

Study of starch metabolism in tomato fruit by using forward and reverse genetic approaches

Jiaojiao Wang

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DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE

Science de la Vie et de la Santé

SPÉCIALITÉ

Biologie Végétale

Par Jiaojiao WANG

Study of Starch Metabolism in Tomato Fruit by Using Forward and Reverse Genetic Approaches

Sous la direction de : Yves GIBON

(co-directeur : Pierre BALDET)

Soutenue le 27th, Novembre, 2020

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Acknowledgement

In Chinese, we would use the phrase "white horse crossing the gap" (白驹过隙) to describe the rapid passing of time. This idiom comes from Zhuangzi, one of my favorite Chinese philosophers. "The life span of a human being is extremely short, like a white horse galloping through a narrow gap, passing away in a flash. However, the Tao (or spirit) can stay in the world forever." These four years are like a white horse crossing a gap, but plant science for me, it may be a kind of way to pursue Tao in a short life. In those four years, I was lucky to meet great supervisors, colleagues and friends in France. When the thesis is completed, I hope to express my deep and sincere gratitude to them.

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Titre : Étude du métabolisme de l'amidon chez les tomates à l'aide d'approches génétiques directes et inverses

Résumé : [4000 caractères maximum, espaces compris]

L'amidon joue un rôle central pendant le développement et la maturation des fruits. Sa dégradation fournit de l'énergie et des sucres aux fruits en maturation tandis que son accumulation répond aux stress environnementaux. La connaissance du métabolisme de l'amidon dans les feuilles s'est considérablement améliorée, principalement grâce à la caractérisation des mutants d'*Arabidopsis* (sans amidon ou excédentaire), mais on en sait très peu sur les fruits. Dans cette étude, nous avons utilisé des approches de génétique directe et inverse pour obtenir des mutants de tomate produisant des fruits avec un excès d'amidon.

Pour mener la démarche de génétique directe, nous avons combiné l'utilisation d'une plateforme robotisée permettant le dosage d'amidon à haut-débit avec celle d'une importante population de mutants EMS, et réalisé un criblage en utilisant des fruits rouges de près de 3000 individus. Trois candidats avec un excès d'amidon ont été trouvés, dont un (P17C12) a ensuite été confirmé avec une mutation récessive. Une grande population BC1F2 a été phénotypée puis utilisée pour localiser la mutation en utilisant une approche de cartographie par séquençage. Les résultats de l'analyse ont montré que la localisation la plus probable de la mutation est à l'extrémité du chromosome 1, avec 1 mutation KO, 2 mutations « faux-sens » et de nombreuses mutations dans des régions non codantes. Nous avons décidé de tester la RecQ hélicase 4 (RECQ4, Solyc01g103960, KO), un membre de la famille des protéines de transport mitochondrial (MCF, Solyc01g095510, « faux-sens »), un transporteur de phospholipides à ATPase (PTA, Solyc01g105700, 5' UTR) et la Rubrédoxine (Solyc01g097910, 3' UTR).

Nous avons créé des mutants homozygotes KO ou faux-sens en utilisant CRISPR/Cas9 pour les gènes candidats RECQ4, MCF et PTA, et des mutants homozygotes avec mutations d'insertion / suppression dans le 5' UTR pour CPuORF, mais aucun n'a montré de phénotype avec excès d'amidon. Pour la Rubrédoxine, en plus de créer des mutants KO et des mutants avec des mutations similaires dans 3' UTR avec CRISPR/Cas9, nous avons également produit des plantes surexprimant la Rubrédoxine car chez mutant P17C12, l'ARNm de la Rubrédoxine était fortement

exprimé dans le fruit vert des lignées BC1F1 et S2. Le développement du mutant KO pour la Rubrédoxine (homozygotes) ne va pas au-delà du stade cotylédon. Les mutants avec des mutations dans 3' UTR, qui n'ont montré aucun changement dans la quantité d'amidon ou les niveaux d'ARNm de Rubrédoxine. Certaines plantes ont montré des expressions 100 fois plus élevées de Rubrédoxine dans les feuilles, mais aucune n'a montré d'augmentation significative de la quantité d'amidon dans les fruits. Finalement, nous n'avons trouvé le phénotype avec excès d'amidon dans les fruits pour aucun des cinq gènes candidats testés, ce qui implique que davantage d'investigations seront nécessaires pour affiner la cartographie et identifier la mutation responsable de ce phénotype.

En utilisant une approche de génétique inverse, nous avons sélectionné une α -amylase (AMY3.2) et trois β -amylases (BAM1.2, BAM3.2 et BAM9), pour lesquelles nous avons créé des mutants KO par CRISPR/Cas9. Nous avons obtenu des mutants KO homozygotes pour AMY3.2 et BAM9, et des mutants KO hétérozygotes pour BAM1.2 et BAM3.2 dans la génération T1. Les mutants de deux lignées transgéniques dépourvues d'AMY3.2 ont montré une teneur en amidon inférieure dans fruits de 20 DAP. L'absence de BAM9 a conduit à une mauvaise fertilisation chez quelques mutants. La quantité d'amidon était remarquablement élevée dans les fruits âgés de 35 DAP dans la plupart des mutants, accompagnés d'un niveau élevé significatif de glucose dans les fruits rouges mûrs. Ces résultats préliminaires suggèrent que BAM9 pourrait jouer un rôle important dans la viabilité du pollen et dans la dégradation de l'amidon des fruits pendant le mûrissement.

Mots clés : [3 minimum]

Amidon; fruit; tomate; mutant EMS; cartographie-par-séquençage; amylase

Title: Study of starch metabolism in tomato fruit by using forward and reverse genetic approaches

Abstract: [4000 characters maximum]

Starch plays a central role during fruit development and ripening. Its degradation provides energy and sugars in ripening fruits whereas its accumulation responds to environmental stresses, which suggests a buffering role. Knowledge of starch metabolism in leaves has dramatically improved, mostly through the characterization of *Arabidopsis* mutants (starch-less or -excessive), but only very little is known in fruits. In this study, we used both forward and reverse genetic approaches to obtain tomato mutants with starch-excess fruits.

For applying forward genetics, we took advantages of the robotic platform and the large EMS mutant population of tomato (Micro-Tom, an ideal model for starch research), and performed a screen by using red fruits of nearly 3,000 individuals. Three starch-excess candidates were found and one (P17C12) could be confirmed as a homozygous mutant with a recessive starch-excess phenotype with Mendelian inheritance analysis. A large BC1F2 population was phenotyped and then used to localize the mutation using the mapped-by-sequencing approach. The sequence data obtained from the two bulks revealed that the mutation was likely located at the end of chromosome 1, a region that harbours 1 KO and 2 missense mutations as well as numerous mutations in non-coding regions. We decided to work with the KO, missense mutations and mutations in 5' UTR and 3' UTR first, which were a RecQ helicase 4 gene (RECQ4, Solyc01g103960), a mitochondrial carrier protein family gene (MCF, Solyc01g095510), a phospholipid-transporting ATPase gene (PTA, Solyc01g096930), a conserved peptide upstream open reading frame (CPuORF, Solyc01g105700), and Rubredoxin (Solyc01g097910), respectively.

To evaluate those mutations, we utilised different bioengineering techniques adapted to each case. We created KO or missense homozygous mutants by using CRISPR/Cas9 for RECQ4, MCF and PTA candidate genes, and homozygous mutants with insertion/deletion mutations in the 5' UTR for CPuORF, but none of them showed a starch-excess phenotype. For Rubredoxin, besides creating KO mutants and mutants with similar mutations in 3' UTR with CRISPR/Cas9, we also overexpressed Rubredoxin under the control of the CMV-35S promoter as we found a significant

increase of Rubredoxin mRNA in the 12 DAP (days after pollination) fruit of BC1F1 and S2 (P17C12), compared to the WT. The homozygous KO mutants could not grow through cotyledon stage, indicating that the lack of Rubredoxin is lethal for phototrophic plants. The mutants with mutations in 3' UTR showed no changes in starch amount or mRNA levels of Rubredoxin. Then, among the transformants overexpressing Rubredoxin, some showed 100 times higher expressions levels of Rubredoxin in leaves, but showed no significant starch increase in 20 DAP fruit. Finally, none of the five candidate genes checked here led to a starch-excess phenotype in fruits, implying that more investigations are required regarding fine-mapping and mining the possible candidate genes.

We also attempted to obtain starch-excess mutants *via* reverse genetics. We selected one α -amylase (AMY3.2) and three β -amylases (BAM1.2, BAM3.2 and BAM9) to create the corresponding KO mutants by CRISPR/Cas9 techniques. We obtained homozygous KO mutants of AMY3.2 and BAM9, and heterozygous KO mutants of BAM1.2 and BAM3.2 in T1 generation. The mutants of two transgenic lines lacking AMY3.2 showed lower starch content in 20 DAP fruits. While the loss of BAM9 led to poor fertilization in some mutants. Furthermore, in most of mutants, starch levels were remarkably high in the ripening 35 DAP fruit, accompanied with a significant high level of glucose in red ripe fruits. Those results lead us to speculate that BAM9 may play important roles both in pollen viability and starch degradation during fruit ripening.

Keywords : [3 minimum]

Starch; fruits; tomato; mapping-by-sequencing; amylase

Biologie du Fruit et Pathologie [UMR 1332 BFP - INRAE Bordeaux-Aquitaine Bât. IBVM 71, av. Edouard Bourlaux - CS 20032 - 33882 Villenave d'Ornon Cedex]

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Abbreviations

3' UTR	three prime untranslated region				
5' UTR	five prime untranslated region				
AMY	α-amylase				
BAM	β-amylase				
CPuORF	Conserved peptide upstream open reading frame				
	Clustered Regularly Interspaced Short Palindromic				
CRIST R/Casy	Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)				
DAP	days after pollination				
EMS	Ethyl methanesulfonate				
IMG	immature green				
InDel	insertion/deletion				
КО	knock-out				
MCF	Mitochondrial carrier protein family				
MG	mature green				
РТА	Phospholipid-transporting ATPase				
qPCR	quantitative polymerase chain reaction				
RECQ4	RecQ helicase 4				
SNP	single nucleotide polymorphism				
WT	wild type				

Introduction

Part I-Introduction

Chapter1.1 Starch Structure, Synthesis and

Degradation

1.1.1 Starch Structure

In plant, starch can be transiently stored in organs like leaf, pollen, fruit and root, in which it turns over within a day or during development, and can be long-stored in some seeds (*e.g.*, rice, maize, wheat...), tubers (*e.g.*, potato) or roots (*e.g.*, cassava) to provide carbon and energy for next generation growth, which are well-known as the main components of staple food for humans and livestock. Thus, according to the storage time, starch is generally categorized as transient and stored. In this part, I am going to introduce starch components, structures, and related properties, emphasize on the differences between transient and storage starch, and also highlight the investigations of starch structure in fruit.

Starch exists as semi-crystalline, insoluble granules composed of two glucan polymers, amylose and amylopectin. Amylose is a less abundant component in starch (10% for leaf and 20–30% for storage organs), whose glucan chains are formed with α -1,4-bonds, and lightly branched with α -1,6-bonds. Amylose exists largely as single helices in the amorphous regions of starch matrix but plays no role in the organization of the matrix, as amylose-free mutants have normal starch contents, granule sizes, and shapes (Jacobsen *et al.*, 1989; Ceballos *et al.*, 2007). Compared to amylose, amylopectin, the other glucan polymer in starch, is more abundant, longer, more branched, and responsible for starch structure.

Amylopectin structure is characterized by several levels that are the basis of the higher order of starch structure. Thus amylopectin is composed of glucan chains linked by α -1,4-bonds, like amylose but with different length, and also branched by α -1,6-bonds but with different frequency and pattern than in amylose (**Fig 1**). According to length and degree of branching, amylopectin chains can be categorized as 3 kinds of chains: unbranched A-chains, branched B-chains assigned as B1-, B2-, B3-, B4-chains for chains spanning one, two, three or four clusters, respectively, and

Part I-Introduction

the only one C-chain containing the single reducing group, which results in a treelike structure. In the region rich in branch points, the linear chains extend to form clusters. Within clusters, adjacent chains form double helices, giving rise to crystalline layers (lamellae, ~6 nm). While between the clusters, as the region containing branch points is less ordered, this results in amorphous lamellae (~3 nm). This 9 to 10 nm periodicity makes up the semi-crystalline structure of starch which is highly conserved in higher plants (Jenkins et al., 1993; Zeeman et al., 2002). There are 3 types (A, B, C) of polymorph depending on the alignment of double helices in crystalline lamellae. A-type polymorph is more dense, generally found in cereal endosperm, and B-type polymorph is less dense thus more hydrated, often found in leaves (Fig 1), while the C-type polymorph is the mixture of both A- and B-type, which is found in some legume species and fruits (MacNeill et al., 2017).



Glucose units are linked into chains via a-1,4-bonds and branched via α-1,6-bonds



Neighbouring chains of amylopectin form double helices.



Amylose chains form single helices



Fig 1 The composition and structure of starch

The structure of two compositions of starch (amylopectin and amylose) and the higher structure of starch granules are showed in Fig 1. The figure is cited from Zeeman et al. (2010).

Higher Order of Starch Structure

The final size and shape of starch granules vary greatly in different tissues and species, ranging from small flat disks of 0.5–2 μ m in diameter in *Arabidopsis* leaves to smooth spheres of up to 100 μ m in tuberous roots (Streb and Zeeman, 2012). Unlike the starch nanometer-scale granules found in leaves, as related to diel turnover, many starch granules found in cereal grains and in sieve tubes show a higher order of structures and reach hundred-nanometer scales. After removing the less crystalline regions of cracked starch granules with α -amylase or acid, scanning electron microscopy analysis revealed a repeated layered structure similar to the growth rings of trees. Those so-called growth rings of starch contain spherical blockets (20–500 nm) in the semicrystalline regions (Gallant *et al.*, 1997) that may be formed by left-handed amylopectin superhelix (Oostergetel and van Bruggen, 1993).

The semi-crystalline structure of starch granules makes them water insoluble and osmotically inactive. Differences in composition, structure, and size of starch granules influence the rate of starch remobilization *in vivo* but also their physical properties *in vitro*, such as gelatinization onset temperature, final viscosity or stickiness, which define their final uses by humans. Thus, there are specific interests in manipulating starch physical properties in crops, through altering the structure of starch, based on better understanding of the relationships between structure and biosynthesis.

Starch Structure in Tomato Fruit

Luengwilai and Beckles (2009) investigated starch granules in pericarp and columella of tomato fruit at different stages. They found that starch in tomato pericarp was mainly polygonal, ranged from 10 to 25 μ m, and the size increased through development, being largest in ripening fruit. Amylopectin content was ranging from 70 to 83%, showed a C-type pattern. Besides, they also compared a high-sugar tomato cultivar with a regular one, to see if carbohydrate metabolism influences starch structure and composition. There was no difference in starch granule structure and composition between those two cultivars, indicating that starch structure and composition are relatively stable traits in tomato fruit. Starch characteristics were similar in the columella except that the granules were bigger in size. In the columella, the largest modal granule size recorded

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was >25 μ m, which is approximately the same modal size as found for large A type granules in wheat endosperm starch at grain maturity (Jane *et al.*, 1994).

Species	Tissues	Allom-	Amylose	Size (average	Shape	Refer-
		orph	content (%)	diameter, µm)		ences
Maize	endosperm	А	23–28	~15	Round, polygonal	1
Rice	endosperm	А	14–29	~6	Polygonal	1
Wheat	endosperm	А	25–26	21–23	Round, lenticular	1
Potato	sieve tube	В	17–27	48–60	Round, oval	1
Arabidopsis	leaf	В	~4	1–2	Irregular discoid	2
Apple	fruit	С	26–2	6–10	Spherical, dome shape	3
Banana	fruit	B/C	16–19.5	20–60	Irregular, elongated oval	4
Kiwifruit	fruit	В	10–19	4–10	Spheroidal	5
Tomato	fruit	С	17–19	13.5–14.3	Polygonal	6

Table 1 Components and structures of starch in different tissues and species

References: 1, Waterschoot *et al.*, 2015; 2, Zeeman *et al.*, 2002 ;3, Stevenson *et al.*, 2006 ;4, Zhang *et al.*, 2005; 5, Li and Zhu, 2018; 6, Luengwilai and Beckles, 2009.

1.1.2 Starch Synthesis

In higher plant, starch is synthesized in the plastids — chloroplasts in leaves and some fruits, or amyloplasts in the starch-storing tissues, like endosperm, tuber and root. Enzymes involved in starch synthesis and degradation have been identified through studies of crops and model species like *Arabidopsis*. These enzymes are well conserved between different plant species, giving us useful references for understanding starch synthesis in fruits. However, as mentioned above, starch displays a well-constructed structure, with big variations in shape, size and granule-number found between tissues and species. This is reflected by the duplication and specialization of starch enzymes, which may display distinct or partially overlapping functions. Starch degradation is also more diverse than starch synthesis, with relatively more steps from starch to the final products maltose and glucose, and thus more enzymes involved, which are also fine-tuned at different levels (regulations will be discussed in Chapter 1.2). I focus more on results obtained in *Arabidopsis*, as starch metabolism has been intensively studied in this species (Smith and Zeeman, 2020). I therefore assume that because leaf- and fruit-starch are both transiently stored, and because fruit

structures accumulating starch were originally leaf tissues, these two forms have more in common than with that found in the endosperm. Besides, the main differences between leaf and endosperm starch will be discussed briefly to indicate how different it could be for starch degradation in different tissues.

In brief, starch synthesis involves 3 main steps, (1) elongation, (2) branching and (3) debranching of glucan chains. The elongation is catalyzed by starch synthase (SSs) enzymes that use ADP-glucose as substrate and add its glucosyl moiety to existing glucose chains with α -1,4-linkage. The enzymes responsible for glucan chain branching are called starch branching enzymes (SBEs); they transfer the terminal part of a chain onto the side of an adjacent chain with α -1,6-linkage. The wrong placed glucosyl chains are removed by starch debranching enzymes (DBEs) *via* hydrolyzing α -1,6 linkages to allow the rise of a structure. SSs, SBEs and DBEs have multiple isoforms playing distinct roles during the synthesis, which are important for the periodic branching pattern of amylopectin and hence the structure of the granule matrix.

