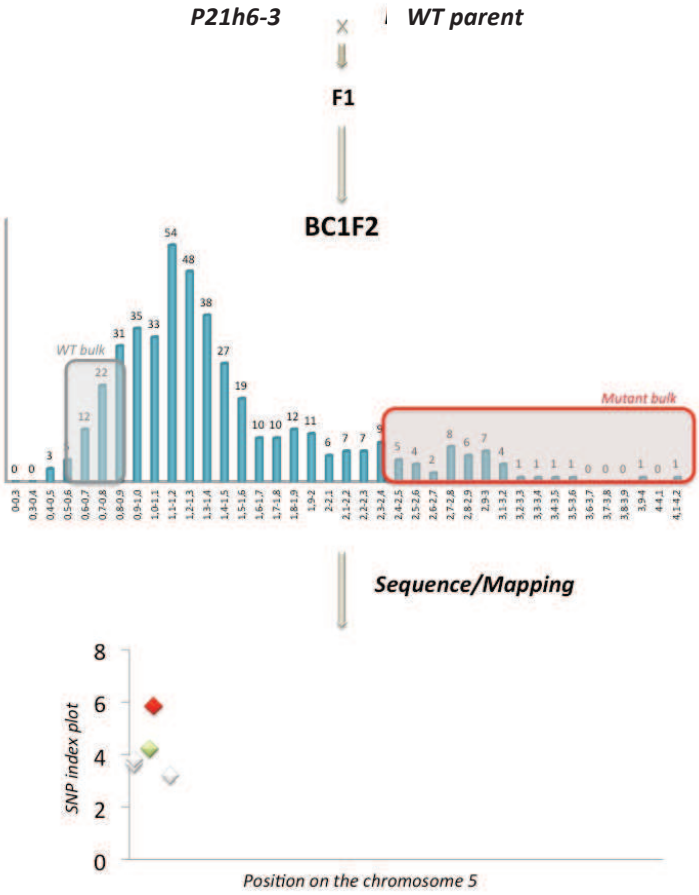


Figure 38 : Overview of the NGS-mapping strategy. The M2/M3 mutant is backcrossed with the WT Micro-Tom to generate the heterozygous BC1F1, which is next, self-pollinate to generate the BC1F2 population. In the case of a recessive mutation, the AsA+ phenotype segregates as 1:3 ratio in the F2 population. Two different bulks were prepared; the WT bulk corresponded to individual plants displaying the WT phenotype and the mutant bulk corresponding to the AsA+ individual plants. Within the two bulks, leaves of each single plant of the bulk were collected and equally pooled to prepare DNA for sequencing. The analysis of NGS sequences allows to detect EMS mutations and comparison of allelic frequencies in the 2 bulks allows identifying the causal mutation. We expect that causal EMS mutation should present highest SNP index plot. (Ratio EMS frequencies in mutant/WT bulk; red start)(Allen *et al.,* 2013)

Chromosome	*EMS detected* *10≤ read depth ≥100*	*SNPs filtered*
Ch01	906	0
Ch02	329	0
Ch03	672	0
Ch04	571	0
Ch05	444	**5**
Ch06	296	0
Ch07	657	0
Ch08	539	0
Ch09	563	0
Ch10	519	0
Ch11	423	0
Ch12	569	0

Table 6: Number of EMS in the "common table". The second column corresponds to the EMS mutations detected after removing the natural polymorphism between Heinz and Micro-Tom. The third column corresponds to the EMS mutations displaying the expected allelic frequencies for a recessive mutation in each bulk (WT bulk: ≤33%; mutant bulk≥90%)

recessive mutation, the frequency expected for the causal mutation in the mutant bulk should be close to 1 (mutant homozygous) whereas in the WT bulk it is around 0,33 (2/3 heterozygous and 1/3 WT homozygous). The recessive causal mutation will be homozygous, whereas the homozygosis of linked SNPs will decrease with distance from the causal mutation (Allen *et al.,* 2013). The position of these linked SNPs and their corresponding allelic frequency can be used as markers to identify the region where is located the causal mutation with a strong likelihood. Combining the classical bulked-segregant (BSA) with the next generation sequencing (NGS) approaches therefore allows decreasing the background noise produced by the numerous non-associated polymorphisms that segregate with the causal mutation in a mutagenized population and therefore facilitates the identification of the causal mutation.

b. Identification of the p21h6-3 causal mutation

This paragraph will describe in more detail the work that has been done to identify the *p21h6-3* causal mutation using BSA-NGS mapping (f*igure 38*). Towards this end, the *p21h6-3* mutant was first single backcrossed with the WT Micro-Tom parent. Among the BC1F2 segregant population of 600 plants, 441 were phenotyped for AsA content in order to define two bulks corresponding to plants with "WT-like AsA" or with "AsA+" phenotypes, respectively. Considering the size of the population analysed (441 plants) and the recessive state of the mutation, 44 plants (10% of the phenotyped population) were pooled for each bulk and further sequenced by NGS. The WGS reads was 383 692 470 pb and 376 559 190 pb for the mutant and the WT bulk, respectively. Regarding the size of the tomato genome (≅950 Mb), a sequencing of 39X depth was obtained, which was compatible with high confidence variant detection. The number of SNPs detected was evenly distributed among the 12 tomato chromosomes. In total around 7000 SNPs were detected (*table 6*). After the use of bio-analytical filters based on the frequencies expected in the case of recessive mutations, only five SNPs were retained (*table 7*). All of them are located in the chromosome 5 and correspond to a linked group in which are present the causal mutation and the linked SNPs. Among the five SNPs, only two corresponded to genes namely *Solyc* (tomato gene identification at SGN (http://solgenomics.net). The first SNP, which corresponded to a C to T transition, is located in the middle of the first intron of the *Solyc05g006660* gene, encoding an

Position	*Depth_Mut*	*Freq allele Mut*	*Depth_WT*	*Freq allele WT*	*Ratio Mut/WT*	*Gene annotation*
302839	39	97,44	30	26,67	3,65	**NA**
355879	71	97,18	47	25,53	3,81	**NA**
1332056	50	100	55	23,64	4,23	**Solyc05g006660**
1610236	33	100	41	17,07	5,86	**Solyc05g007020**
2682086	43	88,37	51	27,45	3,22	**NA**

Table 7: Summary of the Illumina sequencing information and annotation for the 5 SNPs detected in the chromosome 5. Tomato genome annotation allows to clearly identifying 2 genic SNPs but only one is exonic, as a knockout mutation in the Solyc05g007020 (red).

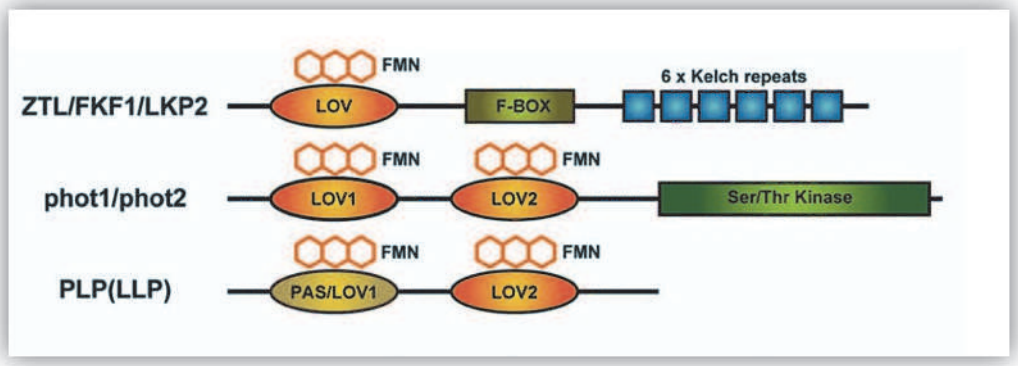


Figure 39: Illustration of functional domains found in proteins of the ZTL/FKF1/LKP2 family, the phototropin proteins, and the PAS/LOV proteins. The LOV domain is able to bind an FMN molecule, which acts as a blue-light-sensing domain. The ZTL/FKF1/LKP2 family proteins contain a LOV domain at the N-terminus region next to a F-box domain and then six Kelch repeats in the C-terminal region. The phototropins Phot1/Phot2 contain two FMN-binding LOV domains named LOV1 and LOV2 in their N-terminal region and a serine/threonine kinase domain in the C-terminal. The PAS/LOV proteins (PLP) contain a PAS domain next to a LOV domain. The LOV domain contain a very conserved cysteine involved in the activation mechanisms of the protein by light, in contrast the PAS domain does not contain this cysteine indicating that there is no light-induced photocycle from this domain (Ito *et al.,* 2012).

"unknown protein" (green). In contrast, the second SNP (red) is detected in the fifth exon of the *Solyc05g007020* gene, and is present at the top of the SNPs plot. It corresponds to a G to A transversion, which results to the appearance of a STOP codon in the protein sequence and consequently to a non-functional protein. At this stage we could conclude that this SNP in the *Solyc05g007020* gene is likely to be the causal mutation for the *p21h6-3* AsA+ phenotype. Additional genetic analyses remain however necessary to unequivocally link the AsA+ trait to that mutation.

c. The Solyc05g007020 protein nature and function

According to the NCBI database and the literature, the *Solyc05g007020* gene encodes a twin LOV (Light, Oxygen, or Voltage) protein. The LOV/LOV proteins are known to be involved in the blue light signalling. A wide variety of physiological processes are regulated by Blue light, which include the circadian clock, the induction of the carotenoid synthesis, the phototropism, and the induction of many gene expression and protein modifications (Lakin-Thomas *et al.,* 1990; Linden *et al.,* 1997). Among the plant photoreceptors, three different classes corresponding to blue-light receptors use a LOV domain as the blue light-sensitive signalling switch (*figure 39*). For the first class, the most known are the phototropins Phto1 and Phot2 which have been identified as the photoreceptors responsible for tropism, chloroplast movement and stomatal aperture (Christie *et al.,* 1998; Kagawa *et al.,* 2001; Sakai *et al.,* 2001). Phototropins have two LOV domains next to a serine/threonine protein kinase domain. The second class of photoreceptors, the ZTL/FKF1/LKP2 family is constituted by three components, the F-box ZEITLUPE (ZTL) protein, the flavin-binding Kelch repeat F-box (FKF1) protein and LOV Kelch protein 2 (LKP2), which consists of LOV, F-box motif and kelch repeat domains. The ZTL regulates the circadian clock whereas the FKF1 induces floral transition under long-day conditions (Nelson *et al.,* 2000; Somers *et al.,* 2000). The third class, the less characterized so far, are the PAS/LOV proteins (PLP), which contain an N-terminal PAS (Per-ARNT-Sim) domain and a C-terminal LOV domain. The LOV protein domains form a subset of the large and diverse PAS superfamily proteins, which are able to bind a flavin mononucleotide (FMN) (Kasahara *et al.,* 2010). The molecular mechanism of the PAS/LOV protein function is as followed. In the dark, the LOV domain binds non-covalently the FMN. The absorption of blue light by the FMN induces the

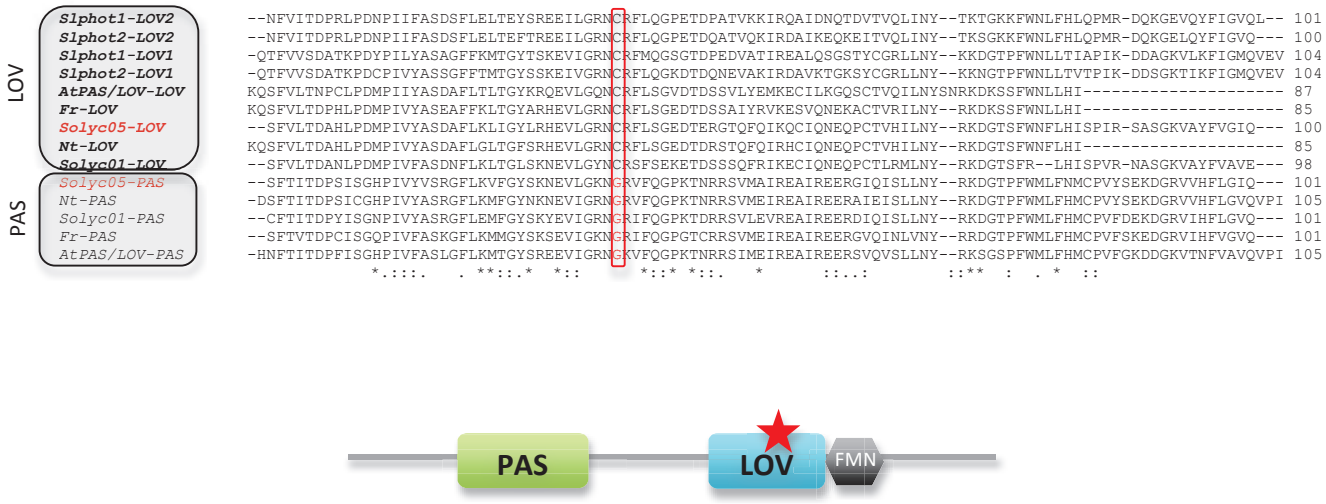


Figure 40: A. Alignments of the amino acid sequence of the LOV and PAS domains. The conserved cysteine residue is marked within the red frame. This highly conserved cysteine is present only in the LOV domain. The sequences used in the alignment include the following: *Solanum lycopersicum* PHOT1 and PHOT2; *Arabidospis thaliana* PAS/LOV; *Fragaria vesca subsp. verca* PAS/LOV; *Nicotiana tomentosiformis* PAS/LOV; Solyc05g007020 (PAS/LOV identify in the present by NGS-mapping) and Solyc01g010480 another PAS/LOV in the tomato genome close to Solyc05g007020. **B.** Illustration depicting the structure of the PAS/LOV protein (398aa). The EMS causal mutation (red star) is located in the LOV domain.

formation of a covalent bond between FMN and a conserved cysteine residue of the LOV domain. This reaction is reversible, and results in a rapid switch leading to the activation of targeted proteins, as for example during the photocycle. The blue-light induces a conformational change of the protein, thus facilitating its interactions with partners. According to Ogura *et al.,* (2008), the difference between the PAS domain and the LOV domain lies on the presence/absence of the key cysteine residue. However, the protein sequence revealed that the Solyc05g007020 protein is not a LOV/LOV protein but a PAS/LOV protein as one of the two domains does not contain the cysteine residue (*figure 40*). The PAS family, including the PAS and LOV proteins, is evolutionary well conserved. The PAS/LOV protein identified in this work does not contain a transmembrane domain suggesting a cytoplasmic localization. In *Arabidopsis thaliana* the LOV1 domain was shown to act as a dimerization site for the phototropin proteins (26). Therefore, the photochemical function of the LOV1 domains may not be necessary to allow them to undergo protein-protein interaction. Using yeast two-hybrid systems, Ogura *et al.,* (2008) have shown that the AtPAS/LOV protein interacts with VTC2, VTC2 like, BLH10A and BLH10B. These interactions seem to be significantly diminished only under high blue light radiation, but not under red, far-red, or green light. These observations suggest that the PAS/LOV may function as photoreceptor. In the dark, the LOV2 domain of the AtPHOT2 protein is able to bind the C-terminal Ser/Thr kinase domain and this results in the inhibition of the activity. When irradiated, LOV2 dissociates from the kinase domain, leading to the activation of the kinase activity. The PAS/LOV protein identified in my work does not contain this Ser/Thr effector domain, suggesting that it may work as effector. It is still difficult at this stage of our study to determine precisely the physiological function of this tomato PAS/LOV protein.

IV. Discussion

The forward genetics approach is largely used to link genetic variations with phenotypic changes in collections of plants displaying large artificially-induced genetic diversity. In that approach, the objective is to discover the allelic variation(s) responsible for single Mendelian traits or variations of quantitative traits previously identified through phenotypic screening of populations displaying genetic diversity (Just *et al.,* 2013). Towards this end, a highly mutagenized EMS Micro-Tom collection was developed at INRA Bordeaux as a source of new genetic diversity that can be used for the identification of genes underlying traits of interest in tomato (Baldet *et al.,* 2013; Just et *al.,* 2013). That EMS collection results from two rounds of mutagenesis with 1% EMS. These were carried out in order to increase the mutation frequencies and reduce the mutant screening effort that corresponded to two treatments. According to the sequencing analysis of several mutants from the EMS Micro-Tom population, it was estimated that 6000 to 7000 mutations are present per individual mutant genome. In this study, the screen of 500 EMS families allowed me to identify mutants displaying an AsA+ phenotype, the AsA content of which being increased by 3 to 5-fold when compared to WT (*figure 36*). Such results illustrate how the screening of a low number of families from an EMS tomato mutant collection can successfully contribute to the isolation of as much as 5 families producing AsA enriched tomato fruits.

Among them, three families are likely to provide new genetic materials for deciphering the mechanisms involved in the regulation of AsA content in tomato fruits. Indeed, the *p17g9-11, p20g7-12* and *p21h6-3* families do not display strong phenotypes at the plant architecture and flower/fruit development levels. An allelic complementation test further confirmed that these three AsA+ phenotypes involve different loci. In contrast, the *P17C5-3* and *P19A6* families display the highest AsA content but this was associated with strong parthenocarpic phenotypes. Interestingly, Bulley *et al.,* 2012 showed that in transgenic lines overexpressing the GGP2 protein, the increase the ascorbate content by 5 to 7 fold was associated with alterations in fruit development such as parthenocarpy, which was characterized by the absence of seeds and of the surrounding jelly of locular tissue. These observations likely suggest a possible link between high AsA content and parthenocarpic fruit development. Several studies have demonstrated that AsA can participate in specific processes linked to

meristem activity, seeds or embryo development. Thus, the treatment of seeds with high AsA concentration can inhibit germination (Canao *et al.,* 1997; Ishibashi and Iwaya-Inoue 2006, our unpublished data). In addition, the AsA content varies as a function of tissue and cell types. For example, AsA content is low in quiescent cells and in desiccated seeds. We can hypothesize that a precise regulation allows maintaining specific AsA content in order to induce adequate biological process. Thus, cells or tissues which need a low AsA content can be very sensitive to any modification in AsA accumulation, and this could interfere with normal development processes like regulation of pollen viability, ovule fertilisation and embryo development. Alternatively, in the 60's many works suggested that the mechanism for AsA regulation of plant growth and development might reside in its interaction with phyto-hormones. Theaotia *et al.,* (1961) and Shanmugavelu (1962) were able to induce the formation of parthenocarpic fruits through the use of gibberellic acid. Such fruits showed a granular pulp and contained more AsA than the WT fruits. Recently, the same relation was characterized between auxin and AsA (Tsaniklidis *et al.,* 2014). Auxin treatment of tomato plants during anthesis affected pollen availability and fertility, which resulted from the inactivation of enzyme involved in AsA recycling. Martinelli *et al.,* (2009) showed also that the modification in auxin related enzyme activities induces the production of parthenocarpic fruits associated with an increase in AsA content. We can reasonably hypothesize that in the *P17C5-3* and *P19A6* mutants, the hormone metabolism mainly related to ABA and auxin is altered due to a causal mutation and, as described above, this could result in an increase of AsA content in the fruits. However, the reverse hypothesis: Is AsA content changes in these mutants at the origin of changes in hormonal signalling and therefore of fruit parthenocarpy is still an open question to be addressed.

In his work, Bulley *et al.,* had difficulties to observe any increase of the AsA content in the leaves of the transgenic tomato plants overexpressing VTC2. This was also true in our study, where the AsA essay on the EMS mutants selected show a clearly AsA+ traits in fruits in contrast to the leaf. This tricky feature could originate from the well-established correlation between the light intensity and the accumulation of AsA in photosynthetic tissues. 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. Before my arrival to the laboratory, the same strategy to screen a population comprising 500 EMS tomato mutants in order to identify plants producing AsA enriched fruits was performed on the basis of the AsA content in the leaf but this approach was unsuccessful (P. Baldet, unpublished data). Taken as a whole, these data suggest that screening mutant plants for AsA-enriched leaf is probably not very discriminatory due to the high variability in leaf ascorbate content and its dependence on light. Finally, the hypothesis of a possible difference in the mechanisms involved in AsA regulation between leaf and fruit is also conceivable.

Because most of the genetic materials and phenotypic characterization were completed for the *p21h6-3* mutant, it was chosen to identify the gene underlying the variations in the AsA phenotypic trait. Mapping-by-sequencing constitutes a powerful approach to accelerate and improve forward genetics in plant (Schneeberger 2014). Associating BSA and NGS-mapping using a BC1F2 segregant population allowed us to identify the single causative SNP using exclusively the SNP position and frequency. Nevertheless, the backcrossing with a parent was necessary to induce segregation of the linked SNP and thus to mark the genome. The highly mutagenized EMS Micro-Tom collection, leading to high SNP frequency may have improved the resolution of the SNP distribution peak and the ability to identify the causal mutation. Another factor that could account for the success in identifying the unique SNP in the *p21h6-3* mutant was the specificity of the chromosome 5 region and its high level of recombination. Indeed, we could identify one single region associated with the AsA+ phenotype that contained 5 SNPs spread on around 2 Mbp This region is located at the extremity of the chromosome 5, which has a high recombination level. In this region, only two SNPs were present in the allele mutant at 100% in the mutant bulk, which are very close in a short region of 278 Mpb. Among them, one displays a low allele frequency in the WT-like bulk, around 17%, which seems to correspond to the causal mutation. In this chromosome 5 region, the recombination rate is estimated to be 20 cM/Mbp (see Suppl. Data in the Tomato consortium published Nature 2012), which is largely sufficient to get recombinants between the causal SNP and the adjacent SNPs in the mutant bulk. Such a high recombination rate is a very favourable condition to breakdown the disequilibrium linkage, and this likely explains how we could obtain only a very tight associated region comprising only one candidate causal SNP. Hence, our strategy demonstrates that a 40X

coverage and about 40-pooled individuals was sufficient for the identification of the candidate gene.

The AsA+ phenotype of *p21h6-3* is caused by a recessive mutation resulting to a stop codon and a non-functional PAS/LOV protein, on *Solyc05g007020* gene. The biological function of this PAS/LOV protein is still unknown. Nevertheless, several proteins containing a LOV domain exist in higher eukaryotes. Most of them are well characterized such as the phototropins as well as the proteins involved in the circadian clock (Briggs *et al.,* 2002; Christie *et al.,* 2002; Cheng *et al.,* 2003; Crosson *et al.,* 2003; Buttani *et al.,* 2008). For the phototropins phot1 and phot2, in vivo mapping of the protein allowed identifying the phosphorylation sites in the LOV domain. The characterization of Arabidopsis mutants harbouring punctual mutations at the phosphorylation site demonstrated that this auto-phosphorylation is essential to trigger phototropin-dependant response (Inoue *et al.,* 2008). These studies also demonstrated that blue-light dependant phosphorylation is necessary to bind interacting partners that are involved in physiological processes. In order to further understand the transduction signal, it would be interesting to determinate if the PAS and LOV domains of the *Solyc05g007020* protein can be phosphorylated. In addition, this post-translational modification appears to modulate the subcellular localization of the phototropins. In the dark, both phot1 and phot2 are tightly associated with the plasma membrane. Blue light triggers the delocalization of a fraction of phot1 to the cytoplasm, whereas a pool of phot2 is delocalized to the Golgi (Kong *et al.,* 2008; Wang *et al.,* 2008; Demarsy *et al.,* 2009). This delocalization process is linked to their physiological function and is highly dependent on the light intensity. Regarding the AsA biosynthesis pathway, some studies have shown that VTC2 is clearly regulated by light, thus suggesting a circadian regulation (Tabata *et al.,* 2002; Dowdle *et al.,* 2007; Patricia Müller-Moulé 2008). Higher transcript levels of circadian clock and photoperiodic pathway genes were detected in *vtc* mutants (Kotchoni *et al.,* 2002). Interestingly, the VTC2:YFP fusion protein was found in both the cytoplasm and the nucleus (Müller-Moulé 2008). AsA biosynthesis pathway is localized in the cytoplasm except for last enzyme GLDH that is associated with the mitochondrial compartment. The presence of VTC2 in the cytoplasm is therefore expected, but not its nuclear localization. YFP protein alone can diffuse into the nucleus due to its small size (27kDA), however, once bound to VTC2 protein, the fusion protein whose size is 76kDa would exceed the limit of size exclusion. The analysis

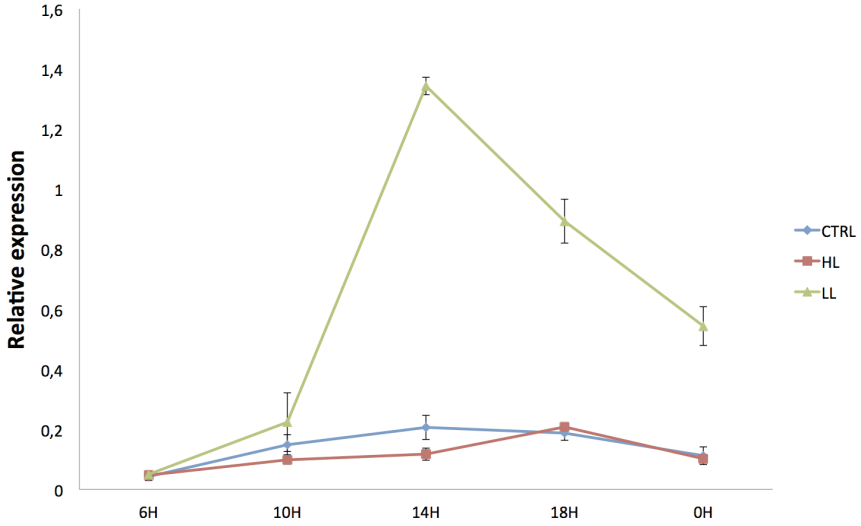
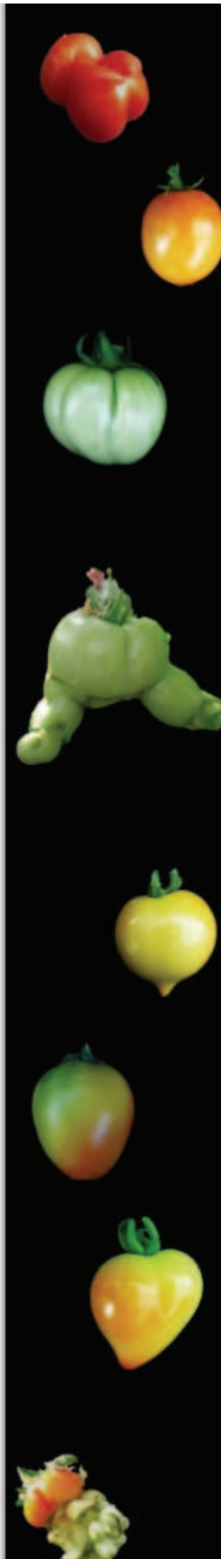


Figure 41: Relative expression of the *PAS/LOV* in the leaves of WT exposed to three light conditions (HL, LL and CTRL). Leavesof MicroTom at "four leaves stage" were harvested all along the day at 6H, 10H, 14H, 18H and 24H. The three light conditions are in the graph: high light (Red), low light (Green) and control (Blue). For each point, three leaves were analysed (±SD).Data were obtained by real-time PCR normalized against Actin and eiF4 reference genes. The relative expressions are in arbitrary unit.

of the VTC2 sequence using PSORT software shows the presence of a specific sequence for nucleus addressing (NLS or Nucleus Localization Sequence) (Müller-Moulé 2008). The daily expression profile of the PAS/LOV protein in the WT shows an expression almost null in control and high light conditions. In contrast, in the low light conditions corresponding to a light reduction of about 70%, the expression picked at 12h UT (*figure 41*). We can hypothesize a possible interaction between the PAS/LOV protein and VTC2 in very specific conditions like low light irradiance. In that case, the PAS/LOV could act as a repressor to inhibit AsA synthesis through the capture of the VTC2 protein or could alter the partitioning of VTC2 in the cytoplasm or the nucleus, as shown by Müller-Moulé (2008). This could be a very simple way to modulate or stop the de novo AsA synthesis in the cell. The PAS/LOV protein identified in our work does not contain any transmembrane domain suggesting that it is not localized at the plasmalemma. To confirm this hypothesis more experiments will be necessary, 1) to demonstrate if there is a real *in vivo* interaction between VCT2 and PAS/LOV proteins, 2) to determine if this interaction is depending on the light conditions, 3) to analyse the VTC2-PAS/LOV localization within the cell according to the light irradiance, and 4) to identify the role of this regulation in a biological system.

Part II

Research of Ascorbic Acid Regulators using a Reverse Genetic Strategy



Part II: Research of Ascorbic Acid Regulators using a Reverse Genetic Strategy

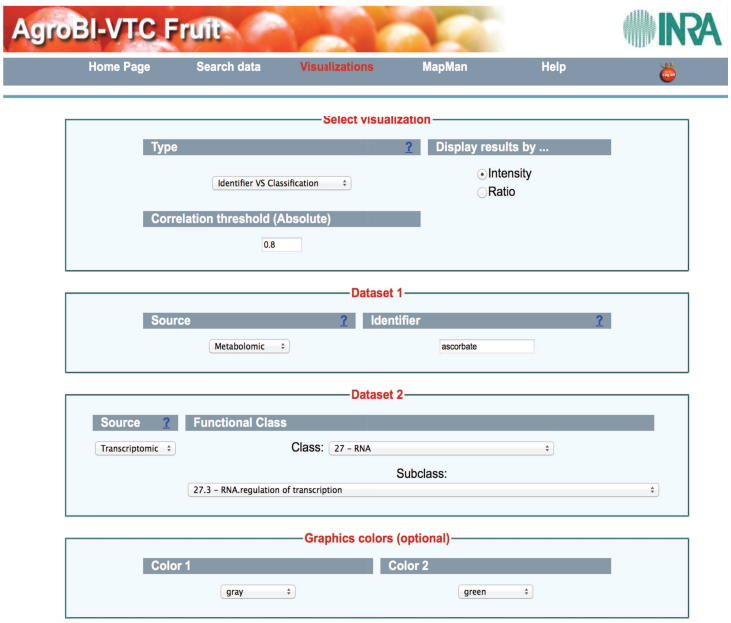
L-ascorbic acid (AsA) is a compound with crucial roles in most organisms including plants and of high nutritional interest for humans. It acts as a water-soluble antioxidant to scavenge reactive oxygen species (ROS) (Noctor and Foyer 1998). In addition to its antioxidant properties, AsA plays an essential role in many biological processes such as cell cycle (Liso *et al.,* 1988; Davey *et al.,* 1999), cell expansion (Fry 1998; Smirnoff N 1996), hormone synthesis and signalling (Pastori *et al.,* 2003; Noctor 2006), biotic and abiotic stress resistance (Noctor and Foyer 1998; Asada K 1999; Barth *et al.,* 2004; Liu *et al.,* 2012). Humans and some other animal species have lost the ability to synthetize this compound due to a loss-of-function mutation in the last step of AsA biosynthesis. Thus, fresh fruits and vegetables represent the major source of vitamin C in the human diet. In fruits like tomato, enhancing AsA content has become a major breeding target to increase fruit nutritional quality. To reach that objective, understanding the mechanism involved in AsA regulation is a prerequisite.

Several classical genetic approaches allowed the identification of QTLs involved in the control of AsA content (Fridman *et al.,* 2004; Rousseaux *et al.,* 2005; Stevens *et al.,* 2007). However, the identification of the genes underlying these QTLs may still represent a long-term objective. Reverse genetics approaches based on the identification of target genes, e.g. through transcriptome and correlation network analyses, and on their functional analysis *in planta*, may represent an alternative and complementary approach to identify genes with key roles in the control of fruit vitamin C content. It is already known that the expressions of genes associated with the same metabolic function are likely to show co-expression patterns (Gao *et al.,* 2013). With the development of the “omics" approaches, at the genome-wide level, large sets of gene expression data have been accumulated, thus allowing the investigation of many gene regulatory mechanisms. Gao *et al.,* (2013) have developed a “weighted gene correlation network analysis” approach based on gene expression and AsA content data in ripening tomato fruits (*Solanum lycopersicum* L.). They have detected clusters of genes that are related to AsA pool size in ripening tomato fruits, among which genes involved in the redox pathways.

However, further studies are necessary to precisely confirm and identify the candidate genes. Additionally, to understand the origin of the differential AsA accumulation in some tomato cultivars, Mellidou *et al.,* (2012) combined fruit metabolite analyses with AsA candidate gene expression. They suggest that in "Ailsa Craig" and "Santorini", respectively AsA-poor and -rich cultivars, complementary or alternative mechanisms are taking place to maintain the AsA pool of the fruit. Alternative routes to synthesize AsA seem to supplement the main L-galactose biosynthesis pathway in the AsA-poor cultivar, while in the AsA-rich cultivar the high AsA accumulation results from an enhanced AsA recycling pathway. In the same manner, Lima-Silva *et al.,* (2012) have analysed the transcript level of genes that positively or negatively correlated with AsA content within a tomato RIL population. Cluster analysis allowed identifying a subset of co-regulated genes mainly involved in hormone signalling.

Prior to these studies, through a collaborative project between several INRA groups interested in tomato fruit vitamin C (the "VTC Fruit" project), a fruit systems biology approach was developed in order to decipher the major relationships between ascorbate content and metabolic pathways. To this end, fruits from four tomato transgenic lines altered in the fruit AsA content as well as at the level of the AsA redox status were thoroughly characterized through phenotypic, transcriptomic, proteomic and metabolomics analyses. The whole data sets were compiled and statistical and bioinformatics tools necessary to store, analyse and integrate experimental data in a Web application called VTC Tool box were developed and implemented (Garcia *et al.,* 2009). The main objective of this study was to use the VTC toolbox to discover new candidate genes that may participate in the regulation of AsA content in tomato fruits. In the present study, after data mining and filtering to target specifically genes of the regulation and signalling pathways, fifteen candidate genes were selected, most of which are transcription factors (TFs) including ARF, ERF, bZIP and C2H2 TFs. These candidate genes display an expression profile that positively or negatively correlate (Pearson correlation) with AsA content in the four transgenic lines. Next step was to confirm the relationship between ascorbate and the expression of these genes and to analyse their possible involvement in the regulation of ascorbate accumulation in the fruit. Indeed, candidate genes differentially regulated in fruits from transgenic lines altered in AsA content and/or redox state are likely regulated by AsA but might in turn control AsA accumulation through feed-back regulation. In order to address these questions, part of

A



B

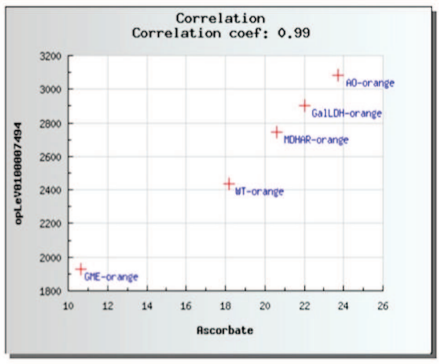


Figure 42: The VTC Fruit project. **A.** Query interface of Agrobi-VTC Fruit database. The MapMan category called "RNA regulation of transcription" was screened to identify genes whose expression correlates with AsA content in the four transgenic lines, which display modification in AsA accumulation in fruit. **B.** Example of graph representing the expression of one the candidate genes as a function of the AsA content. A correlation coefficient was calculated (Pearson); it can be positive or negative depending on the relationship.

my PhD work was based on physiological experiments, firstly to confirm that any modifications of the AsA content can affect the expression of those 15 candidate genes and secondly to check, using a transient transformation system, whether any change in candidate gene expression may induce changes in AsA accumulation.

I. Looking for candidate genes involved in AsA regulation.

a. The VTC fruit project

The "Vitamin C Fruit" (VTC) project was set to understand complex interaction networks between AsA biosynthesis and recycling, and the other main metabolic pathways of the tomato fruits. Towards this end, a RNAi strategy targeting two genes of the biosynthetic pathway, GDP-mannose epimerase (GME), galactonolactone dehydrogenase (GLDH), and two genes of the recycling pathways, monodehydroascorbate reductase (MDHAR) and ascorbate oxidase (AO) was used to generate transgenic lines displaying changes in AsA content and AsA redox state. These 4 transformants and the wild type plants in the cherry tomato WVa106 (West Virginia 106) were characterized at the transcriptomic (TOM2 microarrays), proteomic (2D gels and LC-MS/MS), metabolomics (proton NMP and LC-DAD) and phenotypic levels. Two different stages of fruit development displaying different patterns of AsA accumulation and redox state were chosen to perform this analysis, fruits at 20 days after anthesis (20 DPA) and orange stage (Or). All plants were grown in a greenhouse, at the same location, at Domaine Saint Maurice at INRA Avignon and at the same period of the year in order to minimize the effects of any environmental conditions perturbations. Data from WT and transgenic lines were next integrated in a Web application called VTC Tool box. In order to analyse and interpret the meta datasets, a set of visualization tools, e.g. Kohonen's SOMs, MapMAn in addition to other statistical tools e.g. Pearson correlation, Principal Component Analyses (PCA) was developed in this Web interface.