Precursors of Starch Synthesis

The substrate of starch synthesis is ADP-glucose, whose precursors may differ depending on the tissue. Thus, in photosynthetically active leaf chloroplasts, ADP-glucose generation is directly linked to the Calvin–Benson cycle through conversion of fructose-6-phosphate into glucose-6-phosphate (Glc-6-P), a step catalyzed by phosphoglucose isomerase (PGI), then to glucose-1-phosphate (Glc-1-P), a step catalyzed by phosphoglucomutase (PGM) (**Fig 2**). Glc-1-P and ATP are then converted into ADP-glucose and pyrophosphate (PPi) by ADP-glucose pyrophosphorylase (AGPase). Through those conversions, approximately 30%–50% of photoassimilates of *Arabidopsis* leaves are partitioned into starch (Stitt and Zeeman, 2012). In heterotrophic tissues, sucrose is transported from source tissues and catalyzed to produce hexose-phosphates in the cytosol. Both glucose-phosphates (typically Glc-6-P, although Glc-1-P transport has also been reported) and ATP are transported into the plastid to serve as substrates for the synthesis of ADP-glucose (reviewed by Geigenberger, 2011). In the cereal endosperm, the pathway differs, as most of AGPase activity (typically 80% or more) is found in the cytosol and ADP-glucose is imported directly into the plastid *via* a cereal-specific adenine nucleotide transporter BRITTLE1 (Bt1) (James *et al.*, 2003).

A Leaves



B Heterotrophic Tissues



Fig 2 Schematic representation of the pathway of starch biosynthesis in (A) photosynthetic leaves and (B) heterotrophic tissues.

The abbreviations are listed as below. (1) Metabolites: G1P, Glucose-1-Phosphate; G6P, Glucose-6-Phosphate; ADPglc, ADP-glucose. (2) Enzymes: PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; AGPase, ADP-Glc pyrophosphorylase; SS, starch synthase; SBE, starch branching enzyme; DBE, starch-debranching enzyme; PPase, inorganic pyrophosphatase; SuSy, sucrose synthase; FK, fructokinase; UGPase, UDP-Glc pyrophosphorylase. The circles in blue, orange and green represent the ADP-Glc/ADP translocator, the Glc-6-P/Pi translocator, and the ATP/ADP translocator, respectively. The steps in blue are specific for cereal endosperm. This is adapted from Geigenberger (2011).

Protoin abbroviation	Louisin	Logucin	Departion estalward / Dala attributed
(alternative name)	Locus III Arabidonsis	Locus III tomata	Reaction catalyseu / Role attributeu
(alternative name) Phosphoglucose isomerose (PCI)	Arabiaopsis	tomato	Emu6D <-> Clo6D (novonsible)
r nosphoglucose isomerase (FGI)		a 1 b 4 b a 4000	Fruor <=> Gloor (reversible)
PGI (PGI1)	At4g24620	Solyc04g076090	catalytic, plastidial, mutant has low starch phenotype
Phosphoglucomutase (PGM)			Glc6P <=> Glc1P (reversible)
PGM1 (PGM)	At5g51820	Solyc03g006870	catalytic, plastidial, mutant has low starch phenotype
ADP-glucose pyrophosphorylase (AGPase)			Tetrameric enzyme composed of two small and two large subunits.
(,			Glc1P + ATP <=> ADPGlc + PPi (reversible reaction)
AGPase small subunit 1	At5g48300	Solyc12g011120/	major catalytic subunit, plastidial, mutant has low starch phenotype
(APS1, ADG1)	1.1.05(10)	Solyc0/g056140	
AGPase small subunit-like 2 (APS2)	At1g05610	Solyc08g015670	likely non-functional, plastidial
AGPase large subunit 1 (APL1, ADG2)	At5g19220	Solyc01g079790	major regulatory subunit in leaf, catalytic, plastidial, mutant has low starch phenotype
AGPase large subunit 2	At1g27680	Solyc07g019440	minor regulatory subunit in leaf, catalytic, plastidial
(APL2)	4.4.20210	G 1 01 100500	a construction of a state a
AGPase large subunit 3	At4g39210	Solyc01g109790	regulatory subunit in root, plastidial
(APLS)	Δt2g21500		regulatory subunit in root plastidial
(APL4)	At2g21390		regulatory subulit in root, plasitilar
Starch synthase (SS)			Elongates α -1,4-linked glucose chains at the non-reducing end. (1,4- α -D-Glc)(n) + ADPGlc => (1,4- α -D-Glc)(n+1) + ADP
Granule-bound starch synthase 1 (GBSS)	At1g32900	Solyc08g083320	catalytic, plastidial, amylose synthesis, mutant has no amylose
Soluble starch synthase 1 (SS1, SSI)	At5g24300	Solyc03g083090	catalytic, plastidial, amylopectin synthesis, preferentially elongates chains with 9-10 glucose units
Soluble starch synthase 2 (SS2, SSII)	At3g01180	Solyc02g088000	catalytic, plastidial, amylopectin synthesis, preferentially elongates chains with 13-22 glucose units

Table 2 Genes encoding proteins involved in starch synthesis in Arabidopsis and tomato

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Soluble starch synthase 3 (SS3, SSIII)	At1g11720	Solyc02g080570	catalytic, plastidial, amylopectin synthesis, preferentially elongates chains with >25 glucose units
Soluble starch synthase 4 (SS4, SSIV)	At4g18240	Solyc02g071040/ Solyc07g042830	catalytic, plastidial, amylopectin synthesis, role in starch granule initiation
Glucanotransferase			
1,4-α-glucan branching enzy (BE)	me		Cuts α -1,4-linked glucose chains and transfers the reducing end to another chain creating a α -1,6-linkage (branch point).
Starch branching enzyme 1 (BE1)	At3g20440	Solyc07g064830/ Solyc04g082400	putative glycoside hydrolase unlikely to be involved in amylopectin synthesis
Starch branching enzyme 2 (BE2)	At5g03650	Solyc09g009190	catalytic, plastidial, amylopectin synthesis
Starch branching enzyme 3 (BE3)	At2g36390		catalytic, plastidial, amylopectin synthesis
α-1,6-glucosidase Starch debr (DBE)	anching enzyme		Hydrolyze α -1,6-linkage (branch point) and release the resulting chain into the stroma.
Isoamylase 1 (ISA1)	At2g39930	Solyc07g014590	catalytic, plastidial, amylopectin synthesis, forms complex with ISA2, mutant has altered starch
Isoamylase 2 (ISA2, DBE1)	At1g03310	Solyc09g064800	non-catalytic, plastidial, amylopectin synthesis, forms complex with ISA1, mutant has altered starch

Adapted from Streb and Zeeman, 2012; Moustakas, 2013.

AGPase, which plays an important role in carbon partitioning and starch synthesis (details see Chapter 1.2.2), consists of two large regulatory subunits and two small catalytic subunits. Those 4 subunits synergistically interact with each other to form a heterotetramer that responds to different posttranslational regulations. In *Arabidopsis*, there are two genes coding small subunits and four genes coding large regulatory subunits (**Table 2**), which also showed different spatial patterns of expression, potentially giving rise to AGPase with distinct properties in different tissues of the plant.

Enzymes Involved in Starch Synthesis (SSs, SBEs and DBEs)

There are five SS classes involved in starch biosynthesis: four that are soluble in the stroma (or partially bound to the granule) are responsible for amylopectin synthesis, and one that is almost exclusively granule-bound (so called granule-bound SS, GBSS) is responsible for amylose synthesis. According to a series of SSs mutants, SS1 preferentially elongates short chains of amylopectin (6 or 7 glucose residues), which are further elongated by SS2 to 13–20 glucose residues — sufficient to form double helices with neighboring chains. SS3 is proposed to elongate chains to generate longer, cluster-spanning chains. While SS4 has little roles in chain elongation but more in the initiation of starch granules (Smith and Zeeman, 2020). However, their functions are partially overlapping, and interfering *via* complex formation between enzymes.

SBEs create branches from existing chains *via* glucanotransferase reactions. Most plants have two distinct classes of SBE isoforms, SBE1 and SBE2, which differ considerably in their substrate preferences and products. For example, SBE1 has high activity on long linear chains and can cleave α -1,4 linkages behind existing branch points, whereas SBE2 isoforms preferentially act on outer chains of branched structures (Smith and Zeeman, 2020).

Debranching enzymes can be classified as isoamylase type (ISA1, ISA2, and ISA3) and limit dextrinase type (LDA, also called pullulanase). All are specific for the hydrolysis of α -1,6 linkage but differ in their substrate preferences (Kobayashi *et al.*, 2016). ISA1, ISA3, and LDAs are active hydrolases, while ISA2 lacks conservative substitutions essential for catalysis, appearing to be inactive (Pfister and Zeeman, 2016). However, ISA2 is required to form heteromultimers with ISA1 to improve the enzyme activity or stabilize the enzyme complex. In plants, ISA1 deficiency

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leads to accumulate more branched glucans, which fails to form a crystalline granular and remains soluble as phytoglycogen. It suggests that misplaced or excessive branches may impede the formation and packing of double helices, and hence impede the formation of the semicrystalline matrix. Therefore, selective debranching (trimming) by isoamylase is essential for starch structure. LDA and ISA3 are believed to be primarily involved in starch degradation, but may also contribute to debranching during synthesis (Streb *et al.*, 2008; Fujita *et al.*, 2009; Kubo *et al.*, 2010).

1.1.3 Starch Degradation

For starch degradation in leaves, the breaking of glucan polymers is mainly catalyzed by two types of hydrolytic enzymes, *i.e.* exoamylase (β -amylase) that cleaves α -1,4 linkages, and the debranching enzyme isoamylase that cleaves α -1,6 linkages. Moreover, as starch is compacted into semi-crystalline structures, it is essential to decreases the degree of crystallinity to facilitate and speed up degradation allowing a quick turnover. Phosphorylation at the granule surface, probably by interfering with the packing of double helices, facilitates the opening of the granule surface, thus making starch more accessible for hydrolytic enzymes. However, for full degradation of starch, removing the inhibitory phosphates (dephosphorylation) is also required to allow β -amylase to access (Fulton *et al.*, 2008). The main processes of starch degradation in chloroplast are showed in **Fig 3**, and the involved genes in *Arabidopsis* and tomato are listed in **Table 3**.

Hydrolysis of Glucan

Higher-plant genomes have large numbers of β -amylase like proteins (BAM). Analysis of the genomic data from different species revealed that BAM gene family has increased in size and diversified in structure during evolution (Monroe and Storm, 2018). They have catalytic and non-catalytic members located in several subcellular compartments. The coding genes show different transcriptional regulations between species and tissues, combined with various post-transcriptional regulations, which contributed to high function diversity, and thus need to be further investigated. For example, among the nine β -amylase genes (BAM1–9) present in the *Arabidopsis* genome, none is more than 60% identical regarding the amino acid sequence. Six of these BAM (BAMs 1–4, 6 and 9) are chloroplastic, two (BAM7 and 8) are located in the nucleus, and one (BAM5) in the cytosol (Monroe and Storm, 2018).



Fig 3 The pathway of starch degradation in chloroplast

The size of arrows indicates the estimated flux, and the minor steps in *Arabidopsis* are represented in dash arrows. The figure is cited from Zeeman et al.(2010).

In leaves, two catalytic BAMs (1 and 3) located in the chloroplast play important roles in starch degradation. BAM1 and BAM3 are believed to be partially redundant: *bam1* and *bam3* mutants have a wildtype and a mild starch excess phenotype (also named "sex") in leaves, respectively, but the *bam1/bam3* double mutant has a strong starch excess phenotype. Further investigation revealed that they play distinct roles in starch degradation: BAM3 plays a dominant role in nocturnal starch degradation in leaf mesophyll cells (Lao *et al.*, 1999; Fulton *et al.*, 2008), whereas BAM1 plays a major role in guard cells. Mutants lacking BAM1 fail to degrade starch in guard cell, which impacts

the production of osmotically active metabolites and thus affects stomatal opening (Horrer *et al.*, 2016).

Debranching enzymes, which include 4 members, *i.e.* ISA1, ISA2, ISA3 and LDA, cleave α -1,6 bonds of glucans to produce linear malto-oligosaccharides,. Among them the functions of ISA3 and LDA, which are both required for starch granule degradation, partially overlapped. Loss of LDA alone has no effect on starch metabolism in *Arabidopsis* leaves, whereas loss of ISA3 results in a sex phenotype (Wattebled *et al.*, 2005; Delatte *et al.*, 2006). However, the *isa3/lda* double mutant has a more severe sex phenotype than the *isa3* mutant (Delatte *et al.*, 2006).

Phosphorylation and Dephosphorylation of the Starch Granule

Although the hydrolytic enzymes mentioned above that break the α -1,4 linkages and α -1,6 linkages are major actors of starch degradation, the rate of hydrolysis depends on the crystalline degree of the granule surface. To reduce the crystalline degree and make it more accessible for hydrolytic enzymes, it is essential to phosphorylate the glucose residues with the help of two enzymes, glucan water dikinase [GWD, also called STARCH EXCESS1 (SEX1)] and phosphoglucan water dikinase (PWD). GWD adds the β -phosphate of ATP to the 6th position of the glucose residues, releasing AMP and inorganic phosphate, while the action of PWD requires the prior action of GWD, and prefers the 3rd position (Ritte *et al.*, 2006). The single or double mutants of those two enzymes show that they have relatively different importance in leaf starch degradation. The *gwd* mutant has a very severe sex phenotype (Yu *et al.*, 2001), with the loss of 6-phosphorylation preventing the action of PWD so that starch is almost unphosphorylated (Ritte *et al.*, 2006; Hejazi *et al.*, 2009), while the *pwd* phenotype is mild.

Dephosphorylation of the phosphorylated glucose residues is also necessary to allow the progressive degradation of linear chains by β -amylase. There are three glucan phosphatase family members, SEX4 (Laforin-Like Phosphoglucan Phosphatase), LSF1 (LIKE SEX FOUR 1) and LSF2 (LIKE SEX FOUR 2) that were reported to be related to starch degradation, but only SEX4 and LSF2 are involved in amylopectin dephosphorylation. SEX4 can remove both 6- and 3- phosphates from glucose residues, and LSF2 removes only 3-phosphates. Loss of SEX4 blocks β -amylolysis, therefore *sex4* mutant has elevated starch levels and accumulates linear

phosphorylated glucans in the chloroplast stroma (Santelia *et al.*, 2011). Loss of LSF2 increases the amount of 3-phosphorylation in starch but has little effect on starch levels. However, in the double *sex4/lsf2* mutant, levels of starch and starch-bound phosphate are higher than in *sex4* (Wilkens *et al.*, 2016). Though LSF1 is closely related to SEX4 and LSF2, *lsf1* does not accumulate soluble phosphorylated glucans, and recombinant LSF1 has no detectable phosphatase activity (Schreier *et al.*, 2019). However, its loss elevates starch levels, and *lsf1/sex4* mutant has higher starch levels than either single mutant, indicating that LFS1 plays a different role in normal starch degradation. Recent research suggests that LSF1 interacts with the β -amylases BAM1 and BAM3, and the BAM1-LSF1 complex shows amylolytic but not glucan phosphatase activity. It may facilitate the β -amylolytic attack on the granule surface during starch degradation (Schreier *et al.*, 2019).

Other Enzymes or Proteins Involved in Starch Degradation

Recently, another protein named ESV1 (early starvation1) was found in a screen for *Arabidopsis* mutant in which starch reserves are prematurely exhausted (Feike *et al.*, 2016). Loss of ESV1 accelerates starch degradation and the reserves are exhausted before dawn. ESV1 protein is conserved throughout starch-synthesizing organisms. It contains a series of near-repeated motifs rich in tryptophan, but has no known catalytic activity. It is proposed that ESV1 functions in the organization of the starch granule matrix, and affects starch degradation indirectly, by altering the matrix organization and, thus, the accessibility of starch polymers to starch-degrading enzymes. Recent *in vitro* studies indicate that ESV1 can modulate the actions of GWD and PWD on starch-like surfaces (Malinova *et al.*, 2018).

Plastidial α-amylase may participate in starch degradation when the normal degradation is impeded by losing other necessary enzymes. In *Arabidopsis*, *amy3* mutants do not result in sex phenotypes under normal laboratory conditions (Tatge *et al.*, 1999; Streb *et al.*, 2008). However, AMY3 participates in starch degradation when other starch-degrading enzymes are missing. Soluble branched glucans are accumulated in *isa3/lda* mutant, while loss of AMY3 in *isa3/lda* mutant (thus *isa3/lda/amy3* mutant) prevents the accumulation of soluble branched glucans, confirming that in *isa3/ida* mutant they arise from amylolysis by AMY3 (Tatge *et al.*, 1999; Streb *et al.*, 2008).

Protein abbreviation	Locus in	Locus in tomato	Reaction catalysed / Role attributed
(alternative name)	Arabidopsis		·
a-glucan water dikinase			Phosphorylates glucosyl residues within amylopectin.
(GWD)		a 1 b5 b5 b5	$1,4-\alpha$ -D-Glc + H2O + ATP => phospho- $1,4-\alpha$ -D-Glc + AMP + Pi
Glucan, water dikinase 1 (GWD1_SEX1)	At1g10760	Solyc05g005020	catalytic, plastidial, phosphorylates at C-6 position, mutant has starch-excess phenotype
(01121, 52111)			phonotype
Glucan, water dikinase 2 (GWD2)	At4g24450	Solyc09g098040	catalytic, cytosolic, phosphorylates at C-6 position
Phosphoglucan, water dikinase (PWD, GWD3)	At5g26570		catalytic, plastidial, phosphorylates at C-3 position, dependent on prior C-6 phosphorylation, mutant has starch-excess phenotype
Phosphoglucan			Dephosphorylates glucosyl residues within amylopectin.
phosphatase			
Starch excess 4	At3g52180	Solyc11g007830/	catalytic, plastidial, releases phosphate bound at C-6 and C-3 positions of glucosyl
(SEX4)		Solyc03g111160	residues, mutant has starch-excess phenotype
Like Sex Four 2 (LSF2)	At3g10940	Solyc06g050230	catalytic, plastidial, preferentially releases phosphate bound at C-3 position of glucosyl residues
Like Sex Four 1	At3g01510		non-catalytic (?), plastidial, mutant has starch-excess phenotype
(LSF1) Fyg-amylase			
β-amylase (BAM)			Acts on linear α -1,4-linked glucose chains, liberating β -maltose from the non-reducing end.
			Cannot act on α -1,6-linkages, nor pass them. Cannot pass phosphorylated glucosyl residues.
BAM1 (BMY7 / TR- BMY)	At3g23920	Solyc09g091030/ Solyc08g082810	catalytic, plastidial
BAM2 (BMY9)	At4g00490		catalytic, plastidial
BAM3 (BMY8 / ctBMY)	At4g17090	Solyc08g007130/ Solyc08g077530	catalytic, plastidial, mutant has starch excess phenotype

Table 3 Genes encoding proteins involved in starch breakdown in Arabidopsis and tomato

BAM4	At5g55700		non-catalytic, plastidial, regulatory protein, mutant has starch-excess phenotype
BAM5 (BMY1 / RAM1)	At4g15210	Solyc07g052690	catalytic, cytosolic
BAM6	At2g32290		
BAM7	At2g45880	Solyc01g094580	non-catalytic, nuclear transcription factor
BAM8	At5g45300	Solyc08g005780	non-catalytic, nuclear transcription factor
BAM9 (BMY3)	At5g18670	Solyc01g067660	
Endo-amylase			
α-amylase (AMY)			Acts on α -1,4-linkages releasing linear α -1,4-linked oligosaccharides and branched α -1,4- and α -1,6-linked oligosaccharides (acting between branch points); cannot act on α -1,6-linkages themselves.
AMY1	At4g25000	Solyc03g095710/ Solyc04g078930	catalytic, secreted
AMY2	At1g76130	Solyc04g082090	extracellular
AMY3	At1g69830	Solyc05g007070/ Solyc02g066950	catalytic, plastidial
α-1,6-glucosidase			Starch debranching enzyme (DBE) Acts on α -1,6-linkages (branch points) releasing oligosaccharides into the stroma.
Isoamylase 3 (ISA3)	At4g09020	Solyc06g009220	catalytic, plastidial, amylopectin degradation, prefers short chains, mutant has starch-excess phenotype
Limit dextrinase (LDA, PU1)	At5g04360		catalytic, plastidial, amylopectin degradation, prefers short chains
α-glucan phosphorylase			Acts on the non-reducing end of α -1,4-linked glucose.
(PHS)	A +2 ~20220	$S_{a} = \frac{1}{2} \frac{1}$	$(1,4-\alpha-D-Glc)(n) + P1 \iff (1,4-\alpha-D-Glc)(n) + Glc PP (reversible)$
гпэт	A13g29320	Solyc05g005340/ Solyc05g012510	catalytic, plasticial
PHS2	At3g46970	Solyc09g031970/ Solyc02g077680	catalytic, cytosolic
4-α-glucanotransferase			

Disproportionating enzyme (DPE) Transfers glucose/α-1,4-linked glucan moiety from a donor glucan to an acceptor, releasing the non-reducing end glucose/glucan moiety (reversible)

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DPE1	At5g64860	Solyc04g053120	catalytic, plastidial, donor: maltotriose or longer, acceptor: glucoseoligomers except maltose, mutant has starch-excess phenotype
DPE2	At2g40840	Solyc02g020980	catalytic, cytosolic, donor: β -maltose, acceptor: mixed-sugar polymers
			(<i>e.g.</i> heteroglycan), mutant has starch excess-phenotype

Adapted from Streb and Zeeman, 2012; Moustakas, 2013.

Products and Connection with Cytosol Carbohydrate Metabolism

The products of starch degradation in chloroplast include maltose (the majority), glucose and Glc-1-P. Maltose results from the hydrolysis of β -amylase through the actions directly on starch granule surface or on linear malto-oligosaccharides derived from the catalysis by DBEs or α -amylase. Glucose is released by DPE1 (disproportionating enzyme, α -1,4-D-glucanotransferase), which converts 2 maltotrioses to maltopentaose (five glucoses) and one glucose. Some maltotrioses (with 3 glucose residues) are produced through the β -amylase-catalyzed hydrolysis of linear maltooligosaccharides, and cannot be further hydrolyzed by β -amylase as it cannot remove a maltose unit from the reducing ends. Thus, further mobilization of maltotriose needs the catalysis of DPE1 in the chloroplast (as mentioned before). Besides, DPE1 can also transfer maltosyl residues from maltodextrin in different lengths to malto-oligosaccharides without releasing a free glucose. Mutants lacking DPE1 accumulate malto-oligosaccharides at night and have slightly elevated starch contents (Critchley *et al.*, 2001).

In *Arabidopsis* chloroplasts, Glc-1-P can be also generated in small proportion by plastidial α -glucan phosphorylase (PHS1) from linear malto-oligosaccharides. Also in *Arabidopsis*, *phs1* mutants do not result in sex phenotypes under normal laboratory conditions, but loss of PHS1 in *ss4*, *mex1*, and *dpe2* mutants reduces granule number and alters granule morphology (Malinova *et al.*, 2014, 2017; Malinova and Fettke, 2017).

Exported maltose is metabolized by a cytosolic glucosyl transferase, DPE2, which releases one glucose and transfers the other to an acceptor polysaccharide. The loss of DPE2 blocks the conversion from maltose to sucrose, and the higher amount of maltose feeds back on starch degradation in chloroplasts. As a result, *dpe2* mutants have higher maltose, higher starch, and higher maltodextrin but lower nighttime sucrose than wild-type plants (Chia *et al.*, 2004; Lu and Sharkey, 2004). The polysaccharide acceptor *in vivo* is believed to be heteroglycan, a heterogeneous cytosolic glycan composed of several different, variously linked sugars (Fettke *et al.*, 2004). Then, heteroglycan can be metabolized by a cytosolic phosphorylase, PHS2, to generate Glc-1-P, which is thereafter used for cytosolic sucrose synthesis, glycolysis or oxidative pentose phosphate pathway after conversion into Glc-6-P by PGM.
Starch Degradation in Other Plant Species or Tissues

The relative importance of enzymes or proteins involved in leaf starch degradation may differ between species. The mutants lacking α -amylase, LDA or glucan water dikinase (GWD) are good examples for illustrating those differences. Unlike in *Arabidopsis* leaves, α -amylase plays a significant role in starch degradation in rice leaves. When knocked down, a significant increase of starch accumulation in the young leaf tissues was observed under sugar-supplemented growth conditions. In contrast, the starch content of leaves was reduced in the plants overexpressing α amylase I-1 (Asatsuma *et al.*, 2005). Maize mutants lacking LDA alone (*Zmpu1* mutants) are impaired in transient and storage starch degradation, suggesting that LDA contributes to starch catabolism in maize (Dinges *et al.*, 2003).

Although GWD was suggested to have universal importance in leaf starch degradation in higher plants, the physiological priority of leaf starch in photoassimilate allocation may vary among plant species, thus leading to variously relative importance of enzymes involved in starch degradation in leaves. Thus, a rice mutant accumulating high levels of starch in the leaf blades has been identified as GWD1 mutant. Surprisingly, through it exhibited more than 10-fold higher starch than the wild-type plants throughout the day, the OsGWD1 mutant showed no significant change in vegetative growth, presenting a clear contrast to the reported mutants of *Arabidopsis thaliana*, in which disruption of the genes for GWD leads to marked inhibition of vegetative growth (Hirose *et al.*, 2013).

Unlike starch turnover in leaves, in cereal endosperm there is no evidence that transient glucan phosphorylation occurs during germination. Alpha-amylase plays a central role in starch degradation of during seed germination, but not β -amylase. Maltose and short malto-oligosaccharides produced in the endosperm are degraded to glucose by α -glucosidase (maltase) whereas there is no evidence for a role for α -glucosidase in leaf maltose metabolism (Smith and Zeeman, 2020).

Starch Degradation in Fruit

Because fruit starch and sugar metabolism are involved in fruit quality and also in sink/source relationships, they attracted the interest of scientists since decades. The understanding of the translocation and metabolism of sugars in fruits progressed dramatically in the last few years, the primary and secondary metabolomes were well characterized, but the mechanism of starch breakdown in fleshy fruit remains elusive. Strikingly, although in starchy fruit species such as banana and kiwifruit, starch plays a major role in fruit growth and postharvest quality, it has been little investigated in these species. Indeed, in these perennial plants, genetic manipulations are difficult, as is the use of large mutant collections to study different roles of enzymes or proteins *in vivo*. With such limitations, solid *in vivo* evidence is difficult to obtain and as a consequence, the knowledge on starch breakdown in fruits grows slowly. Researchers mostly build hypotheses based on research conducted on *Arabidopsis* leaves or potato tubers, as well as correlations between enzyme activities, starch content and transcript levels obtained for different genotypes and/or postharvest treatments.

In kiwifruit, a comprehensive analysis of the sucrose to starch metabolic pathway during kiwifruit development combining data on primary metabolites, transcripts, and enzymes revealed that AMY3 and BAM9 transcripts increased significantly along with starch degradation, and may play important roles in starch degradation (Nardozza *et al.*, 2013). Unexpectedly, BAM1 transcripts, a plastidial β -amylase critical in leaf starch degradation (Streb and Zeeman, 2012), did not increase along with AMY3/BAM9. Moreover, BAM3 whose transcripts exhibit a significant increase at the late mature stage and a strongly up-regulated level in ripening kiwifruit, could be a candidate for starch degradation in ripening kiwifruit. Similar results were found in kiwifruit under ethylene treatment in postharvest ripening (Hu *et al.*, 2016). BAM9 and BAM3.1 were up-regulated while the starch degradation rate was increased in response to ethylene treatment. Besides, AMY1 and α -glucosidase (AGL) also showed increased transcript levels. However, those results are only based on correlations, and the corresponding protein levels or possible regulation targets (BAM9 is a non-catalytic protein) still need to be studied.

There are more reports on banana starch degradation, as recently reviewed by Cordenunsi-Lysenko *et al.* (2019). In general, starch degradation during banana ripening appears to be similar to the

process in *Arabidopsis* leaves — it involves few similar enzymes, but there are also noticeable differences. Firstly, in ripening banana the breakdown of starch granules in amyloplasts is assumed to also involve AMY acting mainly in amylose-rich regions (amorphous lamella) (Peroni-Okita *et al.*, 2010). Secondly, large amounts of BAM transcript levels are clearly correlated to a decrease in starch during fruit ripening (do Nascimento *et al.*, 2006). Ten different MaBAM transcripts were observed at various stages of development and ripening in two banana cultivars, and among them, MaBAM9a, MaBAM9b, and MaBAM3c showed high levels of expression following the climacteric crisis in both cultivars, indicating a more clear relationship between gene expression and starch degradation. Besides, a recent extensive study (Xiao *et al.*, 2018) has found further genes potentially associated with starch metabolism in bananas including three GWDs, three phosphoglucan phosphatases, eight BAMs, seven AMYs, two DBEs, two α -glucan phosphorylases, two DPEs, two MEXs (maltose excess transporter, MaMEX1 and MaMEX2), and five pGlcTs (the plastidic glucose translocator).

Tomato, the model plant for fleshy fruit species, also accumulates considerable amounts of starch (up to around 20% of the dry weight) in immature fruits over a long period, until the beginning of ripening at which an almost complete breakdown starts. Surprisingly, there are only few studies dealing with mechanisms of starch degradation in tomato fruit. One attempt was to characterize a GWD gene in a heterozygous mutant. The loss of GWD (even one copy) caused male gametophytic lethality; the mutant pollen had a starch-excess phenotype that was associated with a reduction in pollen germination (Nashilevitz *et al.*, 2009). Further studies conducted in tomato that involved proteomics or transcriptomics, revealed that during fruit ripening, BAM9 protein increases in abundance during the differentiation of chromoplasts (Barsan *et al.*, 2012).

Chapter1.2 Regulation of Transient Starch

1.2.1 Starch Turnover in Leaves and Fruits

In storage organs, starch synthesis and degradation are temporarily separated, usually occurring at different developmental phases, and the associated enzymes may not be present during the same developmental stage. In contrast, starch is only transiently stored as a reserve of carbon and energy in leaves and fruits, its fate being determined within a day in leaves, or during growth in fruits. Thus, accumulation and remobilization of starch probably involve different regulation mechanisms depending on species, organs, tissues or even cell types.

Starch Turnover in Leaves

In Arabidopsis

In *Arabidopsis* grown under artificial controlled growth conditions and in short photoperiods (12h or less), leaf starch accumulation is linear during the day, as well as its remobilization at night. In such conditions, the rate of starch accumulation is adjusted to the light period or to the decreased light intensity, and seem to only result from its synthesis, as no simultaneous degradation was detected (Zeeman *et al.*, 2002). However, regardless that the rate of accumulation changed during the day, the diurnal starch turnover is tightly controlled to prevent the starvation before dawn (Graf *et al.*, 2010; Scialdone *et al.*, 2013; Sulpice *et al.*, 2014; Vasseur *et al.*, 2011).

However, those well-buildup turnover models were re-examined under longer days or natural light-dark transitions (with substantial decreased light mimicking twilight). *Arabidopsis* plants grown in photoperiods as long as 16–18 h displayed a plateauing of starch content toward the end of the light period (Hädrich *et al.*, 2012; Mugford *et al.*, 2014; Figueroa and Lunn, 2016), which was associated with simultaneous synthesis and degradation. Thus, contrary to what has been observed in short photoperiods, starch degradation is activated in the light in long photoperiods, suggesting that there is a mechanism to prevent excessive starch accumulation, in order to favor the export of sucrose to the sink organs or tissues. There is also a lot of evidence indicating that

the adjustment of the onset and rate of starch degradation to the length of the night involve the circadian clock (Lu *et al.*, 2005; Graf *et al.*, 2010), which will be discussed in the next section.

In Other Species

In leaves of many grasses like wheat, barley, maize, rice, *etc.*, besides the accumulation of starch during the day, there is substantial accumulation of sucrose (Gordon *et al.*, 1980; Balaguer *et al.*, 1995), which differs from *Arabidopsis* leaves grown under short photoperiods, as the latter do not accumulate sucrose. Because of this difference in photoassimilate partitioning between starch and sucrose, those plants display different phenotypes when starch synthesis or degradation are impacted. In rice, in which the sucrose to starch ratio is much higher in leaves than in *Arabidopsis*, loss-of-function of a large subunit of AGPase (OsAGPL3, Os05g0580000) leads to a defect in starch biosynthesis in the leaf but does not affect vegetative growth or grain yield under 12-h-light/12-h-dark controlled conditions (Rösti *et al.*, 2007). This suggests that under such conditions rice does not rely on leaf starch as a carbon source during the night. In maize, neither the loss-of-function of the small subunit of AGPase nor RNAi-mediated suppression of a GWD gene affect plant growth in 12-h-light/12-h-dark or natural light conditions, or leads to respectively a decrease or an increase in leaf starch levels (Slewinski *et al.*, 2008; Weise *et al.*, 2012). Based on these results, it appears that grasses accumulating sucrose during the night.

In tomato leaves, Bénard *et al.* (2015) found that starch turnover shows several differences compared to *Arabidopsis* leaves. Firstly, regarding the diurnal pattern of starch in mature leaves of plants bearing fruits and grown under optimal growth conditions, it was shown that starch as well as sucrose and hexoses peak towards the middle of the day and begin decreasing several hours before dusk. Secondly, we have seen previously that in *Arabidopsis* carbon partitioning into starch is adjusted to decreased light intensity by increasing the proportion of carbon fixed by photosynthesis into diurnal starch accumulation. In contrast tomato mature leaves submitted to shading did not increase carbon storage during the day to respond to the decreased cumulated net photosynthesis, and the starch diurnal pattern was no longer detected (Bénard *et al.*, 2015).

Starch Accumulation in Fruits

Strikingly, although daily fluctuations in starch and central metabolites have been intensively studied in leaves, only a few studies investigated fruit diel changes. Thus, growing tomato fruits were found to show diel patterns for numerous metabolites as well as starch, with a relatively small but clear fluctuation, peaking in the early morning and decreasing along the day. In those fruits, the starch diel pattern was barely related to fruit photosynthesis or photorespiration but appeared rather associated with the momentary supply of sucrose from source leaves (Bénard *et al.*, 2015).

Many fleshy fruits show a significant accumulation of starch during growth followed by a quick remobilization at ripening. Thus, starch net accumulation rates were compared between 12 fleshy fruit species thanks to a normalization of time (Roch *et al.*, 2020) (**Fig 4**). Remarkably different patterns were found between climacteric and non-climacteric fruits, the former accumulating starch at higher rates during fruit growth. However, according to the maximum accumulation rate and to the timing of its peak, the fruit species can be clustered into 3 groups. For the first group, which includes clementine, cucumber, and grapevine, there is little starch accumulating during fruit growth. In fruits of the second group, starch accumulates mostly at early developmental stages (before fertilization), but then stays at a very low level; this group comprises eggplant, pepper and strawberry. The third group includes well-known starchy fruits that are also climacteric fruits, like apple, kiwifruit, mango, peach, pear and tomato. Obviously, banana also belongs to this third group of fruits, though the accumulation rate curve is missing in **Fig 4**. For fruits in group three, starch accumulation rates reach the maximum before ripening, but the peaking time varies largely, from early (e.g., tomato or peach) to late (mango) developmental stages.

Among the fruits in group three, when considering the amount of accumulated starch but also the net accumulation and/or degradation rates, there are also noticeable differences. Thus, in tomato, at its peak starch contributes to around 20% of the dry weight in non-mature fruits (Schaffer and Petreikov, 1997), while in apple, kiwifruit and banana it represents 40 to 70% of the dry weight during fruit growth (Zhang *et al.*, 2005; Nardozza *et al.*, 2013; Li and Zhu, 2018). Then, starchy fruits such as kiwifruit, banana and mango usually maintain relatively high level of starch at commercial maturity (full fruit size but still unripe), while for the others, starch has already decreased before the onset of ripening. Because fruits such as apple show conserved temporal

profiles for starch accumulation, starch levels are used to define a ripening index for fruit harvest in several species (Doerflinger *et al.*, 2015).



Fig 4 Starch net accumulation rates in 12 fleshy fruit species.