Name	Unigene	SolyC	Correlation coefficient	Characteristic	Identification	Reference
WRKY1	SGN-U577434	***Solyc01g095630***	-0,98	1 WRKY domain Group III	SlWRKY41	Kurabachev *et al.,* 2013
WRKY2	SGN-U570041	***Solyc02g088340***	0,99	2 WRKY domains GroupI	SlWRKY3	Kurabachev *et al.,* 2013
bZIP1	SGN-U580933	***Solyc04g082890***	0,95	1 bZIP domain	AtbZIP60	Nagashima *et al.,* 2014
bZIP2	SGN-U568869	***Solyc01g079480***	-0,97	1 bZIP domain		
bZIP 3	SGN-U567159	***Solyc06g009640***	-0,97	1 bZIP domain	OCS element binding factor 1 like	Foley et al., 1993
ARF	SGN-U579928	***Solyc03g118290***	0,96	Auxine response factor	ARF2	
C2H2 Zinc finger-1	SGN-U569960	***Solyc01g107170***	-0,96	2 C2H2 domains	ZF2	Hichri *et al.,* 2014
C2H2 Zinc finger-3	SGN-U572337	***Solyc06g075780***	-0,96	2 C2H2 domains	ZAT12 like	Shah *et al.,* 2013 Rizhsky et al., 2004
ERF	SGN-U584756	***Solyc09g075420***	0,96	AP2 superfamily	ERF2	
bHLH	SGN-U575250	***Solyc07g064040***	-0,98	1 dom HLH + TMF DNA db	ILR3	Long *et al.,* 2010
Homeobox	SGN-U581431	***Solyc01g007070***	-0,97	Pox superfamily	Bel1 like homeodomain protein 1	Rutjens *et al.,* 2009
Unknown1	SGN-U581277	***Solyc02g079060***	0,97	eiF3 superfamily	Subunit J like	Gutu et al., 2013
Unknown2	SGN-U579676	***Solyc10g005260***	-0,97	Superfamily polyademylate binding protein	RBP45 isoform like	
Unknown3	SGN-U570562	***Solyc07g064510***	0,97	Superfamily polyademylate binding protein	RBP45	
Unknown4	SGN-U570009	***Solyc08g075840***	-0,95	Replication protein A	Subunit 70kDA	

Table 8: Candidate genes selected. Names were arbitrarily chosen. The "Unigene" corresponds to the gene reference from the SGN (Sol Genomic Network) database. The "Solyc" represents the genomic position. The correlation coefficient was obtained after screening and analysing the VTC-Fruit database (http://www.bordeaux.inra.fr). The "characteristic" and "identification" categories are based on the *in silico* analysis.

b. Screening of VTC fruit database

To discover genes involved in the regulation of AsA content, a MapMan category called “RNA regulation of transcription” was screened to find elements whose transcript levels negatively or positively correlate with AsA content (*figure 42a*). Correlation analyses can be powerful tools to identify the contribution of individual genes in the control of developmental processes or metabolites. The results obtain with VTC Tool box allowed to draw a graph displaying the mathematic relation between a selected gene expression data and the value of AsA content of the WT and the four transgenic lines in the orange fruits (*figure 42b*). A Pearson correlation coefficient was calculated and reflects the relationship between gene expression and AsA content in tomato fruit. To be more restrictive, 1) we chose a threshold for R at ±0,95, 2) we selected only genes displaying a level of gene expression above 1500 (arbitrary units). This strategy allowed selecting fifteen candidate genes (*table 8*). Most of them are transcription factors including ARF (Auxin Response Factor), ERF (Ethylen Response Factor), bZIP (basic Leucine Zipper Domain), C2H2 (Cysteine2 Hystidine2) and WRKY genes that are in a general manner described to be involved in the responses to biotic or abiotic stresses (Lee *et al.,* 2006; Zhang *et al.,* 2010; Zhang *et al.,* 2012b, Alves *et al.,* 2014). Regarding the other four “unknown” candidates, analysis of the peptide sequence allows suggestion of putative functions. The Unknown 1 sequence seems to correspond to subunit 5 of eiF3 complexe. The Unknown 2 and 3 belong to the same RMN superfamily, which includes the RBP polyadenylate binding-protein. RBP proteins are important regulators of eukaryotic gene expression. Based on their homology sequence analysis Unknown 2 could be a RBP45 protein and Unknown 3 could be a RBP45-like protein. The Unknown 4 sequence contains a Zinc Finger CCHC domain, which matches with the 70KDa subunit of the replication protein A. Thus, we can assume that these four “Unknown” candidates may be involved in some cascade of processes during biotic or abiotic stresses. Interestingly, *in silico* analysis showed that C2H2-1, WRKY2, Unk2, ARF and ERF genes co-localize with several AsA QTLs previously identified in tomato (Stevens *et al.,* 2007).

In order to study the relationship between the 15 candidate genes, a correlation network was created. The goal of the construction of gene networks is to draw from experimental data any putative interactions between genes, which can be validated further.

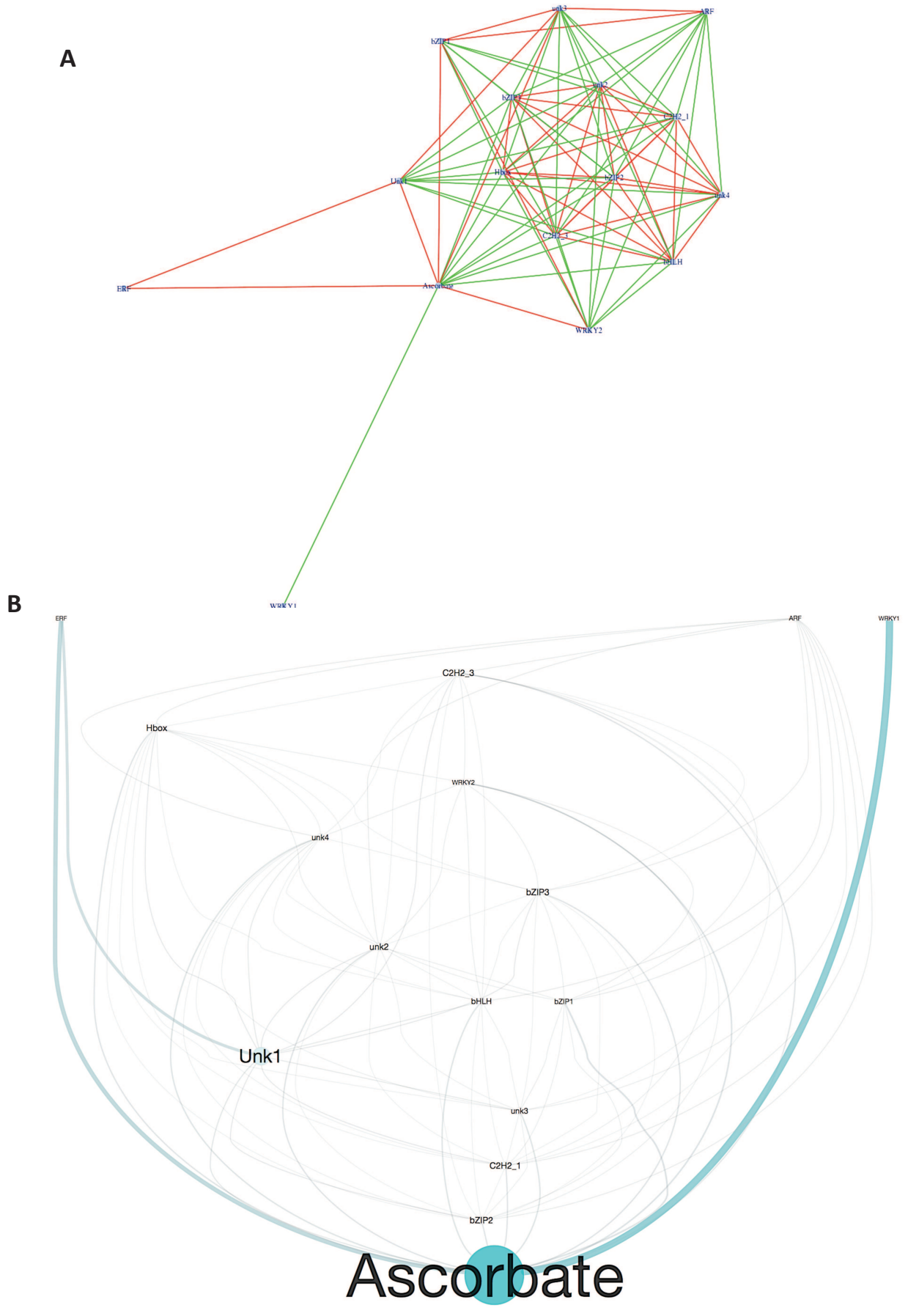


Figure 43: Correlation network between the fifteen candidate genes and AsA. **A.** Correlation graph using "Biostatflow" web site. This figure represents a Fruchterman layout based on Pearson correlation (*P* value=0,05) and a FDR (False Discovery Rate) correction. The interest of this representation is that it is possible to see the direction of the correlation (positive in red or negative in green). The direction of the correlation are similar between genes and AsA, to those obtain with the VTC-Fruit database (table 6). **B.** Correlation graph obtained with "Cytoscape" software. Same parameters were used (Pearson and FDR). The interest of this software is that it is possible to apply different algorithms allowing the rapid development of additional computational analyses and features. Cytoscape is most powerful when used in conjunction with a large database like protein-protein interactions. This representation is based on two algorithms for prioritizing and highlighting the fact that the node size is proportional to their importance, e.g. the AsA hub.

The method used in this analysis was to calculate the correlations between each pair of genes, based on the Pearson correlation coefficient. If the absolute value of the correlation between two genes is greater than *P*-value=0,05, this being either a positive or negative relation, then it is possible to construct the network step by step for each set of genes (biostatflow: *http://biostatflow.org, figure 43a*). To improve the network representation and to help the analysis, two algorithms were used (Cytoscape software; *figure 43b*). The first algorithm called "hierarchical layout" is interesting for representing main direction or "flow" within a network. Nodes are placed in hierarchically arranged layers and the ordering of the nodes within each layer is chosen in such a way that it minimizes the number of edge crossing. The second algorithm is necessary to improve the visual representation where the size of the node is mapped onto the degree, which illustrated the number of links connected to the node. In that representation, the hubs of the network are larger nodes. As shown in a *figure 43a*, the correlation graph realized using "biostatflow" confirmed the direction of the correlation (positive or negative) found with VTC-Fruit database. Only ARF, placed on the outside of the network, does not have a direct link with AsA content. ERF and WRKY1 do not have many links connected with the other candidates, as they are principally linked with AsA. In the center of the network, many genes, bZIP2, bZIP3, Unk2, Unk4, C2H2_1, C2H2_3, Hbox, and bHLH maintain positive correlations in contrast to the genes outside the network. The correlation graph realized with Cytoscape software shows that AsA, the main hub, is connected with several candidate genes directly or indirectly. (*figure 43b*). Interestingly, the Unk1 gene constitutes another important hub in the correlation network. Some of the hub-related genes are transcription factors involved in same response to abiotic and biotic stress, which may explain the high number of edges between them. However, these correlation networks are preliminary data and further observations may be acquired by using other algorithms.

In conclusion, this approach allowed identifying genes whose expression level correlate with AsA content. The functional characterization of the relationship is still in progress. Indeed, we need to determine whether, among the ascorbate-related regulatory genes identified in this study, some genes are involved in the feed-back regulation of ascorbate. If such genes are identified, they would represent likely targets for manipulating through biotechnology or breeding fruit ascorbate content.

Figure 44: Photographs of experiments realized to induce AsA variations in tomato leaves. **A.** Leaves of MicroTom were soaked in either a solution of AsA (10mM) in MS 1/4 or in MS 1/4 (Control) during 30 min, 1h, 2h or 6h. Leaves are under a sodium lamp to induce water evaporation and consequently absorption of the solution in which they were incubated. A fan was used to cool the system, improve evapo-transpiration and prevent heat stress. Three leaves were used in each condition. **B.** Example of the MicroTom used at "four leaves stage" (left). Before starting the experiment, plants were separated to prevent the overlapping of the leaves and placed in three light conditions: High light (HL), Control (CTRL) and Low light (LL). The HL and CTRL correspond to plants exposed outside and inside the greenhouse, respectively (not shown). While the LL condition corresponds to plants shaded as shown, the net reduces by 70% light irradiance (right).

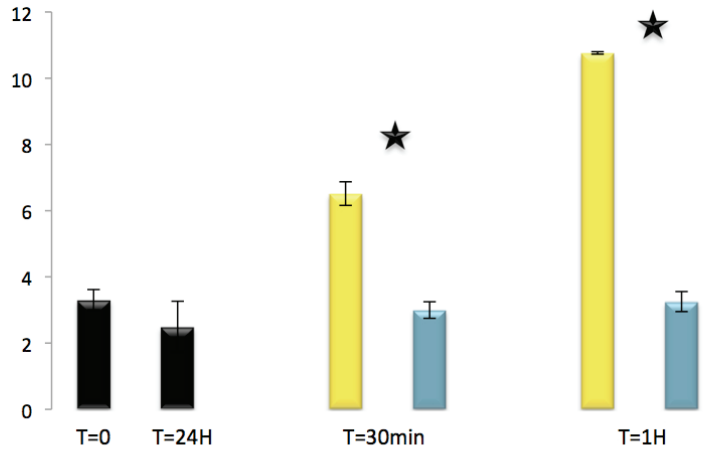


Figure 45: AsA content in tomato leaves soaked in a solution of AsA (10mM) or MS ¼ (control).). Four points are shown in graph. T=0 is the expression when leaves were harvested in the greenhouse. Before starting the experiment, leaves remained in the growth chamber during 24H to recover and to reduce any stress signal induced by the cutting; it corresponds to T=24H. Next step, leaves were soaked in solution of AsA (yellow bar) or MS ¼ (blue bar) during 30 min or 1 hour. For each time, three leaves were analysed in technical triplicate (±SD) and asterisks indicate significant differences between treatments (Student test, P<0,05). AsA content is expressed in nmoles.mg FW^{-1}.

II. Role of candidate genes in ascorbic acid accumulation.

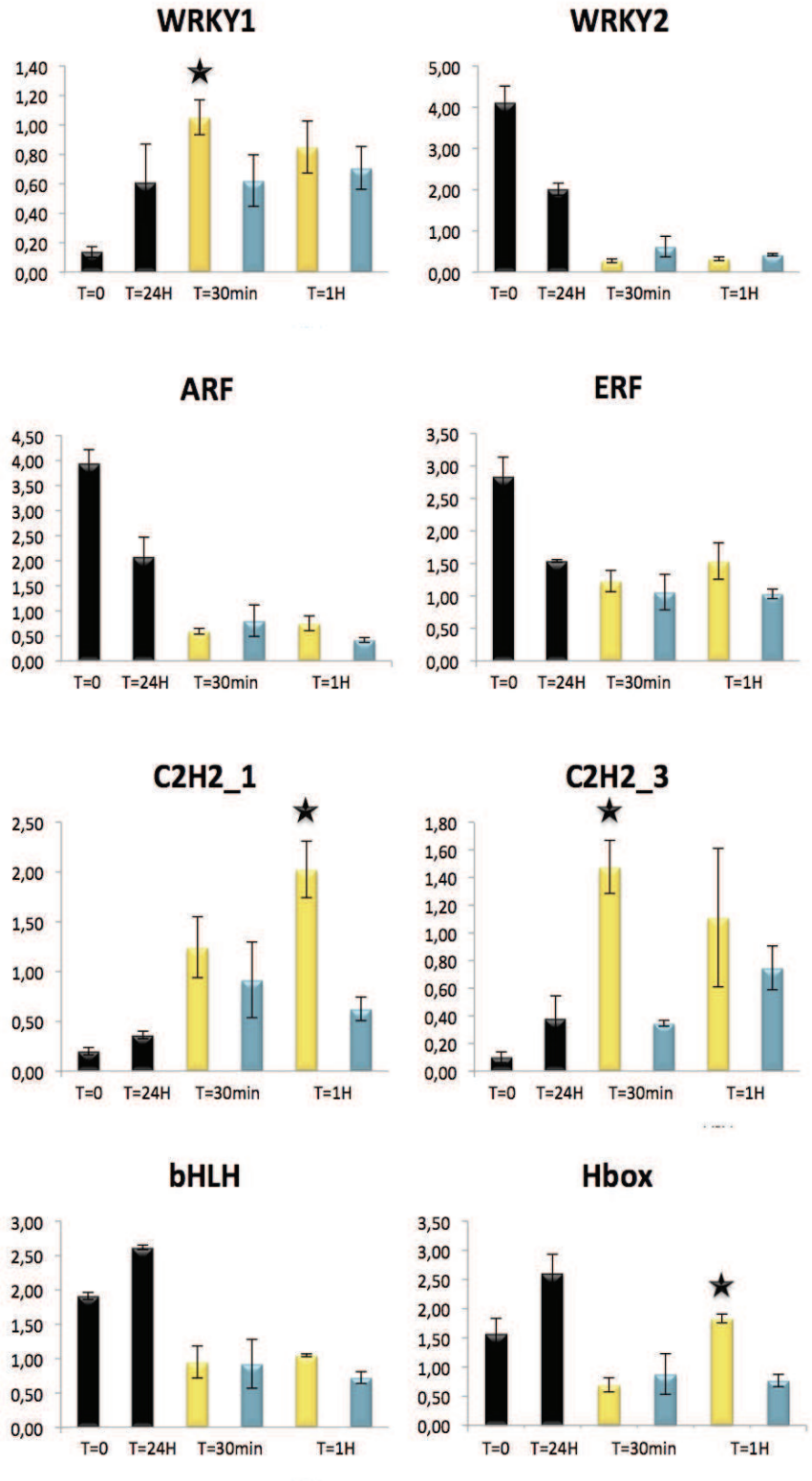
a. Impact of AsA variation on candidate gene expression

The first step of this study was to confirm or infirm *in planta* that a modification of AsA accumulation can influence the transcript level of the candidate genes. Towards this end, physiological experiments were carried out to induce variations in AsA content using artificial or natural methods.

i- Tissue complementation with ascorbate

The supply of AsA to plant organs was performed on fruits and leaves using several methods. The most effective one was using detached leaves maintained under light and in hydrating conditions as shown in *figure 44a*. To set this method we used WT Micro-Tom plants. Young leaves of WT plants were harvested and the petiole of the leaf was maintained in a solution of $MS_{1/4}$ (Murashig and Skoog) inside a growth chamber 24h before starting the analysis. Indeed, previous experiments on detached plants organs, e.g. tomato leaf, demonstrated the existence of a respiration burst corresponding to the cutting stress which lasts about 24h before resuming to physiological respiration (M. Dieuaide personal communication and personal test). Such conditions must be avoided as they can expectedly induce the expression of transcription factors related to stress. After 24h, the leaves were next incubated in AsA (10mM) or $MS1_{/4}$ (control) during 30 min, 1, 2 or 6 hours. AsA assays showed that after 2h and 6h incubations, the amount of AsA reached a level that saturated the foliar tissues, while 30 min and 1 hour incubations induced a linear and gradual increase in AsA content (*figure 45*). For that reason only times 30 min and 1 hour were considered in this analysis. This strategy being efficient to induce AsA supply, the expression of the 15 candidate genes has been examined using real time PCR (*figure 46*). Interestingly, genes displaying an expression level higher at T=0 compared to T=24H, e.g. the *WRKY2*, *ARF* and *ERF* do not appear to be affected by AsA supply. We can assume that these transcription factors are probably related to biological processes independent of the AsA supply. In contrast, *C2H2-1, C2H2-3* and *WRKY1* for which the expression in the leaves at T=0 is very

Patterns of gene expression after specific treatments to induce an artificial AsA variation in tomato leaves



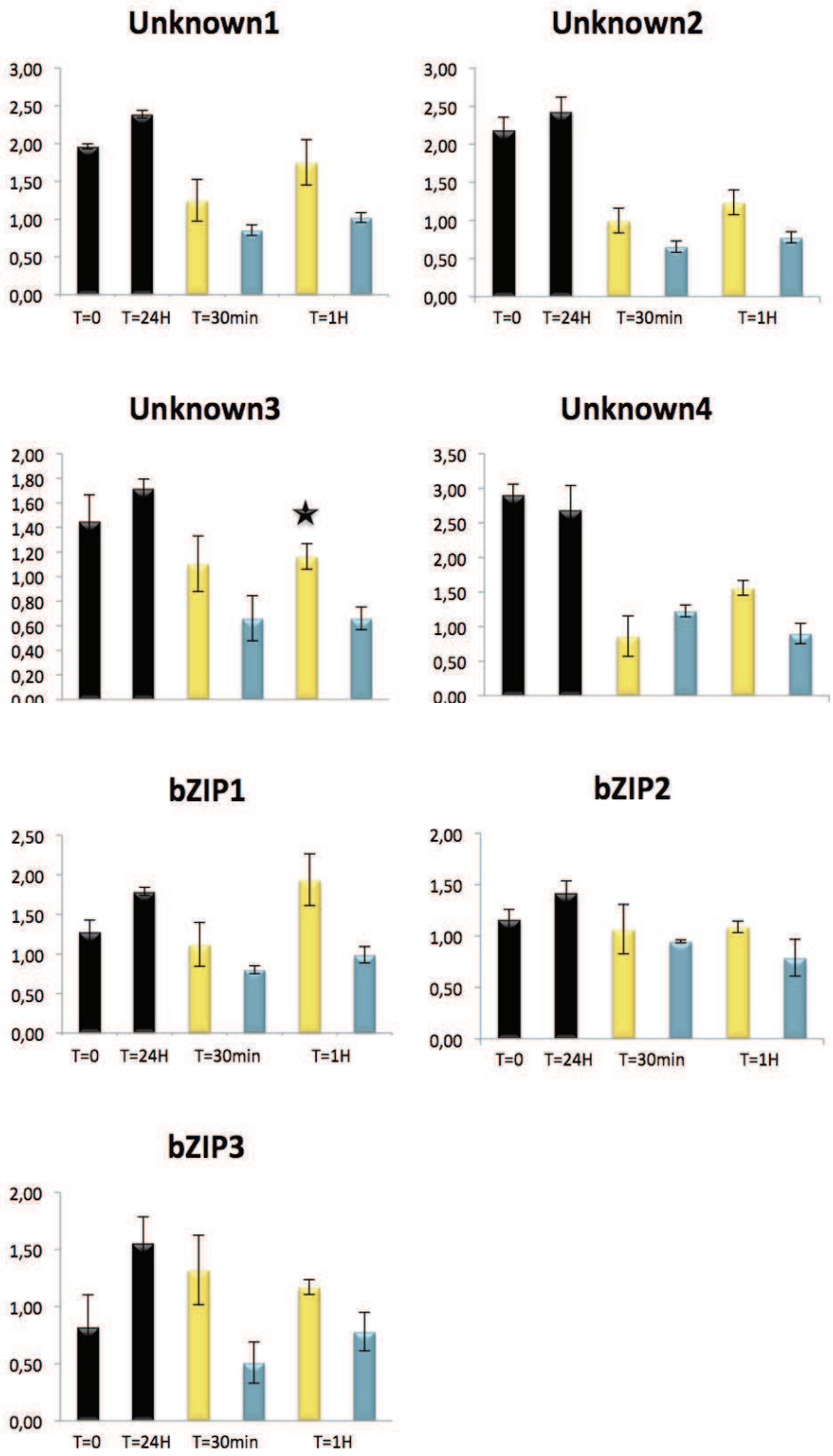


Figure 46: Relative expression of the fifteen candidate genes in the leaves incubated with or without AsA (10mM). Four points are shown in the graph. T=0 corresponds to the time where leaves were harvested in the greenhouse. Before starting the experiment, leaves remained in growth chamber during 24H to recover and to reduce any stress signal induced by the cutting and corresponds to T=24H. Next step, leaves are soaked in solution of AsA (yellow bar) or MS ¼ (blue bar, control). For each time, three leaves were analysed (±SE) and asterisks indicate significant differences between treatments (Student test, P<0,05). Data were obtained by real-time PCR normalized against Actin and eiF4 reference genes. The relative expressions are in arbitrary unit.

low appear to be more affected, as shown by the increase of transcript level when leaves are incubating in the presence of AsA. Among them, *WRKY1* and *C2H2-3* have a rapid response with a stimulation of the expression only at 30 minutes of incubation while *C2H2-1* displays a significant increase of expression after 1hour of incubation. For the remaining genes, the expression level between T=0 and T=24h are sensibly similar. *Unk1, Unk2, Unk4, bZIP2* and *bHLH* display unchanged expression between the control and AsA treatments, whereas *Unk3, bZIP1, bZIP3* and *Hbox* appear to be affected. However, the variation observed for *bZIP1* and *bZIP3* is not significant. *Unk3* and *Hbox* show a response to AsA supply after 1hour of incubation. Thus, the fifteen candidate genes display some difference in the response to AsA supply. They can be stimulated after 30min or 1hour of treatment suggesting a role of AsA in the regulation of their expression level. However, these genes are described to be involved in the responses to biotic and abiotic stresses, their expression changes can result from the cutting stress at the leaf level. In addition, these genes were selected from experiments carried out on fruit while the studies described above were done on leaf, in which AsA regulation is likely different. In order to confirm these first observations, experiment based on natural variation in AsA content in leaves and fruits were next performed.

ii. Ascorbate variation in planta

The objective of this part was to induce AsA variation *in planta* using (i) environmental factors, and (ii) genetic factor. In this two way, AsA- EMS mutants were used as negative control in wich AsA content is decreased by 60 to 80% compared to the WT, respectively. Thus, the screen using reverse genetic approach using TILLING (Targeted Induced Local Lesions IN Genome) strategy (*supplemental data 7*) allows to identy two allelic variants harbouring a knockout mutation in the *ggp2* namely *ggp2-1* and *ggp2-2*.

1. Effect of environmental factors:

- *In the Leaf:*

The influence of irradiance on AsA content has been known for a long time (Davey *et al.,* 2000; Smirnoff and Wheeler 2000; Dumas *et al.,* 2003; Gautier *et al.,* 2009; Massot *et al.,* 2012). Light is known to play crucial role in regulating the AsA

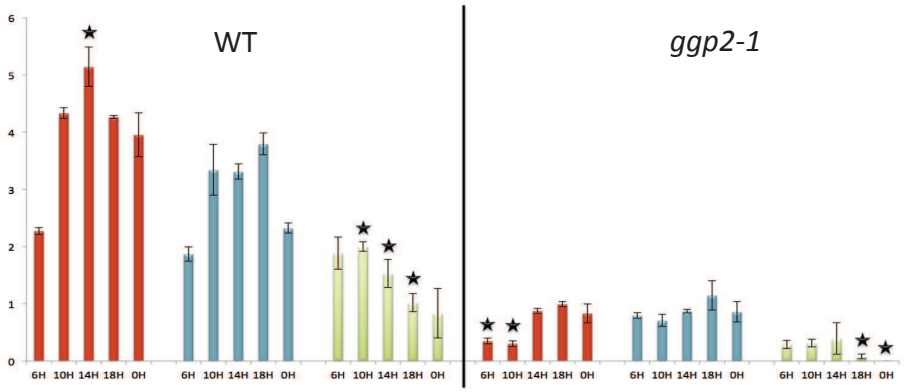
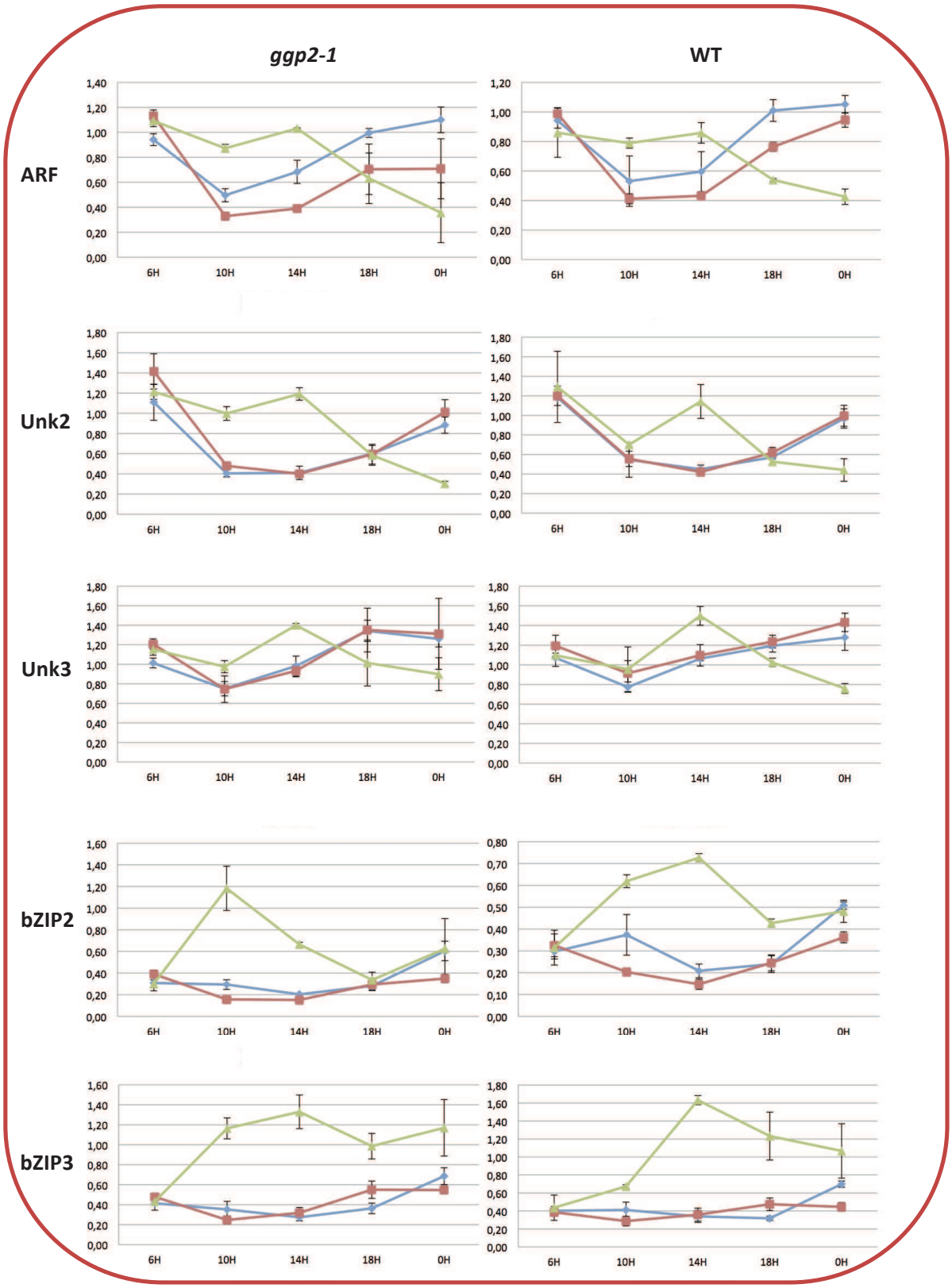


Figure 47: AsA content in tomato leaves from WT and *ggp* (VTC2) mutant after exposure to three light conditions: High light (red bar), Control (blue bar) and Low light (green bar). Samples were harvested all along the day at 6H, 10H, 14H, 18H and 24H. Each plant was used only once to prevent any stress interference. For each time, three leaves were analysed in technical triplicate (±SE) and asterisks indicate significant differences between treatments (ANOVA: Dunnett bilateral test, $P<0,05$). AsA content is expressed in nmoles.mg FW^{-1}.

biosynthesis and metabolism of higher plants (Morimura *et al.,* 1999; Gatzek *et al.,* 2002; Bartoli *et al.,* 2006; Fukunaga *et al.,* 2010; Li *et al.,* 2009; Li *et al.,* 2010b). The AsA content in leaves under high intensity of illumination is usually higher than in shaded leaves (Gautier *et al.,* 2009; Smirnoff N 2011). In order to induce AsA variations in leaves, Micro-Tom plants were exposed to three light intensities during 24 hours as shown in the *figure 44b*. The "high Light" (HL) condition corresponded to plants exposed to full sunlight outside the greenhouse during a summer day without cloud, and ambient temperatures maximum was around 29°C. The "low Light" (LL) condition corresponds to plants maintained under a black net that reduces light irradiance by 70%. The "control" (CTRL) condition corresponds to plants cultured in the greenhouse where the light intensity depends on the maintenance of the temperature of the compartment around 24-26°C (in summer). Indeed, to maintain this temperature occulting curtains are drawn above the culture to avoid the raise of the temperature. The measure of the light intensity in that condition revealed that the ambient light within the green house is still optimal in term of photosynthesis efficiency (D. Just personal communication). To set this experiment Micro-Tom WT plants and the EMS *ggp2-1* mutant were used at the early stage of plant development called "four leaves stage". This stage corresponds to plants at 30 to 35 days after sowing. Indeed, a previous experiment showed that AsA variation in leaves was less important in more mature plants, but it is also noteworthy that in advanced stages of development, the leaves can overlap leading to interferences with the light perception. As showed in *figure 47,* these three light conditions resulted in very distinct daytime AsA accumulation changes. In the WT Micro-Tom, the highest AsA content in "High Light" is almost two times more important than in the CTRL condition. In contrast, the AsA content in "Low Light" is lowered by two times compared to the "CTRL". In *ggp* mutant, the variations between "High Light" and "CTRL" and "Low light" are lower and do not evolve from the low initial AsA content. Thus, this experiment allowed comparing the transcript level of the 15 candidate genes as a function of two conditions that lead to AsA content variations (i) the light intensities difference and (ii) the distinct genetic background leading to the reduction of AsA content close to 80% in the *ggp mutant* compared to the WT (*figure 48*).

If we consider solely the effect of the light variations in the WT, we can distinguish 6 groups in term of response for the 15 genes.

Patterns of gene expression after specific treatments to induce an artificial AsA variation in tomato leaves



(**Group1**): *ARF, Unk2, Unk3, bZIP2* and *bZIP3* genes display similar expression patterns. These genes appear to be stimulated in the "Low Light" condition, with a peak at 14H. In contrast, the transcript in "High Light" and "CRTL" conditions display an inverted curve suggesting a repression of the transcription stronger at 10H and/or 14H. At 14H, the period when AsA content is the highest is also the time corresponding to the zenith in the sun route.

(**Group2**): *bZIP1* and *C2H2-3* gene expressions also show an induction in the "Low Light" condition with a peak at 18H but there is no repression effect in the two other light conditions.

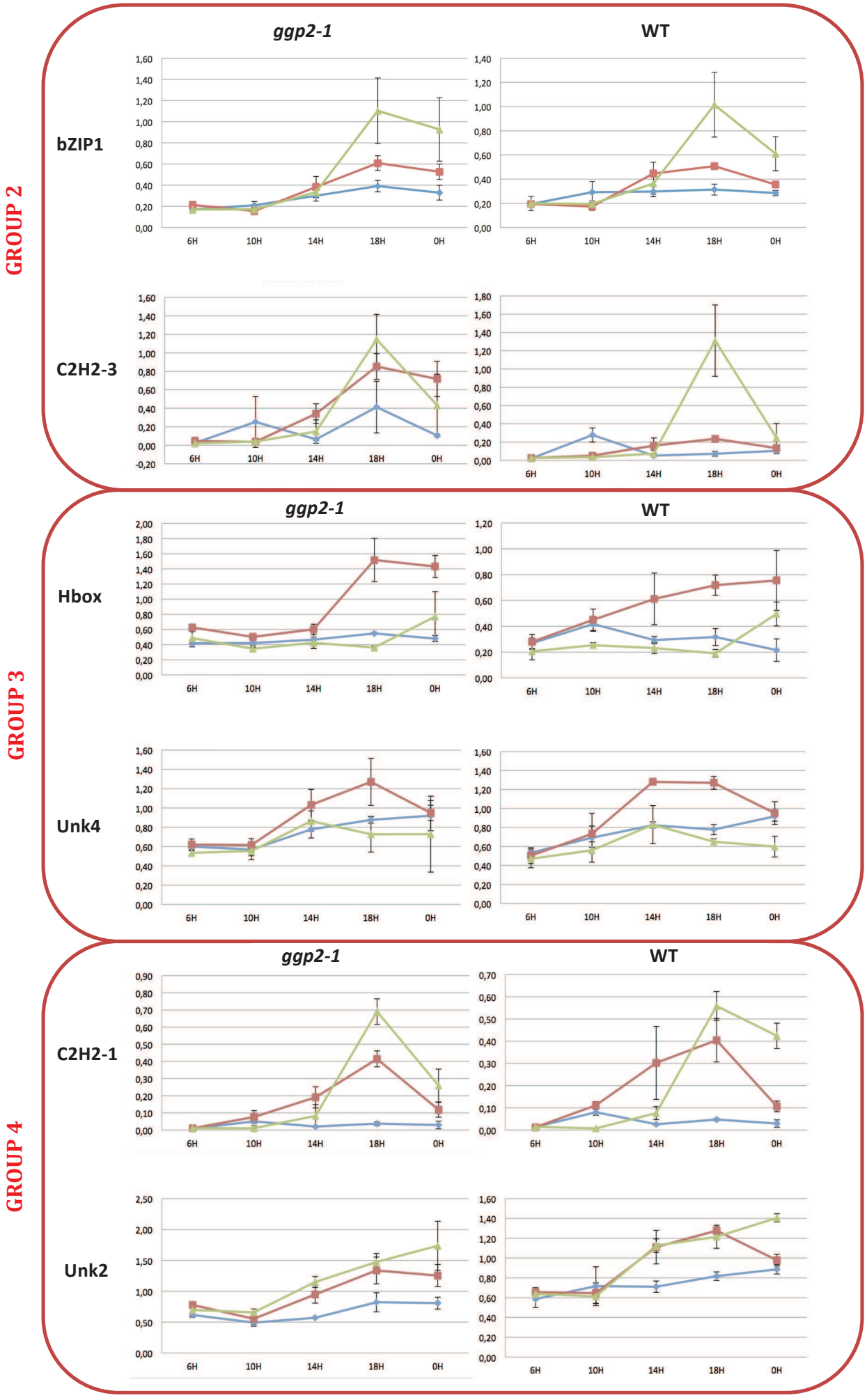
(**Group3**): In the same manner, *Hbox* and *Unk4* gene expression display an increase in "High Light" condition whereas in the two other conditions are relatively stable throughout the day.

(**Group4**): In contrast to the first three categories in which transcription factors display an induction only in one condition, in group 4 the expression of *C2H2-1* and *Unk1* genes are altered in two conditions: "Low Light" and "High Light" while in the "CTRL" the expression remain constant.

(**Group5**): *ERF, bHLH* and *WRKY2* gene expressions display variation in the three conditions in the time course of the day.

At last, (**Group6**) only represented by *WRKY* 1 gene that can be defined as the most difficult to apprehend since three peaks of expression are observed at distinct time in each condition, at 10H in CTRL, 18H in Low Light and 0H in High Light. Interestingly when the experiment was started at 6H, the expression level is similar in the three conditions for each candidate gene and thereafter the expression changes according to the conditions observed. At this step of the analysis it is difficult to establish if the modification of expression originates from AsA variation, light intensity, process related-circadian rhythm or abiotic stress.

Analysis of the "CRTL" condition specially makes possible to examine the variation of transcript levels as a function of AsA accumulation throughout the day period, in a condition where light-related stresses are limited. Four candidates show interesting expression patterns. Among them, *bHLH* and *WRKY2* genes share the same pattern with the AsA accumulation, with a maximum of transcripts levels at 14H and 18H respectively. In contrast, *Unk2* and *ARF* genes show an expression decrease with a minimum at 14H, suggesting an opposite behaviour with that of AsA accumulation.