"Rates expressed in mmol.g⁻¹ FW.day⁻¹ of glucose equivalent were calculated from fit of starch accumulation and fruit growth. Additional fruit datasets were collected for tomato (Biais et al., 2014), strawberry (Cakpo et al., 2020), mango (Tandon and Kalra, 1983) and pear (Oikawa et al., 2015). Time is normalized against total time from anthesis to ripeness. Climacteric species are symbolized with stars and full lines." Cited from Roch *et al.*(2020).

In all these fruit species, in addition to a temporal accumulation, starch also shows spatial distribution patterns. Thus, in tomato fruit, starch accumulates more in the inner pericarp (parenchyma) than in the outer pericarp (Schaffer and Petreikov, 1997). In apple, different spatial starch accumulation/degradation patterns, such as ring or star-shaped patterns, were observed when comparing cultivars (Szalay *et al.*, 2013). In banana, starch was degraded from the fruit center to the outward (Blankenship *et al.*, 1993).

Different species have different strategies in fruit carbon partitioning. For fruits of the first group, such as grape, large amounts of sugars are accumulated instead of starch during growth (Roch *et al.*, 2019). In contrast, in kiwifruit, which belongs to the third group, sucrose, fructose and glucose decrease during early development, then remain stable whereas starch accumulates significantly until reaching a plateau at maturation. In apple fruit, also a member of the third group, starch and sugar accumulation appear coupled. In tomato, another fruit of the third group, carbon partitioning appeared as intermediary between apple and kiwifruit, where sugars (mostly hexoses) remain rather stable during fruit growth while starch accumulates until fruit growth ceases when expressed on a fruit-basis. Additionally, it has been found that starch accumulation responds to abiotic stress, such as shading, water stress or salinity, whereas sugars are more stable and repeatable (Yin *et al.*, 2010; Biais *et al.*, 2014).

We have seen that starch accumulation varies greatly between fruit species and tissues. The influence of the environment and/or agricultural practices may also affect starch accumulation in fruit, but it has been little investigated so far.

Starch Remobilization in Fruits

In tomato, starch that is accumulated during fruit growth is nearly completely degraded at maturation. Experiments based on [³H]-(fructosyl)-sucrose labeling with fruits still attached on their pedicels showed that 25% of the sucrose taken up by the fruit was converted into starch regardless of fruit age, suggesting that tomato fruits perform constant starch turnover throughout development (N'tchobo *et al.*, 1999). Surprisingly, ¹⁴C-pulse-chase analyses of tomato fruit disks indicated that starch synthesis was highest in immature fruit [10 days post anthesis (DPA)] (Luengwilai and Beckles, 2009). It appeared that the rate of starch degradation was maximal at early immature stage and was lowest at maturity. Although those striking results remind us that results obtained *in vitro* should be considered with care, both results implied that starch turnover never ceases in the developing tomato fruit, and that starch remobilization may rather result from a drop in starch synthesis. The underlying mechanisms, which may have evolved to offer flexibility in balancing starch accumulation and utilization in the developing fruit, are largely unknown.

In strawberry, *in vitro* experiments using $[U^{-14}C]$ glucose revealed that although starch content was highest at the earliest developmental stages (below days post anthesis) the incorporation of radioactivity into starch was relatively low (11%) (Souleyre *et al.*, 2004). The incorporation of $[U^{-14}C]$ glucose into starch then increased to a maximum of 20% at 21 days post anthesis but starch had already strongly decreased. These results also show that there is a continuous starch turnover in growing strawberry fruit. Simultaneous starch synthesis and degradation was also found in ripening banana fruit, even though the conversion of hexose monophosphates to starch (0.4 µmol hexose.g⁻¹.h⁻¹ FW) was much smaller than the reverse (5.9 µmol hexose.g⁻¹.h⁻¹ FW) (Hill and ap Rees, 1993).

1.2.2 Regulation of Starch Accumulation

Unlike storage starch that is mobilized at specific moments such as germination or growth recovery, following dormancy, transient starch appears as a more flexible reserve of carbon and energy. Thus, the accumulation of transient starch fluctuates largely when plants face environmental challenges or during shifts in developmental stages, implying that starch plays an important role for plant fitness and growth. While there are in fact many ways of regulating starch levels between the two extremes of storage and transient starch, it seems logical that the underlying regulation is complex and diverse. In general, plants regulate starch metabolism in several ways, as listed below:

- 1. Plants sense the inner carbon/energy balance (supply and demand) and the changes in relative source/sink strength during development to regulate carbon partitioning and allocation.
- 2. Genes encoding proteins involved in the synthesis and degradation of starch (pathway enzymes, enzyme regulators, transcription factors...) are regulated at the transcriptional and post-transcriptional levels.
- 3. By duplicating and then modifying these genes or even recruiting other ones during evolution, plants have considerably broadened the possibilities of regulation.

This complex network for starch regulation underpins the important roles starch plays for plant growth and maintenance but also for coping with environmental stresses. Moreover, for different tissues or species, different regulation strategies have been found. The details of the regulation of synthesis and degradation of starch, and their physiological relevance, are discussed in the following subsections.

Sugar Signaling and Carbon Partitioning

To cope with carbon supply and demand, plants use several sugar signaling pathways sensing carbon availability including hexokinase-1 (HXK1), trehalose-6-phosphate (Tre-6-P) and target of rapamycin (TOR) for high carbon availability, and pathways including sucrose-non-fermenting 1 (SNF1)-related protein kinase 1 (SnRK1) and C/S1 bZIP transcription factors (reviewed by Wingler, 2018) for low carbon availability. Among those sensors, researchers found that Tre6P and SnRK1 interact together to regulate sucrose and starch contents in plants.

Trehalose-6-phosphate Plays a Role in Carbon Partitioning

In plants, sucrose is the main product of photosynthesis and also the most common product transported from source to sink organs through the phloem. The partitioning between sucrose and starch in source organs and the availability of sucrose for sink organs are major determinants for starch accumulation. Cytosolic sucrose correlates with Tre-6-P content, which suggests that sucrose content is regulated by Tre-6-P, but synthesis of Tre-6-P is also affected by changes in sucrose content (Geigenberger et al., 2005; Lunn et al., 2006). Tre-6-P acts as a signaling metabolite coordinating sucrose status, carbon assimilation and development. In Arabidopsis, Tre-6-P is synthesized from UDP-glucose and Glc-6-P by trehalose-6-phosphate synthase1 (TPS1), and then subsequently dephosphorylated by trehalose-6-phosphate phosphatase (T6PP) to produce trehalose. It was envisaged that the circadian clock-controlled starch turnover, which prevents the plant from carbon starvation before dawn, is achieved via the sucrose/Tre-6-P negative feedback circuit, to balance the supply and demand for sucrose. During the day, Tre-6-P plays little role in regulating starch synthesis, but at night, high levels of Tre-6-P inhibit starch degradation, leading to a starch excess phenotype at the end of the night (Martins *et al.*, 2013). Thus, Tre-6-P feedbacks starch degradation at night, contributing to the maintenance of optimal ratios of starch to sucrose in leaves and export of carbon to the rest of the plant. The details of the regulation mechanism involving Tre-6-P are not yet known.

Interaction between SNF1-related protein kinase 1 and Trehalose-6-phosphe Signaling

SNF1-related protein kinase 1 (SnRK1) is regarded as a central integrator of plant stress and carbon signaling cascades that directly phosphorylates key metabolic enzymes and regulatory proteins, and performs extensive transcriptional regulation (*e.g.*, through bZIP transcription factors) (Baena-González *et al.*, 2007; Nunes *et al.*, 2013). There is an important regulatory link between SnRK1 and Tre-6-P. Processes such as starch metabolism during flowering, anthocyanin biosynthesis, and senescence (Wingler *et al.*, 2012), which were known to be under Tre-6-P-regulation, were also identified as SnRK1 targets (Baena-González *et al.*, 2007).

In storage tissues, overexpression of SnRK1 usually leads to increased starch content. In potato, overexpressing SnRK1 under the control of a tuber-specific promoter resulted in an increase of 23 to 30% of the starch content and a decrease of 17 to 56% of the glucose levels. The activity of sucrose synthase and AGPase, two important enzymes involved in carbon partitioning in the tuber was also increased, by approximately 20-60% and three- to five-fold, respectively (Tiessen et al., 2003). SnRK1 may also play an important role in improving quality traits linked to starch. Thus, in sweet potato (Ipomoea batatas), the IbSnRK1 gene is highly expressed in the storage roots and strongly induced by exogenous sucrose. Its overexpression not only increased starch content, but also decreased the proportion of amylose, enlarged the size of the starch granules and improved the degree of crystallinity and gelatinization. In transgenic plants, the genes involved in starch biosynthesis were systematically up-regulated (Ren et al., 2019). Overexpression of SnRK1 in fleshy fruits led to similar results. Thus, the heterologous overexpression of the MhSnRK1 gene from pingyitiancha (Malus hupehensis) in tomato, led to increased activities of sucrose synthase and AGPase, and to increased content of starch in the leaves and red-ripening fruits (Wang et al., 2012). Surprisingly, the transgenic fruit ripened about 10 days earlier than the wildtype, implying that SnRK1 may play different roles in different tissues. Above all, those results emphasize the importance of SnRK1 in the regulation of carbohydrate metabolism and resource partitioning.

ADP-glucose Pyrophosphorylase Plays a Central Role in the Regulation of Starch Synthesis

AGPase catalyzes the before-last step in starch synthesis, by providing ADP-glucose to the reactions of polymerization catalyzed by starch synthases. The genes encoding AGPase but also the enzyme itself are subject to multiple regulations at both transcriptional and post-transcriptional levels, which play a central role in the regulation of starch synthesis.

Transcriptional Regulation of ADP-glucose Pyrophosphorylase

Plant AGPase is a heterotetramer consisting of two large and two small subunits that is usually located in the plastid of leaves and fruits. One exception is the cereal endosperm, in which there are two isoenzymes of AGPase, one located in the plastid and one in the cytosol (Hannah and James, 2008). Four subunits are required for optimal enzyme activity, but the large subunit plays more of a regulatory role, while the small subunit has both catalytic and regulatory properties (Cross *et al.*, 2004). Multiple isoenzymes of AGPase are present at varying levels in different plant tissues. In *Arabidopsis*, there are four genes encoding the large subunit (Apl1–Apl4) and two genes (Aps1 and 2) encoding the small subunit (**Table 2**). In tomato, there are three genes encoding the large subunit and small unit, respectively (**Table 2**)

The temporal and spatial control of these genes varies between tissues, allowing to produce AGPase with different sensitivity to allosteric effectors to respond to the particular metabolic demands for different tissues. The transcript levels of the genes encoding AGPase are increased by sugars and decreased by nitrate and phosphate (reviewed by Geigenberger, 2011), which may allow starch accumulation to cope with fluctuations of the carbon and nutritional availabilities.

In tomato fruit, AGPase related genes AgpL1, AgpL3 and AgpS1 show particularly abundant expression in the parenchyma, and highest expression at immature stages (Shinozaki *et al.*, 2018). The spatiotemporal expression is coordinated with the more abundant AGPase enzyme activities and more starch accumulation at the inner pericarp (Schaffer and Petreikov, 1997). It was also found that AGPase coding genes AgpL1 and AgpS1 are upregulated by sugar, but not by abscisic acid or osmotic stress (Yin *et al.*, 2010).

A series of studies were performed using introgression lines derived from a cross between the Solanum lycopersicum cultivar M-82 and the wild tomato Solanum habrochaites. The mapping of an introgression line characterized by fruits with high Brix(a proxy for sugar content) identified AGPase as a rate-limiting enzyme in starch synthesis in tomato fruit, and that modifying this single rate-limiting enzymatic step can influence fruit sink carbohydrate metabolism, leading to an increase of the net import of photoassimilates into the fruit (Schaffer *et al.*, 2000). It showed an increased level of starch in the immature fruit, accompanied with increased soluble sugar level in the mature fruit. It was then found that this introgression line harbors the S. habrochaites allele of AgpL1, which provides higher AGPase activity, probably because expression of AgpL1 was prolonged, leading to an extended presence of the AgpL1 protein (Schaffer et al., 2000). The fact that the small subunit protein also remained for an extended period during fruit development in the AgpL1H fruit, suggested that the large subunit contributes to stabilize the AGPase heterotetramer (Petreikov et al., 2006). This introgression line showed increased fruit size, carbohydrate concentrations, and carbohydrate contents of the whole fruit. This was related to a large increase in the transient starch reservoir that, upon degradation, accounted for the subsequent increase in soluble sugars (Petreikov et al., 2010).

Posttranscriptional Regulation of ADP-glucose Pyrophosphorylase

The AGPase enzyme is regulated both by the 3-phosphoglycerate (3-PGA) to phosphate (Pi) ratio and by the redox state.

AGPase is allosterically activated by 3-PGA and inhibited by Pi. When concentrations of cytosolic triose-phosphates are high, their export from the chloroplast is impeded, leading to the accumulation of 3-PGA in the plastid and thus increased AGPase activity leading to increased production of starch. Conversely, in low light or darkness, free Pi accumulates inside the plastid, inhibit AGPase and thus restricts carbon allocation to starch (Tiessen and Padilla-Chacon, 2012). This mechanism allows the source tissues of the plant to react to both the cytoplasmic carbon status and light intensity (Hendriks, 2003).

The two catalytic small subunits of AGPase can form a disulphide bridge at a conserved cysteine residue, which inhibits its activity, while its reduction and the subsequent release of the disulphide

bridge lead to activation (reviewed by Geigenberger, 2011). It was demonstrated that two different forms of thioredoxin (Trx) are involved in the redox regulation of plastid metabolism. One is freely linked to ferredoxin (Fdx) and the other bound to NADPH-dependent thioredoxin reductase C (NTRC). Their importance for starch synthesis in photosynthetic and non-photosynthetic tissues differs, with Fdx/Trx being predominant in chloroplasts and NTRC in amyloplasts (Michalska *et al.*, 2009). However, this conclusion was later challenged by a study conducted with an *Arabidopsis ntrc* mutant. Its complementation with NTRC overexpression under the control of a leaf-specific promoter led to wild-type phenotypes, but the mutant phenotype remained unaltered when a root-specific promoter was used (Kirchsteiger *et al.*, 2012). These data thus indicate a role of NTRC in photosynthetic leaves, rather than in roots. In contrast, a recent study showed that in heterotrophic tomato fruit, NTRC downregulation leads to a strong increase in the reductive states of NAD(H) and NADP(H) redox systems and to decreased transient starch accumulation, which leads to a subsequent decrease in soluble sugars at ripeness. The inhibition of starch synthesis was associated with a decrease in the redox-activation state of AGPase (Hou *et al.*, 2019).

During tomato fruit development the redox status can also be manipulated by reducing the activities of either mitochondrial malate dehydrogenase or fumarase *via* targeted antisense approaches, which leads to changes in redox-activation states of AGPase (Centeno *et al.*, 2011). The changes in AGPase redox state were additionally associated with alterations in starch synthesis in immature fruits and subsequent alterations in soluble sugar content in mature fruits (Centeno *et al.*, 2011). However, a similar manipulation in potato (*via* RNA interference to down-regulate fumarase expression under a tuber-specific promoter control) led neither to an increased flux to starch or increased starch levels, nor to an alteration in yield despite displaying similar reductions in both fumarase activity as observed in tomato fruit expressing a similar construct. The fact is that small changes were observed in plastidial malate, but probably too small to alter the redox state and then the activation state of AGPase, thus indicating that the influence of malate on plastidial starch biosynthesis is highly context dependent (Szecowka *et al.*, 2012).

Interestingly, the redox modulation of AGPase also impacts its allosteric activation. Thus, when APS1 is oxidized, the holoenzyme is less sensitive to 3-PGA (Tiessen, 2002; Hendriks, 2003). NTRC-RNAi lines of tomato fruit (Hou *et al.*, 2019) showed AGPase activity comparable to that

of the wild type when 3-PGA was omitted from the enzyme assay, but in the presence of 0.25 to 10 mM 3-PGA, there was a 30% to 50% decrease in AGPase activity.





Because AGPase has multiple regulatory properties, it is widely accepted that it is a rate-limiting enzyme in starch synthesis. According to Smith and Zeeman (2020), control of flux into starch is likely dependent on genetic, developmental, and environmental factors, while AGPase can play an important role, the general use of the term rate-limiting step is misleading. AGPase exerts significant control over the flux into starch in *Arabidopsis* leaves under saturating light, but less under limiting light (Neuhaus and Stitt, 1990). In potato tubers, AGPase exerts a significant control under non-stressed conditions (Geigenberger *et al.*, 1999, 2004; Sweetlove *et al.*, 1999) but less under osmotic stress (Geigenberger *et al.*, 1999). In tomato fruit, a constant starch synthesis (about 25% of the sucrose taken up was converted into starch) was observed throughout fruit development *via* ³H labeling (N'tchobo *et al.*, 1999). Considering that extractible activity of AGPase, which

corresponds to its capacity, decreases along fruit growth (Biais *et al.*, 2014), AGPase would thus be in excess at early stages of fruit development and starch synthesis would rather be controlled by other mechanisms including sucrose supply. A more striking result revealed that when AGPase activity is repressed using a constitutive promoter, the transgenic plants appeared phenotypically normal despite a repression of AGPase activity by 70% that did also not significantly alter starch levels (Ali, 2003). There was only one line, in which AGPase was reduced by 90%, in which starch levels were altered, indicating that under the growth conditions used in the experiment, AGPase activity was in large excess in tomato fruit. It is worth to mention that in this experiment, tomato fruit stored more carbon in the form of soluble sugars than starch, which may suggest that carbon limitation occurred under these growth conditions (in a growth chamber), and that in this case AGPase was not the rate-limiting step.

Regulation of Starch Degradation

Regulation of Starch Degradation in Arabidopsis Leaves

In *Arabidopsis* leaves, the transcript levels of genes involved in starch metabolism are under strong diurnal/circadian regulation. However, the levels of the encoded proteins generally remain relatively constant (Tenorio *et al.*, 2003; Lu *et al.*, 2005), and it was suggested that the diurnal cycling of transcripts is important for medium- to long-term adjustment of protein levels (Gibon *et al.*, 2004). Therefore, post transcriptional regulation appears to play an important role in regulating starch metabolism.

Possible posttranscriptional regulation of starch degradation includes redox regulation, phosphorylation of proteins and forming of functional protein complexes, as reviewed by Streb and Zeeman (2012) and Skryhan *et al.* (2018). Like AGPase, some enzymes participating in starch degradation were also suggested to be redox regulated, including GWD1, SEX4, BAM1, BAM3, AMY3, ISA3, and LDA (Glaring *et al.*, 2012; Silver *et al.*, 2013; Seung *et al.*, 2013). GWD is required for complete starch degradation, and GWD reduction by thioredoxin increases its activity and stromal localization (Mikkelsen et al., 2005). However, *in vivo* experiments showed that redox regulation of GWD is not essential for initiation or modulation of the rate of starch degradation, as being constitutively active, redox-insensitive GWD mutant could fully complement a *gwd–null*

mutant (Scialdone *et al.*, 2013). Those surprising observations imply that other enzymes also need further *in vivo* studies to confirm their redox regulation. Large-scale phosphoproteomic approaches performed in *Arabidopsis* have identified a number of starch metabolic proteins as putative phosphoproteins *in vivo*, including plastidial PGI, PGM1, AGPase, SS3, GWD1 and GWD2, DPE2, AMY3, BAM1 and BAM3, LDA, pGlcT and MEX1 (see Kötting *et al.*, 2010).

In addition of using sugar signals to regulate starch metabolism, plants also use hormones to adjust starch metabolism to cope with environmental changes or developmental shifts. ABA is well known as a key signal in response to different stress conditions. In *Arabidopsis*, ABA-dependent response to osmotic stress is partially triggered by increasing BAM1 and AMY3 gene expression *via* the AREB/ABF-SnRK2 pathway. Both BAM1 and AMY3 promoters contain ABA-responsive element (ABRE) motifs, while none were found in the BAM3 promoter. Application of exogenous ABA induces BAM1 and AMY3 expression and results in increased BAM1 enzyme activity followed by increased starch degradation. The *amy3/bam1* double mutants are hypersensitive to osmotic stress and show impaired root growth, thus mimicking the phenotype of ABA-deficient mutants (Thalmann *et al.*, 2016).

Regulation of Starch Degradation in Fruit

In fruit, the genes participating in starch degradation are temporally expressed, usually coordinating starch degradation with fruit growth. Unlike in leaves in which diurnal fluctuations in transcript levels are not reflected at the protein level, evidence in fruits indicates a more important role for transcriptional regulation during long-term starch turnover. A well-known regulator of levels of transcripts involved in fruit starch degradation is ethylene, even though the details are missing. In starchy fruits like banana and kiwifruit, which accumulate starch along the development and reach a plateau until the climacteric crisis, a commercial practice that has been used for a century consists in using ethylene to promote ripening and starch degradation. Despite this obvious role for ethylene, there is a lack of systematic analysis of ethylene response elements in the promoter regions of genes associated with starch metabolism. Investigations of regulation mechanism have nevertheless recently emerged, shedding light on ethylene regulation of starch metabolism. Thus, Xiao *et al.* (2018) have identified response elements to diverse hormonal classes in the upstream regions of 16 MaBAM genes in banana. Interestingly, only MaBAM3c,

which was highly expressed in the fruit after the climacteric peak, showed a single element of ethylene response in the promoter region. Similar results were found in kiwifruit by performing a transcriptomics-based approach to identify transcriptional regulatory components associated with starch degradation during the ripening phase. Analysis of the regulatory effects of differentially expressed genes identified a zinc finger transcription factor, DNA BINDING WITH ONE FINGER (AdDof3), which showed significant transactivation on the AdBAM3L (β -amylase) promoter. AdDof3 interacted physically with the AdBAM3L promoter. Moreover, transient overexpression analysis showed that AdDof3 up-regulated AdBAM3L expression in kiwifruit (Zhang *et al.*, 2018).

Besides ethylene, ABA could also play an important role in the ripening of climacteric and nonclimacteric fruit (Jia et al., 2011). In tomato, ABA accumulates before the production of ethylene and has been suggested to be required for normal initiation of tomato fruit ripening, while ethylene acts at later stages of that process (Zhang et al., 2009). The role of ABA in regulating fruit primary metabolism has emerged by comprehensive investigations of metabolites on SIAREB1-OE, SIAREB1-RNAi and ABA biosynthesis absent mutant (not). SIAREB1 belongs to a group of bZIP transcription factors known as ABA response element-binding factors (AREBs) and are part of a major ABA-dependent signaling pathway that regulates gene expression in response to stress. Modifications of SIAREB1 expression levels in tomato by overexpressing or silencing resulted into the up-regulation or down-regulation of genes encoding enzymes involved in primary carbohydrate and amino acid metabolism (Bastías et al., 2014). In the not mutant, which is defective in a SINCED gene, a key gene of ABA biosynthesis, the levels of sugars and sugar alcohols were significantly lower, suggesting that impairment of ABA synthesis results in decreased sugar accumulation. ABA also seems to facilitate the degradation of starch to sugars, as the starch content in the not mutant was significantly higher at both the mature green and red ripe stages investigated (Li et al., 2019). However, there are only few studies describing a possible relationship between ABA and starch metabolism in fruits. Underlying mechanisms that link starch and ABA in tomato fruit remain unknown, and more studies are needed.

It is necessary to mention that transcriptional and post-translational regulation of starch biosynthetic enzymes may differ between species, tissue types, and developmental stages. The coordination of this regulation is to a large extent orchestrated by the sugar status, hormones and environmental stimuli. The sensing and signaling mechanisms mediating these processes are largely unknown. Much more work is required to fully understand how the perception of endogenous and environmental conditions can cause changes in starch turnover and how these aspects of regulation are integrated.

1.2.3 Physiological Roles of Transient Starch

Starch can be accumulated in leaves, roots, pollen, fruit and seeds, acting both in the vegetative growth and the reproductive growth. In *Arabidopsis*, transitory starch is synthesized during the light period, and degraded at night to provide substrates for maintenance and growth. The supply of carbon from starch can be vital for normal plant growth. When diel turnover is impaired, *e.g.* in the *Arabidopsis* starch-excess mutants SEX1 and SEX4, plant growth is slowed down and biomass decreases (Zeeman *et al.*, 2002). Starch also accumulates in the guard cells that control stomatal pore size and thus the exchange of water vapor, CO₂, and O₂ between the leaf and the atmosphere. Transitory starch in these cells plays a key role in determining the velocity of stomatal opening in the light. BAM1 was found to be an essential enzyme for starch degradation in guard cells, as the *bam1* knockout mutants were found to have more starch in illuminated guard cells and reduced stomata opening (Valerio *et al.*, 2011).

For many cereal crops including rice, maize, wheat, and barley, starch is the main storage reserve in mature pollen grains and used for supplying energy and a carbon skeleton to support pollen germination and pollen tube growth for proper fertilization. Mutations in the rice plastidic PGM, OspPGM, and the plastidic AGP LS, OsAGPL4, resulted in starch deficiency in pollen grains and induced male sterility (Lee *et al.*, 2016). In tomato, starch is synthesized at an early stage of pollen development (after the first haploid mitosis), and starts to be hydrolyzed during the late two celled stage. The process is complete just before anther dehydration, and the mature pollen grain of tomato is starchless (Pacini and Franchi, 1988). The tomato GWD (LeGWD) heterozygous mutant pollen had a starch-excess phenotype that was associated with a reduction in pollen germination (Nashilevitz *et al.*, 2009).

For fruit development, starch, as a flexible carbon and energy reserve, plays important roles all along fruit growth. For early fruit set, starch was suggested to be a carbon reserve that contributes

to sink strength, thus buffering the fluctuation of energy supply, particularly under mild stress conditions (Ruan *et al.*, 2012). A study of kiwifruit suggested that starch turnover occurs during cell division (Nardozza *et al.*, 2013). Starch (also cell wall polysaccharides) degradation may also be related to the climacteric respiration of tomato fruit that occurs during ripening, as revealed by constraint-based modeling (Colombié *et al.*, 2017). Fruit species that do not store carbohydrate reserves such as starch, for instance muskmelon, must remain attached to the plant for allowing the accumulation of soluble sugars to occur during ripening (Hubbard *et al.*, 1990). In contrast, climacteric fruit species that store starch when fruit is commercially mature, such as banana and kiwifruit, can be harvested before ripening and have a long shelf life. The later net starch degradation in those starch y fruits, which has been attributed to the complex action of a range of enzymes related to starch breakdown at transcriptional and translational levels (Xiao *et al.*, 2018), massively contributes to the final sugar content (Prabha and Bhagyalakshmi, 1998; Nardozza *et al.*, 2013). Petreikov *et al.* (2010) proposed an increase in transient starch accumulation in tomato as a valuable strategy for increasing the sink strength of the developing fruits, the final size and sugar levels of the ripen fruits.

During growth and development, plants face many abiotic stresses related to water (drought or flooding), temperature (heat or cold), light (low/high light or UV light). Starch metabolism appears to play a key role in mitigating the effects of abiotic stress in plants. Thus, when tomato plants were grown under control, shading or water shortage conditions, fruit hexose and sucrose amounts were similar, but fruit starch content showed large fluctuations during fruit growth, which suggested that starch may play a buffering role for carbon supply under different abiotic stresses (Biais *et al.*, 2014). In many cases, plants respond to abiotic stresses by mobilizing starch reserves and releasing soluble sugars to provide energy for growth, and/or by providing sugars as osmoprotectants and substrates for metabolism (intensively reviewed by Thalmann and Santelia, 2017; Dong and Beckles, 2019). Stress-induced starch accumulation has different effects that depend on the tissue type. In the source, it can alleviate excess sugars that repress photosynthesis. While in non-photosynthetic tissues, it can maintain sink strength, and also provide a maximal carbon reserve for the next generation. Dong and Beckles (2019) proposed that starch metabolism is a key convergence point in plant stress response as a mechanism for changing sugar availability.

Through starch-sugar reconversion, starch is able to influence whole-plant carbon allocation, and functions as a buffer against abiotic stress.

Chapter1.3 Strategies for Studying Starch Metabolism in Fleshy Fruits: Forward and

Reverse Genetics

As seen before, transient starch plays import roles in fruit. It is used as carbon and energy reserve during early development, thus buffering possible fluctuations in carbon supply that are linked to the environment, and it contributes to final sugar accumulation. In climacteric fruit it is also involved in the respiration climacteric that occurs at ripening, thus playing an important role for postharvest quality (*e.g.*, shelf life). To achieve those multiple roles, a sophisticated network integrates developmental and environmental signals and orchestrates starch turnover. During last decades, metabolism of transient starch has been intensively studied in leaves and endosperm, which nevertheless show significant differences between them and with fruit and further organs regarding the mechanisms responsible for starch synthesis and degradation. However, even in leaves and endosperm, the regulation of starch degradation remains largely unknown. Thus, it is necessary to study starch metabolism in fruit in order to better understand its regulation and physiological roles during fruit growth and ripening phases, which will ultimately facilitate breeding strategies to improve fruit quality, fitness and postharvest properties through breeding and agricultural practices.

1.3.1 Tomato (var. Micro-Tom) as the Model for Studying Starch Metabolism in Fleshy Fruit

To study starch metabolism in fleshy fruit, tomato can be an excellent model for several reasons. First and most importantly, tomato fruit accumulates starch under optimal growth to large amounts with net accumulation rates and accumulation patterns that are comparable to those found in other starchy fruits like banana, kiwifruit and apple (see Chapter 1.2.1). Second, tomato, which is often considered as the model plant system for fleshy fruit species (Rothan *et al.*, 2019), has numerous

advantages for genetic, molecular and even physiological studies. Thus, rich resources of genomic, transcriptomic and proteomic data are available for tomato fruit, and bioengineering methods are well developed and widely applied, and finally are of advantage for both forward and reverse genetic approaches. Regarding experimentation, unlike most other starchy fruits usually growing in fields, tomato has a relatively short growth period that enables up to 3 to 4 cultivations per year and can easily be cultivated in the greenhouse, under well controlled conditions. Besides, there is a relatively abundant literature on starch regulation, especially on AGPase regulation, in tomato and potato, which are both members of Solanaceae. This knowledge provides a solid basis for studying starch metabolism in tomato fruit.

The "micro" tomato (Micro-Tom) is a miniature tomato mutant, which is dwarf, bushy, and determinate and has dark-green leaves, short internodes, and small fruits. It was bred by crossing the home gardening cultivars Florida Basket and Ohio 4013-3 (Scott and Harbaugh, 1989). Three recessive mutations are mainly responsible for those traits: dwarf (d), self-pruning (sp), and miniature (mnt). The D gene encodes a cytochrome P450 protein involved in brassinosteroid (BR) biosynthesis (Bishop *et al.*, 1996). SP is the ortholog of *Arabidopsis* TERMINAL FLOWER 1 (TFL1) and Antirrhinum CENTRORADIALIS (CEN) and is responsible for continuous growth of shoot apical meristem (Pnueli *et al.*, 1998). The "miniature" mutation associated with the short internode phenotype is likely due to a reduction in gibberellin (GA) signaling (Marti, 2006). However, the responsible gene has not yet been identified (reviewed by Okabe and Ariizumi, 2016). Besides those phenotypes, no significant difference were found for fruit central metabolism when comparing Micro-Tom with other cultivars (Obiadalla-Ali *et al.*, 2004).

Those Micro-Tom traits also provide several extra experimental advantages compared to other tomato cultivars. Firstly, Micro-Tom requires less time and space for cultivation. The life cycle of Micro-Tom from sowing to seed harvesting is shorter (70–90 days), thus allowing more generations per year. Micro-Tom plants can be raised to maturity in a greenhouse or growth chamber condition at a very high density, for instance an area $<4 \text{ m}^2$ is necessary for the culture of a segregating population of 600 plants. Secondly, transformation of Micro-Tom by *Agrobacterium* is well established and has a relatively high efficiency compared to other model plants (Sun *et al.*, 2006). In addition, Micro-Tom can be crossed to many tomato varieties, allowing easy transfer of Micro-Tom-derived mutations to other varieties. As a consequence, Micro-Tom facilitates

experimentation that requires large numbers of individuals and several generations. Also, Micro-Tom can easily be grown under well controlled environments that make experiments more reproducible and this greatly facilitates forward genetic approaches.

1.3.2 Forward Genetics

Forward genetics has proven very valuable to discover and study gene function. Thus a series of *Arabidopsis* starchless or starch-excess (sex) mutants greatly facilitated the research on transient starch in leaves (Caspar *et al.*, 1985; Zeeman *et al.*, 1998). These mutants, carrying random mutations, were usually obtained by chemical or irradiation treatments. Phenotypic screenings were carried on those mutants, followed by the identification of the potentially causal mutations by map-based (positional) cloning. Although this forward genetic approach has been very successful regarding leaf starch metabolism, it has hardly been used to study starch metabolism in fruit. For starchy fruits such as kiwifruit, banana, and apple, it is indeed difficult to create and maintain mutant populations, and because of their long growth cycles mapping the causal genes can be very time-consuming. However, Micro-Tom shows particular advantages for applying forward genetic approaches thanks to its miniature and short life cycle (as mentioned above).

To maximize the chances to find interesting phenotypes, the mutated population needs to be highly mutated and the phenotyping to be robust and amenable to high throughput. Moreover, to identify causal mutations, a well-developed method for mapping (genotyping) is required. Each of these three steps can be the bottleneck in the discovery process. Fortunately, in our unit (UMR1332 BFP, INRAE, Univ. Bordeaux), we maintain a big population of Micro-Tom ethyl methanesulfonate (EMS) mutants and benefit from a high throughput metabolic phenotyping platform (HiTMe).

EMS Mutant Population of Micro-Tom

Ethyl methanesulfonate (EMS) is a commonly used chemical mutagen that generally causes single nucleotide transitions and only rarely induces nucleotide transversion. In Bordeaux, ~8,000 EMS mutant lines have been developed and 30,000 annotations are available for more than 150 phenotypic categories (Just *et al.*, 2013) in the associated Micro-Tom Mutant Database (MMDB, not yet publicly searchable). According to Just *et al.* (2013) , seeds of Micro-Tom were treated

with 1% EMS as described by Dan *et al.* (2007) and Okabe *et al.* (2011), thus generated M1 seeds and M1 plants. In order to increase the mutation frequency, seeds were collected from part of M1 plants and used to perform a second round of 1% EMS treatment. The M2 seeds generated from mutant M1 plants produced from one or two rounds of EMS mutagenesis constructed a population with ~8,000 M2 families. The mean mutation density of the mutant collection was estimated to be close to one mutation per 663 kb by screening 49.9 kb of DNA sequence on 7,296 M2 families by using Endo1 endonuclease. However, this result is probably underestimated because one mutation per 125 kb was found for some genes by TILLING (Targeting Induced Local Lesions in Genomes), and a recent whole genome sequencing of an EMS mutant line from the collection uncovered even higher mutation frequencies.

High Throughput Phenotyping

The HiTMe (High Throughput Metabolic phenotyping) platform is able to quantify metabolites and enzyme activities in very large numbers of samples. It is equipped with three cryogenic grinding units, two 96-channel liquid handling robots, one integrated robotic unit (96-channel liquid handling, centrifuge, heating, microplate reading, *etc.*), and 11 filter-based microplate readers, *etc.* The combination of microplate technology and pipetting robots enables the measurement of metabolic traits such as starch content in up to hundreds of samples per day (for details, https://www.phenome-emphasis.fr/phenome_eng/Installations/Bordeaux-Omic).

Mapping-by-sequencing

In classic forward genetic approaches, the causal gene of a given mutation is identified by mapbased (positional) cloning. It is the process of identifying the mutation by looking for linkage to markers whose physical location in the genome is known. Before the genomic era, it usually took years with large amounts of lab work to find the corresponding mutation. It was necessary to build a physical map, develop markers, followed by cloning, complementation by transformation, and *de novo* determination of the sequence of the entire region of interest to high quality without a previously determined wild-type DNA sequence as a guide (Jander, 2002). Nowadays, thanks to second-generation sequencing, the genotyping procedures have been dramatically shortened and the cost greatly reduced, thus providing a powerful alternative mapping strategy called mappingby-sequencing. It consists in identifying the allelic variant underlying the phenotype of interest by performing whole-genome sequencing. Mapping-by-sequencing, which is very well adapted to tomato, is considered as considerably shortening the time needed to identify a causal mutation (Garcia *et al.*, 2016). When applying mapping-by-sequencing to Micro-Tom EMS mutants, forward genetics becomes remarkably feasible by requiring a limited space for plant culture, reduced time and cost for mapping.