GROUP 2
GROUP 3
GROUP 4
ggp2-1
WT
bZIP1
C2H2-3
Hbox
Unk4
C2H2-1
Unk2
6H
10H
14H
18H
0H

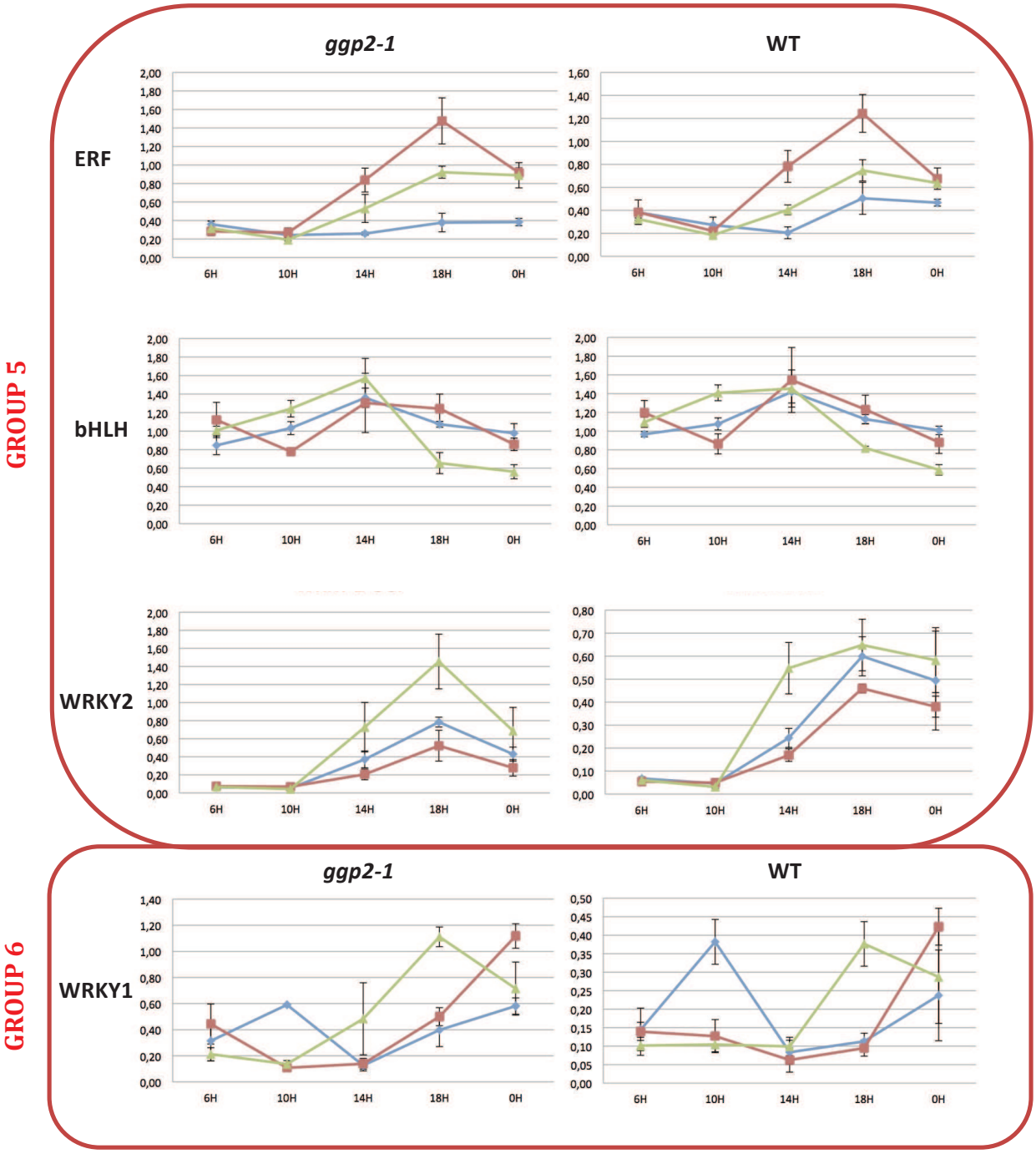


Figure 48: Relative expression of the fifteen candidate genes in the leaves exposed to three light conditions (HL, LL and CTRL). Leaves of MicroTom at "four leaves stage" were harvested all along the day at 6H, 10H, 14H, 18H and 24H. The three light conditions are shown in different colours in the graph: high light (red line), low light (green line) and control (blue line). The candidates are classified in groups with similar expression profiles. For each point, three leaves were analysed (±SD). Data were obtained by real-time PCR normalized against Actin and eiF4 reference genes. The relative expressions are in arbitrary unit.

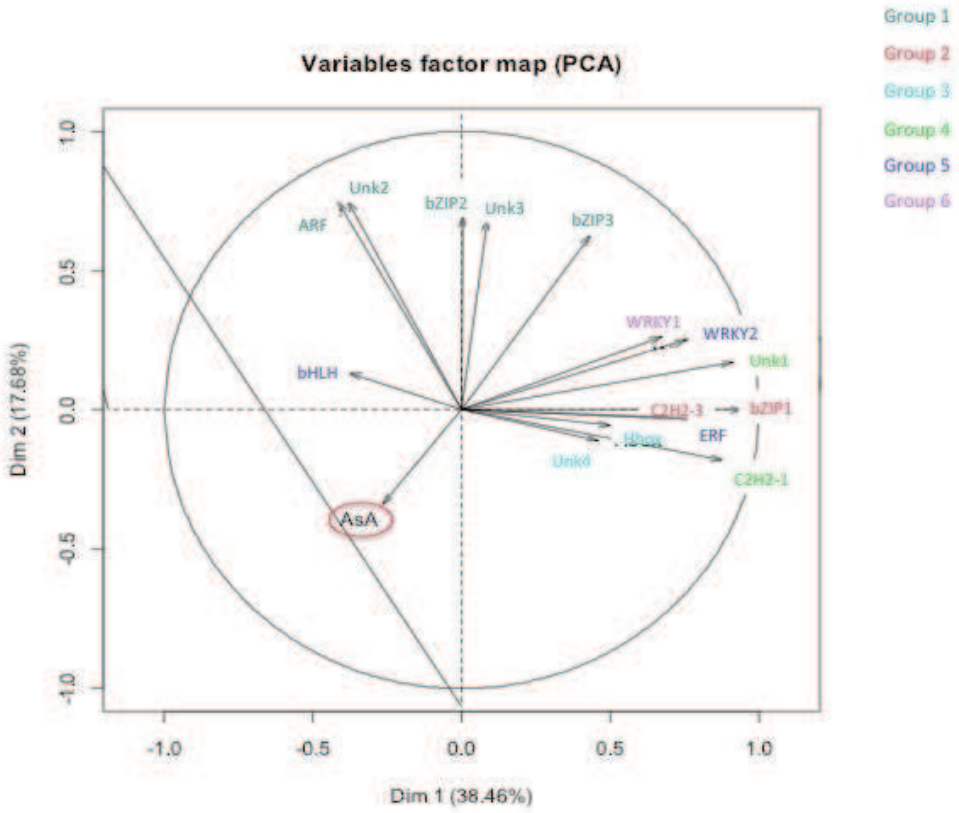


Figure 49: Principal Component Analysis of the HL/LL/CTRL experimental data for the WT and *ggp*. It corresponds to a projection of the initial variables on a 2D plane formed by the first two factors that can explain the variation

The expression patterns in the three conditions are almost similar for most of the candidate genes in the WT and *ggp* mutant leaves. The genetic background affects only *Hbox, bZIP2, C2H2_3, WRKY1* and *WRKY2*. *Hbox, bZIP2, WRKY1* and *WRKY2* display a higher expression in the *ggp* mutant than in the WT. For the *C2H2_3* this observation is also true but only in HL condition. Thus, the expression level is similar in WT and *ggp* at 6H, but then the expression increases for both but is two times higher in *ggp* than in WT, suggesting an effect of the genetic background, and also a possible link with the AsA regulation.

In conclusion, according to our observations performed (1) in three light conditions, (2) in two genetic backgrounds and (3) for several genes, it is possible to draw a putative scheme regarding the regulation of AsA metabolism in tomato leaves. (i) *C2H2-1* and *Unk1* genes appear to be directly expressed in response to stress conditions (HL and LL), but not really in relation to the AsA content in leaves. (ii) *ARF, Unk2, Unk3, bZIP2* and *bZIP3* genes are very interesting because they are only induced in "Low Light" condition, when AsA content is low. Among them, *bZIP3* display expression change in the *ggp* mutant, confirming a link with the AsA metabolism. *ARF* and *Unk2* genes have expression patterns similar to *GME* indicating a possible co-expression and a direct action on AsA biosynthesis pathway. Interestingly, *Unk2* belongs to the superfamily of the polyadenylate-binding proteins (RBP). The RBP proteins are able to interact with the poly-A tail into the nucleus to regulate gene expression during biotic or abiotic stresses. In the same manner, the *Hbox* and *Unk4* genes are more highly induced in the "High Light" condition, than in "Low Light". Between these two genes, the *Hbox* gene is more expressed in the *ggp* mutant. (iii) At last, the *ERF, bHLH* and *WRKY2* genes are induced in the three light conditions but in a distinct way. Hence, in HL for *ERF*, LL for *WRKY2* and in the three conditions for *bHLH.* Among them, *WRKY2* is more expressed in *ggp* background indicating a possible link with the AsA metabolism. The principal component analysis (PCA) shows that the candidate genes that belong to the same group are close in the correlation circle suggesting a similar contribution in the variation observed (*figure 49*).

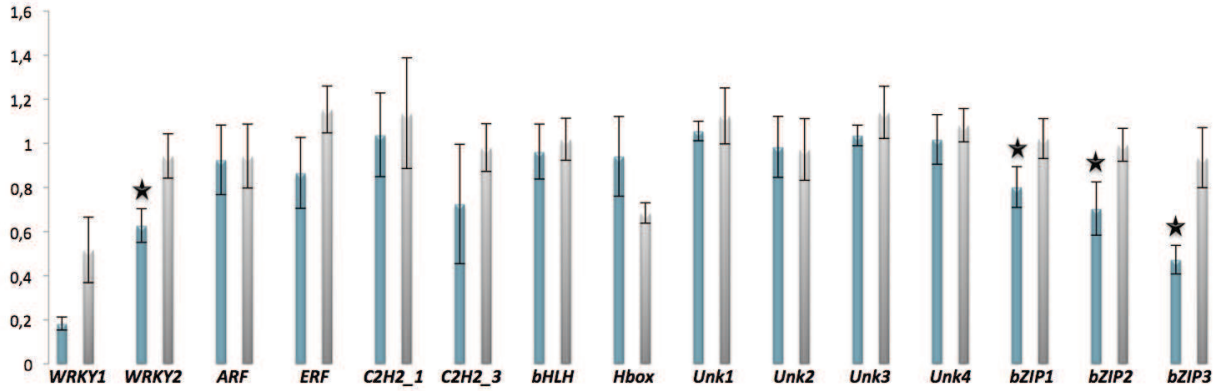


Figure 50: Relative expression of the fifteen candidates genes in tomato fruits shaded (grey bar) or not shaded (blue bar). Fruit of cherry tomato cultivar (*Solanum lycopersicum* cv "West Virginia 106") were harvested at the orange stage after 6 days of treatment. Three biological replicates were analysed per treatment (±SD) and asterisks indicate significant differences between treatments (Student test, P<0,05). Data were obtained by real-time PCR normalized against Actin and eiF4 reference genes. The relative expressions are in arbitrary unit.

	leaf				*fruit*	
	artificial		***natural***			
	AsA	***HL***	***CTRL***	***LL***	***Fruits shaded***	***GGP background***
WRKY1		+	+	+		+
WRKY2		+	+	+	+	+
bZIP1				+	+	
bZIP2		-	-	+	+	+
bZIP3		-	-	+	+	
ARF		-	-	+		
C2H2_1	+	+		+		
C2H2_3	+			+		+
ERF		+	+	+		
Hbox	+	+				+
bHLH		+	+	+		
Unk1		+		+		
Unk2		-	-	+		
Unk3	+			+		
Unk4		+				

Table 9: Summary of the observations obtained during experiments performed on the leaves and fruits. A sign "+" corresponds to up-regulation whereas sign "-" corresponds to a repression. Green colour indicates genes behaving similarly in conditions or experiments going in the same way. Abbreviation: Ascorbate (AsA), HL (high light), LL (low light), CTRL (control)

- *In the Fruit*

The fifteen transcription factors studied were originally selected from experiments carried out in the fruit at the orange stage. *In silico* analysis using the eFP Browser (*http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi*) further showed that most were likely differentially expressed in the leaves and in the fruits suggesting that their role in the regulation of ascorbate-induced responses could be different in these two organs.

In a previous experiment performed at INRA Avignon, cherry tomato (*Solanum lycopersicum* cv "West Virginia 106") plants were grown in a glasshouse under shaded and irradiated conditions (Gautier *et al.,* 2009). The aim was to analyse the regulation of AsA content in response to light irradiance, in fruits and leaves. Reducing light irradiance at the fruit level decreased AsA content in fruit by around 30%. We took the opportunity to investigate the expression of our candidate genes on those shaded and non-shaded fruits (*figure 50*). The results were as followed. The *ARF, bHLH, Unk1, Unk2, Unk3* and *Unk4* are unaffected by light irradiance changes. In contrast, the *bZIP1, bZIP2, bZIP3, ERF, WRKY1* and *WRKY2* display an increase of expression in the shaded fruits, whereas the opposite was seen for the expression of the *Hbox*. The data obtained for the *C2H2-1* and *C2H2-3* are very difficult to consider because they show a large variability between the biological triplicates.

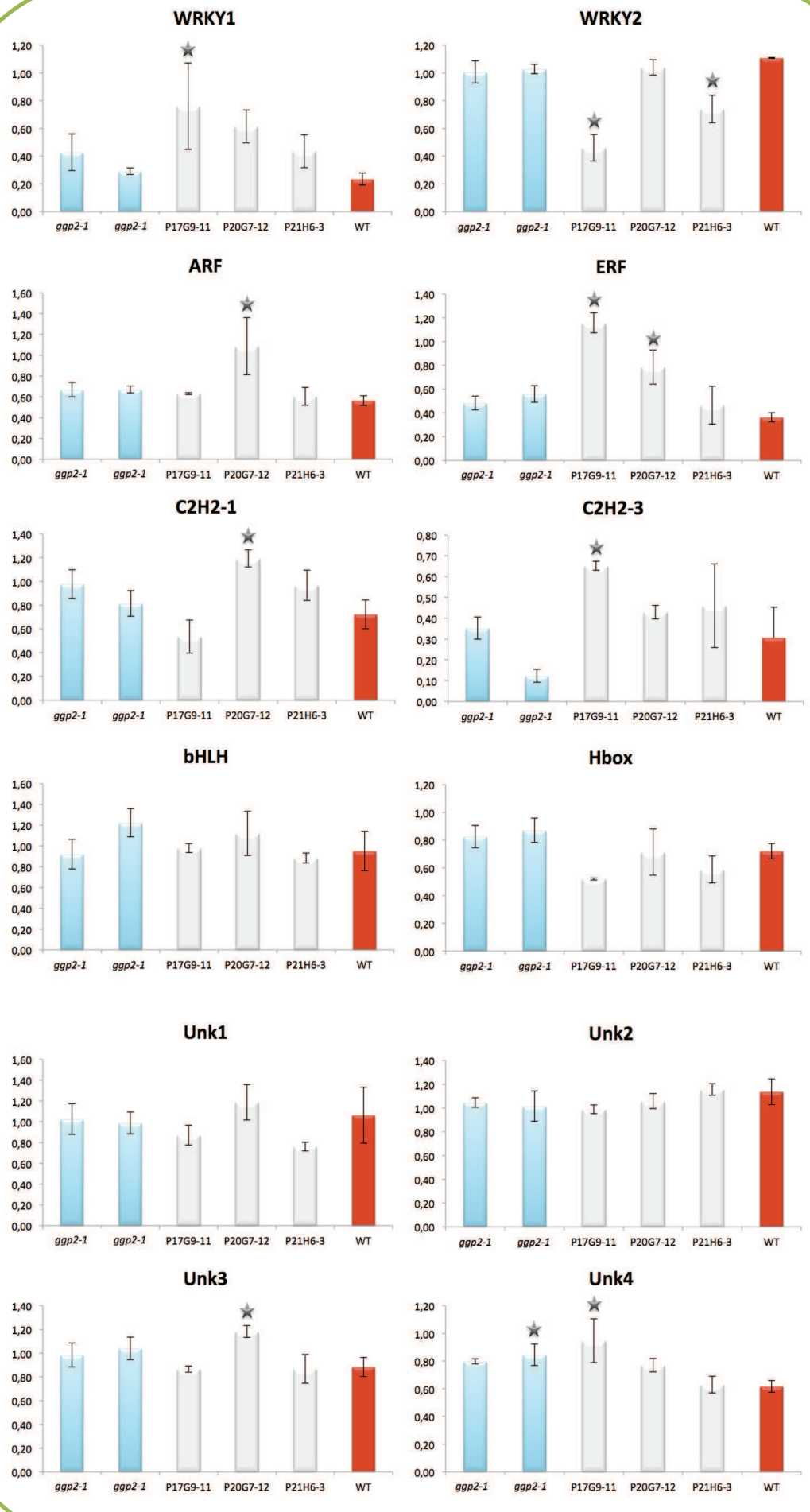
To conclude this part of the study, we can say that the results obtained above are still preliminary and must be analysed in more detail and repeated. One important factor to take into account is that ascorbate itself and the expression of ascorbate-related genes are very sensitive to stress and light conditions, which makes sense given the overwhelming role of ascorbate in the adjustment of the plant to biotic or abiotic stress conditions. *Table 9* summarizes the main observations obtained in our study. Interestingly, the *bZIP2*, *bZIP3* and *WRKY2* are both overexpressed when AsA content is low i.e. in LL condition, in shaded fruits and in the *ggp* background for *bZIP2* only. Such result confirms the negative correlation revealed previously in the screening of the VTC-Fruit database. At this stage it is still very challenging to establish whether some of the AsA-related genes identified in this study can also play a role in the accumulation of ascorbate in the fruit.

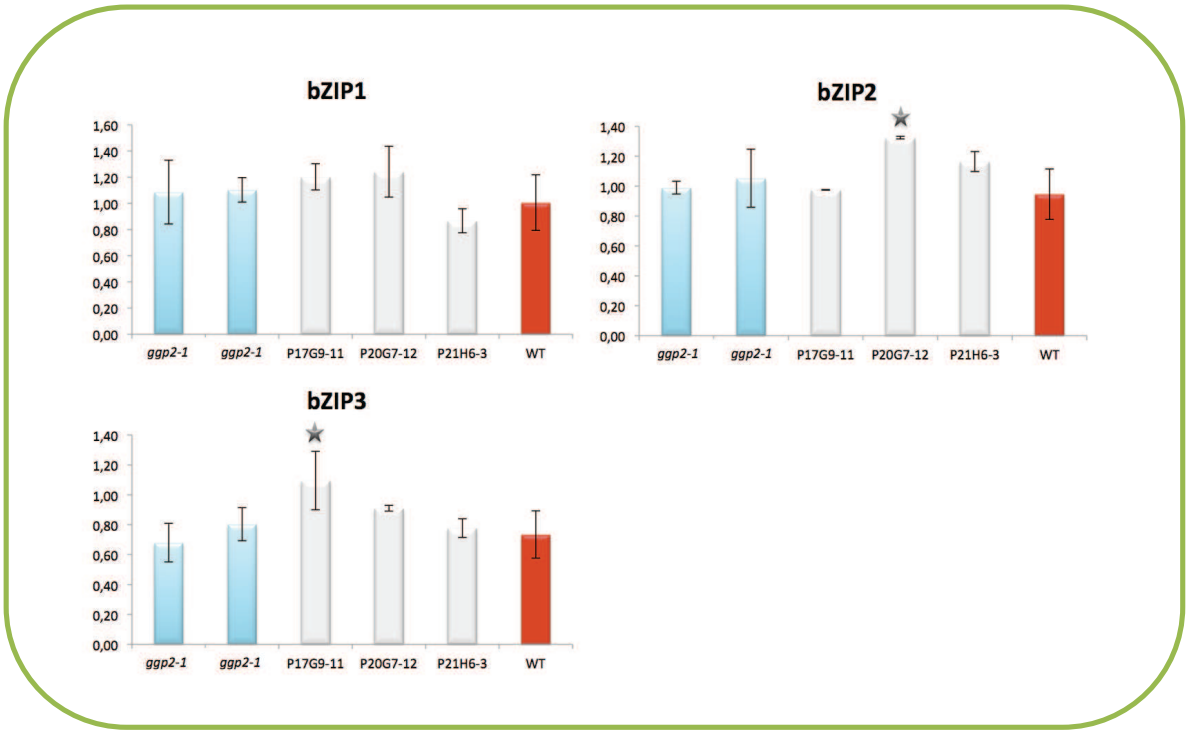
2. Genetic control:

In order to find mutants displaying AsA variations in fruits, the Micro-Tom EMS mutant collection was screened using forward genetic approach (*cf Part I: Identification of Ascorbic Acid regulators using forward genetic approach*) (Just *et al.,* 2013). Five AsA enriched mutants named *P17C5-3, p17g9-11, 19a6-3, p20g7-12* and *p21h6-3* displaying an increase in AsA content were identified. In parallel, this collection was screened using reverse genetic approach using TILLING (Targeted Induced Local Lesions IN Genome) strategy (*supplemental data 7*). The principle is to find allelic variants for a selected gene. In our case, two EMS mutants harbouring a knockout mutation in the *ggp2* gene were identified, namely *ggp2-1* and *ggp2-2*. These two AsA- mutants produce fruits in which AsA content is decreased by 60 to 80% compared to the WT, respectively. These EMS AsA+ and AsA- mutants constitute the perfect tool to study the expression of our candidate genes in genetic background displaying contrasted AsA accumulation. The expression analysis was carried out in fruits at breaker and red ripe stages (*figure 51*).

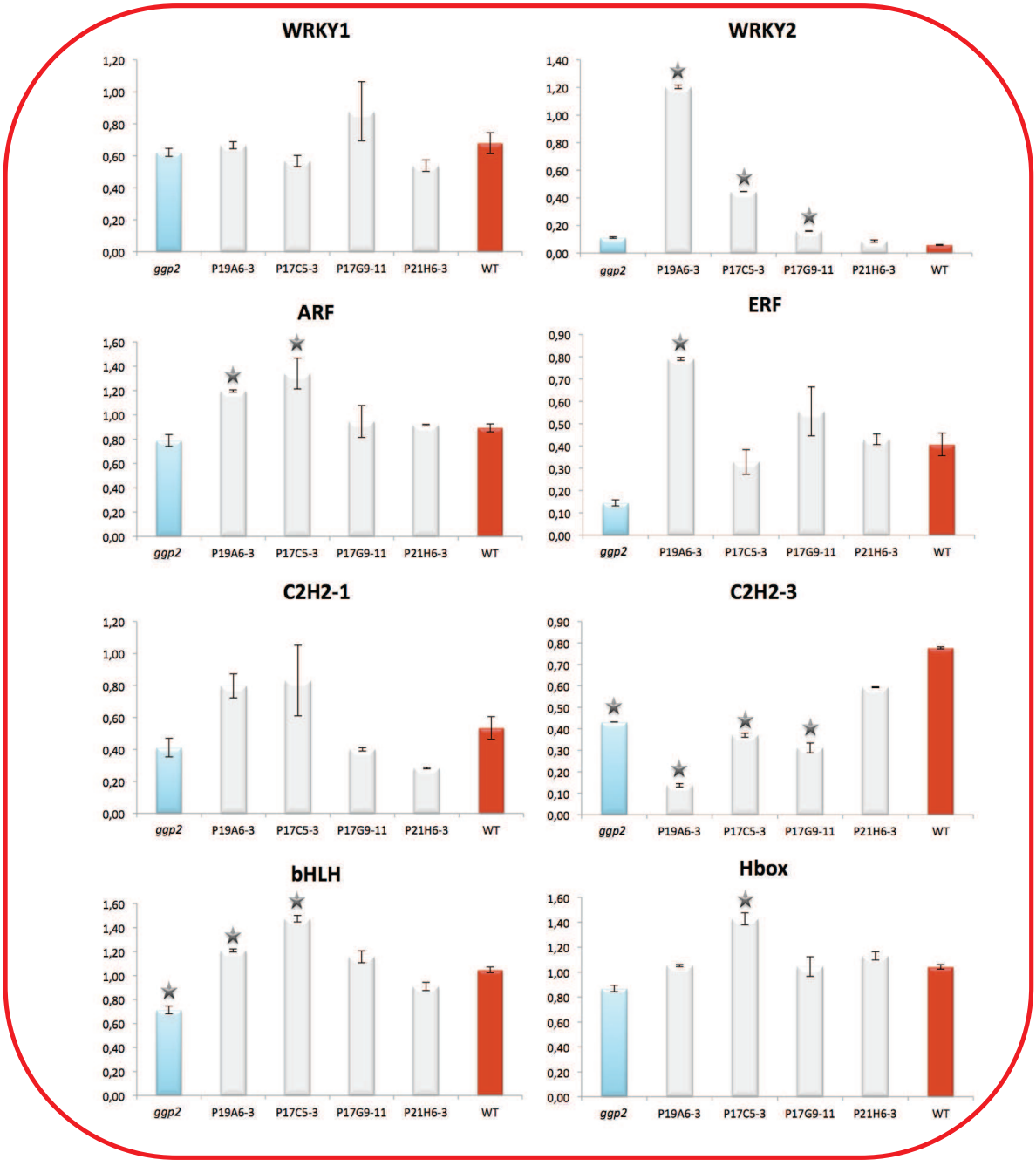
The data obtained in the AsA+ mutants were too tricky to get a clear view of the putative role of the candidate genes. Indeed, the discrepancy in expression variation in these AsA+ mutants is probably due to the identity of the mutation at the origin of the AsA+ phenotype. In order to try to define a tendency, only when half of the AsA+ or the two AsA- mutants display similar expression patterns, a general conclusion was given. According to that we can propose the following features. If we compare the breaker and red ripe stages, we can see that most candidates showed variation at the red ripe stage. The study of the AsA+ mutants shows that accumulation of AsA is higher at early stages of fruit development and also at red ripe stage (*figure 52*). The very young fruits and the immature green fruits displayed higher AsA pool size, presumably to help support higher rates of cellular metabolism during stage of fruit cell division and expansion (De Tullio *et al.,* 1999; Smirnoff *et al.,* 2000; Gilbert *et al.,* 2009). Then, at breaker stage the AsA content declines and rises again during the final ripening stages. So, the difference observed between breaker and red ripe stages could be due to the variation in AsA accumulation. It is interesting to see that *WRKY2, ARF, ERF* and *bZIP1* display variation of expression only in AsA+ mutants. In contrast, *Unk2* is only stimulated in the AsA- at the red ripe stage. *C2H2-3, Unk4* and *bHLH* behave reversely in AsA- and AsA+ compare to the WT. Thus, *bHLH* and *Unk4* are overexpressed in the AsA+ mutants while their expression is lesser in the AsA- mutant. At the opposite, *C2H2-3* appears to be over-

Breaker stage





Red ripe stage



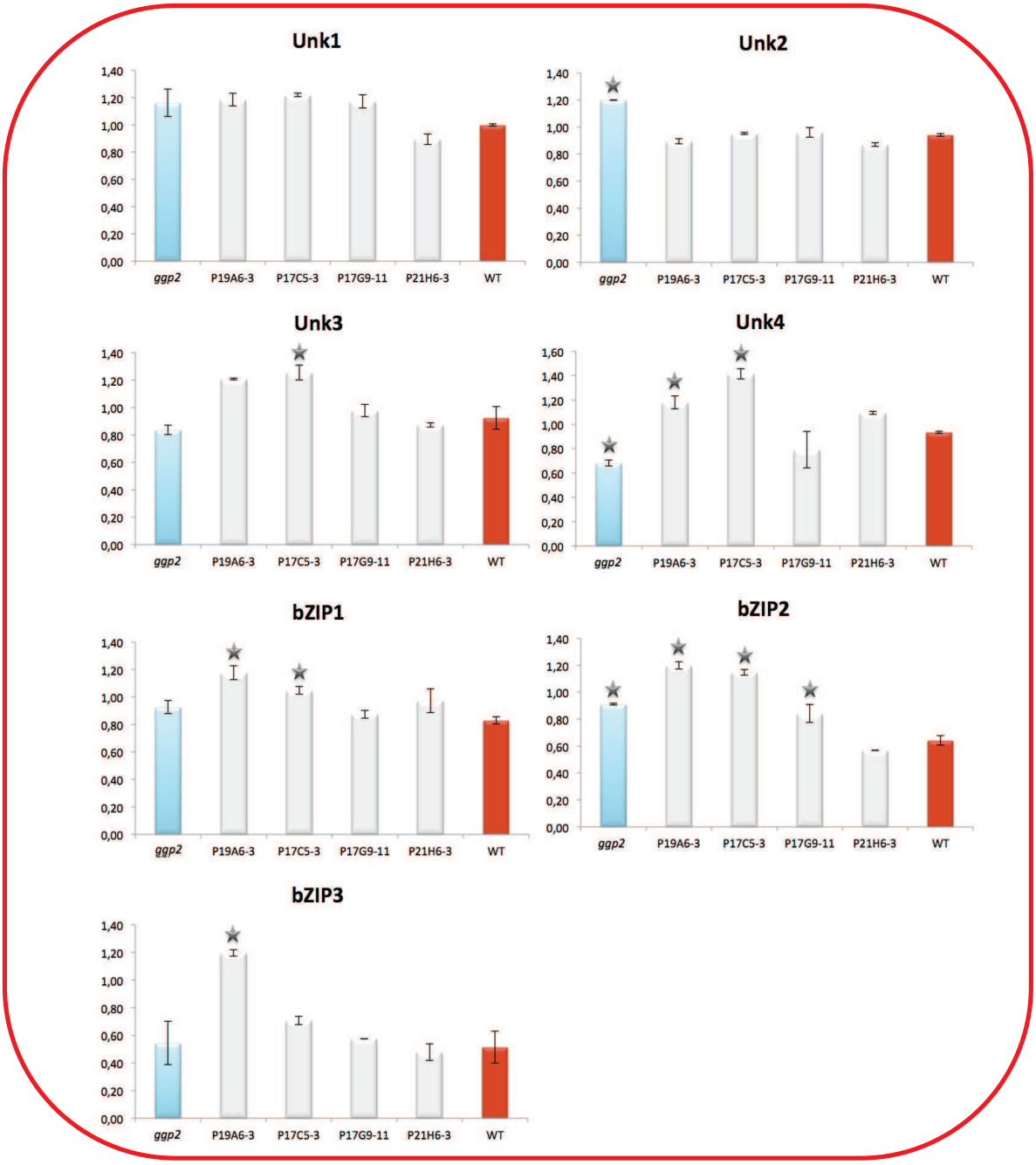


Figure 51: Relative expression of the fifteen candidates genes in tomato fruits at breaker (green frame) and red ripe (red frame) stages from 3 to 4 AsA+ mutants and 1 to 2 AsA- mutants. Fruits are from AsA- mutants (blue bar), AsA+ mutants (grey bar) and WT (red bar). Technical triplicate were analysed per treatment (±SD) and asterisks indicate significant differences between treatments (ANOVA: Dunett bilateral test, P<0,05). Data were obtained by real-time PCR normalized against Actin and eiF4 reference genes. The relative expressions are in arbitrary unit.

expressed in the AsA- and repressed in the AsA+ compared to the WT. For the remaining genes, variations observed are not significant and do not allow to conclude. Among them, *bZIP2* are over-expressed both in AsA- and AsA+ mutant compared to the WT, net genetic effect where the disturbance of homeostasis of ascorbate seems to result in a response to the level of that gene. Taken as a whole, these observations provide information on a possible link between *WRKY2, ARF, ERF, bZIP1, C2H2-3, Unk4, bHLH* and AsA metabolism.

As discussed above, these results are still highly preliminary. A part of this work has been achieved during the last months of my PhD and work is still in progress. Nevertheless, the various strategies used to modulate the ascorbate content of the tissues, in either leaf or fruit and/or in various light conditions, enabled us to highlight a series of genes encoding fruit TFs that are clearly responsive to ascorbate. Among them are genes that could play key roles in the response of the plant to biotic or abiotic stresses (e.g. the WRKY TFs) and possibly in the regulation of the ascorbate biosynthesis and recycling pathway. Investigation of the function of these genes will be done through reverse genetic methods using transient transformation, TILLING or stable transformation with CRISP-R technology. This will allow us testing *in planta* whether their down-regulation could affect ascorbate and to assess the response of the plant challenged with a variety of biotic and abiotic stress conditions.

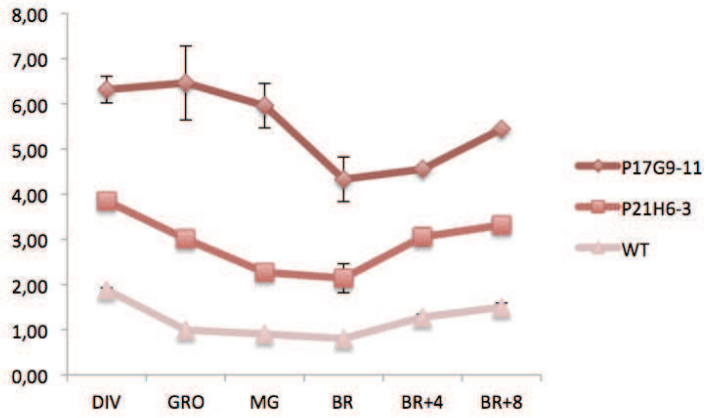


Figure 52: AsA accumulation pattern during tomato fruit development. *p17g9-11* and *P17C5-3* are AsA enriched mutants. Fruits are analysed at different stage of development: cell division (DIV), cell growth (GRO), mature green (MG), breaker (BR), breaker +4 days or orange (BR+4), breaker +8 days or red ripe (BR+8)

III. Discussion

It is well known that AsA content can change according to environmental conditions like light (Li *et al.,* 2009), plant age (Bartoli *et al.,* 2000), and tissue (Valpuesta and Botella 2004). However, the mechanisms controlling AsA pool size are not really described. In this study, we used a top-down systems biology approach to identify transcription factors that could act as putative regulators of the fruit response to ascorbate and/or ascorbate accumulation. Results from a study of four transgenic lines altered in AsA biosynthesis and recycling at transcriptomic, proteomic and metabolic levels were screened to identify 15 candidate genes. Correlation network analysis indicated a strong correlation between these 15 genes except for the ERF and WRKY1 genes that mainly displayed "edge connexions" only with AsA. *In silico* analysis with "String DB database" (*http://string-db.org*) confirmed that the homologous genes in *Arabidopsis thaliana* are able to interact together. For example, *At*WRKY1 homolog can interact with other WRKY, bZIP and ERF transcription factors, and *At*WRKY2 homolog can also interact with a Zing Finger protein. It is conceivable that a complex network can control the response to AsA through a cascade of reactions. However, the correlation analysis did not allow to determine the relationship between AsA and the candidate genes. From the way they were selected, we know that AsA content influences candidate genes (ARF) but we do not know whether among them some TFs can also contribute to the regulation of the pool size of AsA.

During my PhD work, the first question has been addressed in tomato leaves and fruits displaying variations in AsA content. The experiments carried out to induce natural AsA variations in leaves and fruits led to propose some hypotheses regarding the function of the 6 groups of genes, but they will need to be confirmed.

In Group1, *ARF*, *Unk2 and bZIP2* display an increase of expression only in the LL condition. Interestingly, among the fifteen candidate genes only *ARF* and *Unk2* displayed a pattern in CTRL and HL conditions opposite to the AsA accumulation pattern suggesting a possible role of repression. Among them, *bZIP2* is over-expressed in the "AsA-lowered" *ggp* mutant. In a genetic background where AsA content is low, during high light exposure photobleaching were observed (Baldet *et al.,* 2013). It is possible that these candidate genes are involved in the repression of AsA accumulation and maybe, in the case of the *bZIP2*, it could be involved in the response to abiotic stress.

The *bZIP3* gene showed an increase of expression both in LL condition and shaded fruits, suggesting a possible origin from a low AsA content.

In Group2, *bZIP1* and *C2H2-3* display a stable expression in a CTRL condition, which does not correlate with AsA accumulation. The *bZIP1* is stimulated in LL condition and shaded fruits suggesting a role in the repression of AsA metabolism. The *C2H2-3* gene displays an expression stable in CTRL and HL conditions throughout the day. The peak of expression in LL is observed only at 18H. However, in the *ggp* mutant background the *C2H2-3* level in HL condition is higher at 10H. This gene could be involved in the response to abiotic stress but is not really related with the AsA metabolism. Thus, the high intensity of irradiation in HL induces a stress, whose effect is more important in *ggp* mutant where AsA pool is low and in which the protection against oxidative stress is vital. Nevertheless, we cannot exclude a link because the supply of AsA in detached leaves induced an increase of the *C2H2-3* expression.

In Group3, the expressions of *Hbox* and *Unk4* gene are stable in LL and CTRL and display a significant increase only in HL condition. *Unk4* gene does not show any response in the other experiments. In contrast, *Hbox* gene displayed an overexpression also in leaves incubated in AsA solution. However, *Hbox* gene is also overexpressed in the *ggp* mutant in the HL condition. Finally, we can hypothesize that *Unk4* and *Hbox* genes can be stimulated when AsA content increases, probably in the case of *Hbox* in response to light exposure.

In Group4, *C2H2-1* and *Unk1* genes are stable in CTRL conversely to HL and LL regarding the AsA accumulation, which is high in HL and low in LL conditions. Furthermore, *C2H2-1* gene is stimulated in detached leaves incubated in the presence of AsA. These observations suggest that these two candidate genes act in abiotic stress response but are not related with AsA metabolism.

In Group5, *ERF, bHLH* and *WRKY2* genes display expression variations following a circadian rhythm. Among them, *WRKY2* gene is overexpressed in the *ggp* mutant in the LL condition and in shaded fruits. Nevertheless, the variations observed for this candidate gene in the three light conditions suggest a link with AsA metabolism. Indeed, throughout the day the expression of the *WRKY2* gene is more important in LL condition, less important in CTRL and the lowest in HL condition. These observations show that *WRKY2* gene is more affected in LL but the effect varies during the day. In the case of *bHLH* gene, no variation was observed in shaded fruits and leaves incubated with

AsA. In HL/LL/CTRL, no difference appears, just throughout the day. Thus, these data suggest that the *bHLH* gene is not really linked with AsA metabolism. In contrast, it is difficult to conclude for the *ERF* gene.