The whole procedures of mapping-by-sequencing developed on Micro-Tom was documented and detailed by Garcia *et al.* (2016). Taking a recessive trait as an example, a homozygous mutant showing the phenotype of interest after the first screening is selected for confirming the phenotype. After confirmation, the homozygous mutant is crossed with the WT to generate back-crossed F1 (BC1F1) plants, which are then self-pollinated to produce a BC1F2 population. In this population, plants segregate and display WT-like and mutant-like traits according to Mendelian laws. Around 30 to 40 individual plants are selected to construct a WT-like bulk and a mutant-like bulk, based on the phenotype. Then, leaves of the two bulks are used to extract DNA for sequencing to a read depth of $\sim 20-40\times$. Sequences are analysed based on the pipelines with filtering the natural polymorphisms, analyzing allelic frequencies and variant calling. According to Mendelian segregation, the average of allelic frequencies of a target region for the WT-like bulk is around 0.33, while for the mutant-like bulk, it is close to 1. After determining the region of interest, a recombination analysis can be further performed to refine the target region to identify the causal mutation (so called fine-mapping).

Identification of the Causal Mutation

After narrowing down the region of interest, the evaluation of the candidate mutations in a cleanup background (like WT plant) is necessary considering that EMS mutants usually carry on thousands of mutations with possible interferences. EMS produces mutations randomly on the chromosomes, which locate in none-coding regions possibly involved in regulation or in coding regions, resulting in different kinds of mutations. According to the effect on protein sequences, it can be categorized as moderate, missense and knockout mutations. According to the nature of the mutation effect, different identification strategies are required.

To mimic the different mutation effects mentioned above, we can utilize different bioengineering methods, including the generation of missense or KO mutants *via* CRISPR/Cas9 techniques, and the down- or up-regulation of candidate genes by using gene silencing (*via* antisense, sense or RNA interference), or overexpression by fusion with constitutive or tissue-specific expression promoters. All of those genetic engineering tools (CRISPR/Cas9, RNAi, overexpression and so on) allow for a comprehensive examination of the different types of mutations.

CRISPR/Cas9 can generate DSBs (DNA double-strand breaks) *via* RNA-DNA base pairing and the PAM (protospacer adjacent motif) sequence recognition (Adli, 2018), which can be repaired either using error-prone non-homology end joining (NHEJ) or homology-directed repair (HDR) by the organism. Those processes may result InDel mutations or precise substitutions, leading to knockout mutation or precise-genome editing, respectively. The Second generation of CRISPR gene-editing tools are developed to perform base-editing, CRISPR-mediated gene expression regulation, and CRISPR-mediated live cell chromatin imaging *etc.* (Adli, 2018). All of them are achieved by turning nuclease Cas9 to nickase Cas9 (nCas9) or dead Cas9 (dCas9, catalytically inactive Cas9), but still with the capability to recognize specific sequences. The engineered Cas9 can then be fused with other functional enzymes or proteins to enable base editing, gene regulation or chromatin imaging.

In tomato, the first case of applying CRISPR/Cas9 was reported by Brooks *et al.* (2014). Later, Shimatani *et al.* (2017) succeeded in generating homozygous mutant plants with heritable DNA substitutions by using nCas9^{At} fused with either a human codon-optimized PmCDA1 (nCas9^{At}-PmCDA1^{Hs}) or a version codon-optimized for *Arabidopsis* (nCas9^{At}-PmCDA1^{At}). There is also room for improvement for this technique, since part of the offspring of T0 generation also carried InDel mutations, additionally the rate of inducing substitution was much lower than that of InDel mutations. However, it showed the possibility of base editing for crop improvement. CRISPR-Cas9 and related second-generation genome-editing tools increase the feasibility and enlarge the applicable scope of biotechnology.



Fig 6 A general workflow for forward genetic approaches.

The procedures include phenotyping *via* high throughput metabolite measurements, genotyping *via* mapping-by-sequencing, validation of the causal gene *via* bioengineering. The figure is adapted and cited from Garcia *et al.* (2016) and Causse *et al.* (2020).

1.3.3 Reverse Genetics

In contrast to forward genetics, which is a process without prior assumptions, reverse genetic approaches rely on prior knowledge to select candidate genes. Therefore, reverse genetics are considered as being gene-driven rather than phenotype-driven. Both forward and reverse genetics are widely used and are fruitful strategies. Reverse genetics usually takes advantage from discoveries made on model species like *Arabidopsis* to examine a certain set of genes for unanswered questions.

For starch research, it is worth to take this strategy to create transformants *via* genetic engineering, since among leaves and fruits, the basic synthesis and degradation participants are conserved, but the relatively importance and specific roles might differ. Specially, in leaves, starch is under clock control with diel turnover, but in fruits, instead of having a diel turnover, starch exhibits a more stage-depended turnover (introduced in Chapter 1.2.1). Besides, the studies on starchy fruits like banana and kiwifruit mostly based on correlations and *in vitro* experiments, to examine the candidates *in vivo* of tomato *via* genetic engineering may provide more evidence and extra information.

A comprehensive mining of tomato genes homologous to *Arabidopsis* and potato genes involved in starch metabolism, which are listed in **Table 2** and **Table 3**, has been performed by Moustakas (2013). This knowledge on other species and tissues could be a good reference for exanimating starch metabolism in tomato fruit. Besides, as mentioned above, rich resources of genomic, transcriptomic and proteomic data are available for tomato. Thus, the newest version of the tomato annotated genome (SL4.0 and ITAG4.0) was published in 2019 (Hosmani *et al.*, 2019). Transcriptomic data on developmental stages and different tissues/cell types of fruits are also available since 2018 (Shinozaki *et al.*, 2018) in the TEA database (Tomato Expression Atlas, http://tea.solgenomics.net/). To better visualize and explore multiple levels of tomato fruit data mentioned above, an online tool named ePlant (http://bar.utoronto.ca/eplant_tomato/) was constructed by Waese *et al.* (2017). It enables the exploration of subcellular prediction of encoded proteins, transcript levels in different tissues, genome position, sequences *etc.*, thus facilitating data mining and function predicting for many genes. To predict the protein abundance according to the transcript level, a model was built by Belouah *et al.*(2019) to extract information of protein abundance and destiny from transcript levels. The model for the translation of a protein predicted the median values of ~ 2 min, and a protein lifetime of ~ 11 days. Hence, protein concentration is more strongly affected by the rate of translation than that of degradation. It underpins the prediction of protein level based on transcript regardless the dilution effect of fruit growth or the unknown regulation mechanisms.

The subcellular information can be very useful too, as transient starch is synthesized and degraded in plastids. Hence for some proteins, such as BAM members BAM7 and BAM8, the location is not in plastid but in nucleus, implying that they do not directly participate in starch granule attacking like the other members (BAM1 and BAM3), more likely playing as indirect regulators (Reinhold *et al.*, 2011).

After integrating all the available information and useful references, genetic engineering methods described above can be used to manipulate the candidate genes *in vivo*, followed by characterizing of the derived phenotypes to dissect the functions of those genes in fruit starch metabolism.

1.3.4 Aims and Strategies for the Thesis

In many fruits such as banana, kiwifruit and apple *etc.*, starch, as carbohydrate reservoir, provides carbon and energy during fruit growth, and contributes to large amount of sugar at ripening. Additionally, previous studies of the Meta group (Biais *et al.*, 2014; Colombié *et al.*, 2017) suggested that starch plays a buffering role under mild stressed environment (shading and water stress) in tomato fruit, and that its degradation enhances respiration during ripening. Although starch plays important roles in fruit, we know little about its metabolism and regulation in fruit. We used tomato (var. Micro-Tom) as a model for studying starch metabolism and possible regulations, as it accumulates considerable amount of starch during fruit growth, and is easier for bioengineering manipulation than other starchy fruits (banana, kiwifruit, apple, *etc.*).

The success of a series of starch mutants (*sex*, *sex4*...) in *Arabidopsis* encouraged us to take advantages of the big EMS mutant population of Micro-Tom in our unit (BFP, Bordeaux) to also apply forward genetics for screening novel starch mutants in tomato. At the same time, as starch metabolism shows large diversifications for different tissues and species, we took prior knowledge

as references to select candidate genes that may play important roles in starch degradation, to generate starch mutant *via* reverse genetics.

With those two strategies, we aimed at finding mutants accumulating abnormal amount of starch, and identifying the causal mutations, and study the functions of responsible candidate genes or regions to reveal novel mechanisms of starch metabolism and regulation. We also aimed at generating new starch mutants to provide new understanding of how starch participating in fruit growth and ripening.



Fig 7 The schematic presentation of the strategies and aims of thesis



Part II-Results

Chapter2 Screen for Novel Starch Mutants

in Tomato (var. Micro-Tom) Fruits

2.1 Introduction

As introduced in Chapter 1, starch is an important reservoir of carbon and energy for fruit development (reviewed by Roch *et al.*, 2019), contributing to final sugar accumulation and central metabolism of fruits, and also affecting postharvest qualities (brix, shelf storage *etc.*) (Schaffer and Petreikov, 1997; Centeno *et al.*, 2011; Nardozza *et al.*, 2013). Besides, it buffers the environmental changes during fruit growth and may associate with climacteric respiration at ripening (Biais *et al.*, 2014; Colombié *et al.*, 2017). However, little is known about starch metabolism in fruit.

Encouraged by the success of a series of starchless or starch-excess (sex) mutants of *Arabidopsis* (Caspar *et al.*, 1985; Lin *et al.*, 1988; Zeeman *et al.*, 1998), we applied forward genetics to screen for starch related mutants in tomato fruit. In this chapter, we used a highly mutated tomato population (EMS mutant population of Micro-Tom, Just *et al.*, 2013) and robust and high throughput biochemical phenotyping, and then guided by a previous developed method based on second-generation sequencing for mapping (so-called mapping-by-sequencing, Garcia *et al.*, 2016) for searching the causal mutations. After screening nearly 6000 mutant individuals, we selected 3 starch-excess mutants, and then performed Mendelian inheritance analysis for those three. We selected one homozygous mutant P17C12 with a recessive starch-excess phenotype to be further mapped. The procedures and results of the whole screening, confirmation and mapping are presented here.

2.2 Materials and Methods

Plant Material

Experiments were performed on a dwarf determinate tomato cultivar cv. Micro-Tom. At INRAE Bordeaux, a highly mutagenized EMS (ethyl methanesulfonate) collection of Micro-Tom has been developed as described by Just *et al.* (2013). Briefly, seeds of Micro-Tom were treated with 1% EMS as described by Okabe *et al.* (2011). Treated seeds were sown and mutant plants were cultivated in the greenhouse to produce mutated seeds. This first batch of mutated seeds was pooled and submitted to a second 1% EMS treatment, in order to increase the mutation frequencies. The seeds of the second round of EMS treatment were cultivated and leaves were harvested on each single mutant plant for DNA extraction and stored at -80°C in 96 well microplates. Thus, the plate number and the position on the plate give the name of a single mutant plant corresponding to one EMS treated seed. For example, the mutant in plate 17, row C, column 12, was named P17C12, and all the seeds harvested from this plant, are sorted as progenies from one family, P17C12.

During the 2012–2013 period, 500 mutant families of the EMS Micro-Tom population and WT plants with 12 plants per genotype have been cultivated in the green house (Bournonville, 2015), the whole representing about 6000 plants. Fruits at ripening stage (3–4 fruits per plant) were harvested, ground in liquid nitrogen and stored at -20°C until used for specific assays. Thereafter, A. Mirto (PhD 2016 Caserta University-Italy), in the frame of an ERASMUS exchange program, stayed at UMR BFP in two periods (2015–2016) during which he screened samples of 250 families for differences soluble sugars (glucose, fructose, sucrose) and/or starch (corresponding to mutants in plates P17, P20 and P21). This investigation allowed selecting 11 mutant families displaying altered starch and/or sugar contents compared to WT fruits. In order to confirm these phenotypes, 12 plants of each of the 11 selected families and WT were grown again in 2016. This time fruits at the immature green (IMG), breaker (Br), and red ripe (RR) stages were collected and assayed for metabolite contents. Regarding starch levels, only three over the 11 families displayed a significant increase in IMG and RR fruits. The work described above was a part of A. Mirto PhD study.

Methods

Greenhouse Settings, Plant Growth Conditions and Management

The greenhouse settings and tomato growth conditions are described in detail by Rothan *et al.* (2016). In brief, plants were grown under a long day photoperiod with 15 h/9 h day/night. Temperature settings were 18°C during the night and 23°C during the day. During the day, if natural irradiance was decreased below 4000 lux, supplemental lighting would be provided, and if natural irradiance was too high (more than 450 W/m²), a shading screen would be unrolled over the plant culture. The relative humidity was generally set at around 70%.

Since Micro-Tom is a cultivar with poor self-pollination ability, it is necessary to perform manual pollination, otherwise most of flowers would abort or this might result in an abnormal fruit development leading to small and seedless fruits. Therefore, at anthesis (fully open flower), manual pollination of the flower was carried out using the tip of an electric toothbrush device. The flower was then tagged at its petiole using a straw ring dated at the pollination day, hence allowing to harvest fruits at exact developmental stages defined as days after pollination (DAP). In order to reduce source/sink competition between trusses and to allow optimal fruit development, when the first three fruits started to develop on the first two trusses (total of 6 to 8 fruits per plant), the extra flowers as well as the extra inflorescences were removed.

Seeds were obtained from fruits harvested at the red ripe stage. After removing the outside locular tissue, they were treated with a hypochlorite solution at 2.5% of active chloride for 15 min and then rinsed with water three times for 10 min to eliminate the remaining active chloride, and placed under a hood overnight to get dry. Dry seeds are stored in paper bags at 8–10°C in the presence of a desiccant to prevent rehydration.

Analysis of Sugar and Starch Contents

For the extraction of sugars and starch, 96-well micronic tubesTM equipped with screw cap and silicon-O-ring were used. About 15 to 25 mg fresh powder of tomato fruit pericarp was weighted precisely. A batch of analysis consisted of 6 aliquots as biological standards, which were used on every batch of a given experiment, 6 blanks (without sample), and 84 randomly placed tubes with
samples, for a total of 96 wells. The map of the plate was recorded (sample names and powder weight) for calculations.

Extraction of sugars: This extraction is robotized using a designed Robot (Hamilton Microlab STAR Liquid Handling System), allowing the extraction of 4 plates in 4 hours. The protocol was as follows. The first extraction was carried out with 250 μ L 80% v/v ethanol and HEPES/KOH 10 mM (pH6) added to all tubes that were shaken for 30 sec. Then plates were moved to an incubator at 80°C for 20 min, followed by a centrifugation step at 5,000 rpm for 10 min. The robot transferred the first supernatant (S1) into new tubes. The second extraction was carried out with 150 μ L 80% v/v ethanol and followed the same steps to obtain the second supernatant (S2), which was pooled to S1. The third extraction was carried out with 250 μ L of 50% v/v ethanol and the supernatant S3 was pooled to S1 and S2 (in total 650 μ L), and finally stored at -20°C for metabolite assays.

Extraction of starch: starch, which is insoluble in 70% or 50% ethanol, is contained in the pellet left following the ethanol fractionation. For that reason, the pellet was re-suspended in 400 μ L NaOH (0.1 M) and vigorously mixed, and then incubated at 90°C for 30 min. The extract was then neutralized before adding degradation enzymes whose optimal pH is 4.9. After adding 80 μ L neutralization mix (acetate/NaOH, 0.1 M, pH=4.9, plus HCl 0.5 M), it was necessary to check the pH, which should be around 4 to 5. Then we added 100 μ L of starch degradation mix per sample, which contained amyloglucosidase (100 U/mL) and α -amylase (140 U/mL) in 50 mM acetate/NaOH buffer (pH=4.9), to reach the final volume 580 μ L. The plates were incubated at 37°C for 10 to 16 hours to reach full degradation.

Determination of glucose and fructose: The method used for the determination of hexose was adapted from Stitt *et al.* (1989) for robotic analysis. The assay solution contained HEPES buffer (0.1 M HEPES/KOH, pH 7.0, with MgCl₂ 3 mM), ATP (3 μ M), NADP (1.4 μ M), and G6PDH (grade II, 3.4 U/mL). A pipetting robot was used to transfer solutions into a 96-well microplate, and each well was filled with 10 to 50 μ L of the supernatant (S1+S2+S3) and 160 μ L of determination mix. The plate was then placed in a SAFAS MP96 microplate reader and the reaction was measured at 340 nm and at 37°C (1 reading per min). Enzymes were added successively, as following (once OD stabilized): (1) 1 μ L hexokinase (900 U/mL solution in 0.1 M HEPES/KOH

buffer) for measuring glucose; (2) 1 μ L phosphoglucose isomerase (1000 U/mL solution in 0.1 M HEPES/KOH buffer) for measuring fructose.



Fig 8 Principle of the determination of hexoses (Adapted from Roch, 2018)

Firstly, glucose is catalyzed by HK (hexokinase) and G6PDH to produce NADPH, which can be detected at 340 nm with SAFAS MP96 microplate reader. The enzyme PGI (phosphoglucose isomerase) is then added to the mixture to convert F6P (fructose 6-phosphate) to G6P (glucose 6-phosphate) for the measurement of fructose.

Determination of starch: starch was degraded into glucose as mentioned above (section of "Extracting of starch"), then centrifuged the micronic tubes and used $10-50 \,\mu$ L to measure glucose as described above.

Calculations:

For NADPH:

$$NADPH \ \mu mol \ = \frac{\Delta OD}{\varepsilon \times l'} = \frac{\Delta OD}{6.22 \times 2.85} \ \mu mol$$

 $\varepsilon = 6.22 \text{ mL} \cdot \mu \text{mol}^{-1} \cdot \text{cm}^{-1}$, extinction coefficient of NADPH at 340 nm.

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 $l'= 2.850 \text{ cm} \cdot \text{mL}^{-1}$, was calculated by a standard curve produced by measuring ΔOD with a series of known concentrations of NADH with the microplate we used in the experiments.

For hexoses:

$$\mu mol \cdot gFW^{-1} = \frac{\Delta OD}{6.22 \times 2.85} \mu mol \times \frac{650 \ \mu L}{X \ \mu L \ supernatant} \times \frac{1000}{Y \ mg \ fresh \ weight}$$

In the formula, 650 μ L is the total solution volume for hexose extraction per sample. X μ L is the volume used for hexose measurement, normally from 10 μ L to 50 μ L. Y mg is the amount of each sample used for hexose extraction which was recorded at preparation procedures. Multiplying by 1000 is to convert units from mg to g.

For starch:

$$\mu mol \ Glucose \ Equivalent. \ gFW^{-1}$$

$$= \frac{\Delta OD}{6.22 \times 2.85} \mu mol \times \frac{580 \ \mu L}{X \ \mu L \ supernatant} \times \frac{1000}{Y \ mg \ fresh \ weight}$$

In the formula, 580 μ L is the total solution volume for starch extraction per sample. X μ L is the volume used for starch (converted into glucose) measurement, normally from 10 μ L to 50 μ L. Y mg is the amount of each sample used for starch extraction which was recorded at preparation procedures. Multiplying by 1000 is to convert units from mg to g.

Apply Mapping-by-sequencing for Starch-excess Mutant P17C12

In 2017, from June to September, 200 individuals of BC1F2 (self-pollinated offspring of BC1F1) of P17C12 as well as 12 individuals of P17C12 S2 (second generation of self-pollination), 12 plants of BC1F1 (backcross with WT once) and 12 WT plants were cultured. For each plant, the pericarp of 3 fruits was sampled at 20 DAP and 35 DAP. Starch and sugars were measured as described above. According to the results obtained for starch at 20 DAP and 35 DAP, ~30 plants with fruit showing a starch-excess phenotype at 20 DAP were selected for the mutant-like bulk, and ~30 plants with a WT-like fruit starch phenotype were selected for the WT-like bulk. For each bulk, 5 leaf disks of 5 mm in diameter were taken using a cork borer from each selected plant, then

pooled into 2 segregate 50 mL tube to extract DNA, as described previously by Garcia *et al.* (2016). Later, DNA quality and integrity were controlled by using an agarose-gel and Nanodrop.

The DNA samples obtained for the two bulks were sent to the GeT-PlaGe sequencing platform (Genotoul, Toulouse) to perform Illumina HiSeq2000 sequencing with a depth of ~20-40×. Before sequencing, quality was checked by fluorometric measurement with the Quant-iT dsDNA Assay Kit. The sequencing data was analysed following the steps described and well-documented by Garcia et al. (2016). In brief, the Heinz 1706 tomato genome sequence (SL3.0) was used to map the raw reads of the mutant- and WT-like bulks of P17C12 by using the BWA v0.7.15 aligner. Then we used SAMtools v1.3.1 to perform variant calling for the two bulks, generating output .vcf files of total variants. Additionally, to filter the natural polymorphisms between Heinz 1706 and Micro-Tom, the sequencing and mapping of Micro-Tom WT plants were required as a control. To analyse the remaining EMS variants, an in-house Python script was developed to compare the variants for each bulk and generate SNP files (1), calculate the variant allelic frequencies for each bulk (2), indicate the variants present in the Micro-Tom WT line (3) and indicate the localizations in tomato genes according to the Heinz 1706 genome annotation. When obtained only EMS mutations, to specifically identify the causal mutation, two filtering parameters were further considered: the read depth that should be between 10 to 100 to remove false-positive variants, and allelic frequency that expected for a recessive mutation in two bulks (for mutant-like bulk it tends toward 1, and for WT-like bulk it tends toward 0.33).

Fine Mapping

For recombinant analysis, the plant material was obtained from the same BC1F2 culture of P17C12. However, instead of pooling the leaves of the selected plants for the bulk construction, this time leaves for each plant were harvested individually and placed in 96-well plates for large-scale analysis. DNA extraction and quality control were carried out as described above. The SNP markers of EMS mutations were used to genotype the BC1F2 populations. The Kompetitive Allele Specific PCR (KASP) assay was used to detect the homozygous WT, homozygous mutant or heterozygous at different EMS mutation loci (LGC Genomics; http://www.lgcgroup.com/our-science/genomics-solutions/genotyping/). All the primers were designed with the Batch-Primer3 software and are listed in **Table 4**.

Position	Name of primer	Sequences	
	Reverse primer	AGACACCACTCATGACCCCCTTGT	
Ch01C11280174T	Forward Primer 1	AGCCTTCATATGTACATTTCACAAGTGAAC	
	Forward Primer 2	GAGCCTTCATATGTACATTTCACAAGTGAAT	
	Reverse primer	AATTTGTGTGCACGCAATTCTGAAC	
Ch01G20163672A	Forward Primer 1	GAACTTTCAAATCCTGATAGAGTCCTTGAG	
	Forward Primer 2	GAACTTTCAAATCCTGATAGAGTCCTTGAA	
	Reverse primer	GGGATGTGGTCTTATGCAAGATGGA	
Ch01G40105420A	Forward Primer 1	GGTAATTCTTCTAATGTATTTTCAGGTGTCTGG	
	Forward Primer 2	GGTAATTCTTCTAATGTATTTTCAGGTGTCTGA	
	Reverse primer	GTGGAAGGGAGAGAACTCAAAATGT	
Ch01C60643457T	Forward Primer 1	CAATCTTTGAGACATAAAATGATCTTACCCC	
	Forward Primer 2	CAATCTTTGAGACATAAAATGATCTTACCCT	
Ch01G86626712A	Reverse primer	CCTGTCTCCGAATCCCATATTGACA	
	Forward Primer 1	GGACTCGCCTTCACATTCCG	
	Forward Primer 2	GGGACTCGCCTTCACATTCCA	
	Reverse primer	CCAGTTTTGTCAGAGAAAACGTATTTT	
Ch01G87809674A	Forward Primer 1	GGGCTCTTAACATAAATGAGGATTTGG	
	Forward Primer 2	AGGGCTCTTAACATAAATGAGGATTTGA	
	Reverse primer	TGAGACGAACACATTTTCTGATCATTC	
Ch01G88201115A	Forward Primer 1	AAAAAGATAGTTGCCGTACAGGTTCTG	
	Forward Primer 2	TCAAAAAGATAGTTGCCGTACAGGTTCTA	
	Reverse primer	CCATTCCACCATCTCTCATTGCTGT	
Ch01C88241063A	Forward Primer 1	GTTGAAAAGGGCGAAATTAAACCC	
	Forward Primer 2	GTTGAAAAGGGCGAAATTAAACCA	
	Reverse primer	CATTCTCACCTCAGTGACTTCAGTGA	
Ch01C89975551T	Forward Primer 1	CCATGAGTGTGTGTATGTATAGAGTCATCAAAGAAC	
	Forward Primer 2	CCATGAGTGTGTGTATGTATAGAGTCATCAAAGAAT	
	Reverse primer	TCATTGTATCCACTAAACTGTGTGTCGA	
Ch01A92333692T	Forward Primer 1	TCATTCCCTTCAAGGATTAATCCTACAA	
	Forward Primer 2	TTCATTCCCTTCAAGGATTAATCCTACAT	
	Reverse primer	TGTAGTCTCCATTTTCCTCCAAAAGC	
Ch01C9366900/T	Forward Primer 1	CAATTTCTGAAGCCCAAGAGTAATAGGTAAC	

Table 4 Primers used for the fine-mapping of P17C12 mutant

	Forward Primer 2	CAATTTCTGAAGCCCAAGAGTAATAGGTAAT
	Reverse primer	TGGTGAATACATTTATCCTATTTTGAACC
Ch01T96712294A	Forward Primer 1	CTCATGTATGGAATAATCCATGTGGCT
	Forward Primer 2	CATGTATGGAATAATCCATGTGGCA
	Reverse primer	TCGGAATCGGTATAAACCGATACCG
Ch01C96794828T	Forward Primer 1	GAAATGGAACGAAATCGAGATTTGAC
	Forward Primer 2	GAAATGGAACGAAATCGAGATTTGAT

Note: The position of the mutation was indicated like Ch01C11280174T, meaning that it is in chromosome 1, at the position 11280174 (aligned with SL3.0) with a mutation from C (cytosine) to T (thymine). According to the methods of KASP, we used different 5' reported dyes to indicate the alleles for each tested individual (homozygous mutant alleles or WT alleles, or heterozygous alleles). We used FAMTM dye to indicate the WT allele, a tail (or a probe) with sequence "5'-GAAGGTGACCAAGTTCATGCT-3' " would be attached to the 5' end of Forward Primer 1. To indicate mutant allele, VIC[®] dye was chosen by adding tail with sequence "5'-GAAGGTCGGAGTCAACGGATT-3' " to the 5' end of Forward Primer 2.

2.3 Results

First Screening for Mutants with Starch- and Hexose-excess Fruits

Fruit powder from nearly 250 families of three groups (P17, P20 and P21) have been used to detect mutants displaying abnormal starch accumulation. For all the cultures performed during spring, summer and autumn seasons, WT plants which were randomly placed among the mutant plants were used as controls. For each plant, the pericarp of three red ripe fruits was sampled to assay starch and major soluble hexoses (glucose and fructose). It is noteworthy that for the three groups of WT plants used here, starch measured at red ripe stage was very low, but with a relatively large SD value (0.36 ± 1.34). This clearly indicated that in red ripe tomato fruit starch has been almost totally degraded, but displayed a large variation in this culture season (**Fig 9 A**). The big variations were also observed in glucose and fructose contents in those mutants (**Fig 9 B, C**).

Among the mutant plants displaying higher levels of starch or hexoses, 3 families were selected: P17C12, P21C7 and P21H6. A subsequent culture was performed with 12 plants for each of those 3 families to confirm the phenotypes.



Fig 9 Concentrations of starch (A), glucose (B) and fructose (C) in screened tomato fruits

The red ripe fruits of nearly 6000 EMS mutant plants belonging to three groups (P17, P20 and P21) were used for measuring starch and hexose contents in pericarps to screen for starch mutants. Data are presented for each individual.

Second Screening for Confirmation of Three Mutant Families

In the second screening of the P17C12, P21C7 and P21H6 mutant families, mature leaves and fruits at two developmental stages were individually collected from the 12 plants of each family to better characterize the starch and hexose phenotypes.



Fig 10 Concentrations of starch (A) and hexoses (B) during fruit ripening of mutants and WT Mean values (μ mol glocose.eq.g⁻¹ FW for starch, and μ mol g⁻¹ FW for hexoses) are given with SD (n=3). Abbreviations: L, leaf; IMG, immature green; Br, breaker; RR, red ripe.

Part II-Results

In general, starch levels found in mature leaves of the mutants were comparable to WT, indicating that the mutations did not affect leaf starch metabolism. In contrast, among the 12 plants cultured for each family, two P17C12, three P21C7 and one P21H6 plants produced fruits with higher starch content (**Fig 10**) than that in the WT, whatever the stages. In WT, starch levels decreased sharply from 19.7 ± 1.4 at immature green (IMG) stage to 2.6 ± 0.2 at breaker and then to almost nil (*i.e.* 0.29 ± 0.02) at red ripe. In the mutants, the starch content followed the same pattern. The IMG fruits of the P17C12, P21C7 and P21H6 plants displayed the highest difference with around 2- to 5-times more starch than in the WT. This difference remained significant at breaker stage and also at red ripe stage even though starch levels were much lower than at IMG stage.

In contrast to starch, the hexose concentrations of mutant fruits displayed no significant difference with the WT at IMG stage. Nevertheless, for the P21C7-6 plant, the hexose content was strongly increased at RR, with 70.9% and 48.5% more glucose and fructose, respectively. According to their higher starch accumulation at IMG and breaker stages, three individual plants, namely P17C12-6, P21C7-9 and P21H6-11, were used for self-pollination and back-crossing with the WT.

Mendelian Inheritance of the Mutations Affecting Starch

The first progeny of back-cross between mutant and WT was called BC1F1, while the first progeny of mutant from a self-pollination was called S1. The fruit samples were harvested at two stages, immature green stage around 20 days after pollination (DAP), and red ripe stage, to measure starch and hexose content.

For the P17C12 mutant, IMG fruits of S1 plants had about 2 times higher starch content than the WT fruits (**Fig 11**), while BC1F1 fruits showed no significant difference compared with the WT. For hexoses, IMG fruits from BC1F1 and S1 of P17C12 were comparable to the WT. At red ripe stage, the starch content was higher in P17C12 S1 fruits than in WT fruits, but the difference between S1 and WT was less significant than at IMG stage. In contrast, remarkable differences were found for hexose contents, as S1 red ripe fruits contained 2 to 3 times more fructose and 3 times more glucose than the WT. Starch and hexose contents were similar in red ripe fruits of BC1F1 of P17C12 and WT plants. At this stage we could conclude that: firstly, as BC1F1 progenies produced fruits displaying starch and hexose contents similar to the WT, the causal

mutation of the P17C12 mutant should be recessive; secondly, since all the fruits of the S1 progenies displayed higher starch content at IMG stage, and higher hexose contents at red ripe stage, the parent P17C12 was homozygous regarding the mutation responsible for the corresponding phenotypes.

In addition to the starch-excess phenotype found in fruit, the P17C12 mutant showed other phenotypes, such as poor fertility leading to limited numbers of fruits, small fruits with few seeds and thicker pericarp, and relatively taller plants. However, we have no idea about how those phenotypes associated together. Those phenotypes also reminded us that starch-excess phenotype may be one of many consequences other than the only and direct consequence of the mutation.

Unlike the P17C12 mutant, BC1F1 and S1 plants of the P21C7 and P21H6 mutants exhibited large fluctuations of starch and hexose contents that were reflected by large SD values (**Fig 12** and **Fig 13**), suggesting that the mutants selected to produce F1 and S1 were not homozygous. Compared to P17C12, the other two mutants, P21C7 and P21H6 were more ambiguous. The possible explanation is that we planted 12 plants for S1, BC1F1 and WT plants, which was a small population, hence it may not be enough for observing a clear segregation, especially considering the fact that the starch amount fluctuates even in WT fruits (as described before). They may be heterozygous dominant mutants of starch phenotype in fruits, but it requires a new culture including more plants for each group to confirm.

As the P17C12 mutant showed clear homozygous recessive phenotypes, it was selected to apply the mapping-by-sequencing strategy, and to generate a BC1F2 population from the BC1F1 plants mentioned above, and thereafter to determine the segregation of the starch-excess phenotype and constructing bulks for sequencing.



Fig 11 Concentrations of starch and hexoses in immature green and red ripe fruits of S1, F1 plants of P17C12 and WT plants

- A. Concentrations of starch in immature green fruits of S1, F1 plants of P17C12 and WT plants
- B. Concentrations of hexoses in immature green fruits of S1, F1 plants of P17C12 and WT plants
- C. Concentrations of starch in red ripe fruits of S1, F1 plants of P17C12 and WT plants
- D. Concentrations of hexoses in red ripe fruits of S1, F1 plants of P17C12 and WT plants

Abbreviations: BC1F1, the first generation of the plant (BC1F1) back-crossed with the WT parent; S1, the first generation of plants from self-pollinated mutant; WT, wild type plants.



Fig 12 Concentrations of starch and hexoses in immature green and red ripe fruits of S1, F1 plants of P21C7 and WT plants

- A. Concentrations of starch in immature green fruits of S1, F1 plants of P21C7 and WT plants
- B. Concentrations of hexoses in immature green fruits of S1, F1 plants of P21C7 and WT plants
- C. Concentrations of starch in red ripe fruits of S1, F1 plants of P21C7 and WT plants
- D. Concentrations of hexoses in red ripe fruits of S1, F1 plants of P21C7 and WT plants

Abbreviations: BC1F1, the first generation of the plant (BC1F1) back-crossed with the WT parent; S1, the first generation of plants from self-pollinated mutant; WT, wild type plants.



Fig 13 Concentrations of starch and hexoses in immature green and red ripe fruits of S1, F1 plants of P21H6 and WT plants

- A. Concentrations of starch in immature green fruits of S1, F1 plants of P21H6 and WT plants
- B. Concentrations of hexoses in immature green fruits of S1, F1 plants of P21H6 and WT plants
- C. Concentrations of starch in red ripe fruits of S1, F1 plants of P21H6 and WT plants
- D. Concentrations of hexoses in red ripe fruits of S1, F1 plants of P21H6 and WT plants

Abbreviations: BC1F1, the first generation of the plant (BC1F1) back-crossed with the WT parent; S1, the first generation of plants from self-pollinated mutant; WT, wild type plants.

Starch Accumulation in WT Fruits during Development

I found that the starch content of WT fruits at IMG was 2 times higher (~50 μ mol.glucose.eq.g⁻¹ FW) without fruit thinning than previously measured by A. Mirto (~25 μ mol.glucose.eq.g⁻¹ FW). In order to perform a solid and precise phenotyping of the P17C12 BC1F2 big population, I firstly examined starch content in development stages of WT plants from March to June of 2017. I

cultivated nearly 20 WT plants and designed a series of harvesting through the whole development of tomato fruits, with a sampling gap of 5 to 7 days. As fruits at 6 DAP are too small (~0.2 g), only 3 samples with numerous fruits were collected. After harvesting 6 DAP fruits, I did fruit thinning to keep 6–8 fruits per plant, then marked the flowers when they were pollinated. For the other fruit stages, I harvested at least 3 fruits per sample and 5 samples per stage.

With all the controls mentioned above, for fruits of Micro-Tom plants grown under optimal growth conditions, the starch content in the pericarp reached a very high level (~135 μ mol.glucose.eq.g⁻¹ FW) at 16 DAP (**Fig 14 B**), then decreased until the red ripe stage to reach nearly zero. Fruits from 28 DAP to 42 DAP showed a sharp decrease in starch. In contrast to the big variation in starch content during fruit development, the contents of glucose and fructose were relatively stable; they started to increase after 35 DAP in parallel, when starch was quickly decreasing.