At last, in Group6, the *WRKY1* gene displays a peak of expression in the three light conditions, at 10H for the CTRL, at 18H for LL and at 24H for HL conditions. In the *ggp* mutant this peak are two times higher than in the WT. We can suppose that the *WRKY1* gene can act as a signal or in response to a signal at a specific time of the day and maybe linked with AsA metabolism.

Finally, we can assume that *bZIP1, bZIP2, bZIP3, ARF, Hbox, Unk2, Unk4* and *WRKY2* genes can be involved in AsA regulation and/or be influenced by AsA content. In contrast, this is not the case for the *C2H2-1* and *C2H2-3* genes. At this stage of my work, these observations are still preliminary and really need to be confirmed. Variations in AsA content using artificial and natural methods in leaves and fruits are linked with abiotic stress responses, which are difficult to get rid of for this analysis. Indeed, the literature strongly suggest that AsA and those candidate genes get involved in response or signalling to biotic or abiotic stresses (Smirnoff N 1996; De Tullio *et al.,* 1999; Gatzek *et al.,* 2002; Anthonius *et al.,* 2003; Pastori *et al.,* 2003; Conklin and Barth 2004; Halliwell 2006; Badejo *et al.,* 200). The light irradiance and the cutting effect are probable factors at the origin of abiotic stress. However, even though AsA as a major antioxidant is used to protect plant against oxidative stress and ROS, many other antioxidant compounds, among them anthocyanins, glutathione, polyphenols may act as alternative antioxidants. Analysis of the candidate genes using mutants altered in AsA accumulation can be a useful way to eliminate stress induction during the experiments.

Expression of the candidate genes in AsA+ and AsA- mutants show that 8 of them, *bZIP1, bZIP2, ARF, ERF, bHLH, WRKY2, Unk2, Unk4* display high variation suggesting a possible link with AsA metabolism. For the last remaining, *WRKY1, C2H2-1, Hbox* and *Unk3* genes the modification of their expression is only observed in one of the AsA+ mutant. This can be explained by the identity of the causal mutation responsible for the AsA+ phenotype.

Even if not all the experiments described above to investigate the function of the 15 candidate genes could not give a clear conclusion regarding their putative regulatory role in AsA response, several scientific publications bring some clues and indications to

understand the mechanisms involving these candidate genes. In the case of Unk1, which is predicted to correspond to a subunit of the eiF3 superfamily, Yahalom *et al.,* (2008) demonstrated that the COP9 signalosome is involved in the fine regulation of eiF3e expression in specific light conditions, an element necessary for the normal development of Arabidopsis plants. When present in excess, eiF3e can act as a negative regulator in the translation. We can suggest that eiF3e is essentially related to the role of light in plant development but not in AsA response, this explains why no variation of Unk1 expression in AsA- and AsA+ mutant lines is observed.

Rizhsky *et al.,* (2004) demonstrated that an homologue of our *C2H2-3 in Brassica carinata* is required for the expression of the cytosolic ascorbate peroxidase *Apx1* in stress response. Furthermore, they showed that *C2H2-3* is additionally essential for controlling the expression of another *C2H2* and *WRKY25*, two TF putatively involved in the response of plants to oxidative stress. However, constitutional expression of C2H2 and WRKY transcription factors in tomato transgenic line did not enhance the expression of *Apx1* in the absence of stress suggesting additional factor(s). Bc*C2H2-3* is expressed in response to elevated expression of different transcripts related to ROS-metabolism and hormone signalling. Interestingly, in a transgenic line over-expressing C2H2-3, a replication protein homologue to our Unk4 protein displays a higher transcription.

WRKY transcription factors are implicated in the regulation of plant growth and development (Uleker and Somssich 2004; Mao *et al.,* 20011) and also in the response to various abiotic and biotic stresses (Pandey and Somssich 2009; Rushton *et al.,* 2010). Liu *et al.,* 2013, showed that in the plant *Dendranthema grandifulium* the expression of *DgWRKY3* an homolog of our candidate *WRKY2*, is increased under salinity or dehydration stress conditions but not by abscisic acid (ABA). The *DgWRKY3*-overexpression in tobacco plants results in lowering the accumulation of malonyldialdehyde (MDA), hydrogen peroxide (H_2O_2), GSH and also AsA, accompanied by higher activities of antioxidant enzymes like SOD, POD, CAT and APX. Thus, the WRKY transcription factor can play as a positive regulator to mediate tolerance of plants through glutathione-ascorbate cycle.

Finally, we can suggest according the litterature that the fifteen candidate genes selected are involved in many biological processes such as growth, development and response to biotic or abiotic stresses. All these biological processes involve several plant growth regulators such as abscisic acid (ABA), gibberellic acid (GA), salicylic acid (SA) and jasmonic acid (JA). Many studies revealed that AsA accumulation could be modulated by these plant growth regulators, which *via* specific signalling pathways, act using transcription factors like the ERF, ARF, Hbox, bZIP, C2H2 and WRKY families. Indeed, the activity of ascorbate peroxidase (APX), a key enzyme of the recycling pathway, is increased in the presence of ABA and GA treatments (Barth *et al.,* 2006). In contrast, APX activities can be inhibited by SA and 2,6-dichloroisonicotinic acid, two enhancers of plant defence responses (Navabpour *et al.,* 2003). In the systemic acquired resistance (SAR), SA acts as a signal in the general plant-resistance response that can be induced during local infection by a virulent pathogen. SAR stimulates the concerted expression of a battery of genes known as pathogenesis-related genes (PR). Interestingly, AsA-deficient Arabidopsis mutants (*vtc*) appear more sensitive to abiotic stresses but more resistant to biotrophic pathogen, which involve the SAR response. Thus, *vtc* mutants are able to activate numerous defence genes including those encoding PR protein (Pastori *et al.,* 2003). Reciprocally, SA-deficient plants infected by a RNA virus react better as they show lighter symptoms and low ROS accumulation, and such a response was attributed to a higher AsA content (Wang *et al.,* 2011). JA and methyl jasmonate (MeJA) are two important signalling compounds in the response to abiotic stress in plants. Emerging evidences indicate that JA participates in the regulation of AsA metabolism. Several genes related to AsA biosynthesis and recycling pathways such as GMP, GGP, GME for the biosynthesis, MDHAR and DHAR for the recycling as well as APX, are up-regulated during treatments with JA and MeJA (Sasaki-Sekimoto *et al.,* 2005; Wolucka *et al.,* 2005). In tomato as in Arabidopsis, the mutations that impair JA metabolism and signalling influence the foliar AsA content. However, JA effect on AsA accumulation varies with plant species, as JA enhances AsA accumulation in Arabidopsis whereas this is the reverse in tomato, demonstrating hence the complexity of the relation of JA within the AsA regulation (Suza *et al.,* 2010).

To end, we can say that all these experiments demonstrated that any AsA variation in leaf or fruits could influence the expression for most of the 15 candidate genes selected. Nevertheless, the remaining question is to determine if these genes are implicated in the regulation of AsA pool in our model, the tomato fruits. To decipher the relationship between the candidate genes and AsA metabolism, the next step would be to determine if any variation in their expression could result in changes of the AsA pool size. Given the time remaining to address this question and validate these genes, I decided to develop a strategy based on fruit protoplast transformation instead of a strategy using stable transformation that is too much time consuming. Currently, the preparation of the vectors for the transformation is in progress following the protocol described by Sakamoto *et al.,* (2013). Another way to address this question was to use the EMS Micro-Tom collection developed in our laboratory. This approach is in progress and constitutes an interesting part of the perspectives of this project.

Part III

Antioxidant potential in postharvest fruit quality.

Part III: Antioxidant potential in postharvest fruit quality.

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, preserves, sauce, paste, etc. During the ripening process, several important changes occur in the ultra-structure and the metabolic content of the fruit among which the pigment synthesis, 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 (Vit C) contents increase during the ripening phase whereas hydroxycinnamic and chlorogenic acid contents decline (Hunt and Baker 1980; Senter *et al.,* 1988; Buta and Spaulding, 1997; Cano *et al.,* 2003). However, many tomato fruits produced for fresh consumption are harvested before complete ripening when they are still firm and turning. During postharvest storage, and to extend the shelf-life, the fruit respiratory metabolism is slowed down by maintaining at low temperatures and/or a high carbon dioxide atmosphere, and in some countries exposed to exogenous ethylene to induce colour and ripeness just before delivering to the fresh food market (Kalt *et al.,* 1999). It has been shown that the ripening process at such low storage temperatures is affected, resulting in the alteration of the final nutriment composition of the fruits (Madhavi and Salunkhe 1998; Javanmardi and Kubota *et al.,* 2006).

Storage of tropical or subtropical products like tomato fruits below a critical temperature induces the appearance of chilling injury (CI). CI are physiological disorders caused by exposure to low but non-freezing temperatures (ca.>10°C), which induces symptoms like arrest of the ripening process, pitting, water soaking, and increase of disease susceptibility. The severity of chilling injury depends on numerous intrinsic (e.g. cultivar, prior growing conditions, and exposure to stress) and extrinsic factors (e.g. temperature, duration of exposure, surrounding relative humidity, sanitation and level of mechanical injury). Thus, CI can occur before or after the harvest of the fruits, during transportation, storage or displaying at the market and it appears generally when fruits or vegetables are transferred to non-chilling temperatures (Morris 1982; Luengwilai *et al.,* 2012). Recently, oxidative stress has been associated with the appearance of chilling damage in fruits (Hodges *et al.,* 2004). Oxidative stress occurs

when the generation of ROS exceeds the capacity of the tissue/cell to maintain a cellular redox homeostasis (Hodges *et al.,* 2004). To prevent this oxidative damage of cellular components such as membranes, proteins and nucleic acids, plants have developed several mechanism of defence based on antioxidant potential. In tomato, antioxidant potential is derived from the association of many antioxidant compounds including lipophilic compounds like the main pigments lycopene, and β-carotene, the tocochromanols (Vit E) and also hydrophilic compounds like phenolic acids, flavonoids, and ascorbic acid (Vit C).

Among the water-soluble compounds, ascorbic acid is one of the most abundant, which can accumulate to high levels in the fruits. Stevens *et al.,* (2008) have shown that increase of MDHAR activity, an enzyme involved in the AsA reduction, induced a decrease of tomato firmness during CI. In the same manner, over-expression of GGP, an enzyme of the AsA biosynthesis pathway, resulted in the better protection of tobacco plants subjected to chilling stress. These transgenic tobacco plants, in addition to accumulate more AsA, accumulated less hydrogen peroxide (H_2O_2), displayed lower levels of ion leakage and malonyldialdehyde content thus reflecting a better maintenance of the integrity of the cellular membrane. Furthermore, pepper fruits (*Capsicum annuum. L*) treated with H_2O_2 during storage were significantly more resistant to postharvest damage. Hydrogen peroxide treatment induces an increase of AsA content in addition to activities of antioxidant enzymes such as APX and DHAR (Bayoumi 2008). So, biofortification of fruits such as tomato with improved AsA content can constitute an important challenge, notably with the high expectations of the consumers for a product with very good visual aspect and nutritional values.

Currently, in breeding programs, many cultivars were selected for traits valued by producers such as resistance to biotic and abiotic stress, uniformity, appearance, firmness and extended shelf-life (Shewfelt 2000). However, in the fruits, these traits are usually not linked with nutritional and organoleptic traits. Stevens (1986) showed that breeding selection for higher soluble solids in tomato fruits compromised the yield, which was below the profitably threshold for the tomato crop. Reinforcement of this breeding strategy comes from the fact that consumers appeared conflicted in their

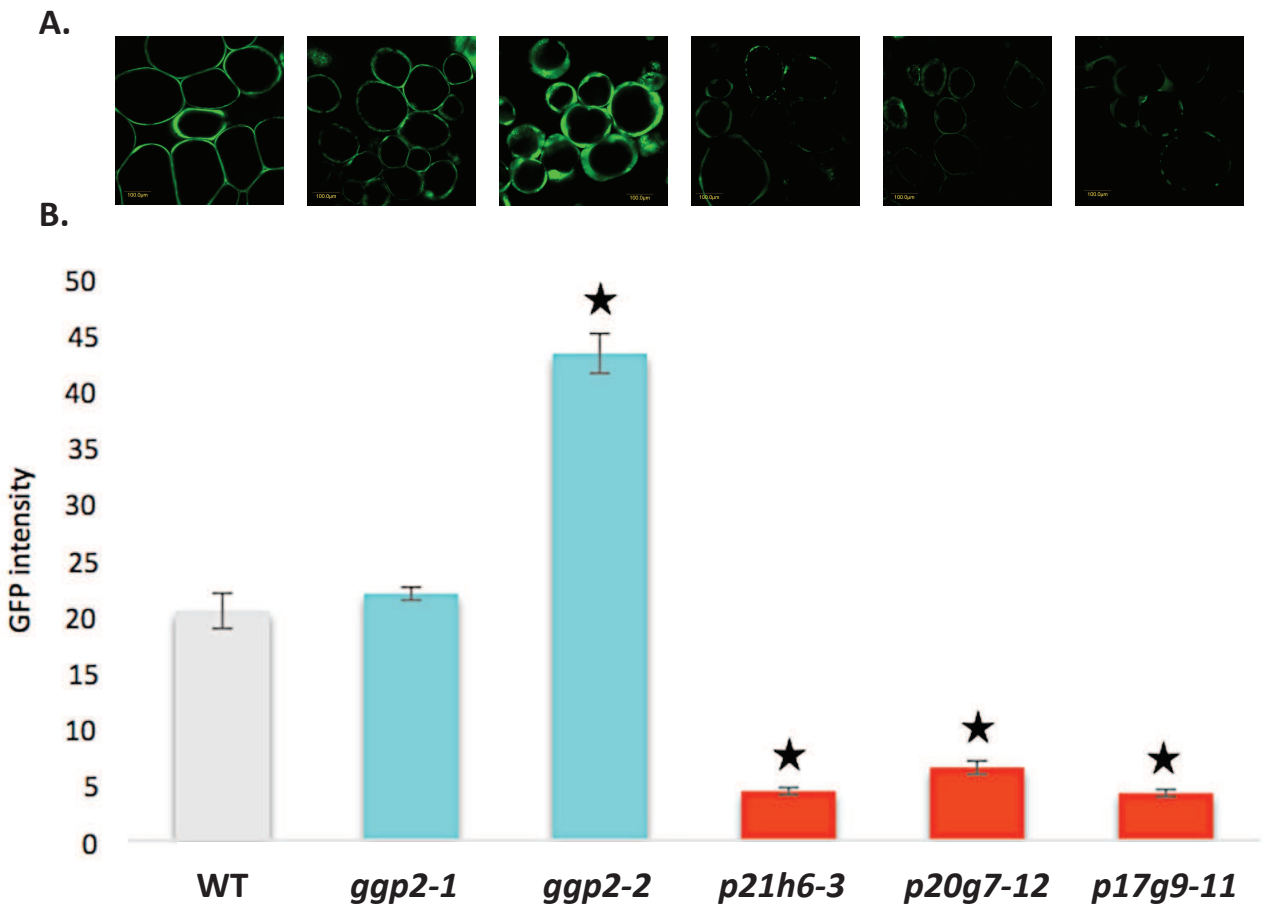


Figure 53: Antioxidant potential in mutants lines. **A.** Confocal Imaging of fruit cell pericarp after wounding stress. In green, fluorescence emission of DCF exited under blue light. **B.** Graph showing the means of GFP intensities (±SD; n= 10) asterisks indicate significant differences between lines (ANOVA: Dunnett test, P<0,05).

desires: while taste was given high importance, fruits of poor appearance will not be chosen even if the taste can be "guaranteed" (Beckles 2011). As a result, the cost-benefit ratio currently tilts in favour of non-taste-related traits. Thus, biofortication of cultivated tomato using AsA enriched trait is related to two major economic issues: improving both fruit nutritional quality and postharvest fruit resistance, which can respond to the expectations of consumers but also of the tomato producers. The objective of the present study was to investigate the postharvest fruit quality of several mutant lines enriched in AsA content. Towards this end, abiotic stress and biotic stress were applied on AsA+ and AsA- mutant lines in order to evaluate their resistance or susceptibility and thus the valuable quality attribute that represents the improvement of AsA content in fruits.

I. Antioxidant potential in the AsA+ mutants.

The screen of a Micro-Tom EMS mutant collection allowed identifying several families altered in fruit AsA accumulation. Thus, we had two mutants displaying a significant decrease of AsA content, around 80% compared to WT and five mutants enriched in AsA, respectively called AsA- and AsA+ mutants. In this study, among the five AsA+ mutants only three have been used, namely *p17g9-11, p20g7-21* and *p21h6-3* because the other two displayed a parthenocarpic fruit phenotype. Furthermore, the two AsA- mutants corresponded to knockout mutations of the GGP2 enzyme of the AsA biosynthesis pathway, named *ggp2-1* and *ggp2-2*. Hence, the AsA- and AsA+ mutants constitute powerful tools in order to determine if the biofortification of tomato fruits with a protective molecule such as AsA can improve the fruit antioxidant potential. Towards this end, mature green fruits have been peeled to mimic a wounding stress (*figure 53*). This abiotic stress induces production of ROS that can be revealed *in situ* by staining using the dihydrodichlorofluorescin diacetate (H2DCF-DA) reagent. H2DCF-DA is a non-fluorescent lipophilic ester that can easily cross the plasma membrane and enter the cytosol, where it is rapidly cleaved by unspecific esterases. One of the reaction products is the non-fluorescent alcohol H2DCF. The oxidation by ROS of this molecule induces production of a DCF fluorochrome, which emits green fluorescence light when excited with blue light. The intensity of the fluorescence is considered to reflect ROS level. Observations of fruit slices incubated in H2DCF-DA using confocal imaging,

allowed us to estimate the intensity of the fluorescence in each mutant and to compare it with the WT, and consequently to quantify ROS level. The intensity of the fluorescence appears to be less important in the AsA+ mutants. In contrast, the fluorescence is 4 to 10 times more important in the WT and AsA- plants. These observations demonstrate that after abiotic stress, here wounding, the ROS production is less important in AsA+ than in AsA- and WT. So, increasing AsA content in fruits can improve the antioxidant potential and improve the postharvest fruit quality.

II. Postharvest fruit quality of AsA+ mutants.

The connections between the antioxidant potential and the postharvest quality have already been made in several fruit species like apple (Davey *et al.,* 2007). In order to decipher the impact of changes in the antioxidant potential during postharvest, the resistance of AsA+ and AsA- mutants to abiotic and biotic stresses have been investigated.

a. Resistance to an abiotic stress: chilling stress

The common practice of cold storage of fruits allows to get extended market periods, long distance transportations, and a more steady supply of commodities in the market. However, below 12,5°C corresponding to an optimum temperature, tomato quality may be ruined by chilling injury. Previous studies have shown that the severity of response to chilling injury is higher in green than in red fruits and it increases as a function of storage time and temperature (Morris 1982). Thus, to evaluate the resistance of mutants enriched in AsA to a chilling stress, mature green fruits have been stored at 2°C during five weeks inside a cold room in the dark. During and after cold storage, some parameters have been checked to evaluate the chilling susceptibility of the mutants compared to the WT. Dysfunction of cell membrane at chilling temperature is though to be the primary event that ultimately leads to CI symptoms (Lyons 1973, Sharom 1994). Evidence for membrane damage as the cause of CI includes the increase of membrane permeability then followed by browning, loss of firmness, and after back to non-chilling temperature, uneven ripening. During cold storage, peroxidation of the membrane lipids has been observed using IVIS (In Vitro Imaging System).

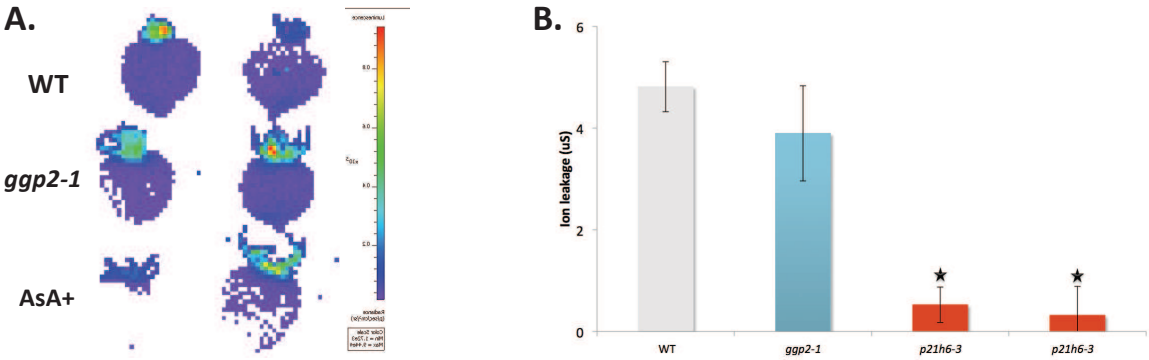


Figure 54: Peroxidation of the membrane lipids of mature green fruits in response to a chilling stress. **A**. After 10 days of storage at 2°C the luminescence is quantified using IVIS (In Vitro Imaging System). Luminescence reflects intensity of lipid peroxidation. Here is shown only *p21h6-3* fruits, named as AsA+. The *p20g7-12* fruits display also a similar imaging profile. **B.** Ion leakage of fruit after five weeks of storage at 2°C. (±SD; n= 15 fruits) Asterisks indicate significant differences between lines (ANOVA: Dunnett test, P<0,05).

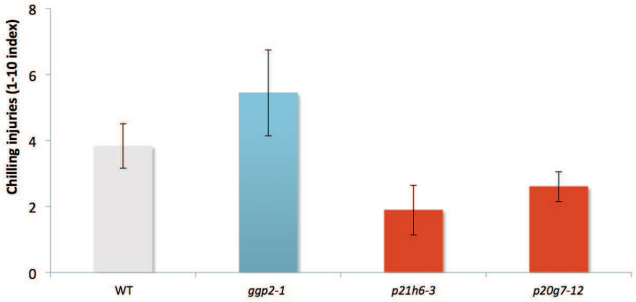


Figure 55: Susceptibility of mature green fruits stored at 2°C during five weeks. Chilling injury is estimated using chilling index. A number comprised from 1 to 10 was given for each fruit analysed according to the severity of the symptoms. (±SD; n= 15 fruits) Asterisks indicate significant differences between lines (ANOVA: Dunnett test, P<0,05).

Figure 56: 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.

This approach has shown that AsA+ mutants have less important peroxidation of membrane lipids than AsA- and WT. Furthermore, after five weeks of storage, the measure of ion leakage demonstrated that membrane permeability is twice to three times less important in AsA+ mutants than WT and AsA- mutants, respectively (*figure 54*). After five weeks of storage when browning appeared, fruits were back at room temperature and CI symptoms have been evaluated using CI index (*figure 55*). It appears that the severity of symptoms is less important in the AsA+ mutants than in the AsA- and WT. After two weeks at room temperature, WT and AsA- display uneven ripening whereas AsA+ mutants have maintained ripening process to reach red ripe stage (*figure 56*). In addition, some fruits from *ggp2-1*, one of the AsA- mutant, and from the WT displayed decay symptoms. To conclude, these observations indicate that the AsA+ mutants are more resistance to chilling stress compared to WT and the AsA- mutants.

b. Resistance to a biotic stress due to fungus.

The losses due to postharvest diseases may occur at any time during postharvest handling, from harvest up to consumption. These postharvest diseases are at the origin of the lessening in fruit quality and quantity, as some diseases make the fruits unsuitable for sale at the same time for consumption. Infected fruits mostly develop disease symptoms after harvest and during storage at cold temperature and transportation (Prusky *et al.,* 2013). Postharvest fungal pathogens exploit three main routes to penetrate the host tissue: (i) through wounds caused by biotic and/or abiotic agents during growth and storage; (ii) through natural openings such as lenticels, stem ends and pedicel-fruit interphase, and (iii) by direct breaking of the host cuticule. Many factors can lead to favour fungus attack including handling methods, commodity type, postharvest environment, maturity stage, and cultivar susceptibility. In order to determine if the increase of the antioxidant potential can promote resistance to pathogen infections, mutant lines were infected by two different types of fungi infected mutants.

Figure 57: Red ripe tomato fruits from different varieties displaying infection symptoms after attack by *Colletotrichum coccodes* (left) and *Botrytis cinerea* (right) leading to anthracnose and grey rot diseases, respectively.

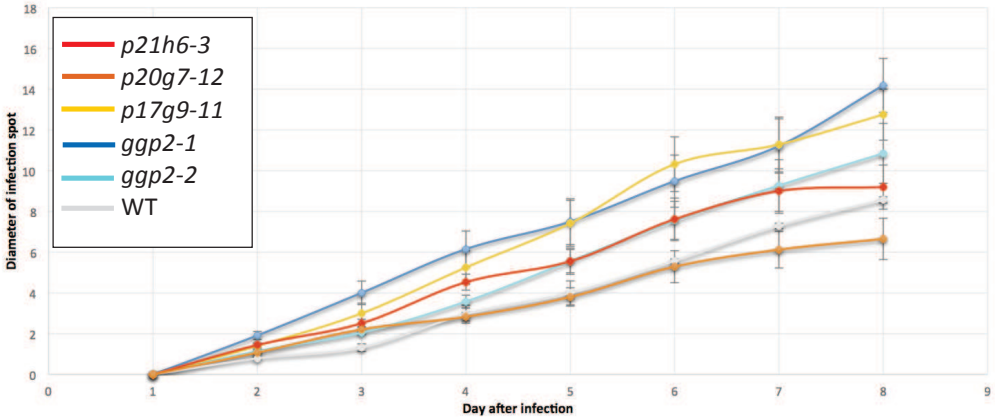


Figure 58: Infection of tomato fruits at the red ripe stage by *Colletotricum coccoedes*. The diameter of infection was measured during 8 days after infection. Results obtained on three AsA+ lines: *p21h6-3, p20g7-12* and *p17g9-11*; Two AsA- lines: *ggp2-1* and *ggp2-2* and WT are shown in this picture. Data are means ± SD of 30 infection spots. Diameters of infection spot are in centimetre.

i- *Colletotrichum coccodes*

Colletotrichum coccodes is a hemi-biotrophic plant pathogen that causes a disease named anthracnose on tomato. At the fruit level, the fungus colonizes unripe/green fruits, leaving the host tissues symptomless for long periods. This development of the fungus corresponds to the quiescent biotrophic stage. Then, *Colletotrichum coccode* activates its colonization during fruit ripening and senescence, this corresponding to its active necrotrophic stage (Prusky 1996). The infection process begins when conidia germinate on the surface of host tissue to produce germ tube and an appressorium. Then, the appresoria geminate to produce infection hyphae and cause anthracnose (*figure 57*). To investigate whether the AsA+ mutants are more resistant to *Colletotrichum coccodes* infection, fruits at the red ripe stage were infected. The infection was analysed during 8 days after the infection by measuring the diameter of infection. As shown in the *figure 58*, the AsA-, AsA+ and WT display similar susceptibility. These results suggest that AsA content does not influence infection efficiency, suggesting that AsA is not involved in the resistance to infection by *Colletotrichum coccodes* or at least not alone.

ii- *Botrytis cinerea*

Botrytis cinerea is a necrotrophic ascomycete and an important plant pathogen, inducing the disease known as “noble rot” in grape or “grey rot” in other fruits like tomato or strawberry. This disease appears from quiescent infections established before postharvest. This fungus colonises necrotic and non-necrotic tissues, able to remain quiescent in the base of the floral receptacle. Several months later when fruits are harvested, the infections develop at a stem end rot in ripe fruits, and are commonly called grey rot. Grey rot is the most important pre-harvest and postharvest disease for fresh-market tomato (Mari *et al.,* 1996). Botrytis displays the capacity to kill host cells through the production of toxins, ROS and the induction of an oxidative burst. This pathogen is non-specific to plant organs. In my work, the resistance of AsA+ mutants to infection by *Botrytis cinerea* was carried out on leaves and fruits. Inoculations were only

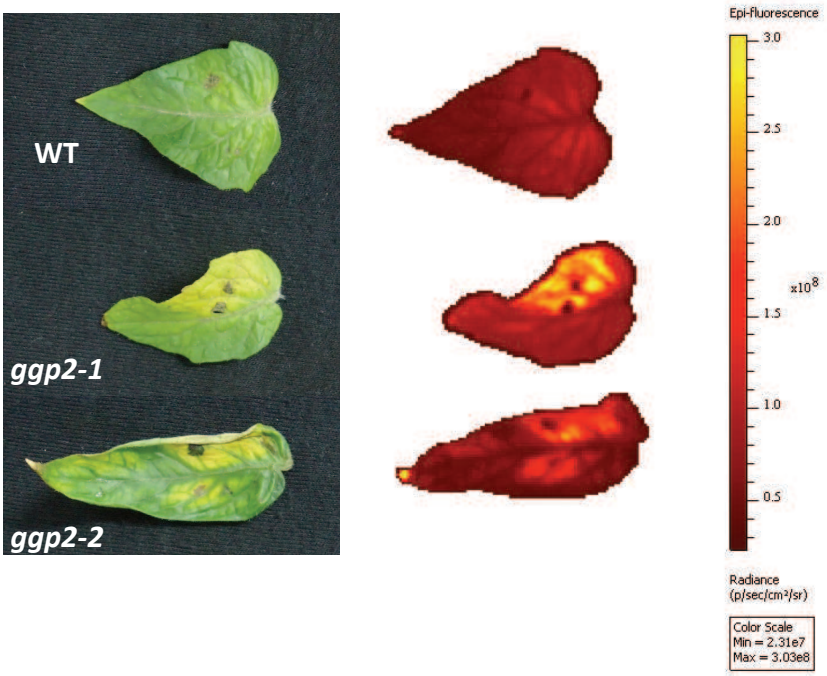


Figure 59: Infection of tomato leaves by *Botrytis cinerea* for the two AsA- lines (*ggp2-1 and ggp2-2*) and the WT. Leaves infected display necrotic areas where the fungus develops and the hypersensitive response occurs. The Observation of DCF staining was realized using IVIS system (In Vitro Imaging System). Presence of ROS is illustrated by the yellow colour and located where fluorescence values reach the level above the value of 3.10^8 on the scale of colour intensity.

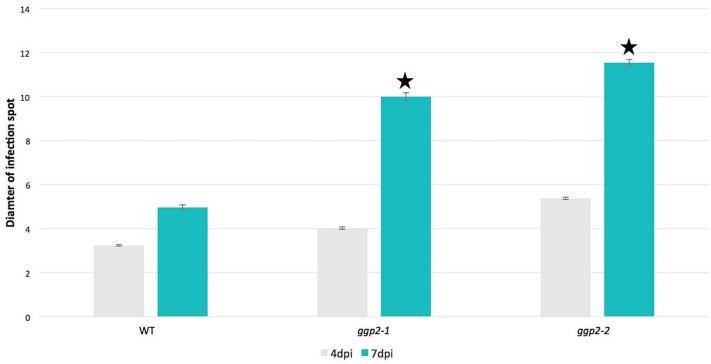


Figure 60: Infection of tomato leaves by *Botrytis cinerea* for the two AsA- lines (*ggp2-1 and ggp2-2*) and the WT. Diameter of infection was measured 4 and 7 days after infection. (±SD; n= 30) asterisks indicate significant differences between lines (Student test, P<0,05). Diameters of infection spot are expressed in centimetre.

performed on detached leaves in the AsA- and WT plants. Indeed, none of the AsA+ mutants have shown an increase of AsA accumulation in leaf organs. Observations of the infection 3 days after inoculation, showed that the two AsA- lines produce ROS in contrast to the WT (*figure 59*). Furthermore, 4 and 7 days after inoculation, the diameter of infection spots were more important in the AsA- mutant compared to the WT (*figure 60*). After 7 days, most of AsA- leaves displayed full necrosis. Thus, the two AsA- mutants appear more susceptible than the WT to *Botrytis* infection in leaves. In fruits, the infection at the red ripe stage was analysed during 6 days in two AsA+ mutants, *p21h6-3* and *p20g7-12*, the AsA- mutant *ggp2-2* and the WT (*figure 61*). The kinetic of the infection is quite difficult to comment as the tendency is similar between one of the AsA+, the AsA- mutants and the WT. However, a significant difference between the AsA+ *p21h6-3* mutant and the WT is observed. According to these observations, it is impossible to conclude about any kind of resistance of the AsA+ mutants to *Botrytis*. It is interesting to note that the observations obtained on leaves are not confirmed on fruits.

III. Discussion

Though the results obtained on the postharvest quality of the tomato fruit are rather preliminary, it is tempting to discuss and propose some working hypotheses. The agricultural practices to extend vegetable and fruit shelf life can trigger physiological disorders before their consumption. These physiological disorders are responsible for the loss of the postharvest fruit quality due to fruit senescence and pathogen infection. The associated oxidative stress is linked with the production of reactive oxygen species, which can alter cellular component such as membrane, protein and nucleic acid integrity. In that case, to scavenge the ROS, the antioxidant potential of the fruits can play a prominent role. Among the antioxidant compounds present in the plant cell in general and in the fruits in particular; ascorbic acid (Vitamin C) displays a major interest due to the fact that its content can be very high in fruit tissues (Lee *et al.,* 2000). Numerous approaches can be considered to ameliorate abiotic and biotic stress sensitivity. For enhancing stress resistance, treatments such as temperature modulation including intermittent warming, extreme atmosphere with high or low O2 levels, high CO2 levels, growth regulators, anti-transpiring,

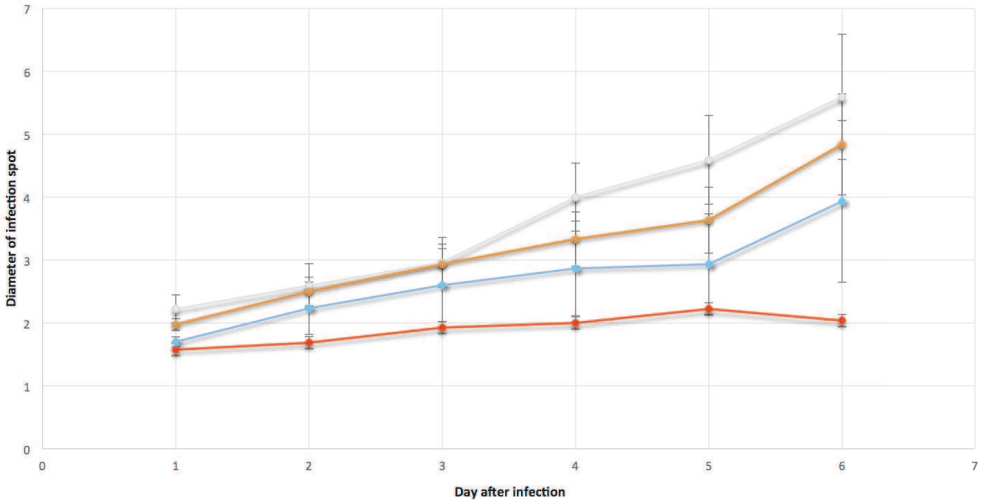


Figure 61: Infection of tomato fruits at the red ripe stage by *Botrytis cinerea*. The diameter of infection was measured during 6 days after infection. Results obtained for two AsA+ lines: *p21h6-3* (red line) and *p20g7-12* (orange line); one AsA- line: *ggp2-2* (blue line) and WT (grey line) are shown in this picture. Data are means ± SD of 30 infection spots. Diameters of infection spot are expressed in centimetre

antioxidant dips, nitric oxide and ethanol, and in the case of biotic stress the use of synthetic fungicides and antagonistic microorganisms have been evaluated (Mari et al., 1996; Toiven, 2003; Sharma *et al.,* 2009; Droby *et al.,* 2009; Toiven and Hodges 2011). However, due to the complexity of the mechanisms involved in the stress response, designing an effective treatment for the several abiotic and biotic stresses that a fruit or vegetable may encounter during harvest, handling, storage and distribution, appear as difficult as unrealistic. Secondly, the simple modification of the harvest and handling protocols can reduce stress exposure. One of the most successful strategies resulting in significant improvements and shelf life quality is the application of plastic packaging films or wraps to prevent desiccation. At last, another option is the use of molecular engineering strategies based on the knowledge of fruit physiology, genomic and also metabolic that can help to produce better plant resistance. A large number of genes, proteins and also quantitative trait loci (QTL) associated with stress tolerance have been described in the literature and marker-assisted selection can be used for selecting stress resistance genes (Collins et al., 2008). However, this strategy requires the perfect or better knowledge of the stress response because, like for the treatment designs, and due to the complexity of the processes involved, it appears difficult to select profitable traits. To finish, the selection of germplasms or cultivars can be a powerful approach to improve postharvest stress resistance. Several studies have shown that according to the cultivar, broccoli and spinach can be more or less susceptible to yellowing during storage (Toivonen and Sweeney 1997; Hodges *et al.,* 2011). In addition, Toiven *et al.,* (2003) have demonstrated that some apple cultivars are more resistant to browning in response to cutting due to the high concentration of apoplastic antioxidant, resulting in a decrease of ROS level. In the present study, tomato mutants enriched in AsA into the fruit have been discovered. These AsA+ mutants display an increase of their fruit antioxidant potential. The analysis conducted regarding the postharvest resistance of tomato fruits is very remarkable. The storage at chilling temperature of tomato fruits showed that mutants enriched in AsA are more resistant than mutants impoverished in AsA as well as the WT. The membrane integrity of the AsA+ fruits was conserved, and allowed the progress of a normal ripening process. At the opposite, in the AsA- and WT fruits, the peroxidation of membrane lipids was observed and consequently chilling injuries were more severe inducing uneven ripening. In contrast, the study of the resistance to biotic stress using hemi-biotroph and necrotic fungi, is less clear and

instructive. In fruit, the resistance or susceptibility of the three AsA+ mutants, two AsA- mutants and the WT did not appear dependant of the level of AsA accumulation. In contrast, this was real in the leaf since for detached leaves, the two AsA- mutants are clearly more susceptible to *Botrytis cinerea* infection.

Nevertheless, many work in that research field are described in the literature and this allows proposing hypotheses to explain the difference observed in the response between fruits and detached leaves to biotic stress. Plants have several means of struggle to respond to biotic and abiotic stress such as hypersensitive response (HR) and systemic acquired resistance (SAR). The type of response depends on the pathogens. Among them are biotrophic pathogens that acquire nutrition directly from living plant cells, necrotrophic that kill the plant cells and feed on the dead cells and hemi-biotrophic corresponding to an early biotrophic phase followed by a switch to necrotrophy (Van Kan *et al.,* 2014).

The HR mechanism is characterized by the rapid death of the cells in the local region surrounding the infection due to an oxidative burst, that is, a rapid production of ROS. These processes are called the programmed cell death (PCD). The HR mechanism allows restricting the growth and the spread of the pathogens to other parts of the plant. Thus, the perception of necrotrophic pathogen like Bo*trytis cinerea* induces programmed cell death in the host tissues, which interestingly is necessary for the successful infection. It has been shown that AsA metabolism is linked with PCD, as it is involved in the reduction of ROS. During leaf senescence, a process that corresponds to a certain type of PCD, the expression of senescence-down-regulated genes (SDGs) decline, and at the early stage of leaf senescence, other genes called senescence-associated genes (SAGs), are up-regulated. In such a case, AsA is known to influence the senescence of plants by modulating the expression of SAGs (Barth *et al.,* 2004). Thus, the low accumulation of AsA in the organs, like in the AsA- mutants, accelerates senescence processes, while it is the reverse when AsA content is increased. Furthermore, ROS are able to promote the expression of senescence-associated genes. At the same time, AsA is involved in transcriptional regulation of SAGs. Treatment of leaves with silver nitrate, a ROS reagent, induces the up-regulation of two SAGs (LSC54 and LSC94) until the subsequent treatment with AsA, which reverses the process (Navabpour *et al.,* 2003). Supplement of AsA will reduce the ROS accumulation, alleviate oxidative damage to photosynthetic tissue, and consequently delay the process of senescence. Thus, during

PCD of the leaves, AsA regulates the ROS level and/or the expressions of SAGs to reduce ROS accumulation and high oxidative damage of the photosynthetic apparatus of the tissue, and consequently delays the processes of senescence. According to these data, in the present detached tomato leaves inoculated with *Botrytis cinerea*, we can assume that the PCD observed in the AsA- mutants is more severe because of the reduction of AsA content. Conversely, we can hypothesize that leaves displaying higher AsA accumulation can be less affected by PCD.

The HR is located at the position of the infection and commonly precedes a slower systemic response at the whole plant level, which subsequently leads to SAR. The SAR is defined as the "whole-plant" resistance response. In SAR mechanism, conserved microbial structures are recognized and this recognition induces the expression of a wide range of genes such as pathogenesis-related genes (PR). SAR requires the accumulation of endogenous salicylic acid (SA). However, it has been shown that SA can inhibit APX (ascorbate peroxidase), an enzyme using AsA to reduce hydrogen peroxide. This supports the hypothesis that SA-induced defence responses are mediated, in part, through elevated ROS level or coupled perturbations of the cellular redox state (Durner and Klessig 1995). Arabidopsis mutants displaying defects in various defence-related signalling pathways were tested for analysing in the response in the resistance to various pathogens (Thomma *et al.,* 1999; Glazebrook 2005). The observations led to conclude that the SA-dependent defence is active against biotrophic pathogens. In our case, we can hypothesize that during the infection with *Colletotrichum coccodes*, no resistance associated with a higher AsA accumulation was observed because in the presence of this hemi-biotrophic pathogen, the SA pathway is activated by high level of ROS and there is no need of AsA.

The results obtained on tomato fruits infected with *Botrytis cinerea* are still unclear. In the literature, it has been shown that resistance to Botrytis depends on JA signalling and camalexin production (Thomma *et al.,* 1998; Ferrari *et al.,* 2003; Lorenzo *et al.,* 2004). However, the link between JA and AsA is very complex. In tomato and Arabidopsis, mutations that impair JA metabolism and signalling can influence AsA accumulation, suggesting that endogenous JA may regulate AsA metabolism. Furthermore, JA enhances AsA accumulation in Arabidospis, whereas in tomato, it is the reverse (Suza *et al.,* 2010). Since the WT appeared to be less resistant to Botrytis infection than the AsA + and AsA- mutants, we can consider that any variation in AsA

accumulation in the AsA-related mutants might have an influence on JA metabolism and consequently enhance the response, even if no effect in AsA accumulation was observed.

The biofortication of the fruits presents two main issues: improving postharvest fruit resistance and, consequently, improving postharvest fruit quality by preventing alteration of its nutritional quality. Increasing fruit AsA content would allow increasing resistance to chilling stress and probably to the abiotic stress that occurs during postharvest processing. The link between the higher AsA accumulation and the resistance to biotic stress is still not clear. Even preliminary, our results are very encouraging but they need to be confirmed. In that sense, it is possible that adjusting the concentration of pathogen during inoculation can permit to see differences in term of plant/tissue resistance or susceptibly. Nevertheless, the link between AsA metabolism and the pathways involved in response to biotic stress are really complex and are dependent on the pathogen considered. At last, our study confirmed that the biofortication of the fruits with AsA-enriched trait could, beyond the increase of this antioxidant compound, confer health-promoting effects, and improve postharvest fruit quality during an abiotic stress.

Perspectives

The vitamin C (AsA) is an essential naturally organic compound for both human and plants. It acts as a water-soluble antioxidant to scavenge reactive oxygen species (ROS) and to prevent oxidative damage, ROS compounds which are involved in many biological processes. However, human and some other mammalian species are not able to synthesize this secondary metabolie due to a loss-of-function in the last enzyme of the biosynthesis pathway (Linster and Schaftingen 2007). Plant-derived vitamin C, mostly found in fruits and vegetables, represents the major source in the human diet (Davey *et al.,* 2000).

The common postharvest practices used to extend shelf life of fruits and vegetables allow responding to the global demands of local and large-scale production for international distribution of fresh produce. However, inadequate management of postharvest technologies can result in major losses in nutritional and quality attributes, which may cause financial loss for all players along the supply chain, from growers to consumers. The physiological disorders that appear, such as pitting, water-soaking, pathogen susceptibility, and uneven ripening are often related with an oxidative stress in which the antioxidant properties of the vitamin C play a prominent role (Lee *et al.,* 2000; Hodges *et al.,* 2004; Davey *et al.,* 2007; Stevens *et al.,* 2008). Moreover, AsA is much more sensitive to degradation when the commodity is subjected to adverse handling and storage conditions (Hunt and Baker 1980; Senter *et al.,* 1988; Buta and Spaulding, 1997; Cano *et al.,* 2003; Prusky *et al.,* 2013). Hence, biofortication fruit with vitamin C constitutes a double challenge to improve both the postharvest and the nutritional qualities of the fruit. The objective of this study was (i) to confirm that increasing fruit AsA content can improve its postharvest quality, and (ii) to understand the mechanisms involved in the regulation of AsA accumulation in the fruits.

Increasing AsA content in tomato fruits, does it result in the improvement of postharvest fruit quality?

The screen of a tomato EMS mutant collection allowed the identification of five mutants displaying significant AsA+ phenotypes. The discovery of tomato mutants enriched in AsA represent a powerful means to address the above question. Indeed, the characterization of these mutant fruits in response to wounding and chilling stresses

clearly demonstrated that AsA+ fruits are more resistant to stress and therefore more fitted to withstand unfavourable postharvest storage conditions.

Fruits harvested at red ripe stage inoculated with necrotrophic and hemi-biotrophic fungi showed contrasting behaviours. First, AsA can act efficiently in plant as immune-modulator in the response to biotic stress, and second its function is different regarding to the pathogens (Noctor and Foyer 1998; Asada K 1999; Barth *et al.,* 2004; Liu *et al.,* 2012; Prusky *et al.,* 2013). Observations reported for the AsA-deficient mutants *vtc1* and *vtc2* suggest that AsA could regulate stress response through a complex sequence of biochemical reactions involving the activation or suppression of key enzymatic activities and defence proteins but also the production of various chemical defence compounds (Pastori *et al.,* 2003; Colville *et al.,* 2008) However, there is still a large gap to fill in order to elucidate the precise role of AsA in the processes of tolerance that plants have developed to face pathogens during their development. Hence, our preliminary results do not bring simple evidences that could help to get a better knowledge about the role of AsA during fungus infection in the fruits. The discrepancy of the response to fungus infection that was observed could be explained by the fact that the concentration of pathogen used was too high. We can envisage that using lower concentration of pathogen can help us to observe some tolerance or resistance phenotypes. Besides, it is well known that the biosynthesis of AsA and that of cell wall polysaccharides share a common pathway (Gilbert *et al.,* 2009; Voxeur *et al.,* 2011). Recently, a link between the AsA biosynthesis and the wax biosynthesis pathway has been described (Zhu *et al.,* 2014). It is thus tempting to suggest that in the AsA+ mutants both the cell wall and the cuticle formations could be affected and interfere with the resistance to pathogens. Thus, the lack of significant difference between AsA+ mutants and AsA- mutant or WT can originate from the fact that the fruit epidermis was scratched before being inoculated.

In contrast, the results of the chilling stress experiment are convincing. Indeed, mature green fruits from the AsA+ mutants are more resistant to chilling stress compared to AsA- mutants and WT. In the AsA+ mutant fruits, such a resistance was the result from a reduced peroxidation of membrane lipids, which is the first physiological disorder observed during chilling stress. The preservation of the membrane integrity

allows the prevention of membrane leaking and consecutively water-soaking, loss of firmness and pitting. After chilling treatment and back at room temperature, the AsA+ mutant fruits displayed a normal ripening process whereas it was significantly impaired in AsA- mutants and in the WT. In addition, the uneven ripening observed was associated with the presence of decay symptoms, suggesting an exacerbated susceptibility of the AsA- and WT fruits to postharvest pathogen attacks consecutive to chilling stress. Interestingly, the fact that the three AsA+ mutants analysed so far displayed resistance to chilling stress strongly suggests that the AsA+ trait is at the origin of chilling resistance. However, only external parameters were observed. It is important to estimate the nutritional quality after a chilling stress to validate the quality attributes of the AsA+ fruits. Furthermore, it can be interesting to analyse all these parameters and the behaviour of the AsA+ mutant fruits when common practices of the fresh market and habits of the consumers are used. This corresponds in the case of tomato, to store the fruits at 12°C on the stand and at 4°C in the fridge, respectively. In that frame, a culture including three AsA+ and two AsA- mutants and the WT parent is currently in progress to investigate internal and external physiological disorders that appear after 8 days of storage at 4°C and 12°C.

How to identify the mechanisms involved in the regulation of ascorbate accumulation in fruits?

In the present study, two approaches were developed in order to understand the mechanisms involved in the regulation of AsA accumulation in tomato. The first strategy based on reverse genetics allowed selecting fifteen candidate genes, mainly transcription factors. The *in silico* investigation showed that these candidate TFs are interconnected, suggesting a participation in similar cascade of reactions in response to biotic or abiotic stresses (http://string-db.org; http://biostatflow.org; Cytoscape software). Furthermore, according to the literature, for some of these TFs or homologs we could hypothesise a link with the AsA metabolism (Yahalom et al., 2008; Rizhsky *et al.,* 2004). Nevertheless, the physiological experiments performed in the present work on leaves and fruits did not allow concluding whether these TF control the fruit AsA level or the reverse. Indeed, the variations of the transcript levels observed might also be due to the abiotic stresses that the leaf or fruit underwent during these experiments, among them the “cutting effect”, high temperature, or over-irradiance.

To validate definitively the role of these TFs, we have considered two strategies to modify the expression of the candidate TF genes and to analyse the impact on AsA content in fruits and/or responses to AsA. Genetic engineering approaches have been widely adopted to modify AsA biosynthesis and metabolism in various plant species. Stable transformation is a method for assessing the function of genes; however; this approach is highly time-consuming. Alternative is to use transient expression approaches for rapid *in vivo* analysis of gene expression. Several studies have previously shown that transient expression system is an efficient method to induce change in AsA accumulation. As an example, transient expression of the phosphomannomutase (*PMM)* in tobacco using viral vector-mediated ectopic expression led to a 20-50% increase in AsA content (Qian *et al.,* 2007). Recently, Sakamoto *et al.,* (2013) have developed a very attractive method consisting in performing transient transformation of tomato fruit protoplasts. They showed that transient over-expression of the L-galactose-1-phosphate phosphatase (*GPP*) in tomato fruit protoplasts resulted in the increase of the AsA content by two fold compared to the WT protoplasts. In the perspectives of my study, the over-expression and the silencing of the TF candidates using the protoplast strategy has been started. Indeed, I already succeeded in fruit protoplast preparation, the transformation procedure is working and for 5 of the TFs, the construction vectors have been obtained. The next step will be to transform the fruit protoplasts with both constructions and to perform AsA assay.

The second strategy is to use our EMS Micro-Tom population to perform TILLING (Targeted Induced Local Lesions IN Genomes) for some of the candidate TFs. In the recent years, TILLING proved successful in tomato for the identification of allelic series for target genes including knock-out and strong missenses mutations (Okabe *et al.,* 2011; Baldet *et al.,* 2013; Just *et al.,* 2013). Hence, TILLING constitutes a powerful strategy to determine gene function and analyse changes that occur in plants when the corresponding gene is mutated. The success of this strategy has been validated both in plants (Perry *et al.,* 2003; Till *et al.,* 2003a; Till *et al.,* 2003b; Cooper *et al.,* 2008; Xin *et al.,* 2008) and animals (Winkler *et al.,* 2005; Moens *et al.,* 2008). The interest of this approach is the possibility to obtain allelic series on a specific gene including truncation mutations e.g. splicing site mutations or nonsense mutations. It is also possible to isolate several point mutations on the same gene, which display different consequences on the protein function going from gene knockout to missense mutations due to a single base

change in a given codon. Amino acid substitutions due to missense mutations can be conservative (similar function) or non-conservative (function modification). Thus, the mutation can produce dominant-negative mutants, which are very useful for assessing the biological function of a protein. In the purpose of my study, TILLING is an appropriate strategy to study the function of candidate TFs. Currently, three genes among the 15 TFs have been selected, *WRKY2, ARF* and *ERF*, and for each several EMS mutants have been identified. The characterization of those mutants is in progress.

The second approach developed in the time course of my PhD work was based on forward genetic screening of an EMS tomato mutant collection. Screening the INRA EMS Micro-Tom population permitted the finding of several mutants producing fruits enriched in AsA. These AsA+ mutants were then used as new plant materials to discover elements involved in the regulation of AsA metabolism. For one of the 5 selected mutants, namely *p21h6-3*, and thanks to an NGS-mapping strategy, we were able to identify the causal mutation, at the nucleotide resolution. Within a BC1F2 segregant population from *p21h6-3*, two bulks including 44 plants displaying the AsA+ and WT phenotypes were sequenced with a depth close to 39X coverage of tomato genome. Finally, the causal mutation was identified and shown to induce a stop codon in a PAS/LOV protein. The function of the PAS/LOV proteins remains unclear, although the LOV domain is well characterised as being involved in light signalling, more precisely in the blue light response (Christie *et al.,* 2008). Using a double-hybrid screening, Ogura *et al.,* (2008) revealed a possible interaction of the *Arabidopsis thaliana* homolog of our PAS/LOV protein with VTC2, a key enzyme of the AsA biosynthesis pathway. Interestingly, the *VTC2* gene expression appears to be strongly regulated by light signalling (Tabata *et al.,* 2002; Dowdle *et al.,* 2007; Müller-Moulé 2008). Confocal imaging experiments have shown that the VTC2 protein can be located in both the cytoplasm and the nucleus, thanks to the presence of a Nucleus Localization Sequence (NLS) (Müller-Moulé 2008). Nevertheless, the signification and consequences of this differential localization within the cell is still unknown. In contrast, our *SlPAS/LOV* does not contain any NLS or transmembrane domain, suggesting an exclusive cytoplasmic localization of the protein. Furthermore, analysis of the *SlPAS/LOV* expression revealed a higher expression in low light conditions. It is tempting to make the hypothesis that SlPAS/LOV protein is able to interact with SlGGP2, homolog to AtVTC2, thus leading to

the reduction of the AsA biosynthesis flux either (i) by inducing a change of SlGGP2 location in either the cytoplasm or the nucleus or, (ii) by scavenging SlGGP2, resulting in the suppression of its enzymatic activity. To investigate this hypothesis and to identify the target protein of the SlPAS/LOV protein, a collaborative project with Matias Zurbriggen at the University of Freiburg (Germany) has been set up. This group has an excellent expertise in mammalian and plant synthetic signalling, especially of light-responsive systems. They have developed molecular tools to analyse cellular processes at the spatio-temporal level as well as regulation networks of gene expression and signalling (Cabello *et al.,* 2014).

The third part of this prospective work will be the continuation in the identification of the causal mutation by NGS-mapping strategy for the other AsA+ mutants. Actually, the culture of 500 plants corresponding to the BC1F2 segregant population for the *p20g7-12* mutant has been performed, the red ripe fruits harvested, and the final AsA assay is planned. The next step will be the constitution of the AsA+ and WT bulks using the same number of plants and sequencing depth (40X) and same bio-informatic tools. Remarkably, allelic tests confirmed the existence of distinct loci between all the selected AsA+ mutants, and moreover, as for the *p21h6-3* mutant, the AsA content of the fruits of the *p20g7-12* is strongly dependent on the light irradiance.

Besides being a powerful tool to study the mechanisms involved in AsA accumulation and a source of alleles for plant breeding, the EMS Micro-Tom population will be an original way to explore the question about a possible link between AsA level in the fruit and the incidence of fruit parthenocarpy. Taken as a whole: 1) the role of AsA as a co-factor for the hormone biosynthesis (Smirnoff 1996; Pastori *et al.,* 2003; Gallie *et al.,* 2013), 2) several studies showing that hormonal treatments result in both parthenocarpy and higher AsA fruit content (Shanmugavelu 1961; Martinelli *et al.,* 2009; Tsaniklidis *et al.,* 2014), 3) studies demonstrating parthenocarpic phenotypes associated with higher AsA content resulting either from the over-expression of genes of the AsA pathway or as shown in the present data obtained in the P17C5-3 and P19A6 families, makes it very exciting to address this fascinating question (Bulley *et al.,* 2012).

Materials and Methods

Experimental conditions

Plant materials

Experiments were performed on a dwarf determinate tomato cultivar cv. Micro-Tom, which was originally bred for home gardening purposes. This tomato plant displays two recessive mutations (Meissner et al., 1997; Dan et al., 2007; Campos et al., 2010). At INRA Bordeaux (France), a highly-mutagenized EMS (ethyl methanesulfonate) collection has been developed on Micro-Tom as previously described (Rothan and Causse 2007; Just et al., 2013). During the experiments, three genetic background have been used and analysed:

- **Wild Type** plants: Micro-Tom parent
- **AsA enriched or AsA+** plants**:** The screen of the EMS mutant collection allowed isolating several mutants displaying an increase of AsA content in fruits. These mutants are called *p17g9-11, P17C5-3, P19-A6, p20g7-12* and *p21h6-3*.
- **AsA lessened or AsA-** plants: Two mutants displaying a knockout mutation on the *SlGGP2* gene (SGN-U579800) encoding GDP-L-galactose phosphorylase were identified using TILLING approach in the EMS mutant collection. These two mutants are respectively named *ggp2-1* and *ggp2-2*.

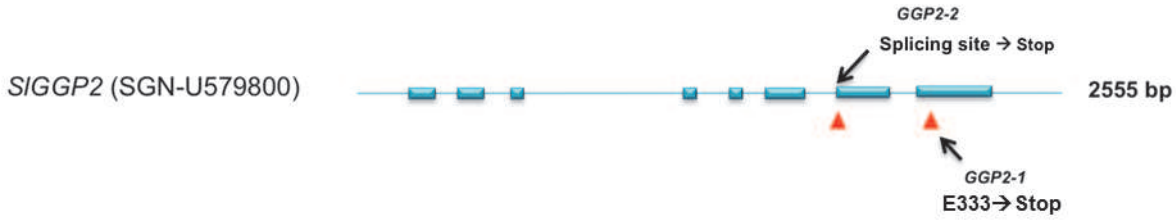


Figure 62: Representation of the mutations in the SlGGP2 gene. Predicted size of the coding DNA sequence is indicated on the right of the gene. Blue boxes represent the exons. Knock-out mutations are indicated by red triangles.

1. Growth conditions

Plant were grown in a greenhouse under 14h light/10h dark, with a temperature between 18°C to 28°C and 60% to 80% of relative humidity. Plants were watered twice a week with a solution 1 (ph 5,8, oligo elements, 3,5mM KNO_3, 1mM K_2SO_4, 2mM KH_2PO_4, 6mM $Ca(NO_3)_2$ and 2mM $MgSO_4$) until first the fruit set on the first truss, then with a solution 2 (pH 5,8, oligo elements, 4mM KNO_3, 1,5 mM K_2SO_4, 1,5mM KH_2PO_4, 4mM $Ca(NO_3)_2$, 1,5mM $MgSO_4$) until fruits ripe. Flowers were regularly vibrated to

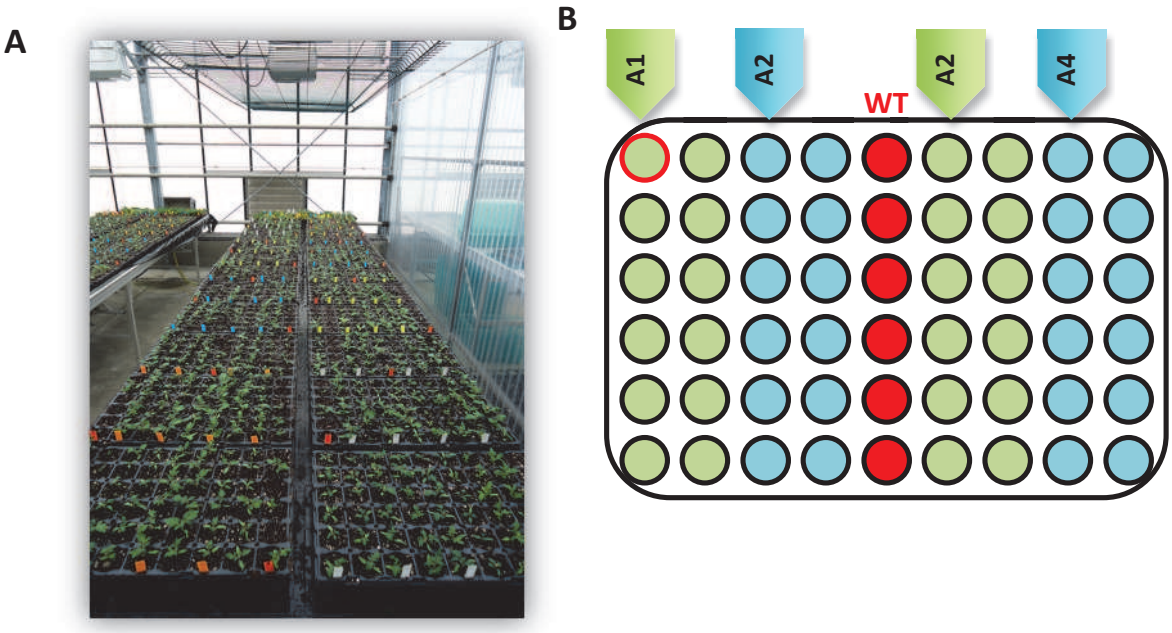


Figure 63: Screening of the EMS tomato mutant collection. **A.** Photo of the P17 culture 3 weeks after sowing. Each black plate contains 54 plants, the whole table contains 24 black plates, in total 1296 plants per table. **B.** Schema of the plant positioning. Around 100 families were sown per culture, which are numbered A to H with A1,A2, A3 to A12. For each family 12 plants were sown and numbered as A1-1, A1-2, A1-3 to A1-12. For example, plant circle in red has as a name: P17A1-1.

ensure optimal self-pollination and therefore normal fruit development. Seeds were collected then thoroughly washed using tap water, dried under air flow and finally stored at 4°C with a desiccant until use.

2. Experiments

i. EMS mutant collection screening

Plant materials

Mutants affected in AsA content were isolated from the EMS mutant tomato (*Solanum lycopersicum*) collection generated in the miniature Micro-Tom cultivar at INRA Bordeaux (FRANCE).

Culture:

500 EMS mutant families were used. 12 plants were sown per families and finally around 6000 plants were cultured (*figure 63*). The 500 families corresponded to five cultures carried out and spread out during 6 months in year 2012. The family name started with P17, P18, P19, P20 and P21 and then a numbered letter which corresponded to ELISA plate format from A1 to H12 (96 in total per plate). For each culture WT were randomised as shown if the figure below. The culture density was around 230 plants per square meter. According to the size of this population P17, P18 and P19 were sown in June 2012 while P20 and P21 were sown July 2012 in order to shift the harvest of red ripe fruits. During the culture, the first mature leaves were removed at two periods just after the flowering stage; this was done to prevent any pathogen problem mainly due to the high density of plant culture that avoids a good air flow.

Sampling:

To collect the samples, three fruits at the red ripe stage have been pooled for each plant and snap frozen in liquid nitrogen before stored at -80°C. The red ripe stage was chosen using visual estimation. The AsA assay of the whole 500 mutants families was carried out between November and January. After harvesting the fruits, plants were cut back to get a new growth. According to the results of the AsA assays, the plants displaying AsA+ and AsA- phenotypes were then kept and re-potted in 1L pot. All the rest of the culture was destroyed. This second growth allowed harvesting a second set of red ripe fruits on the same plant in order to perform a second AsA assay and confirm the first phenotype observed. This confirmation was carried out during spring 2013.

ii. Incubation of leaves in presence of AsA solution

Experiment setting:

Micro-Tom plants were growth in 300mL pots. Once reached the "four-leaves stage", the leaves were harvested and the petiole was bevel cut in order to increase water contact/surface. Only one leaf was harvested per plant to prevent any stress effect due to the cut. Before starting the experiment, the harvested-leaves were soaked in a $MS_{1/4}$ solution during 24H inside a growth chamber (temperature: 22°C- day/18°C- night; photoperiod: 16H-days/8H-night; light intensity 150 microE m-2 sec-1). Thereafter, leaves were incubated in a solution of ascorbate (10mM) in $MS_{1/4}$ or in $MS_{1/4}$ (control) during 30min, 1H, 2H or 6H under light supplied by a sodium lamp. The heat produced by the sodium lamp was enough to induce evapotranspiration of the leaves and then to induce absorption of the solution. To prevent heat stress, a fan was used to homogenize the ambient temperature. The incubation vessels were black eppendorf 2ml tubes in order to protect AsA from light degradation.

Sampling:

Analysis was performed when leaves have been harvested in the greenhouse (T=O), 24H after recovery in the growth chamber (T=24H) and then in the time course of the incubation in AsA or $MS_{1/4}$ solution from T=30 min to T= 6H). For each time, three samples were collected; one leaf corresponds to one sample resulting in three biological replicates for each time. Only the leaflets were snap frozen in liquid nitrogen and stored at -80°C. These sample were used for AsA assays and gene expression analysis.

iii. Light exposure of leaves

Plant materials:

This study was performed on the dwarf Micro-Tom tomato cultivar, with two genetic backgrounds: WT (control) and *ggp2-2* mutant.

Experiment setting:

Micro-Tom plants were grown in 300mL pots. This experiment was performed in summer 2013 during a day of clear sky in order to have homogenous and constant high light intensity. At 6h am, the plants at the "four-leaves stage" are dispatched in three different conditions of light intensities:

- **Control**: The plants stay in the greenhouse where light and temperature are controlled by the climatic program set for the green house.
- **Low light**: The plants are under black net, which reduces by 70% the light intensity.
- **High-light:** The plants are placed outside of the greenhouse. During the day, the temperature at the zenith was around 29°C.

To prevent overlapping of the leaves, a respectable distance was maintained between plants. During the day, leaves have been harvested at 6H, 10H, 14H, 18H and 24H.

Sampling:

For each time, three leaves were harvested, the leaflet was snap frozen in liquid nitrogen and stored at -80°C. Only one leaf has been harvested per plant to prevent stress effect due to the cutting. In these samples, AsA content and gene expression were analysed.

iv. Postharvest analysis

Plant materials

The study was performed on the dwarf Micro-Tom tomato cultivar, with three genetic backgrounds: WT (control), AsA+ (P17C5-3, *p17g9-11* and *p20g7-12* mutants) and AsA- (*ggp2-1* and *ggp2-2* mutants).

Chilling injury:

This experiment has been performed in the frame of the COST STM program during spring 2014 in the Noam Alkan laboratory at the Agricultural Research Organization

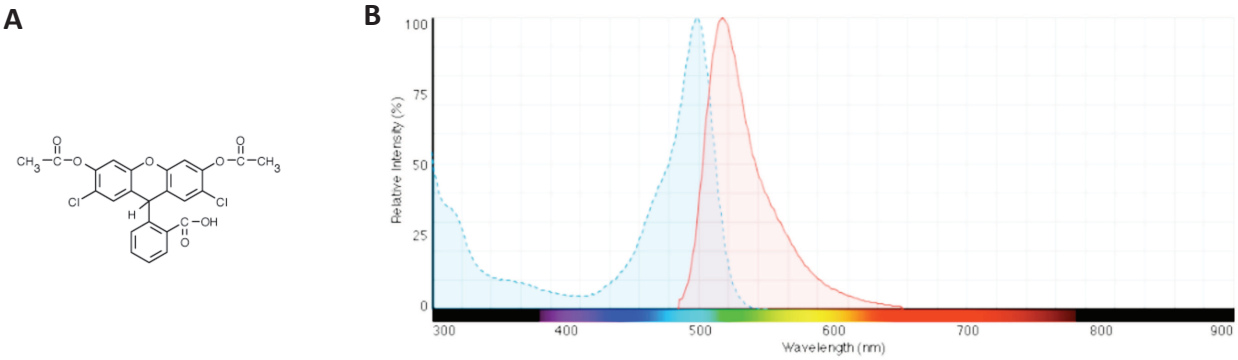


Figure 64: 2'7'-dichlorodihydrofluorescein diacetate. **A.** Chemical structure **B.** Fluorescent spectrum with in bleu excitation (λ=490nm) and red emission (λ=520nm) spectra.

Volcani Center (Israël). A minimum of 15 immature green fruits were harvested for each plants and stored at 2°C in the dark in a cold chamber during 5 weeks and then back to room temperature for two weeks.

Ion leakage assay:

Fruits are soaked in 10mL of double distillated water. After 1hour the electrical conductivity was measured using a conductimeter HI 98311 (Hanna Instruments).

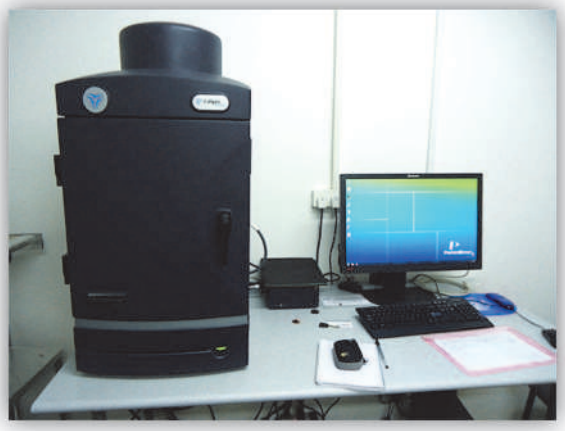
Fungus inoculation:

Two fungus strains were used: *Colletotrichum coccodes* and *Botrytis cinerea*. The cultivation of the fungi was carried out on potato dextrose media (PDA) implemented with chloramphenicol (0,25g/L) at room temperature. When growth is optimum, conidia were harvested by pouring into the Petri dishes 10ml of sterile water and gently dispersing with a small sterile rake. The solution was next filtered with cheese cloth and the concentration was adjusted at 10^6 spores/mL. The inoculation was carried out on red ripe fruits and in the case of *B. cinerea* onto leaves. In order to accelerate the infection, fruit and leaves were previously scratched and a drop of *inoculum* was layed down. During the experimentation to prevent desiccation, fruit and leaves were stored in box containing a sheet of Whatman saturated with water. Every day, the infection area was checked until the fruit or leaves are totally infected.

DCF staining:

The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species in living cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCF-DA is converted to the highly fluorescent 2'7'-dichlorofluorescein (DCF) (*figure 64*).

A



B

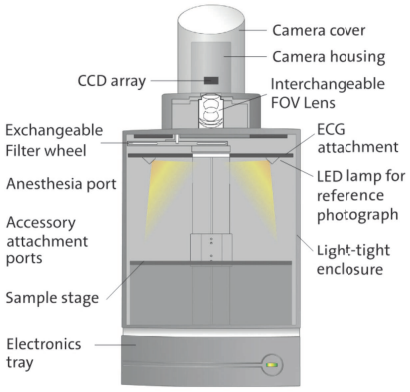


Figure 65: In Vivo Imaging System or IVIS. **A.** Picture of the IVIS Lumina LT from Caliper Life science. **B.** Components of the IVIS.

IVIS Lumina LT - Standard Excitation and Emission Filter Sets

FLUOROPHORES	STANDARD HIGH RESOLUTION EXCITATION FILTER SET (BUILT-IN)	STANDARD EMISSION FILTER SETS
GFP, YFP and PKH26	430 nm, 465 nm, 500 nm, 535 nm, 570 nm, 605 nm, 640 nm, 675 nm, 710 nm, 745 nm	515-575 nm
Cy 5.5, DsRed, tdTomato and VivoTag 680		575-650 nm
Indocyanine Green and VivoTag 750		695-770 nm
Multiple fluorophores Spanning 500-900 nm Broad Imaging Solution		810-875 nm

In this study the ROS study was realised on fruit slices after wounding and leaves inoculated with *Botrytis cinerea*. In that aim, fruits slides and leaves were incubated during 15 min in a solution of DCF (20µM) and then washed twice with double distillated water. This staining needs to be performed in the dark to prevent alteration of the DCF-emission stability. After staining, fluorescence was observed using confocal imaging (fruit slices) or an In Vivo Imaging System (infected leaves).

In Vivo Imaging System or IVIS:

IVIS® Lumina LT from Caliper Life Science is a toolset for non-invasive *in vivo* imaging (*figure 65*). It can be used for the widest range of applications with the highest level of intensity for bioluminescence, fluorescence throughput 2D or tomographic imaging. The system includes a highly sensitive CDD camera, light-tight imaging chamber and complete automation. It was equipped with filters that can be used to image reporters that emit from green to near-InfraRed. Data analysis was performed on a separate computer workstation, using Living Image 4.0 software, which yields high-quality, reproducible, quantitative results incorporating instrument calibration, background subtraction and the image algorithms.

In this study IVIS was used firstly to evaluate the peroxidation of membrane lipids. In that aim, the bioluminescence of fruits was analysed using a "DS red" emission filter (575-650nm). Secondly, IVIS was used to analyse ROS content after DCF staining of leaves infected by *Botrytis cinerea.* To measure DCF fluorescence a combination of 465nm excitation filter and Cy5.5 emission filter with 10 seconds of exposure was used.

Molecular biology analysis

1. RNA extraction

For extracting and handling of RNA, all solutions are treated with DEPC (diethyl pyrocarbonate, Sigma) 0,1% (v/v) or prepared with DEPC-treated water. Additionally, materials is cleaned with chloroform, rinsed with ethanol 100% and sterilized by autoclaving for 20min at 120°C.

The total RNA extraction was performed from 100mf of fresh powder to which is added 1mL of Tri®-Reagent (Sigma). The resulting mixture was vortexed one minute and centrifuged for 10 minutes ar 12,000g at 4°C. The supernatant was transferred to a clean tube and left at room temperature for 5 minutes. 200µL of chloroform are added, vortexed one minute and left another 5 minutes at room temperature. The tubes are then centrifuged 15 minutes at 12,000g at 4°C. Two phases form, the aqueous phase (upper) containing the RNA was recovered (400µL). After addition of 400µL of isopropanol, tubes are inverted several times to mix gently the solution, and left at room temperature for 10 minutes. The RNA were precipitated and recovery by centrifugation, 1à minutes at 12,000g at 4°C. The supernatant is removed and the pellet is washed with ethanol 70%-H20 DEPC. After remove the ethanol, the pellet is air-dried before being taken up in 30µL of water-DEPC.

The quality of RNA was analysed by electrophoresis on agar gel 1,5% (p/v) migrating in TAE 0,5X (TAE 50X, Tris-acetate 2M, EDTA 50mM, pH 8,0). The concentration was estimated using NanoVue system (GE Healthcare) and the ration of absorbance $A_{260/280}$ and $A_{260/230}$ were checked. The possible contamination of RNA with genomic DNA was controlled by PCR using primers specific of *ACTINE* gene. If contamination were detected, elimination was performed with a Turbo DNase treatment according the manufacturer's protocol.

2. Reverse transcription

The synthesis of complementary DNA (cDNA) is performed with reverse transcriptase iScript® according to the manufacturer's protocol (Bio-Rad) from 1µg of total RNA. RNA is mixed with 1µL iScript reverse transcriptase and 4µL of 5X iScript reaction mix to 20µL of final volume. Before start retrotranscription RNA were incubated at 75°C 5 minute of a way to linearized it. The stabilization of RNA/random

primer structure is obtained after 5 minute at 25°C, then cDNA was synthesis during 30 minutes at 42°C. A final step of 5minutes at 85°C allow to denatured the reverse transcriptase before cDNA use. The cDNA quality was check as previously with PCR on *ACTINE* gene.

3. Real Time PCR (RT-PCR)

The RT-PCR was performed in order to evaluate the transcripts accumulation of the 15 candidates genes in the different experiments. The PCR amplification is realized in the presence of SYBR Green, a fluorescent intercalating agent of DNA, which can detect the presence/accumulation of double-stranded nucleic acids in the reaction medium at each PCR cycle. It maximum wavelength of excitation is 497nm while the maximum wavelength of emission is 520 nm. The real-time recording of the emitted fluorescence allows to visualize the exponential phase of the PCR reaction, in which the signal is proportional to the amount of target present in the initial sample. The integration of the fluorescence signal by the iCycler program (Bio-Rad) gives the number of cycles (Ct noted for threshold cycle) for the measured signal is equal to a certain threshold ("Ct threshold"). In his study, threshold value of 300 has been applied to all the genes studied.

The RT-PCR was realised using Kit GoTaq® Master Mix (Promega) on the cDNA obtained previously and diluted of 1/10e. The reaction mix is 25µL final and consists of 2X mater mix (GoTaq® Hot Start polymerase, MgCl2, dNTP, buffer, SyBrGreen), 5µL of cDNA and 10µM of each primer. The amplification reactions were in a thermocycler Bio-Rad iCycler Optical System equipped with a tungsten halogen lamp (excitation range 400 to 700 nm), for applications using SYBR green chemistry. Samples are denaturised 3 minute at 95°C, then amplification was performed for 40 cycles including denaturing cycles (15 seconds at 95°C) follow by initiation/polymerization (25seconds at 60°C). To finish, a step of amplicons dissociation performed between 60°C to 95°C will allow to verify the presence of one or more amplicon species. The amplicon product is follow by the incorporation of SyBrGreen. This RT-PCR does not allow to quantify the number of molecules of target RNA but only compare relative expression between candidate genes.

The primers are designed to amplify the 3'UTR region, which are specific of the gene targeted. The primer efficiency was previously verified firstly with classic PCR and secondly using standard range of 7 serial dilution of cDNA. 100% of efficiency is ideal

and indicates that the number of copy duplicate during each PCR cycle. Furthermore, the fusion curves are used to check the synthesis of a single PCR product and thus the primers specificity. In the *supplemental data 6* as show the list of the primer used, which display an efficieny comprise between 88% and 100%.

The results were processed and standardized according the "GeNorm" method (Manual version 1.4, 2003 10 November). The raw results are the average of Ct values (sample Ct) and the Ct standard deviations (SD sample Ct). For each experimental condition the relative quantity of transcripts (Q) is determined by the following formula: Q=E (min Ct-sample Ct) where E is the efficiency of amplification (estimated at 100%, E=2) and min Ct is the lower Ct value. Then the standard deviation of the relative amount (Q SD) is calculated using formula: SD Q = Q X lnE.SD sample Ct. The relative amounts of the samples are then normalized (qnorm) from the relative amounts of the constitutive genes, in this case endogen genes of *ACTINE* and *eIF4A* according the formula: QNorm = Q/(geometric mean (Q *ACTINE* + Q *eIf4a*)). The standard deviation are calculated from the two endogen genes using the formula:

$$SD\ Q_{Norm} = Q_{Norm} \cdot \sqrt{((SD\ Q / Q)^2 + (SD\ Q_{Actine} / 2.Q_{Actine})^2 + (SD\ Q_{Eif4a} / 2.Q_{Eif4a})^2)}.$$

Biochemical analysis

Standard curve:

A.

Stock solution (µL)	0	10	20	30	40	60
TCA 6% (µL)	200	190	180	170	160	140
Concentration (nmoles)	0	5,05	10,1	15,15	20,2	30,5

B.

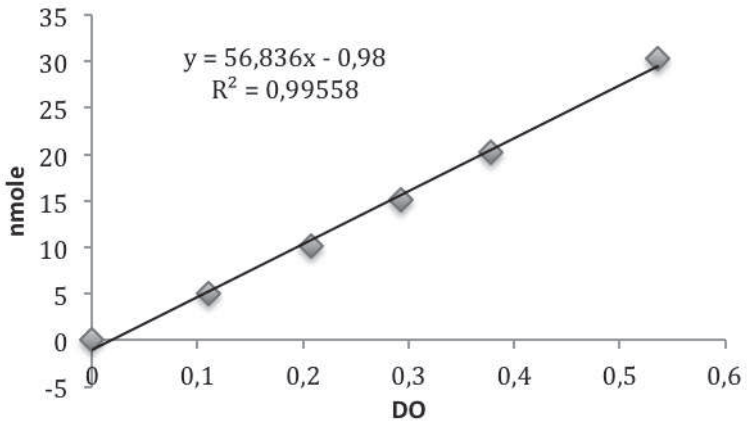


Figure 66: Standard curve. A. Concentration of the six points constituting the standard curve B. Representation of the standard curve.

Solutions:

N-ethylmaleimide (NEM)
Concentration: 0,5%
Solvent: DDW

Dithiothreitol (DTT)
Concentration: 5mM
Solvent: phosphate buffer
0,4M pH: 7,4

Trichloroacetic acid (TCA)
Concentration: 6%
Solvent: DDW

Reagent A
31% **H_3PO_4** (w/v)
4,6% **TCA** (w/v)
0,6% **$FeCL_3$** (w/v)
Solvent: DDW

Reagent B
4% **2,2-dipyridil** (w/v)
Solvent: ethanol 70%

Colour reagent
Proportion:
2,2ml A → 0,8ml B

Ascorbate assay

The ascorbate (AsA) assay was performed according the method described by Stevens *et al.,* (2006). In this study, only the ascorbate total was essayed in order to estimate the pool of AsA in the samples studied.

To evaluate total AsA content, 100mg (fruits) or 30mg (leaves) of fresh power previously stoked at -80°C were extracted using 400µL of Trichloroacetic acid 6% (TCA). After vortexed, samples were centrifuged 25 minutes at 12 000g at 4°C. The supernatant was recovered and ready to assay. The entire extraction step needs to preform 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 mg/mL) was diluted in TCA 6% according the *figure 66.* This range allow to obtain linear standard curve comprise between 0 and 1 of DO_{550nm}.

For each sample, biologic triplicates were analysed. 20µL of supernatant (or standard) were deposed in 96 well plate ELISA and 20µL of dithiothreitol (DTT) 5mM are added to reduced the oxidized AsA. Plate was next incubated 20 minutes at 37°C under gentle stirring. Then, 10µL of N-ethylmaleimide (NEM) 0,5% (w/v) are additional. After one minute at room temperature, 80µL of colour reagent was added and incubated 40 minutes at 37°C. The reduction of the Fe^{3+} contained in the reagent by AsA will lead to the formation of 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 550nm using a Multiscan Ascent lector (Thermo) (*figure 66*).

The concentration of AsA is expressed in $nmoles.mg.FW^{-1}$. 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:

(((DO x a)-b)/ supernatant x TCA)/ fresh powder)

Genomic analysis

General pipeline for NGS-mapping analysis

The NGS mapping for the P21H6-3 mutant was performed as described in the figure .

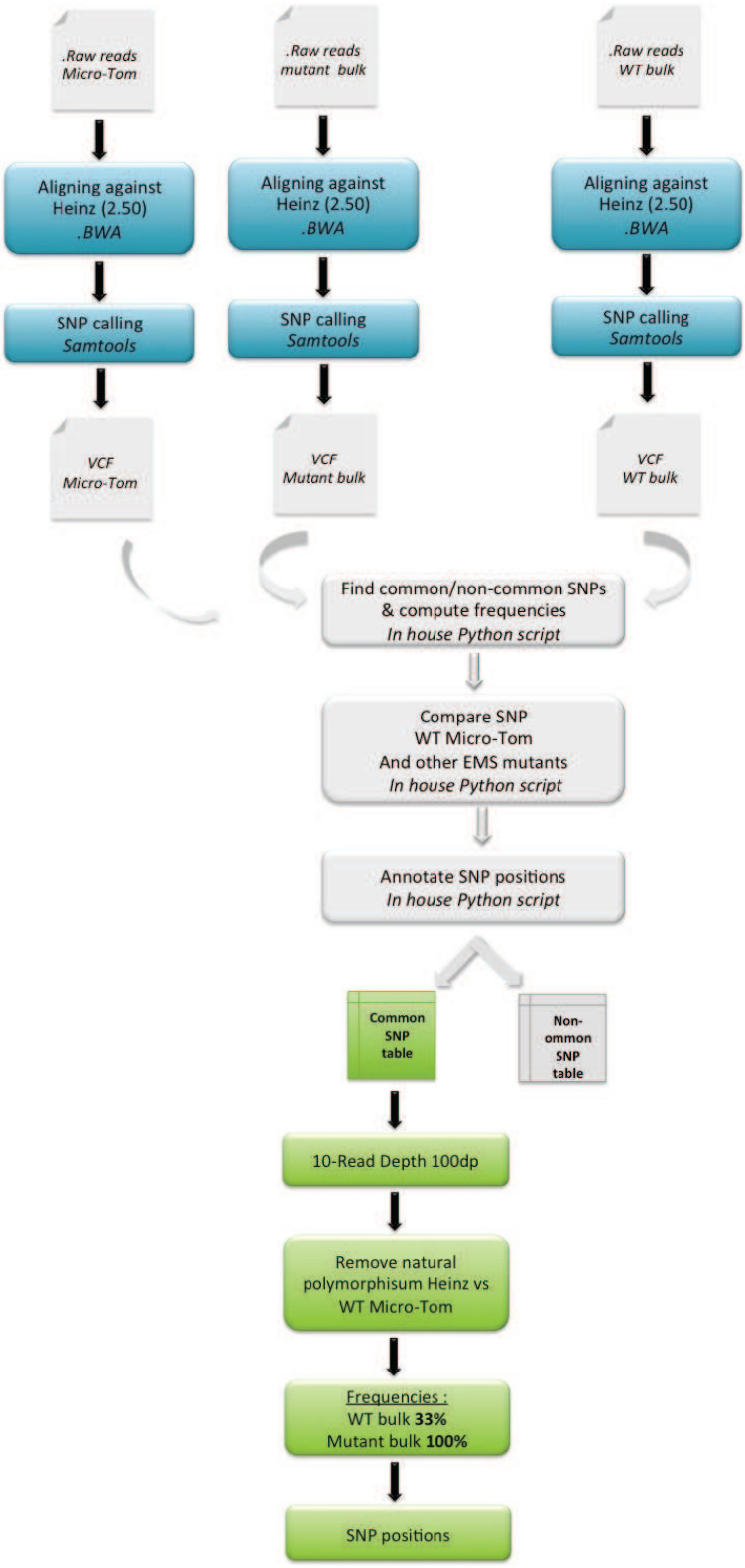
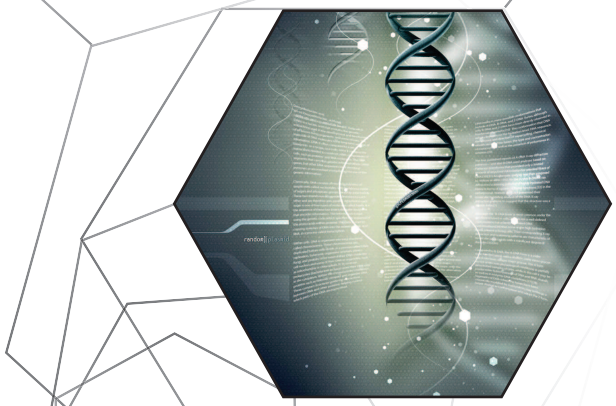
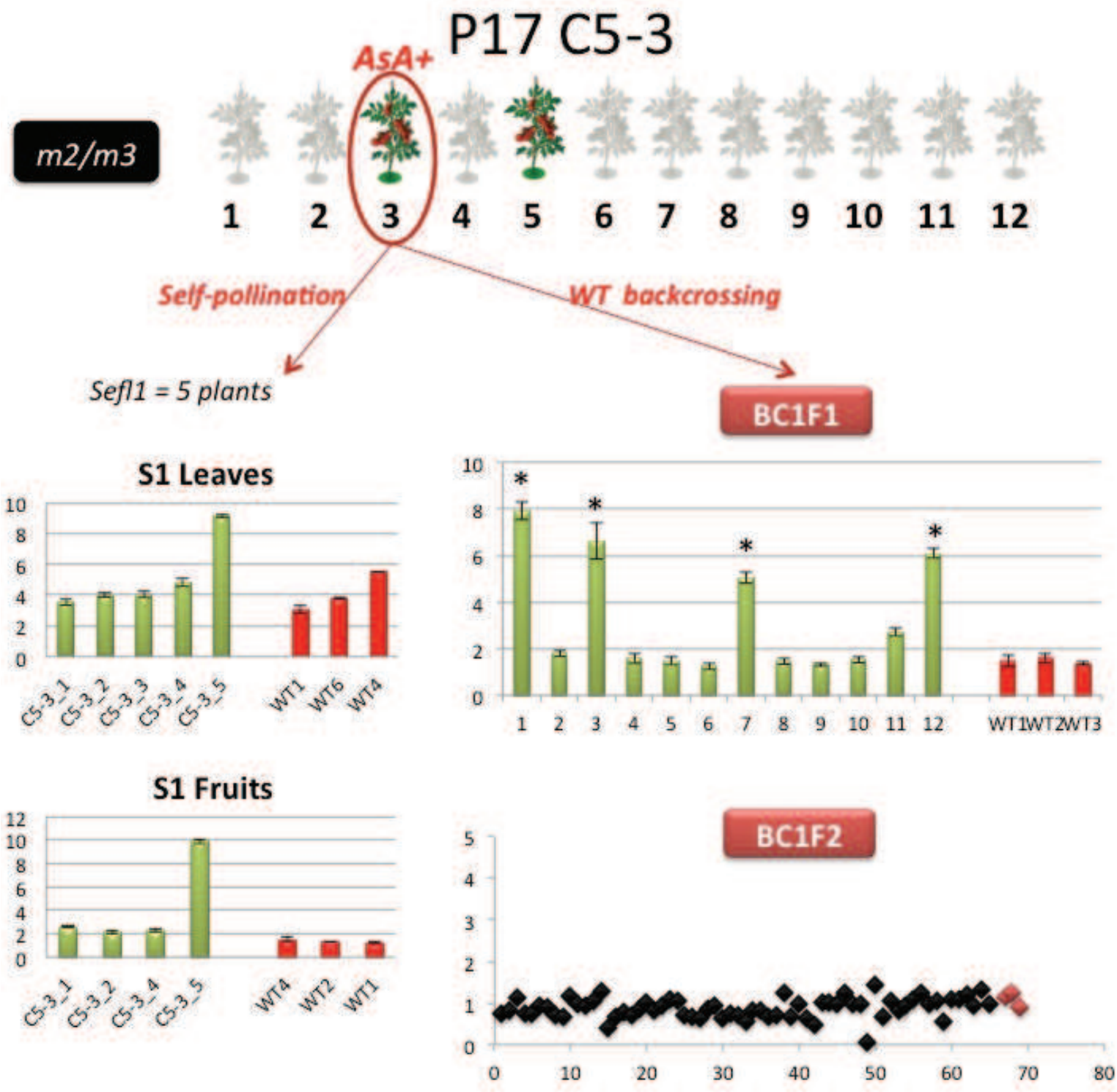


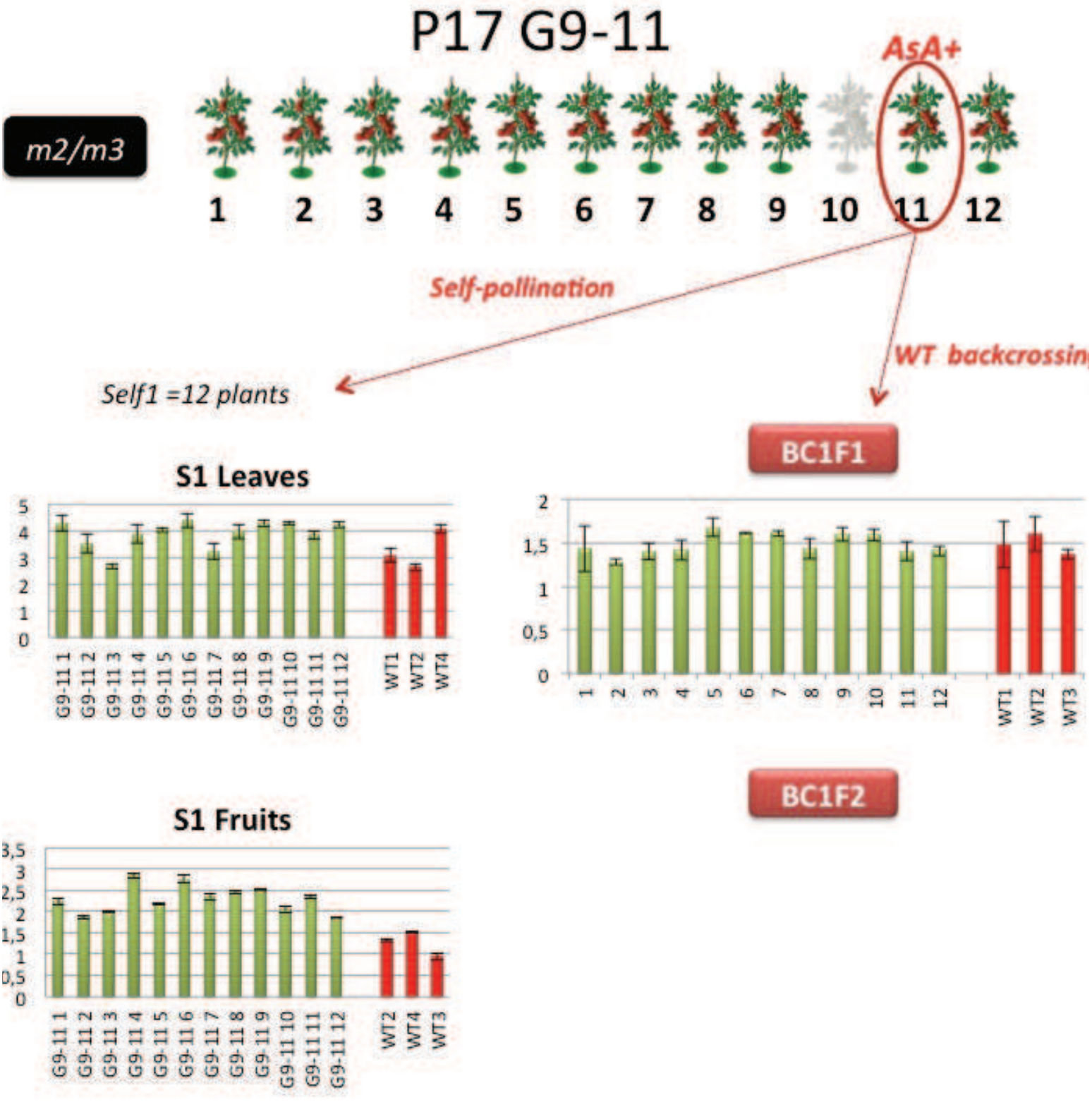
Figure 67: Overview of the bioinformatic workflow used for the NGS-mapping analysis. Firstly, the WT and mutant bulks were sequenced by Next generation Sequencing. The output files were independently aligning on the 2.50 Heinz version reference genome. After aligning, variants detection was performed using *Samtools*. Thus, VCF files were produced for each bulk. The two VCF files were compared to find common and non-common SNPs based on the position, and to estimate their allelic frequencies in the WT and mutant bulks. In the case of a recessive mutation (e.g. *p21h6-3*), the expected frequency in mutant bulk is about 100% while in WT bulk the expected frequency is about 33%. So, only "common SNP" table is studied. Among them, the SNPs displaying a read depth comprises between 10 and 100 were selected. In order to remove SNPs due to natural polymorphism between Micro-Tom and Heinz or sequencing background noise, the SNPs were compared to the ones present in the WT Micro-Tom, and also others EMS mutants sequenced in the laboratory. Finally, only SNPs showing the frequencies expected in the case of a recessive mutation were selected. *In Grey : Bioinformatic pipeline ; Green : Filters to identify causal mutation*

Supplemental

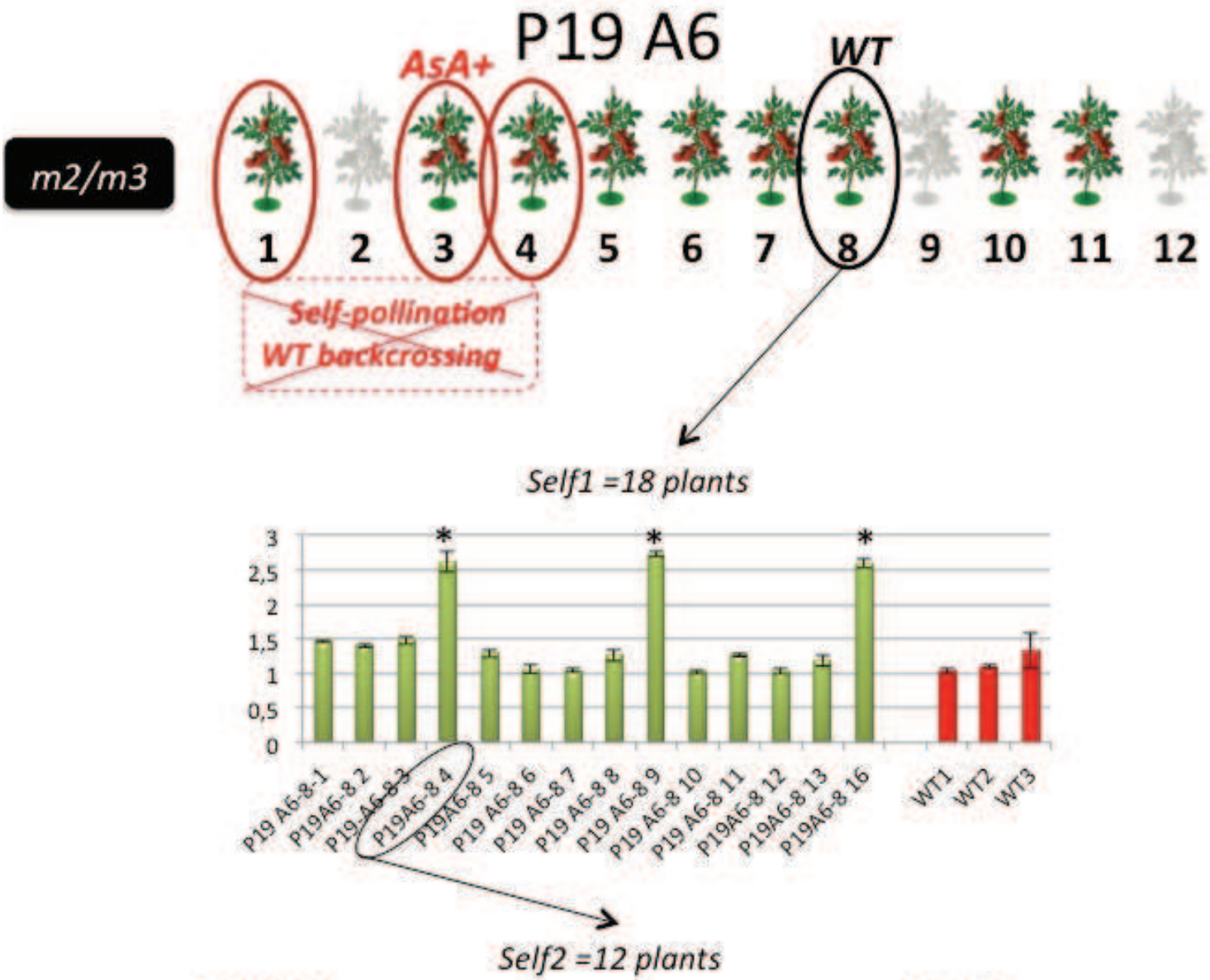


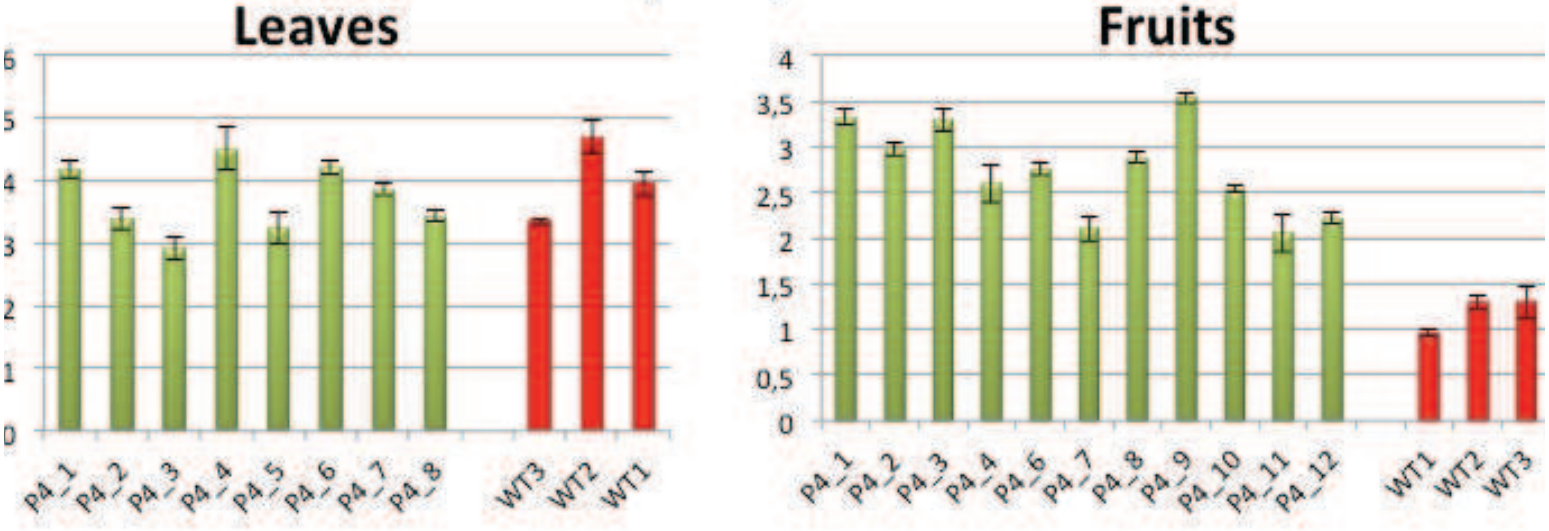


Supplemental data 1 : 12 M2/M3 seeds were sown for the P17C5 mutant family. Among them only two plants were studied (colored). Among the 12 plants, those displaying an absence of fruit or a delayed maturation were not analyzed (grey). Plants displaying a strong AsA+ phenotype (4 nmoles.mg FW^{-1}) have problem to product seeds after self-pollination. From P17C5-3, 5 progenies (S1) were analyzed in self1 generation in leaf and fruits. The P17C5-3 plant was back-crossed with the WT parent. In the BC1F1 generation around 50% of the plants (*) display an AsA+ phenotype associated with fruit parthenocarpy (fruit analysis). The BC1F1 WT-like plants were used to generate a BC1F2 segregant population as their fruits have seeds. No AsA+ phenotype was found in this BC1F2 population (fruit analysis). The P17C5-3 mutation seems to be associated with the parthenocarpic phenotype. For each assay three fruits/leafs are pooled and analyzed in technical triplicate. Leaves were harvested at the "four leaves stages". The AsA values were expressed in nmoles.mg FW^{-1} (± SD).

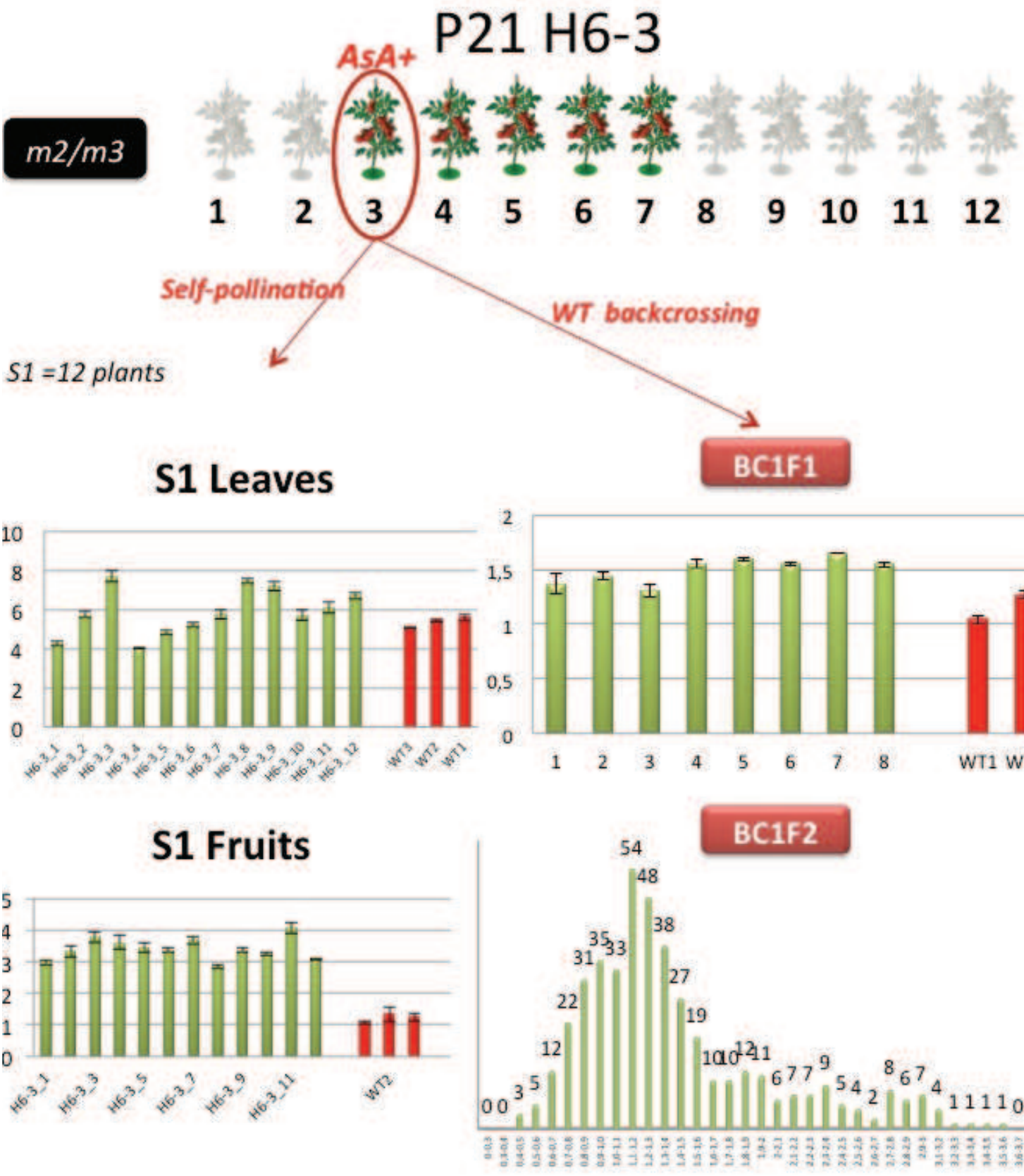


Supplemental data 2 : 12 M2/M3 seeds were sown for the P17G9 mutant family. Among them only 11 plants were studied (colored). Among the 12 plants, those displaying an absence of fruit or a delayed maturation were not analyzed (grey). Plant P17G9-11 displaying a strong AsA+ phenotype (2-3 nmoles.mg FW^{-1}) was isolated. 12 progenies from P17G9-11 were analyzed in self1 generation for leaf and fruits. The P17G9-11 plant was back-crossed with WT parent. In the BC1F1 generation all the plants show a WT-like phenotype (fruit analysis). The BC1F2 segregate for the mutation in 1:3 ratio (16 plants analyzed for fruits). The P17G9-11 mutation seems to associated with a single locus. For each assay three fruits/leafs are pooled and analyzed in technical triplicate. Leaves were harvested at the "four leaves stages". AsA values were expressed in nmoles.mg FW^{-1} (± SD).

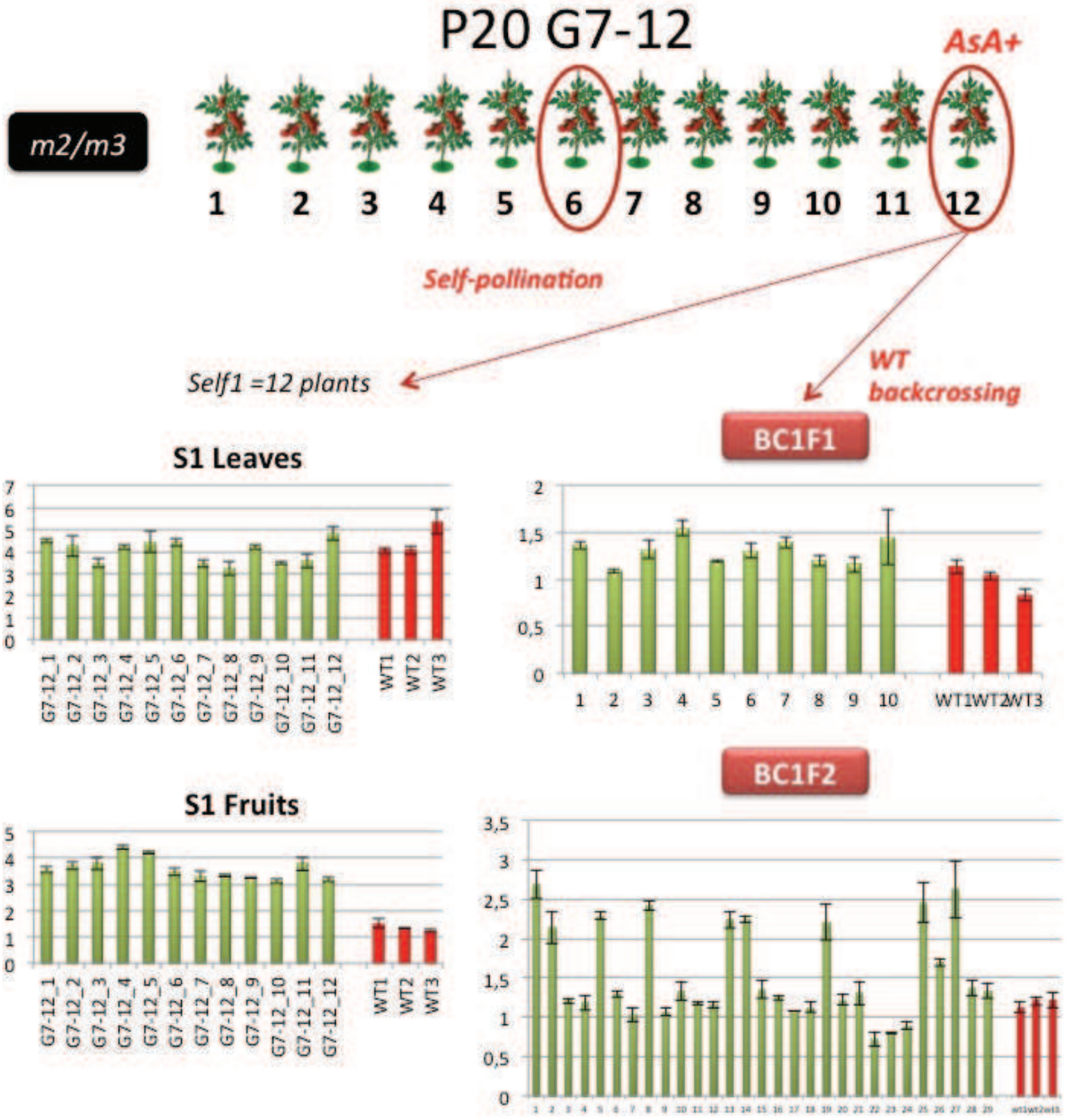




Supplemental data 3 : 12 M2/M3 seeds were sown for the P19A6 mutant family. Among them only 9 plants were studied (colored). Among 12 plants, those displaying an absence of fruit or a delayed maturation were not analyzed (grey). Three plants displaying a strong AsA+ phenotype (4 to 5 nmoles.mg FW^{-1}) presented fruit parthenocarpic phenotypes (mutant 1, 3 and 4). One mutant (mutant 8) displaying a WT phenotype was selected with assumption that it was heterozygous for the mutation. 18 seeds from P19A6-8 were sown in self1 generation. Among three displaying an AsA+ phenotype, two produced parthenocarpic fruits. For the plant remaining, 12 self1 were sown and all of them were AsA+ in fruit suggesting an recessive mutation. For each assay three fruits/leafs are pooled and analyzed in technical triplicate. Leaves were harvested at the "four leaves stages". AsA values were expressed in nmoles.mg FW^{-1} (± SD).



Supplemental data 4 : 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 3 displaying a strong AsA+ phenotype (4 nmoles.mg FW^{-1}) was isolated. 12 progenies from P21H6-3 were analyzed in self1 generation in leaf and fruits. The P21H6-3 mutant was back-crossed with WT parent. In the BC1F1 generation all the plants showed a WT-like phenotype (fruit analysis). The BC1F2 segregate for the mutation in 1:3 ratio (440 plants analyzed for fruits). The P20H6-3 mutation seems to be associated with a single locus. For each assay three fruits/leafs are pooled and analyzed in technical triplicate. Leaves were harvested at the "four leaves stages". AsA values were expressed in nmoles.mg FW^{-1} (± SD).

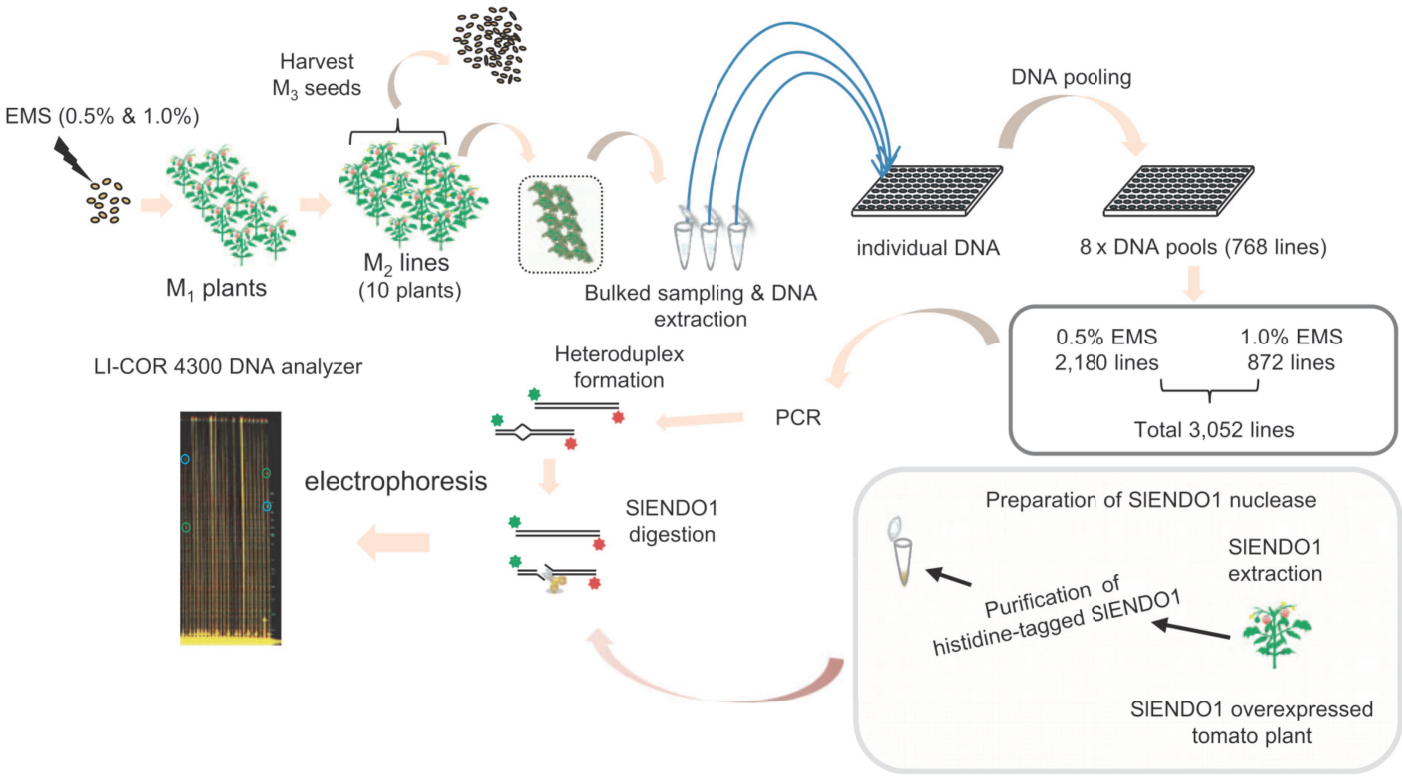


Supplemental data 5 : 12 M2/M3 seeds were sown for the P20G7 mutant family. Among them only 11 plants were studied (colored). Among 12 plants, those displaying an absence of fruit or a delayed maturation were not analyzed (grey). Mutants (6, 12) displaying a strong AsA+ phenotype (3-4 nmoles.mg FW^{-1}) were isolated. 12 progenies from P20G7-12 were analyzed in self1 generation in leaf and fruits. The P20G7-12 plant was back-crossed with WT parent. In the BC1F1 generation all the plants showed a WT-like phenotype (fruit analysis). The BC1F2 segregate for the mutation in 1:3 ratio (29 plants analyzed for fruits). The P20G7-12 mutation seems to be associated with a single locus. For each assay three fruits/leafs are pooled and analyzed in technical triplicate. Leaves were harvested at the "four leaves stages". AsA values were expressed in nmoles.mg FW^{-1} (± SD).

Name	*Primer Sequence (5'→3')*	*Accession number*
ARf-qF1	TCATACGCTGCACCTCCTCT	SGN-U579928
ARF-qR1	TGAGGGTGACATGATGCTTG	
ACT-qF1	GGACTCTGGTGATGGTGTTAG	SGN-U579547
ACT-qR1	CCGTTCAGCAGTAGTGGTG	
bHLH.1-qF1	GCCTACAAAGGTTGCCTTCA	SGN-U575250
bHLH.1-qR1	TGCAGTAAGGGGAGTTGAGC	
bZIP.1-qF1	ATCTTTTCCCATAGGTGGATCA	SGN-U580933
bZIP.1-qR1	TCCTTCAGATGTCAAAGCAAGA	
bZIP.2-qF1	AACAGGTGCTCCTTGTATCCTC	SGN-U568869
bZIP.2-qR1	CATAAAGGAGAACTTCGGGAAA	
bZIP.3-qF1	TCAGAAACAAAAGCTACATTGCTAA	SGN-U567159
bZIP.3-qR1	TCAGAAACAAAAGCTACATTGCTAA	
C2H2.1-qF1	CCAATTCTCCAAACCCTAACC	SGN-U569960
C2H2.1-qR1	AGCTTCAATCGACATCGAGAA	
C2H2.3-qF1	CTTATCCTCTTAGGACTTGGCTAGAA	SGN-U572337
C2H2.3-qR1	AAAGAAATCCGATCAATTAAGGTAAA	
ERF.1-qF1	CCCACACCAAAACCAAAACTA	SGN-U584756
ERF.1-qR1	CAAGAATTGCACCACCACAC	
eiF4-qF1	AGTGGACGATTTGGAAGGAAG	SGN-U578071
eiF4-qR1	GCTCCTCGATTACGACGTTG	
Hbox.1-qF1	TCGCTAAACAAGAACATGATGAA	SGN-U581431
Hbox.1-qR1	TTGTTTCTTGATTCTTTACACTACCA	
Unk.1-qF1	CCGTCGGGTCAAATAAGAAA	SGN-U581277
Unk.1-qR1	GCAGATCAACCGACAAATCA	

Unk.2-qF1	AGGTTTTTCAGATGAATGCAGAG	SGN-U579676
Unk.2-qR1	AAACAGACAGCTGACCAGAACA	
Unk.3-qF1	ACGACGTTCAAATTTCCAGTG	SGN-U570562
Unk.3-qR1	TGTCCCACAAAAACTCACAATC	
Unk.4-qF1	GGGAATACTCCAAGGCAACA	SGN-U570009
Unk.4-qR1	TGAGCCATGATGAGCCACTA	
WRKY.1-qF1	TGAACCTGAAATCACATTTGACA	SGN-U577434
WRKY.1-qR1	TTGCACAAAACTTACTTCCTGCT	
WRKY.2-qF1	TTGCAGCATTAGTAGCATCAGG	SGN-U570041
WRKY.2-qR1	GGACCAAGCATGTGGTTTTC	

Supplemental data 6: Sets of RT-PCR primers used to amplify candidates genes and references genes with the corresponding accession number



Supplemental data 7: Outline of Micro-Tom TILLING platform. Seeds were mutagenized two times with 0.5 or 1.0% EMS. To avoid ambiguities caused by chimerism of mutant plants in the M1 generation, plants were self-fertilized. The M2 progeny of 10 seeds per line was used for screening. The DNA pool and M3 seeds were collected from each M2 line (10 plants/line). Several 8x DNA pools were used for the screening. PCR was performed using 5'end-labeled gene-specific primers to target the desired region, and hetero-duplexes were formed by heating and cooling the PCR products. Recombinant SlENDO1 nuclease was used to cleave base mismatches, and products that represent induced mutations were visualized by denaturing PAGE as fluorescent spots (blue and green circles). To detect the mutation in the corresponding 8x DNA pool which contains several mutants DNA, a deconvolution was performed using the same PCR procedure with individual DNA/family in order to identify the family harbouring the mutation of interest. Recombinant SlENDO1 was extracted and purified from SlENDO1-overexpressing transgenic tomato plants (According to Okabe *et al.,* 2011).

References

Abe A, Kosugi S, Yoshida K, Natsume S, Takagi H, Kanzaki H, Matsumura H, Yoshida K, Mitsuoka C, Tamiru M, Innan H, Cano L, Kamoun S, Terauchi R (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. *Nat Biotechnol* **30:**174-178

Adisa V.A (1986). The influence of molds and some storage factors on the ascorbic acid content of orange and pineapple fruits. *Food Chem.* **22:** 139-146

Agar, I.T., Massantini, R., Hess-Pierce, B., Kader, A.A., (1999). Postharvest CO2 and ethylene production and quality maintenance of fresh-cut kiwifruit slices. *J. Food Sci.* **64:** 433-440

Agius F, Gonzalez-Lamothe R, Caballero JL, Munoz-Blanco J, Botella MA, Valpuesta V. (2003). Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nature Biotechnology* **21:** 177-181

Aharoni A, Keizer LC, Van Den Broeck HC, Blanco-Portales R, Muñoz-Blanco J, Bois G, Smit P, De Vos RC, O'Connell AP. (2002). Novel insight into vascular, stress, and auxin-dependent and -independent gene expression programs in strawberry, a non-climacteric fruit. *Plant Physiol* **129**(3): 1019-31

Aharoni A,Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004). The SHINE clade of AP2 domain transcription factors activates wax biosyn- thesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. *Plant Cell* **16:** 2463–2480

Aharoni A, Leopold C. P. Keizer, Hetty C. van den Broeck, Gregory Bois, Patrick Smit, Ric C. H. De Vos and Ann P. O'Connell (2002). Novel Insight into Vascular, Stress and Auxin Dependent and Independent Gene Expression Programs in Strawberry, a Non-Climacteric Fruit. *Plant Physiology* **129:** 1019-103

Ahn T, Schofield A, Gopinadhan P. (2002). Changes in antioxidant enzyme activities during tomato fruit development. *Physiology and Molecular Biology of Plants* **8:** 241–249.

Alhagdow M, Mounet F, Gilbert L, Nunes-Nesi A, Garcia V, Just D, Petit J, Beauvoit B, Fernie AR, Rothan C, Baldet P. (2007). Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase affects plant and fruit development in tomato. *Plant Physiology* **145:** 1408-1422

Alimohammadi M, De Silva K, Ballu C, Ali N, Khodakovskaya M (2011). Reduction of inositol (1,4,5)–trisphosphate affects the overall phosphoinositol pathway and leads to modifications in light signalling and secondary metabolism in tomato plants. *Journal of Experimental Botany* **63:** 825-835

Allen R, Nakasugi K, Doran R, L Millar R, Waterhouse A, Peter M. (2013). Facile mutant identification via a single parental backcross method and application of whole genome sequencing based mapping pipelines. *Frontiers in plant science* **4:** 362

Alves M.S, Dadalto S.P, Goncalves A.B, Souza G.B, Barros V.A, Fietto L.G. (2014).Transcription Factor Functional Protein-Protein Interactions in Plant Defense Responses. *Proteomes* **2:** 85-106

Anderson JW, Foyer CH, Walker DA. (1983). Light-dependent reduction of dehydroascorbate and uptake of exogenous ascorbate by spinach chloroplasts. *Planta* **158:** 442-450

Antonius G, Hawkins L, Kochhar T, (2003). Foliar phenolic variation in wild tomato accessions. *Bulletin of Environmental Contamination and Toxicology* **70:** 9-16

Aoki,K., Y.Ogata, K.Igarashi, K.Yano, H.Nagasaki, E.Kaminuma and A.Toyoda (2013) Functional genomics of tomato in a post- genome-sequencing phase. *Breed. Sci.* **63:** 14–20

Apel K. and Hirt H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* **55:** 373-399

Arrigoni O, Clabrese G, De Gara L, Bitonti MB, Liso R. (1997). Correlation between changes in cell ascorbate and growth of Lupinus albus seedling. *Journal of Plant Physiology* **150:** 302-308

Arrigoni O, De Tullio MC. (2000). The role of ascorbic acid in cell metabolism: between gene-directed functions and unpredictable chemical reactions. *Journal of Plant Physiology* **157:** 481-488

Arrigoni O, De Tullio MC. (2002). Ascorbic acid: much more than just an antioxidant. *Biochimica et Biophysica Acta* **1569:** 1-9

Asada K (1994) Production and action of active oxygen in photo- synthetic tissue. *In* CH Foyer, PM Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. *CRC, Boca Raton*, FL, pp 77-104

Asada K. (1999). THE WATER-WATER CYCLE IN CHLOROPLASTS: Scavenging of Active Oxygens and Dissipation of Excess *Photons Annual Review of Plant Physiology and Plant Molecular Biology* **50:** 601-639

Augustin, J., (1975). Variations in the nutritional composition of fresh potatoes*. J. Food Sci.* **40:** 1295–1299

Austin R, Vidaurre D, Stamatiou G, Breit R, Provart N, Bonetta D, Zhang J, Fung P, Gong Y, W.Wang P, McCourt P , S.Guttman D. (2011). Next-generation mapping of Arabidopsis genes. *The plant journal* **4:** 715-25

Badejo A and Tanaka N. (2008). Analysis of GDP- D -Mannose Pyrophosphorylase Gene Promoter from Acerola (Malpighia glabra) and Increase in Ascorbate Content of Transgenic Tobacco Expressing the Acerola Gene. *Plant Cell Physiol* **49:** 126-132

Badejo A, Wada K, Gao Y, Maruta T, Sawa Y, Shigeoka S, Ishikawa T. (2011). Translocation and the alternative D-galacturonate pathway contribute to increasing the ascorbate level in ripening tomato fruits together with the D-mannose/L-galactose pathway. *Journal of Experimental Botany* **63:** 229-239

Baldet P, Bres C, Okabe Y, Mauxion J.P, Just D, Bournonville C, Ferrand C, Mori K, Ezura H, Rothan C. (2013). Investigating the role of vitamin C in tomato through TILLING identification of ascorbate-deficient tomato mutants. *Plant Biotechnology* **63:** 229-239

Bangerth F. (1976). Relationship between calcium content and the content of ascorbic acid in apple, pear and tomato fruits. *Qual. Plant.* **26:** 341-348

Barth C, Moeder W, Klessig DF, Conklin PL. (2004). The timing of senescence and response to pathogens is altered in the ascorbate-deficient Arabidopsis mutant vitamin c-1. *Plant Physiology* **134:** 1784-1792

Barth C, De Tullio M, Conklin PL. (2006). The role of ascorbic acid in the control of flowering time and the onset of senescence. *J. Exp. Bot.* **57:** 1657–1665

Bartoli CG, Pastori GM, Foyer CH. (2000). Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiology* **123:** 335-343

Bartoli CG, Yu J, Gomez F, Fernandez L, Mcintosh L, Foyer Christine (2006). Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in Arabidopsis thaliana leaves. *J. Exp. Bot.* **8:** 1621-1631

Bartoli CG, Tambussi EA, Diego F, Foyer CH. (2009). Control of ascorbic acid synthesis and accumulation and glutathione by the incident light red/far red ratio in Phaseolus vulgaris leaves. *FEBS Letters* **583:** 118-122

Batini P, Ederli L, Pasqualini S, Antonielli M, Valenti V .(1995). Ef- fects of ethylenediurea and ozone in detoxificant ascorbic-as- corbate peroxidase system in tobacco plants. *Plant Physiol. Bio- chem.* **33:** 717–723

Bayoumi Y. (2008). Improvement of postharvest keeping quality of white pepper fruits (Capsicum annuum, L.) by hydrogen peroxide treatment under storage conditions. *Acta Biolica Szegediensis* **52:** 7-15

Beck E, Burkert A, Hofmann M. (1983). Uptake of L-Ascorbate by Intact Spinach Chloroplasts. *Plant Physiology* **73:** 41-45

Beckles D. (2011). Factors affecting the postharvest soluble solids and sugar content of tomato (Solanum lycopersicum L.) fruit. *Postharvest Biology and Technology* **63:** 129-140

Batini P, Ederli L, Pasqualini S, Antonielli M, Valenti V. (1995). Ef- fects of ethylenediurea and ozone in detoxificant ascorbic-as- corbate peroxidase system in tobacco plants. *Plant Physiol. Bio- chem.* **33:** 717–723

Bernier, G., Havelange, A., Houssa, C., Petitjean, A., and Lejeune, P. (1993). Physiological signals that induce flowering. *Plant Cell* **5:** 1147–1155

Bertin N. (2005) Analysis of the tomato fruit growth response to temperature and plant fruit load in relation to cell division, cell expansion and DNA endoreduplication. *Annals of Botany* **95:** 439–447

Biedinger, U., Youngman, R.J., Schnabl, H., (1990). Differential effects of electrofusion and elctropermeabilization parame- ters on the membrane integrity of plant protoplasts. *Planta* **180**: 598 — 602

Bielski BHJ (1982). Chemistry of ascorbic acid radicals. *In* PA Seib, BM Tolbert, eds, Ascorbic Acid: Chemistry, Metabolism, and Uses. *American Chemical Society* pp 81-100

Blazquez M, Soowal L, Lee I, Weigel D. (1983). LEAFY expression and flower initiation in Arabidopsis **3844:** 3835-3844

Botella-Pavia P, Besumbes O, Philips M, Carretero-Paulet L, Boronat A, Rodriguez-Concepcion M. (2004). Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. *Plant Journal* **40:** 188-199

Bourdon M, Frangne N, Mathieu-Rivet E, et al. (2010). Endoreduplication and growth of fleshy fruits. *Progress in Botany* **71**: 101–132

Briggs W and Christie J (2002). Phototropins 1 and 2: versatile plant blue-light receptors. *Trends in Plant Science* **7:** 1360-1385

Brown PM, Caradoc-Davies TT, Dickson JM, Cooper GJ, Loomes KM, Baker EN. (2006). Crystal structure of a substrate complex of myo-inositol oxygenase, a di-iron oxygenase with a key role in inositol metabolism. *Proc Natl Acad Sci USA* **103**: 15032–15037

Buchanan-Wollaston, V. (1997). The molecular biology of leaf senescence. *J. Exp. Bot*. 48: 181-199

Bulley SM, Rassam M, Hoser D, Otto W, Schunemann N, Wright M, MacRae E, Gleave A, Laing W. (2009). Gene expression studies in kiwifruit and gene over-expression in Arabidopsis indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis. *Journal of Experimental Botany* **60:** 765-778

Bulley S, Wright M, Rommens C, Yan H et al. (2012). Enhancing ascorbate in fruits and tubers trough over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase. *Plant Biotechnology* **10:** 390-397

Buta, J. G., & Spaulding, D. W. (1997). Endogenous levels of phenolics in tomato fruit during growth and maturation. *Journal of Plant Growth and Regulation* **16:** 43–46

Buttani V, Cao Z, Losi A, Gartner. (2008). A Blue Light Inducible Two-Component Signal Transduction System in the Plant Pathogen *Pseudomonas syringae pv*. tomato. *Biophysical Journal* **94**: 897-905

Cabello J.V, Lodeyro A.F, Zurbriggen MD. (2014). Novel perspectives for the engineering of abiotic stress tolerance. *Nature Protocols* **9**(3): 622

Cano A, Acosta M, Arno M.B. (2003). Hydrophilic and lipophilic antioxidant activity changes during on-vine ripening of tomatoes (*Lycopersicon esculentum* Mill.) *Postharvest Biology and Technology* **28**: 59-65

Caris-Veyrat, C., Amiot, M.J., Tyssandier, V., Grasselly, D., Buret, M., Mikolajczak, M., Guilland, J.C., Bouteloup-Demange, C., and Borel, P. (2004). Influence of organic versus conventional agricultural practice on the antioxidant microconstituent content of tomatoes and derived purees; consequences on antioxidant plasma status in humans. *J. Agric. Food Chem.* **52:** 6503-6509

Carrari F, Baxter C, Usadel B, Urbanczyk-Wochniak E, Zanor MI, Nunes-Nesi A, Nikiforova V, Centero D, Ratzka A, Pauly M, Sweetlove LJ, Fernie AR. (2006). Integrated analysis of metabolite and transcript levels reveals the metabolic shifts that underlie tomato fruit development and highlight regulatory aspects of metabolic network behavior. *Plant Physiology* **142:** 1380-1396

Casano LM, Martin M, Sabater B. (1994). Sensitivity to superoxide dismutase transcript levels and activities to oxidative stress is lower in mature-senescent than in young barley leaves. *Plant Physiol* **106:** 1033-1039

Causse M, Lecompte L, Baffert N, Duffé P, Hospital F. (2001). Marker-assisted selection for the transfer of QTLs controlling fruit quality traits into tomato elite lines. *Acta Hortic.* **546:** 557-564

Chandra H, Shanmugaraj B, Srinivasan B, Ramalingam S. (2012). Influence of Genotypic Variations on Antioxidant Properties in Different Fractions of Tomato. *Journal of Food Science* **77**

Chen Z, Young TE, Ling J, Chang SC, Gallie DR. (2003). Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 3525-3530

Chen L, Song Y, Li S, Zhang L, Zou C, Yu D. (2012). The role of WRKY transcription factors in plant abiotic stresses. *Article in press*

Cheng P, He Q, Yang Y, Wang L, Liu Y. (2003). **Functional conservation of light, oxygen, or voltage domains in light sensing**. *PNAS,* **100:** 10

Chevalier C. (2007). Cell size control and fruit development. *Annual Plant Reviews* **32:** 269–290

Christie, J.M., Reymond, P., Powell, G.K., Bernasconi, P., Raibekas,A.A., Liscum, E. and Briggs, W.R. (1998) Arabidopsis NPH1: a flavoprotein with the properties of a photoreceptor for photo- tropism. *Science* **282**: 1698–1701

Christie J, Swartz T, Bogomolni R, Briggs W (2002). Phototropin LOV domains exhibit distinct roles in regulating photoreceptor function. *The Plant Journal* **32:** 205-219

Citterio S, Sgorbati S, Scippa S, Sparvoli E. (1994). Ascorbic-acid effect on the onset of cell-proliferation in pea root. *Annual Meeting Abstracts of Scientific Paper,* **92:** 601-607.

Collins, N. C., F. Tardieu, and R. Tuberosa. (2008). Quantitative trait loci and crop performance under abiotic stress: Where do we stand? *Plant Physiology* **147:** 469–486

Colville L, Smirnoff N. (2008). Antioxidant status, peroxidase activity, and PR protein transcript levels in ascorbate-deficient Arabidopsis thaliana vtc mutants. *Journal of Experimental Botany* **59:** 3857-3868

Conklin PL, Pallanca JE, Last RL, Smirnoff N. (1997). L-ascorbic acid metabolism in the ascorbate-deficient Arabidopsis mutant vtc1. *Plant Physiology* **115:** 1277-1285

Conklin PL, Norris SR, Wheeler GL, Williams EH, Smirnoff N, Last RL. (1999). Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 4198-4203

Conklin PL, Barth C. (2004). Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant, Cell and Environment* **27**: 959-970

Cooper J.B, Heuser J.E, and Varner J.E. (1994). 3,4-Dehydroproline inhibits cell wall assembly and cell division in tobacco protoplasts. Plant Physiol. **104:** 747-752

Cooper, J.L. et al. (2008). TILLING to detect induced mutations in soyben. BMC Plant Biology **8:** 9

Córdoba-Pedregosa MdC, Córdoba F, Villalba JM, González-Reyes JA. (2003). Zonal changes in ascorbate and hydrogen peroxide contents, peroxidase, and ascorbate- related enzyme activities in onion roots. *Plant Physiology* **131:** 697-706

Crosson S, Rajagopal S, Moffat K. (2003). The LOV domain family: photo- responsive signaling modules coupled to diverse output domains. *Biochemistry* **42:** 2–10

Daruwala, R., Song, J., Koh, W.S., Rumsey, S.C., and Levine, M. (1999). Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. *FEBS Lett.* **460:** 480–484

Davey MW, Persiau Ge´stergaard J, Gilot C, Han Y, Bauw G, Van Montagu M. (1999). Ascorbate biosynthesis in Arabidopsis cell suspension culture. *Plant Physiology* **121:** 535–544

Davey MW, Van Montagu M, Inze D, Sanmartin M, Kanellis A, Smirnoff N, Benzie IJJ, Strain JJ, Favell D, Fletcher J, et al .(2000). Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. *J. Sci. Food Agric.* **80:** 825–860

Davey MW, Keulemans J. (2004). Determining the potential to breed for enhanced antioxidant status in Malus: mean inter- and intravarietal fruit vitamin C and glutathione contents at harvest and their evolution during storage. *J. Agric. Food Chem.* **52:** 8031–8038

De Boer, J.M., Borm, T.J.A., Jesse, T., Brugmans, B., Tang, X., Bryan, G.J., Bakker, J., van Eck, H.J. and Visser, R.G.F. (2011). A hybrid BAC physical map of potato: a framework for sequencing a heterozygous genome. *BMC Genomics* **12:** 594

De Cabo RC, Gonza´lez-Reyes JA, Co´rdoba F, Navas P. (1996). Rooting hastened in onions by ascorbate and ascorbate free radical. *Journal of Plant Growth Regulation* **15:** 53–56

De Koning H, Diallinas G. (2000). Nucleobase transporters. *Mol. Membr.. Biol.* **17:** 75–94

De Tullio MC, Paciola C, Dalla Vechia F, Rascio N, D'Emerico S, De Gara L, Liso R, Arrigoni O. (1999). Changes in onion root development induced by the inhibition of peptidyl-prolyl hydrox- ylase and influence of ascorbate system on cell division and elongation. *Planta* **109:** 424–434

De Tullio MC, Ciraci S, Liso R, Arrigoni O. (2007). Ascorbic acid oxidase is dynamically regulated by light and oxygen. A tool for oxygen management in plants? *Journal of Plant Physiology,* **164:** 39-46

DeLong, J.M. and Prange, R.K. (2003). "Superficial scald - A postharvest oxidative stress disorder.", in Hodges, D.M. (ed.) - Postharvest Oxidative Stress in Horticultural Crops, *Haworth Press*, Inc, pp. 91-112

Demarsy E, Fankhauser C. (2009). Higher plants use LOV to perceive blue light. *Curr Opin Plant Biol.* **12:** 69–74

Dowdle J, Ishikawa T, Gatzek S, Rolinski S, Smirnoff N. (2007). Two genes in Arabidopsis thaliana encoding GDP-L-galactose phosphorylase are required for ascorbate biosynthesis and seedling viability. *Plant Journal* **52:** 673-689

Droby, S., Wisniewski, M.E., Macarisin, D. and Wilson, C. (2009) Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biol. Technol.* **52:** 137–145.

Dumas, Y., M. Dadomo, G. Di Lucca, and P. Grolier. (2003). Effects of environmental factors and agricultural techniques on antioxidant content of tomatoes. *J. Sci. Food Agr.* **83:** 369– 382.

Durner J, Klessig DF. (1995). Inhibition of ascorbate peroxidase by salicylic-acid and 2,6-dichloroisonicotinic acid, 2 inducers of plant defense responses. *Proc Nat Acad Sci U S A* **92:** 11312–11316

Elstner EF, Oswald W. (1994). Mechanism of oxygen activation during plant stress. *Proc Roy Soc Edinburg* **102B:** 131–154

Eltayeb A.E.K, Badawi G.H, Kaminaka H, Sanekata T, Shibahara T, Inanaga S. (2007). Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. *Planta* **225:** 1255-1264

Esteve, M., Farre, R., Frigola, A. (1995). Changes in ascorbic acid content of green asparagus during the harvesting period and storage. *J. Agric. Food Chem.* **43:** 2058–2061.

Falkowski PG. (2006). Evolution. Tracing oxygen's imprint on earth's metabolic evolution. *Science* **311:** 1724–1725

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM. (2003). Arabidopsis local re- sistance to *Botrytis cinerea* involves sal- icylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. *Plant J.* **35:**193–205

Foyer, C.H., Lopez-Delgado, H., Dat, J.F., and Scott, I.M. (1997). Hydrogen peroxide– and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. *Physiol. Plant.* **100:** 241–254.

Foyer CH, Noctor G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* **155:** 2–18.

Foyer CH, Noctor G. (2012). Managing the cellular redox hub in photosynthetic organisms. *Plant, Cell and Environment* **35:** 199–201

Franceschi VR, Tarlyn NM. (2002). L-Ascorbic acid is accumulated in source leaf phloem and transported to sink tissues in plants. *Plant Physiology* **130:** 649-656

Fridman E, Carrari F, Liu YS, Fernie AR, Zamir D. (2004). Zooming in on a quantitative trait for tomato yield using interspecific introgressions. *Science* **305:** 1786–1789

Fridovich J. (1998). Oxygen toxicity: a radical explanation. *J. Expt. Biol*. **210:** 1203-1209

Fry SC. (1986). Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annu Rev Plant Physiol* **37:** 165-186

Fry SC. (1998). Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *The Biochemical Journal* **332** (2): 507–515.

Fukunaga K, Fujikawa Y, Esaka M. (2010). Light regulation of ascorbic acid biosynthesis in rice via light responsive cis-elements in genes encoding ascorbic acid biosynthetic enzymes. *Bioscience, Biotechnology, and Biochemistry* **74:** 888–891

Galiana-Balaguer L, Rosello S and Nuez F .(2006). Characterization and selection of balanced sources of variability for breeding tomato (*Lycopersicon*) internal quality. *Genetic Resources and Crop Evolution* **53:** 907-923

Gan, S., and Amasino, R. (1997). Making sense of senescence*. Plant Physiol.* **113:** 313–319

Gao C, Ju Z, Li S, Zuo J, Fu D, Tian H, Luo Y, Zhu B. (2013). Deciphering Ascorbic Acid Regulatory Pathways in Ripening Tomato Fruit Using a Weighted Gene Correlation Network Analysis Approach*. JIPB* **55:** 1080-1091

Gara Ld, Pinto MCd, Arrigoni O. (1997). Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. *Physiologia Plantarum,* **100:** 894-900

Garcia V, Stevens R, Gil L, *et al.* (2009). An integrative genomics approach for deciphering the complex interactions between ascorbate metabolism and fruit growth and composition in tomato. *Comptes Rendus Biologies* **332:** 1007–1021

Gatzek S, Wheeler GL, Smirnoff N. (2002). Antisense suppression of L-galactose dehydrogenase in Arabidopsis thaliana provides evidence for its role in ascorbate synthesis and reveals light modulated L-galactose synthesis. *Plant Journal* **30:** 541-553

Gautier H, Diakou-Verdin V, Bénard C, Reich M, Buret M, Bourgaud F, Poëssel JL, Caris-Veyrat C, Génard M. (2008). How does tomato quality (sugar, acid, and nutritional quality) vary with ripening stage, temperature, and irradiance? *Journal of Agricultural and Food Chemistry,* **56:** 1241-1250

Gautier H, Massot C, Stevens R, Serino S, Génard M. (2009). Regulation of tomato fruit ascorbate content is more highly dependent on fruit irradiance than leaf irradiance. *Annals of Botany* **103:** 495-504

George G M, van der Merwe M J, Nunes-Nesi A, Bauer R, Fernie A R, Kossmann J and Lloyd J R. (2010). Virus-Induced Gene Silencing of Plastidial Soluble Inorganic Pyrophosphatase Impairs Essential Leaf Anabolic Pathways and Reduces Drought Stress Tolerance in Nicotiana benthamiana. *Plant Physiology* **154**(1): 55-66

Gest N, Gautier H, Stevens R (2013). Ascorbate as seen throught plant evolution: the rise of a successful molecule ? *J. Expl. Bot. **64:** 33-53*

Gest N, Garchery C, Baldet B, Causse M, Duboscq R, Gautier H, Riqueau G, Sauvage C, Stevens R. (2013). Variation in tomato fruit ascorbate levels and consequences of manipulation of ascorbate metabolism on drought stress tolerance. *Acta Horticulturae*. *In press.*

Gilbert L, Alhagdow M, Nunes-Nesi A, Quemener B, Guillon F, Bouchet B, Faurobert M, Gouble B, Page D, Garcia V, Petit J, Stevens R, Causse M, Fernie AR, Lahaye M, Rothan C, Baldet P. (2009). GDP-D-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. *Plant Journal* **60:** 499-508

Giovannoni J. (2004). Genetic regulation of fruit development and ripening. *The Plant Cell* 16: 170–S180

Glazebrook J (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43:** 205–227

Goggin DE, Emery RJN, Powles SB, Steadman KJ. (2010). Initial characterisation of low and high seed dormancy populations of Lolium rigidum produced by repeated selection. *Journal of Plant Physiology* **167:** 1282–1288

Gonzalez-Reyes JA, Alcain FJ, Caler JA, Serrano A, Cordoba F, Navas P. (1995). Stimulation of onion root elongation by ascorbate and ascorbate free radical in *Allium cepa* L. *Protoplasma* **184:** 31–35

Grace SC, Logan BA. (1996). Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. *Plant Physiology,* **112:** 1631-1640

Graham, W.D., Stevenson, M.H., (1997). Effect of irradiation on vitamin C content of strawberries and potatoes in combination with storage and with further cooking in potatoes. *J. Sci. Food Agric.* **75:** 371–377.

Green MA, Fry SC. (2005). Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-O-oxalyl-L-threonate. *Nature (London)* **433:** 83-87

Hager A, Holocher K. (1994). Localization of the xanthophyll-cycle enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease. *Planta* **192:** 581–589

Halliwell B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol* **141:** 312–322

Hancock RD, McRae D, Haupt S, Viola R. (2003). Synthesis of l- ascorbic acid in the phloem. *BMC Plant Biology* **3:** 7

Hancock RD, Walker PG, Pont SDA, Marquis N, Vivera S, Gordon SL, Brennan RM, Viola R. (2007). l-Ascorbic acid accumulation in fruit of *Ribes nigrum* occurs by in situ biosynthesis via the l-galactose pathway. *Functional Plant Biology* **34:**1080–1091.

Handa AK, Singh NK, Biggs MS. (1985). Effect of tunicamycin on in vitro ripening of tomato pericarp tissue. *Physiol. Plant* **63:** 417–424

Hartwig B, James GV, Konrad K, Schneeberger K, Turck F. (2012). Fast isogenic mapping-by-sequencing of ethyl methanesulfonate-induced mutant bulks. *Plant Physiol* 2012, **160:**591-600

Hetherington PR, Marshall G, Kirkwood RC, Warner JM. (1998). Absorption and efflux of glyphosate by cell suspensions. *Journal of Experimental Botany* **49:** 527–533

Hidalgo A, Gonza´lez-Reyes JA, Navas P. (1989). Ascorbate free radical enhances vacuolization in onion root meristems. *Plant, Cell and Environment* **12:** 455–460

Hodge S, Ward J, Galster A, Beale M, Powell G. (2011). The effects of a plant defence priming compound, β -aminobutyric acid, on multitrophic interactions with an insect herbivore and a hymenopterous parasitoid. *BioControl* **56**(5): 699-711

Hodges, D.M. and C.F. Forney. (2000). The effects of ethylene, depressed oxygen and elevated carbon dioxide on antioxidant profiles of senescing spinach leaves. *J. Expt. Bot.* **51:** 645–655

Hodges, D.M. (2001). Chilling effects on active oxygen species and their scavenging systems in plants, In: A. Basra (ed.). Crop responses and adaptations to temperature stress. *Haworth Press, New York*. p. 53–76

Hodges M, Flesch V, Ga´ lvez S, Bismuth E. (2003). Higher plant NADP-dependent isocitrate dehydrogenases, ammonium assimilation and NADPH production. **Plant Physiology and Biochemistry 41:** 577–585

Hodges D.M., Lester G.E., Munro K.D. & Toivonen P.M.A. (2004). Oxidative stress: importance for postharvest quality. *HortScience* **39:** 924–929

Horemans N., Foyer C.H. & Asard H. (2000). Transport and action of ascorbate at the plant plasma membrane. *Trends in Plant Science* **5:** 263–267

Hunt, G. M., & Baker, E. A. (1980). Phenolic constituents of tomato fruit cuticles. Phytochemistry, **19:** 1415–1419

Inoue, S., Kinoshita, T., Matsumoto, M., Nakayama, K. I., Doi, M., and Shimazaki, K. (2008). Blue light-induced autophosphorylation of phototropin is a primary step for signaling. *Proceedings of the National Academy of Sciences of the United States of America* **105** (14): 5626-5631

Ioannidi E, Kalamaki MS, Engineer C, Pateraki I, Alexandrou D, Mellidou I, Giovannonni J, Kanellis AK. (2009). Expression profiling of ascorbic acid-related genes during tomato fruit development and ripening and in response to stress conditions. *Journal of Experimental Botany,* **60:** 663-678

Isherwood, F.A., Chen, Y.T., and Mapson, L.W. (1954). Synthesis of L-ascorbic acid in plants and animals. *Biochem. J.* **56:** 1-15

Ishibashi Y, Iwaya-Inoue M. (2006). Ascorbic acid suppresses germination and dynamic states of water in wheat seeds. *Plant Production Science* **9:** 172 – 175

Ishikawa T, Dowdle J, Smirnoff N. (2006). Progress in manipulat- ing ascorbic acid biosynthesis and accumulation in plants. *Physiology Plantarum* **126:** 343–355

Ishikawa T and Shigeoka S. (2008). Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. *Biosci. Biotechnol. Biochem.* **72** (5): 1143-54

Ito M, Kodama H, Komamine A, Watanabe A. (1998). Expression of extensin genes is dependent on the stage of the cell cycle and cell proliferation in suspension-cultured Catharanthus roseus cells. *Plant Molecular Biology,* **36:** 343-351

Ito S, Song Y.H, Imaizumi T. (2012). LOV Domain-Containing F-Box Proteins: Light-Dependent Protein Degradation Modules in Arabidopsis. *Molecular Plant,* **5:** 573-582

Ivanov KI, Puustinen P, Merits A, Saarma M, Ma¨kinen K. (2001). Phos- phorylation down-regulates the RNA binding function of the coat protein of potato virus A. *J. Biol. Chem.* **276**: 13530–13540

Jain, A.K., and Nessler, C.L. (2000). Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. *Mol. Breeding* **6:** 73-78

James G.V, Patel V, Nordstrom K.J, Klasen J.R, Salomé P.A, Weigel D, Schneeberger K. (2013). User guide for mapping-by-sequencing in Arabidopsis. *Genome Biology* **14:** R61

Javanmardi, J. and C. Kubota, (2006). Variation of lycopene, antioxidant activity, total soluble solids and weight loss of tomato during postharvest storage. *Postharvest Biol. and Tec.* **41**(2): 151-155

Jimenez, A., Creissen, G., Kular, B., Firmin, J., Robinson, S., Verhoeyen, M., and Mullineaux, P. (2002). Changes in oxidative processes and components of the antioxidant system during tomato fruit ripening. Planta **214:** 751-758

Just D, Garcia V, Fernandez L, Bres C, Mauxion J, Petit J, Jorly J, Assali J, Bournonville C, Ferrand C, et al. (2013). Micro-Tom mutants for func- tional analysis of target genes and discovery of new alleles in tomato. *Plant Biotechnol*. **30:** 225–231

Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M. (2001). Arabidopsis NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response*. Science* **291:** 2138–2141

Kalt, W., Forney, C. F., Martin, A., & Prior, R. L. (1999). Antioxidant capacity, Vitamin C, Phenolics, and anthocyanins after fresh storage of small fruits. Journal of Agriculture and Food Chemistry **47:** 4638–4644

Kasahara M, Torii M, Fujita A, Tainaka K. (2010). FMN binding and photochemical properties of plant putative photoreceptors containing two LOV domains, LOV/LOV proteins. *J. Biol. Chem.* **285**: 34765-34772

Kato K., Onuki M., Fuji S., Hanada K., (1998). The first occur- rence of Tomato yellow leaf curl virus in tomato (*Lycopersi- con esculentum* Mill.) in Japan. *Annals of the Phytopatho- logical Society of Japan* **64:** 552-559

Kerk NM, Feldman LJ. (1995). A biochemical-model for the initiation and maintenance of the quiescent center-implications for organization of root-meristems. *Development,* **121:** 2825-2833

Khodakovskaya M, Sword C, Perera I, Boss W, Brown C, Sederoff H. (2010). Expression of inositol-1,4,5-triphosphate metabolism affects drought tolerance, carbohydrate metabolism, and phosphate-sensitive biomass increases in tomato. Plant Biotechnology Journal **8**: 170–183.

Klein, B. P., & Perry, A. K. (1982). Ascorbic acid and vitamin A activity in selected vegetables from different geographical areas of the United States. *Journal of Food Science*, **47:** 941–945, 948

Kong Y.W, Cannell I.G, de Moor C.H, Hill K, Garside G.P, Hamilton T.L, Meijer H.A, Dobbyn H.C, Stoneley M, Spriggs K.A, Willis A.E, Bushell M. (2008). Ascorbic acid and vitamin A activity in selected vegetables from different geographical areas of the United States. *Journal of Food Science*, **47:** 941–945, 948

Kotchoni SO, Larrimore KE, Mukherjee M, Kempinski CF, Barth C. (2009). Alterations in the endogenous ascorbic acid content affect flowering time in Arabidopsis. *Plant Physiology* **149:** 803–815

Kotchoni SO, Shonukan OO. (2002). Regualtory mutations affecting the synthesis of cellulase. *World J Microbiol Biotechnol* **18:** 487-491

Kvaratskhelia M, George SJ & Thorneley RNF. (1997). Sali- cylic acid is a reducing substrate and not an effective inhibitor of ascorbate peroxidase. *J. Biol. Chem*. **272:** 20998–21001

Lakin-Thomas P, Cote GG, Brody S. (1990). Circadian rhythms in *Neurospora crassa:* biochemistry and genetics. *Crit. Rev. Microbiol* .**17:** 365-416

Landry L.G and Pell E.J. (1993). Modification of Rubisco and altered proteolytic acitivty O3-stressed hybrid poplar (Populus maximowizii x trichocarpa) *Plant Physiol.* **101:** 1355-1362

Larrigaudière C, Lentheric I, Pintó E and Vendrell M. (2001). Short-term effects of air and controlled-atmosphere storage on antioxidant metabolism in "Conference" pears*. J. Plant Physiol.* **158:** 1015–1022

Larry R and Joanne L. (2007). Genetic resources of tomato. In: Razdan MK, Mattoo AK, eds. Genetic improvement of solanaceous crops. **Vol. 2.** Tomato. Enfield, *NH: Science Publishers*

Lee S.K and Kader A.A. (2000). Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biology and Technology* **20**: 207-220

Lee S.C, Choi H.W, Hwang I.S, Choi D.S, Hwang B.W. (2006). Functional roles of the pepper pathogen-induced bZIP transcription factor, CAbZIP1, in enhanced resistance to pathogen infection and environmental stresses. *Planta* **224**: 1209-1225

Li Q and Schultes NP. (2002). *Arabidopsis thaliana* locus At5g62890, a nucleobase-ascorbate transporter family member, is preferentially expressed in carpel transmitting tract and tapetal cells. *Plant Sci*. **163:** 233-240

Li M, Ma F, Zhang M, Pu F. (2008). Distribution and metabolism of ascorbic acid in apple fruits (Malus domestica Borkh cv. Gala). *Plant Science* **174:** 606-612

Li Y, Sperry J.S, Shao M. (2009). Hydraulic conductance and vulnerability to cavitation in corn (*Zea mays* L.) hybrids of differing drought resistance. *Envi and Expl Bot* **66**: 341-346

Li F, Wu QY, Sun YL, Wang LY, Yang XH, Meng QW. (2010). Overexpression of chloroplastic monodehydroascorbate reductase enhanced tolerance to temperature and methyl viologen-mediated oxidative stresses. *Physiol Plant* **139**: 421-434

Liang, Y, Lu J, Shang S. (1996). Effect of gibberellins on chemical composition and quality of tea (*Camellia sinensis*.L.). *J. Sci. Food Agric*. **72:** 411–414.

Lim P.O., Woo H.R., and Nam H.G. (2003). Molecular genetics of leaf senescence in Arabidopsis. *Trends Plant Sci.* **8**: 272–278

Lima-Silva V, Rosado A, Amorim-Silva V, *et al*. (2012). Genetic and genome-wide transcriptomic analyses identify co-regulation of oxidative esponse and hormone transcript abundance with vitamin C content in tomato fruit. *BMC Genomics* **13:** 187

Lin, L.S., and Varner, J.E. **(1991).** Expression of ascorbic acid oxidase in zucchini squash (*Cucurbita pepo* L.). *Plant Physiology* **96:** 159-165

Lin T *et al.* (2014). Genomic analyses provide insights into the history of tomato breeding. *Nature* **46**(11): 3117

Linden,H., Ballario,P. and Macino,G. (1997). Blue light regulation in Neurospora crassa. *Fungal Genet. Biol.* **22:** 14-150

Linster CL and Van Schaftingen E. (2007). Vitamin C: Biosynthesis, recycling and degradation in mammals. *FEBS* **274**(1)**:** 1-22

Linster CL, Adler LN, Webb K, Christensen KC, Brenner C, Clarke SG. (2008). A second GDP-L-galactose phosphorylase in Arabidopsis en route to vitamin C: covalent intermediate and substrat requirements for the conserved reaction. *Journal of Biological Chemistry* **283:** 18483-18492

Lisiewska Z, Kmiecik W. (1996). Effect of level of nitrogen fertilizer, processing conditions and period of storage for frozen broccoli and cauliflower on vitamin C retention. *Food Chem*. **57**: 267–270

Liso R, Innocenti A.M, Bitonti M.B, Arrigoni O. (1988). Ascorbic acidinduced progression of quiescent centre cells from G1 to S phase. *New Phytologist.* **110:** 469-471

Liu L, Li Y, Hu N, He Y, Pong R, Lin D, Lu L, Lauw M. (2012). Comparison of Next-Generation Sequencing Systems. *Journal of Biomedecine and Biotechnology* **2012:** 11

Liu Q, Zhong S, Li et al. (2013). Overexpression of a chrysanthemum transcription factor gene, DgWRKY3, intobacco enhances tolerance to salt stress. *Plant Physiology and Biochemistry* **69:** 27-33

Liso R, Innocenti A.M, Bitonti M.B, Arrigoni O. (1988). Ascorbic acidinduced progression of quiescent centre cells from G1 to S phase. *New Phytologist* **110:** 469- 471

Loewus FA, Jang R, Seegmiller CG. (1956). The conversion of 14C-labeled surgars to L-ascorbic acid in ripening strawberries. *J. Biol. Chem.* **222:** 649-664

Loewus FA, Loewus MW, Seib PA. (1987). Biosynthesis and metabolism of ascorbic acid in plants. *Critical Reviews in Plant Sciences* **5:** 101 - 119

Loewus FA. (1988). Ascorbic acid and its metabolic products. *The Biochemsistry of plant,* **14:** 85-107

Lorence, A, Chevone B.I, Mendes P, Nessler C.L. (2004). myo-inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. *Plant Physiol.* **134:** 1200-1205.

Lorenzo O, Chico JM, Sanchez-Serrano JJ, Solano R. (2004). *JASMONATE- INSENSITIVE1* encodes a MYC tran- scription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* **16**:1938**–**50

Luengwilai K, Beckles D.M, Saltveit M.E. (2012). Chilling-injury of harvested tomato (Solanum lycopersicum L.) cv. Micro-Tom fruit is reduced by temperature pre-treatments. *Postharvest Biology and Technology.* **63:** 123-128

Lyons J.M. (1973). Chilling injury in plants. *Annual Review of Plant Physiology.* **24**: 445-466

Madhavi D.L, and D.L Salunke (1998). Tomato. In Handbook of vegetable science and technology: production, storage and processing (Salunkhe D.K. & Kadam S.S., ed). *Marcel Dekker, New York,* pp. 171-201

Malacrida C, Valle E, Boggio S. (2006). Postharvest chilling induces oxidative stress response in the dwarf tomato cultivar Micro-Tom. *Physiologia Plantarum* **127**: 10–18

Mao L, Wood TC, Yu Y, Budiman MA, Tomkins J, Woo SS, Sasinowski M, Presting G, Frisch D, Goff S. (2000). Rice transposable elements: a survey of 73,000 sequence-tagged-connectors (STCs). *Genome Res.* **10**: 982–990.

Mao, Y., Tournier, A.L., Bates, P.A., Gale, J.E., Tapon, N., Thompson, B.J. (2011). Planar polarization of the atypical myosin Dachs orients cell divisions in Drosophila. *Genes Dev.* **25 (2):** 131-136.

Mari M, Guizzardi M, Pratella G.C. (1996) Biological control of grey mold in pears by antagonistic bacteria. *Biological Control* **7**: 30–37.

Martinelli F, Uratsu SL, Reagan RL, Chen Y, Tricoli D, Fiehn O, Rocke DM, Gasser CS, Dandekar AM. (2009). Gene regulation in parthenocarpic tomato fruit. *Journal of Experimental Botany* **60:** 3873-3890.

Maruta T, Yonemitsu M, Yabuta Y, Tamoi M, Ishikawa T, Shigeoka S. (2008). Arabidopsis phosphomannose isomerase 1, but not phosphomannose isomerase 2, is essential for ascorbic acid biosynthesis. *J Biol Chem* **283**: 28842–28851

Mascher M, Jost M, Kuon J, Himmelbach A, Abfaig A, Beier S, Scholz U, Graner A, Stein N. (2014). Mapping-by-sequencing accelerates forward genetics in barley. *Genome Biology* **15:** R78

Massot C, Stevens R, Genard M, Longueness J-J, Gautier H. (2012). Light affects ascorbate content and ascorbate-relates gene expression in tomato leaves more than in fruits. *Planta* **235**: 153–163

Mach J. (2013). COP9 signalosome-regulated proteolysis: turning off ascorbic acid synthesis when the lights go out. *Plant Cell* **25:** 359

Mieda T, Yabuta Y, Rapolu M, Motoki T, Takeda T, Yoshimura K, Ishikawa T, Shigeoka S (2004). Feedback inhibition of spinach L-galactose dehydrogenase by L-ascorbate. *Plant Cell Physiol.* **45**: 1271–1279

Mehlhorn H. (1990). Ethylene-promoted ascorbate peroxidase activity protects plants against hydrogen peroxide, ozone and paraquat. *Plant Cell Environ.* **13**: 971–976

Mellidou, I., Keulemans, J., Kanellis, A. K., and Davey, M. W. (2012). Regulation of fruit ascorbic acid con- centrations during ripening in high and low vitamin C tomato culti-vars. *BMC Plant Biol*. **12:** 239

Mitchell, G.E, McLauchlan, R.L, Isaacs A.R, Williams D.J, Nottingham, S.M. (1992). Effect of low dose irradia- tion on composition of tropical fruits and vegetables. *J. Food Comp. Anal.* **5:** 291–311

Miyaji T, Kuromori T, Takeuchi Y, Yamaji N, Yokosho K, Shimazawa A, Sugimoto E, Omote H, Ma JF, Shinozaki K, Moriyama Y. (2015). AtPHT4;4 is a chloroplast-localized ascorbate transporter in Arabidopsis. *Nat Coomun.* **6:** 5928

Moens C.B, Donn T.M, Wolf-Saxon E.R, Ma T.P (2008). Reverse genetics in zebrafish by TILLING. *Plant.Sci.* **7**(6)**:** 454-459

Morimura Y, Iwamoto K, Ohya T, Igarashi T, Nakamura Y, Kubo A, Tanaka K, Ikawa T. (1999). Light-enhanced induction of ascorbate peroxidase in Japanese radish roots during postgerminative growth. *Plant.Sci.***142:** 123-132

Morris, L.L. (1982). Chilling injury of horticultural crops. *HortScience* **17:** 161–162

Mozafar A (1993). Nitrogen fertlizers and the amount of vitamins in plants: A review. *J. Plant Nutr.* **16(12):** 2479-2506

Müller-Moule P, Conklin PL, Niyogi KK. (2002). Ascorbate deficiency can limit violaxanthin de-epoxidase activity in vivo. *Plant Physiol.* **128:** 970–977

Müller-Moule P (2008). An expression analysis of the ascorbate biosynthesis enzyme VTC2. *Plant Mol. Biol.* **68:** 31-41

Nagy S. (1980). Vitamin C contents of citrus fruit and their products: *a review. J. Agric. Food Chem*. **28:** 8–18

Navabpour S, Morris K, Allen R, Harrison E, A-H-Mackerness S, Buchanan-Wollaston V. (2003). Expression of senescence- enhanced genes in response to oxidative stress. *Journal of Experimental Botany* **54**: 2285–2292

Noctor G, Foyer CH. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology,* **49:** 249-279

Noctor G. (2006). Metabolic signalling in defence and stress: the central roles of soluble redox couples. *Plant, Cell and Environment* **29:** 409-425

Ogura, Y. et al. (2008). Blue light diminishes interaction of PAS/LOV proteins, putative blue light receptors in Arabidopsis thaliana, with their interacting partners*. J. Plant Res.* **121**: 97–105

Okabe Y, Asamizu E, Saio T, Matsukura C, Ariizumi T, Bres C, Rothan C, Mizoguchi T, Ezura H. (2011). Tomato TILLING Technology: Development of a Reverse Genetics Toll for the Efficient Isolation of Mutants from Micro-Tom Mutant Libraries*. Plant and Cell Physiology* **52**(11): 1994-2005

Pallanca JE, Smirnoff N. (1999). Ascorbic acid metabolism in pea seedlings. A comparison of D-glucosone, L-sorbosone, and L-galactono-1, 4-lactone as ascorbate precursors. *Plant Physiology,* **120:** 453-461

Pallanca JE, Smirnoff N. (2000). The control of ascorbic acid synthesis and turnover in pea seedlings. *Journal of Experimental Botany,* **51:** 669-674

Pandey, S.P. and Somssich, I.E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* **150:** 1648–1655

Pantos C. E and Markakis P. (1973). Ascorbic acid content of artificially ripened tomatoes. *J. Food Sci.* **38:** 550

Parsons HT, Yasmin T, Fry SC. (2011). Alternative pathways of dehydroascorbic acid degradation in vitro and in plant cell cultures: novel insights into vitamin C catabolism. *Biochem J.* **440**: 375-383

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH. (2003). Leaf vitamin c contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. *Plant Cell* **15:** 939-951

Pateraki I, Sanmartin M, Kalamaki MS, Gerasopoulos D, Kanellis AK. (2004). Molecular characterization and expression studies during melon fruit development and ripening of L-galactono-1,4-lactone dehydrogenase. *Journal of Experimental Botany* **55:** 1623-1633

Perry J.A, Wang T.L, Welham T.J, Gardner S, Pike M, Yoshida S, Parniske M. (2003). A TILLING Reverse Genetics Tool and a Web-Accessible Collection of Mutants of the Legume *Lotus jasmonicus. American Society of Plant Biologists* **131:** 866-871

Potters G, Gara Ld, Asard H, Horemans N. (2002). Ascorbate and glutathione: guardians of the cell cycle, partners in crime? *Plant Physiology and Biochemistry* **40:** 537-548

Potters G, Horemans N, Caubergs RJ, Asard H. (2000). Ascorbate and dehydroascorbate influence cell cycle progression in a tobacco cell suspension. *Plant Physiol.* **124**: 17–20

Prusky D. (1996). Pathogen quiescence in postharvest diseases. *Annu. Rev. Phytopathol*. **34:** 413-434.

Prusky D, Alkan N, Fluhr R, Tesfaye M. (2013). Quiescent and necrotrophic lifestyle choice during postharvest disease development. *Ann Rev Phytopathol*. **51**: 155-176

Quian W et al. (2007). Mole- cular aspects of leaf senescence. Molecular and functional analysis of phosphomannomutase (PMM) from higher plants and genetic evidence for the involvement of PMM in ascorbic acid biosynthesis in Arabidopsis and *Nicotiana benthamiana. Plant J.* **49**(3)**:** 399-413

Quirino B.F, Noh Y.S, Himelblau F, Amasino R.M. (2000). Mole- cular aspects of leaf senescence. *Trends Plant Sci.* **5:** 278-282

Radzio, J.A., Lorence, A., Chevone, B.I., and Nessler, C.L. (2003). L-Gulono-1,4-lactone oxidase expression rescues vitamin C-deficient Arabidopsis (vtc) mutants. *Plant Mol. Biol.* **53:** 837-844.

Rautenkranz AAF, Li L, Machler F, Martinoia E, Oertli JJ. (1994). Transport of ascorbic and dehydroascorbic acid across protoplasts and vacuole membranes isolated from barley (Hordeum vulgare L. cv. Gerbel) leaves. *Plant Physiology* **106:** 187–193.

Rayle DL, Cleland RE. (1992). The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiol* **99:** 1271–1274

Razavi F, Keulemans J, Davey M.W. (2005). A study of the L-ascorbate biosynthetic capacity of apple fruit. Commun. *Agr. Appl. Biol. Sci.* **70**: 213–216

Rick, C.M. (1995). Tomato. Lycopersicon esculentum (Solanaceae). In Evolution of Crop Plants, Second Edition., J. Smartt and N.W. Simmonds, eds (Essex, UK: Logman scientific and Technical), pp. 452-457

Rizhsky L, Davletova S, Liang H, Mittler R. (2004). The zinc-finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in Arabidopsis. *J. Biol. Chem.* **279:** 11736–11743

Rodríguez GR, Muños S, Anderson C, Sim S-C, Michel A, Causse M *et al*. (2011). Distribution of SUN, OVATE, LC, and FAS in the tomato germplasm and the relationship to fruit shape diversity. *Plant Physiol.* **156:** 275–285

Rousseaux MC, Jones CM, Adams D. (2005). QTL analysis of fruit antioxidants in tomato using Lycopersicon pennellii introgression lines. *Theor. Appl. Genet.* **111**: 1396–1408

Rushton P.J, Somssich I.E, Ringler P, Shen Q.J. (2010). WRKY transcription factors. *Trends Plant Sci.* **15**: 247–258

Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K. (2001). Arabidopsis nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. *Proc. Natl Acad. Sci.* USA **98:** 6969-6974

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakurai N, Suzuki H, Hirai MY, Noji M, Saito K, et al. (2005). Coordinated ac- tivation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. *Plant J* **44:** 653–668

Sakamoto S, Fujikawa Y, Esaka M.. (2013). Analysis of Ascorbic Acid Biosynthesis Using a Single Transient Gene Expression System in Tomato Fruit Protoplast. *Biosci. Biotechnol. Biochem* **77**(3)**:** 673-675

Senter, S. D., Horvat, R. J., & Forbus, W. R. (1988). Quantitative variation of total phenols in fresh market tomatoes at three stages of maturity. *Journal of Food Science* **53:** 639–640

Shanmugavelu K.G., Rangaswami G. (1962) Tryptophan and indole compounds in banana ovaries. *Nature* **194:** 775-776

Sharma G, Nautiyal BP, Nautiyal AR. (2009). Seedling emergence and survival in *Cinnamomum tamala* under varying micro-habitat conditions: conservation implications. *Tropical ecology* **50 (**1**):** 201-209

Sharom M., Willemot C., Thompson J. E. (1994). Chilling injury induces li- pid phase changes in membranes of tomato fruit. *Plant Physiology* **105 (**1**):** 305–308

Smirnoff N. (1996). The function and metabolism of ascorbic acid in plants. *Annals of Botany* **78:** 661-669

Smirnoff N, Pallanca JE. (1996). Ascorbate metabolism in relation to oxidative stress. *Biochemical Society Transaction* **24:** 472-478

Smirnoff N. (2000a). Ascorbate biosynthesis and function in photoprotection. *Philosophical Transactions of the Royal Society of London B Biological Sciences* **355:** 1455-1464

Smirnoff N. (2000b). Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Current Opinion in Plant Biology* **3:** 229-235

Smirnoff N, Wheeler GL. (2000). Ascorbic acid in plants: Biosynthesis and function. *Critical Reviews in Plant Sciences,* **19:** 267-290

Smirnoff N. (2011). Vitamin C: the metabolism and functions of ascorbic acid in plant. *Adv. Bot. Res.* **59:** 107-177

Stevens MA. (1986). Inheritance of tomato fruit quality components. *Plant Breeding Reviews* **4:** 273-311

Shewfelt R.L (2000). Consumer friendly specifications for a global marketplace. *Food Aust.* **52:** 311–314

Siendones E, Gonza´ lez-Reyes JA, Santos-Ocan˜ a C, Navas P, Co´ rdoba F (1999). Biosynthesis of ascorbic acid in kidney bean. L-Galactono- g-lactone dehydrogenase is an intrinsic protein located at the mito- chondrial inner membrane. *Plant Physiol* **120:** 907–912

Stevens R, Buret M, Garchery C, Carretero Y, Causse M. (2006). Technique for rapid, small-scale analysis of vitamin C levels in fruit and application to a tomato mutant collection. *J. Agric. Food Chem.* **54:** 6159-6165

Stevens R, Buret M, Duffe P, Garchery C, Baldet P, Rothan C, Causse M. (2007). Candidate genes and quantitative trait loci affecting fruit ascorbic acid content in three tomato populations. *Plant Physiol.* **143:** 1943-1953

Stevens R, Page D, Gouble B, Garchery C, Zamir D, Causse M. (2008). Tomato fruit ascorbic acid content is linked with monodehydroascorbate reductase activity and tolerance to chilling stress. *Plant, Cell and Environment* **31:** 1086-1096

Sturgeon BE, Sipe HJ, Barr DP Jr, Corbett JT, Martinez JG, Mason RP. (1998). The fate of the oxidizing tyrosyl radical in the presence of glutathione and ascorbate. Implications for the radical sink hypothesis. *Journal of Biological Chemistry* **273:** 30116–30121

Suza WP, Avila CA, Carruthers K, Kulkarni S, Goggin FL, Lorence A. (2010). Exploring the impact of wounding and jasmonates on ascorbate metabolism. *Plant Physiology and Biochemistry* **48**: 337–350

Szarka A, Horemans N, Bánhegyi G, Asard H. (2004). Facilitated glucose and dehydroascorbate transport in plant mitochondria. *Arch Biochem Biophys* **428:** 73–80

Tabata K, Takaoka T, Esaka M. (2002). Gene expression of ascorbic acid-related enzymes in tobacco. *Phytochemistry* **61:** 631-635

Takahama U. (1993). Regulation of peroxidase-dependent oxidation of phenolics by ascorbic acid: different effects of ascorbic acid on the oxidation of coniferyl alcohol by the apoplastic soluble and cell wall-bound peroxidases from epicotyls of Vigna angularis. *Plant and Cell Physiology* **34:** 809-817

Tedone L, Hancock R, Alberino S, Haupt S, Viola R. (2004). Long-distance transport of L-ascorbic acid in potato. *BMC Plant Biology* **4:** 16

Telesinski A, Nowak J, Smolik B, Dubowska A, Skrzypiec N. (2008). Effect of soil salinity on activity of antioxidant enzymes and content of ascorbic acid and phenols in bean (Phaseolus vulgaris L.) *Plants. J. Elementol* **13**: 401–409

Thomas H, Ougham HJ, Wagstaff C, Stead AD. (2003). Defining senescence and death. *Journal of Experimental Botany* **54**: 1127–1132

Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, et al. (1998). Separate jasmonate-dependent and salicylate-dependent defense-res- ponse pathways in Arabidopsis are es- sential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA* **95**: 15107–11

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M.-J. and Broekaert, W.F. (1999). Requirement of functional Ethylene-Insensitive 2 gene for ef®cient resistance of Arabidopsis to infection by Botrytis cinerea. *Plant Physiol.* **121:** 1093-1101

Thompson, J.E., Legge, R.L., and Barber, T.F. (1987). The role of free radicals in senescence and wounding. *New Phytol.* 105: 317-344

Tikunov, Y. M., Molthoff, J., De Vos, R. C. H., Beekwilder, J., Van Houwelingen, A., Van der Hooft, J. J. J., et al. (2013). NON-smoky glycosyltransferase1 prevents the release of smoky aroma from tomato fruit. *Plant Cell* **25:** 3067–3078

Till B.J, Colbert T, Tompa R. *et al.* (2003a) High-throughput TILLING for functional genomics. *Methods Mol. Biol.* **236**, 205–220

Till B.J, Reynolds S.H, Greene E.A *et al.* (2003b) Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Res.* **13**, 524–530.

Tomato Genome Consortium. (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **485:** 635–641

Tohge T, Alseekh S, Fernie AR. (2014). On the regulation and function of secondary metabolism during fruit development and ripening. *Journal of Experimental Botany* **65**: 4599–4611

Toivonen P. M. A., Hodges D. M. (2011). Abiotic Stress in Harvested Fruits and Vegetables, AbioticStress in Plants Mechanisms and Adaptations, ed. A. Shanker, Available from: http://www.intechopen.com/books/abiotic-stress-in-plants-mechanisms-andadaptations/ abiotic-stress-in-harvested- fruits-and-vegetables

Toivonen, P.M.A. (2003a). Effects of Storage Conditions and Postharvest Procedures on Oxidative Stress in Fruits and Vegetables. *Postharvest Oxidative Stress in Horticultural Crops*, D.M. Hodges (Ed.), 69-90, Food Products Press, ISBN 1-56022- 962-4, New York

Toivonen, P.M.A. (2003b). Postharvest Treatments to Control Oxidative Stress in Fruits and Vegetables. In: *Postharvest Oxidative Stress in Horticultural Crops*, D.M. Hodges, (Ed.), 225-246. Food Products Press, ISBN 1-56022-962-4, New York

Toivonen, P.M.A. & Sweeney, M. (1997). Differences in Chlorophyll Loss at 13°C for Two Broccoli (Brassica oleracea L.) Cultivars Associated With Antioxidant Enzyme Activities. *Journal of Agricultural and Food Chemistry* **46**(1): 20-24

Tóth, S. Z., Nagy, V., Puthur, J. T., Kovács, L., and Garab, G. (2011). The physiological role of ascorbate as photosystem II electron donor: protection against photoinactivation in heat-stressed leaves. *Plant Physiol.* **156:** 382–392.

Tsaniklidis G, Delis C, Nikoloudakis N, Katinakis P, Passam H.C, Aivalakis G. (2014). L-Ascorbic acid metabolism in parthenocarpic and seeded cherry tomatoes.*Plant Growth Reg* **72:** 141-153

Tsukaguchi H, Tokui T, Mackenzie B, Berger U. V, Chen X. Z, Wang Y, et al. (1999). A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature* **399**: 70–75

UL Hassan AND A. L. Lehninger, (1956) *J. Biol. Chem*. 223-I23

Ulker B ans Somissich IE. (2004). WRKY Biosynthesis of L-ascorbic acid in plants: new pathways for an old antioxidant.*Curr. Opion Plant Biol.* **7**(5): 491- 8

Valpuesta V and Botella MA (2004). WRKY transcription factors: from DNA binding towards biological function. *Trends Plant Sci.* **9**(12): 573-7

Van Kan J.A, Shaw M.W, Grant-Dowton R.T. (2014). Botytris species: telentless necrotrophic thugs or endophytes gone rogue ? *Molecular Plant Pathology* **15**(9): 957-961

Venkatesh J and Park S.W. (2014). Role of L-ascorbate in alleviating abiotic stresses in crop plants. *Botanical Studies* **55:** 38

Wagner C, Sefkow M, Kopka J. (2003). Construction and application of a mass spectral and retention time index database generated from plant GC/EI-TOF-MS metabolite profiles. *Phytochemistry* **62:** 887–900

Wang N, Fang W, Han H, Sui N, Li B, Meng Q. W. **(2008).** Overexpres- sion of zeaxanthin epoxidase gene enhances the sensitivity of tomato PSII photoinhibition to high fight and chilling stress. *Physiologia Plantarum* **132**(3): 384–396

Wang X, Li W, piqueras r, cao K, Deng XW, Wei N. (2009). Regulation of COP1nuclear localization by the COP9 signalosome via direct interaction with CSN1. *Plant J.* **10:** 1365-313

Wang SD, Zhu F, Yuan S, Yang H, Xu F, Shang J, Xu MY, Jia SD, Zhang ZW, Wang JH, Xi DH, Lin HH (2011) The roles of ascorbic acid and glutathione in symptom alleviation to SA-deficient plants infected with RNA viruses. *Planta* **234**: 171–181

Wang S, Liang D, Li C, Hao Y, Ma F, Shu H. (2012). Influence of drought stress on the cellular ultrastructure and antioxidant system in leaves of drought-tolerant and drought-sensitive apple root- stocks. *Plant Physiol. Biochem.* **51:** 81–89

Wang J, Yu Y, Quan R, Zhang H, Ma L, Deng X.W, Huang R. (2013) Arabidopsis CSN5B Interacts with VTC1 and Modulates Ascorbic Acid Synthesis. *Plant Cell* **10**

Watada, A.E, Aulenbach, B.B, Worthington, J.T. (1976). Vitamins A and C in ripe tomatoes as affected by stage of ripeness at harvest and supplementary ethylene. *J. Food Sci*. **41:** 856–858

Wheeler, G.L., Jones, M.A., and Smirnoff, N. (1998). The biosynthetic pathway of vitamin C in higher plants. *Nature* **393:** 365-369.

Wilson, JX. Asard, H.; May, JM.; Smirnoff, N., editors. (2004). Vitamin C. Functions and biochemistry in animals and plants. *London: Bios Scientific Publishers* p. 97-113

Xin Z, Wang M.L, Barkley N.A, Burow G, Franks C, Pederson G, Burje J. (2008). Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biology* **8**: 103.

Winkler S, Schwabedissen A, Backasch D, Bokel C, Seidel C, Bonisch S, Furthauer M, Kuhrs A, Cobreros L, Brand M, *et al*. (2005) Target-selected mutant screen by TILLING in Drosophila. *Genome Res* 2005, **15**(5)**:** 718-723

Wismer, W.V. (2003). Low temperature as a causative agent of oxidative stress in postharvest crops. In *Postharvest Oxidative Stress in Horticultural Crops*, (D.M. Hodges, ed.) Food Products Press, New York, NY. pp. 55–68

Wituszyńska W and Karpińsk S. (2013). Programmed Cell Death as a Response to High Light, UV and Drought Stress in Plants. Stress, *Plant Responses and Applications in Agriculture, Chapter 7*

Woese K, Lange D, Boess C, Bogl K.W. (1997). A comparaison of organically and conventionally grown foods. *J. Sc. Food Agric.* **74**: 281-293

Wokes F, Organ JG. (1943). Oxidizing enzymes and vitamin C in tomatoes. *Biochemical Journal* **37:** 259-265

Wolucka BA and Van Montagu M. (2002). GDP-mannose 3,5-epimerase forms GDP-L-gulose, a putative intermediate for the *de novo* biosynthesis of vitamin C in plants. *J. Biol. Chem.* **278**: 47483-47490

Wolucka, B.A., and Van Montagu, M. (2003). GDP-mannose 3',5'-epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosynthesis of vitamin C in plants. *J Biol Chem* **278:** 47483-47490

Wolucka BA, Goossens A, Inze D. (2005). Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. *J. Exp. Bot.* **56**: 2527–2538

Worthington V. (2001). Nutritional quality of organic versus conventional fruits, vegetables and grains, *J. Altern. Complement. Med.* **7** (2001): 161 – 173

Wu Y, Perry A.K, Klein B.P. (1992). Vitamin C and B- carotene in fresh and frozen green beans and broccoli in simulated system. *J. Food Qual.* **15**: 87–96

Yabuta Y, Mieda T, Rapolu M, Nakamura A, Motoki T, Maruta T, Yoshimura K, Ishikawa T, Shigeoka S. (2007). Light regulation of ascorbate biosynthesis is dependent on the photosynthetic electron transport chain but independent of sugars in Arabidopsis. *J. Exp. Bot.* **58:** 2661–2671

Yahalom A, Kim TH, Roy B, Singer R,Von Arnim AG, Chamovitz DA. (2008). ArabidopsiseIF3e is regulated by the COP9 signalosome and impacts development and protein translation. *Plant J.* **53**: 300-11

Yahia EM, Contreras-Padilla M, Gonazalez-Aguilar G. (2001). Ascorbic acid content in relation to ascorbic acid oxidase activity and polyamine content in tomato and bell pepper fruits during development, maturation and senescence. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology* **34:** 452-457

Yoshimura K, Yabuta Y, Ishikawa T, Shigeoka S. (2000). Expression of spinach ascorbate peroxidase isoenzymes in response to oxidative stresses. *Plant Physiol*. **123**: 223–233

Zechmann B, Stumpe M, Mauch F. (2011). Immunocytochemical determination of the subcellular distribution of ascorbate in plants. *Planta* **233**: 1–12

Zepplin M, Elvehjein C.A. (1944). Effect of refrigeration on retention of ascorbic acid in vegetables. *Food Res.* **9**: 100–111

Zhang J and Kirkham B. (1996) Antioxidant responses to drought in sunflower and sorghum seedlings. *New Physiologist* **132**(3): 361-373

Zhang H, Zhang D, Chen J, Yang Y, Huang Z, Huang D, Wang XC, Huang R. (2004). Tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to Ralstonia solanacearum. *Plant Mol. Biol.* **55:** 825–834

Zhang WY, Lorence A, Gruszewski HA, Chevone BI, Nessler CL. (2009). AMR1, an Arabidopsis gene that coordinately and negatively regulates the Mannose/L-Galactose ascorbic acid biosynthetic pathway. *Plant Physiol.* **150**: 942-950

Zhang Z, Huang R. (2010). Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor TERF2/LeERF2 is modulated by ethylene biosynthesis. *Plant Mol. Biol.* **73**: 241-249

Zhang C, Liu J, Zhang Y, Cai X, Gong P, Zhang J, Wang T, Li H, Ye Z. (2011). Overexpression of SlGMEs leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. *Plant Cell Reports* **30:** 389–398

Zhang Z, Wang J, Zhang R, Huang R. (2012). The ethylene response factor protein AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in *Arabidopsis*. *Plant J.* **71:** 273–287

Zhang H, Ni L, Liu Y, Wang Y, Zhang A, Tan M. (2012b). The C_2H_2-type Zinc Finger Protein ZFP182 is Involved in Abscisic Acid-Induced Antioxidant Defense in Rice. *Journal of Integrative Plant Biology.* **54**(7)**:** 500-510

Zhu, C., G.W. Zeng, J.S. Hu, M.F. Zhang, and X.M. Jing. (2001). Aging-resistance of ultra-dried onion seeds and its free radical scavenging. J. Zhejiang. *Univ. Sci.* **27:** 139-144

Zhu C, Sanahuja G, Yuan D et al. (2013). Biofortification of plants with altered antioxidant content and composition: Genetic engineering strategies. *Plant Biotechnology Journal* **11:** 129-141

Zhu L, Guo J, Zhu J, Zhou C. (2014). Enhanced expression of EsWAX1 improves drought tolerance with increased accumulation of cuticular wax and ascorbic acid in transgenic Arabidopsis. *Plant physiology and Biochemistry* **75:** 24-35

Abstract

The ascorbic acid (AsA) is an essential antioxidant in both plants and humans. Plant-derived AsA is the major source of vitamin C in the human diet. In addition to its effect on tomato nutritional value, increasing tomato AsA content would likely affect postharvest storage and resistance to pathogens of the fruit. While AsA metabolism is well characterized, the mechanisms involved in its regulation remain poorly understood. Recent studies in Arabidopsis leaves indicate that few regulatory proteins can regulate this pathway at transcriptional and post-transcriptional levels. Still nothing equivalent has been described in fruits. In that aim, a forward genetic approach has been carried out to investigate the regulation of AsA in tomato (*Solanum lycopersicum*) fruit. The screening of an EMS tomato mutant population in the miniature cultivar Micro-Tom for identifying mutant lines with AsA-enriched fruits was done. Among the 500 M2 mutant families screened, four mutant lines with higher AsA content ranging from 2.5 to 4 fold were selected. These mutant lines have been characterized for postharvest traits quality and showed promising results. A method based on NGS-mapping allowed the identification of the putative AsA-enriched related gene. Thus, the screening of EMS mutants led to original findings such as the discovery of new unexpected proteins regulating AsA in plants, and particularly in fruits. Our work confirms at the molecular level the direct interaction between light signalling component and the regulation of the AsA biosynthesis pathway.

Keywords: ascorbic acid, EMS mutant, tomato fruit, NGS-Mapping, postharvest fruit quality

Résumé

L'acide ascorbique (AsA) est un antioxydant essentiel à la fois pour l'homme et les végétaux. L'AsA provenant des plantes représente la source principale de vitamine C dans l'alimentation quotidienne. Au delà de son impact nutritionnel, augmenter la teneur en AsA dans le fruit de tomate serait susceptible d'influencer la qualité des fruits après la récolte, en terme de conservation mais également de résistance à des pathogènes. Bien que le métabolisme de l'AsA soit bien caractérisé, les mécanismes impliqués dans sa régulation restent jusqu'à présent peu compris. Des études récentes menées sur des feuilles d'*Arabisdopsis thaliana* montrent que certaines protéines seraient capables de réguler la teneur en AsA, en agissant au niveau transcriptionnel ou post-transcriptionelle. A ce jour, ce type de régulation n'a pas été encore décrit chez les fruits. Dans ce but, une approche de génétique directe a été développée afin d'étudier les mécanismes impliqués dans la régulation de la teneur en AsA et ceci dans le fruit de tomate (*Solanum lycopersicum*). L'analyse d'une population de mutants EMS de tomate Micro-Tom a permis l'identification de lignées de mutants présentant des teneurs en AsA de 2,5 à 4 fois plus importantes que celles observées dans les fruits de tomate sauvage. La caractérisation de ces lignées a conduit à des résultats prometteurs pour l'étude de la qualité des fruits après la récolte. Une stratégie de NGS-mapping a permis l'identification des mutations causales responsables du phénotype AsA observé. Ainsi, le criblage de mutants EMS a permis la découverte de nouvelles protéines inattendues, permettant de confirmer au niveau moléculaire l'existence d'une interaction directe en la signalisation lumineuse et la régulation de la voie de biosynthèse de l'AsA.

Mots clefs: Acide ascorbique, mutant EMS, fruit de tomate, NGS-Mapping, qualité des fruits après la récolte