Fruits at different development stages

Fig 14 Starch and hexose contents in development fruits of Micro-Tom WT plants

- A. Fruits harvested in different development stages of Micro-Tom WT plants;
- B. Starch and hexose contents in development fruits of WT plants.

Mean values (expressed as μ mol.glucose.eq.g⁻¹ FW for starch content or μ mol.g⁻¹ FW for hexose content) are given with SD. Pericarp of fruits were harvested from 6 days after pollination (DAP) to 50 DAP with at least 3 samples per stage.

Culture of the BC1F2 Population of P17C12 for Performing the Mappingby-sequencing

Before cultivating a large number of BC1F2 plants, we cultivated a small BC1F2 population of 24 plants to check if the starch-excess phenotype reappeared in this generation, which would indicate

the segregation of the mutant phenotype. After observing that ~ 5 plants showed a 1.5-times higher level of starch than WT control plants, we confirmed there was a segregation of the starch-excess trait in the BC1F2 population of P17C12. Then, I carried out a culture of 200 BC1F2 plants of P17C12, as well as 12 plants for WT, BC1F1 and S2, as controls. To optimize the source-sink relationship, flower- and fruit-thinning were performed to limit the fruit number to about 6 to 8 per plant. In order to know the exact stage of the fruits, as well as to be able to compare the mutant fruit with the WT, the flowers were marked at anthesis (*i.e.* after having been pollinated with a vibrating electric toothbrush). This allowed to sample the fruits at 20 and 35 DAP more accurately, and thus to reduce the fluctuation of the starch content, as starch varies quite dramatically along fruit growth and development. I decided to sample the fruits at 35 DAP instead at red ripe stage, because I observed that in the 35 DAP fruit of the P17C12 mutant, the starch content was still high, probably resulting from more synthesis of starch at IMG stage or a delay in the remobilisation of starch, which occurred at the beginning of ripening. The differences of starch content at 35 DAP between mutants and WT were more significant than that at red ripe stage (Fig 15 D), thus those remarkable differences could be considered as part of the starch-excess phenotype of P17C12 mutant, further to provide additional information to help us distinguish WT-like and mutant-like plants.

For the 20 DAP fruits of control plants, S1 plants had higher starch amount, while those from the BC1F1 plants displayed similar starch content to WT (**Fig 15 B**). Similar results were also observed in the analysis of Mendelian inheritance of P17C12 (**Fig 11**), indicating that the homozygous starch-excess phenotype was reproducible in different cultures.

The analysis of the large BC1F2 population showed that the starch amount at 20 DAP displayed a normal distribution (**Fig 15, A**). The average amount of starch for the whole BC1F2 plants at this stage was 129 ± 31 (µmol.glucose.eq.g⁻¹ FW), there were 37 over 200 plants that had fruits with starch levels higher than 159 µmol.glucose.eq.g⁻¹ FW, which was equal to mean + 1 SD, while nearly half had starch level less than the mean (129 µmol.glucose.eq.g⁻¹ FW). This normal distribution of starch amount in fruits at 20 DAP implied that the trait we were interested in maybe a QTL trait, other than Mendelian trait, which showed more clearly when we examined starch content in a big population. It is interesting to notice that fruits from the F1 contain 20% more

starch than the WT in a significant manner, thus suggesting that the phenotype is not strictly recessive.



Fig 15 Starch levels in mature green fruits of a population of 200 individuals of P17C12 BC1F2 and in controls

A. Distribution of starch levels in 20 DAP fruits of the P17C12 BC1F2 population.

B. Starch levels in 20 DAP fruits of P17C12 S2, F1 and WT plants.

C. Distribution of starch levels in 35 DAP fruits of the P17C12 BC1F2 population.

D. Starch levels in 35 DAP fruits of P17C12 S2, F1 and WT plants.

Abbreviations: F1, the first generation of progeny for back-crossed (BC1) with the WT genotype plants; S2, the second generation of progeny for self-pollinated mutant; WT, wild type; DAP, days after pollination. Asterisks indicate significant differences (Student's t test, P < 0.01); ns, no significance.

For the 35 DAP fruits, starch content in the BC1F2 plants was not normally distributed (**Fig 15 C**). Furthermore, as expected, S2 plants had a delay in starch degradation, which resulted in a higher difference compared to F1 and WT fruits. However, the delay varied, as for some S2 plants starch content at 35 DAP was as high as that at 20 DAP, and for others it was only 50%. This variation found within the S2 population may be the reason for such a non-normal distribution of starch amount of BC1F2 at 35 DAP.

For the construction of two bulks, the starch data obtained for 20 DAP fruits was used as the main reference regarding S2, F1 and WT plants. For the starch-excess bulk, we selected the plants bearing the fruits with the highest starch levels (*i.e.* as in fruits of S2 plants), and for the WT-like bulk, we chose the plants bearing fruits with starch levels similar to the WT and F1 plants. For each bulk, 30 to 40 plants were selected. At the same time, the level of starch at 35 DAP was used as complementary information to filter out some bias plants. The plants and the corresponding starch results for two stages are listed in **Table 5**. After preparing the two bulks as described in Chapter 2.2, DNA samples were sent to Toulouse-GeT-PlaGe sequencing platform to perform the sequencing.

Bulk	Plant	Starch at	Starch at
		20 DAP	35 DAP
Mutant	3	172.1	190.2
Mutant	13	176.5	91.4
Mutant	17	175.6	108.2
Mutant	18	171.0	122.7
Mutant	20	164.5	18.7
Mutant	22	166.3	75.7
Mutant	25	185.4	109.9
Mutant	31	189.8	44.5
Mutant	36	153.7	83.6
Mutant	48	187.4	59.2
Mutant	51	210.4	32.8
Mutant	53	196.1	108.6

 Table 5 The plants selected for constructing 2 bulks

Mutant	56	162.1	62.8
Mutant	58	167.7	53.0
Mutant	60	176.3	89.7
Mutant	72	170.9	58.0
Mutant	80	159.1	59.3
Mutant	94	184.7	55.5
Mutant	98	152.0	100.8
Mutant	104	178.5	75.7
Mutant	115	164.3	57.7
Mutant	119	185.3	50.4
Mutant	122	170.5	49.5
Mutant	125	177.3	13.3
Mutant	126	167.2	30.9
Mutant	134	174.1	NA
Mutant	140	169.3	90.4
Mutant	150	169.6	65.1
Mutant	151	181.0	32.0
Mutant	153	163.0	61.1
Mutant	156	159.4	69.7
Mutant	158	160.6	64.8
Mutant	167	179.2	46.3
Mutant	170	192.0	NA
Mutant	185	181.7	NA
Mutant	188	174.6	112.9
WT	21	93.2	34.8
WT	95	103.0	25.3
WT	103	95.5	32.4
WT	106	98.4	41.7
WT	107	113.3	19.7
WT	111	107.7	34.9
WT	113	84.4	33.5
WT	116	94.0	9.9

WT	117	111.9	15.5
WT	124	90.6	38.9
WT	135	112.0	25.2
WT	136	106.5	36.4
WT	142	97.6	16.7
WT	146	112.1	31.2
WT	154	117.1	16.3
WT	155	104.9	14.1
WT	161	105.7	24.8
WT	162	110.8	21.3
WT	163	88.9	28.1
WT	164	110.6	17.8
WT	166	93.2	31.2
WT	168	114.2	15.7
WT	169	108.4	7.3
WT	171	106.8	29.6
WT	172	76.7	22.9
WT	173	107.9	34.1
WT	174	107.8	9.3
WT	177	92.3	32.6
WT	178	94.5	19.6
WT	180	102.9	27.1
WT	182	87.8	41.4
WT	184	109.0	19.6
WT	186	99.0	52.4
WT	191	84.4	30.2
WT	192	91.1	13.9

Analysis of Mapping-by-sequencing and Fine-mapping for P17C12 Mutant

According to the NGS quality check performed by the sequencing platform (GetPlace, Genotoul, Toulouse), the fragments at sizes around 40,000 bp were over 95.6%, which indicated that the DNA samples had good integrity.

After assembling the sequenced fragments against the Heinz 3.0 genome sequences, and performing the variant calling frequency analysis described in methods Chapter 2.2, the differences in SNP frequencies between the WT-like bulk and the mutant-like bulk were scrutinised (**Fig 16**). According to the Mendelian segregation law, for a homozygous recessive phenotype controlled by a single gene, the frequency of the causal SNP would be ~33% in the WT-like bulk, while for the mutant-like bulk, it would approach 100%. However, as the previous experiments showed in practice and especially when the trait under study was quantitative, experimental noise almost always results in false positives or negatives getting included in one or the other bulk, which results in an allelic frequency of the causal mutation ranging from 60% to 100% in the mutant-like bulk and between 20% and 40% in the WT-like bulk (Bournonville, 2015; Petit *et al.*, 2016). In our case, the pattern of allelic frequency of the mutant- and WT-like bulks displayed no difference along the 12 chromosomes, except at the end of chromosome 1 (from 8.5×10^7 to 1.0×10^8 bp) where a clear and distinct difference of SNP frequencies was observed between the two bulks (**Fig 16, ch01**).



Fig 16 Frequency analysis of SNP mutations for WT- and mutant-like bulks of the P17C12 BC1F2 culture.

Variant calling frequency analysis after sequencing the wild-type-like and mutant-like (starchexcess) bulks. Variations in SNP frequencies along the 12 tomato chromosomes are shown using a 10-EMS-mutations sliding window for the WT- like bulk (yellow line) and the mutant-like bulk (cyan line). When checking this narrow region, there were only one knock-out (KO) mutation with high effect, two missense mutations with moderate effect and other limited mutations in non-coding region with possible modified effect (listed in **Table 6**). In the non-coding regions, there were mutations found either in the 5' UTR and 3' UTR regions, or within intergenic or intronic regions. The possible mutations were listed mainly according to the mutation effects which are discussed below.

Solyc	Protein	Mutation type	Mutation effect	Mutation
Solyc01g103960.3	RecQ helicase 4	Knock out	HIGH	c.1096A>T
Solyc01g095510.3	Mitochondrial carrier protein family	Missense	MODERATE	p.Asp43Asn
Solyc01g096930.3	Phospholipid-transporting ATPase	Missense	MODERATE	p.Gly452Ser
Solyc01g105700.3	Conserved peptide upstream open reading frame	5' UTR	MODIFIER	c75G>A
Solyc01g097910.2	Rubredoxin protein family	3' UTR	MODIFIER	c.*82G>A

Table 6 Selected candidate mutations of P17C12 starch-excess mutant

To break the linkage among mutations within chromosome 1, a fine mapping was carried out to narrow down the putative region and identify the possible causal mutation. Thus, SNP markers found by NGS were used *via* the KASP method described in "Materials and Methods" in this Chapter.

The results of the QTL analysis of fine-mapping showed the possible regions of the causal mutation (**Fig 17**). The starch-related trait was mapped in chromosome 1 between the SNP marker C89975551T and the end of the chromosome. The best estimate of the location of the QTL is given by the chromosomal location that corresponds to the highest LOD score. Maximum LOD score is at 75 cM and the confidence interval is between 70 and 84 cM. A92333692T was the closest marker to the QTL while C89975551T and T96712294A were the two flanking markers.

In order to confirm which of these candidate genes could be responsible of the starch phenotype, I developed several strategies like CRISPR/Cas9 or other genomic engineering techniques to mimic those mutations in a clear genomic background like WT.



Fig 17 QTL mapping of the starch variation in the P17C12 mutant

The Kosambi genetic distance expressed in cM is indicated on the left. Marker identifiers and positions are indicated on the right. 1-LOD and 2-LOD support intervals of QTL is marked by box and thin bars, respectively. The result of the QTL mapping is plotted as a likelihood-ratio statistic test (LOD score) against the chromosomal map distance, measured in recombination units. The vertical dotted line represents a threshold value, above which a likelihood-ratio test provides a statistically significant fit to a model of the data (3.0). Results shown are the output of simple interval mapping (MapMaker/Exp v3.0 Lander *et al.*, 1987; MapMaker/QTL v1.1, Paterson *et al.*, 1988)

2.4 Discussion

Phenotyping Starch-related Traits in Micro-Tom Fruit

In Micro-Tom fruit, starch accumulation shows a temporary and spatial pattern and is more abundant in the columella, placenta, pericarp parenchyma and seeds (Obiadalla-Ali et al., 2004). When expressed on a fresh weight basis it was peaking during the immature stage, and completely degraded at the red ripe stage, as also observed in other growth conditions (Obiadalla-Ali et al., 2004) and in another cultivar, *i.e.* the S. lycopersicum L. cv F144 (Schaffer and Petreikov, 1997) and S. lycopersicum L. cv. Moneymaker (Luengwilai and Beckles, 2009). However, in our experiments that were performed under optimal growth conditions and in which the sink/source ratio was controlled via flower and fruit thinning, the amount of starch reached high levels (~135 umol.glucose.eq.g⁻¹ FW) during the summer, much higher than in previous reports (Obiadalla-Ali et al., 2004). During the last three years we found that in the P17C12 mutant the starch level showed considerable fluctuation between culture seasons and this was even true for the WT. Starch accumulation in fruit is indeed affected by several factors including the stage of development, source/sink relationships and environmental conditions, comparing our results to previous observations in Micro-Tom fruits (Obiadalla-Ali et al., 2004). By contrast, starch metabolism varies little considering the age in leaves of Arabidopsis, which makes starch studies easier in leaves (Smith and Zeeman, 2020) than in fruit.

Therefore, to better phenotype the starch content and avoid false positives that would be caused by other factors during screening, we paid extra effort on cultivation and sampling. Firstly, we used only pericarp as material to avoid the possible interfering of the columella, which contains higher concentrations of starch. Then, as starch accumulates along fruit growth to peak during the immature stage at around 18–20 DAP and then decreases quite sharply, there was only a short period that was suitable for sampling. To reduce as much as possible the differences caused by using inappropriate sampling stages, I marked the pollination date for every flower so to obtain harvest dates that would match the starch plateau situated between 18 and 20 DAP. It is necessary to mention that, the accumulation patterns shown in **Fig 14** were measured in summer fruits, which needed around 42 days to develop and ripe, while for fruits in winter, the growth period represented

around 50 days, which implied that the sampling date was shifted for days according to the developmental stages. Finally, starch amount in tomato fruit largely depends on source/sink relationships (Osorio *et al.*, 2014), as also observed for the Moneymaker cultivar in our group (Biais *et al.*, 2014). To minimize those effects, I also performed flower and fruit thinning during the pollination stage, leaving 6 to 8 fruits on two trusses to make sure that I would have enough sample for analysis and seed collecting.

However, this more robust screening method was established after the first screening that was performed on red ripe fruits by A. Mirto (a visiting PhD student) in 2016. The reason why he chose red ripe fruits to screen for starch-excess mutants was to take advantages of a previous project which aimed to screen ascorbate-excess mutant in red ripe tomato fruit carried by C. Bournonville (2015) (details see chapter 2.1 Material and Methods). It was also the reason why he performed a second screening on selected mutants with fruits in different stages to confirm the phenotypes of starch-excess. It was not surprising that mutants selected according to the starch content in red ripe fruits also showed higher starch amounts at green stages, implying there was a stable and clear correlation between the starch-excess phenotype in immature and starch- or hexose-excess phenotypes at the red ripe stage. This observation also agreed with the previous reports which found that higher starch amount during development finally contribute to sugar contents in ripening in different environment conditions or cultivars (Schaffer and Petreikov, 1997; Yin *et al.*, 2010).

Putative Causal Mutations for P17C12 Mutant

SNPs in the Coding-region

When the EMS treatment leads to SNP mutations in coding regions that affect the integrity of the encoded protein (stop codon, non-functional amino acid sequence, *etc.*), which may result in the appearance of knock-out (KO) or missense mutations with high possibilities to affect the function of the encoded protein, those SNP mutations are considered to result in high effect. In the case of P17C12, in the selected region of interests, there are one KO mutation in the coding region of enzyme RECQ4, and two missense mutations of MCF and PTA in the coding region.

Part II-Results

A Knock-out SNP of RecQ helicase 4

RECQ4 (RecQ helicase 4) is a DNA helicase enzyme belonging to RECQ helicases, which are evolutionarily conserved and involved in numerous functions such as telomere stability, DNA replication, recombination and repair (Kaiser et al., 2017). Arabidopsis contains two orthologues, RecQ4A and RecQ4B due to recent gene duplication (Mieulet et al., 2018), which are both functional. However, the *Recq4ab* double mutants but not the single mutants show a higher (sixfold) increase of crossover frequency in intraspecific Arabidopsis crosses (Séguéla-Arnaud et al., 2015). To date only one tomato orthologue (Solyc01g103960) was reported (Mieulet et al., 2018; Maagd et al., 2020). In rice, pea and tomato, a single recq4 mutation increases crossovers about three-fold, suggesting that manipulating RECQ4 can be a universal tool to increase recombination in plants (Mieulet et al., 2018). A recent study (Maagd et al., 2020) showed that manipulating RECO4 with CRISPR/Cas9 increased crossover frequency significantly in interspecific hybrids: a 1.8-fold extension of the genetic map when measured by analysing SNP markers in the progeny (F2) plants. However, there is no report about the role of RECQ4 in starch metabolism. According to previous studies of RECQ4 functions, it is very unlikely that RECQ4 would be involved in starch metabolism. However, we cannot exclude a pleiotropic effect of the mutation that may lead to starch excess. Considering that RECQ4 was the only candidate with a KO mutation, I decided to examine its relationship with starch.

Missense Mutation of the Gene Encoding a Mitochondrial Carrier Family (MCF) Protein

The other two candidate genes with missense mutations were also in my priorities for function checking, even though according to the literature, they seemed to have little relation with starch metabolism. The member of the mitochondrial carrier family (MCF) proteins, which consists of six membrane spanning helices, exhibits typical conserved domains and forms a homodimer within the membrane (Haferkamp and Schmitz-Esser, 2012; Lee and Millar, 2016). However, contrary to what their name suggests, the localization and physiological functions of MCF are not limited to mitochondria as they are found in several compartments such as the glyoxysome, peroxisome, plastid, and the plasma membrane. MCF proteins catalyse the specific transport of various substrates such as nucleotides, amino acids or dicarboxylates. In plants, MCF is a large family of proteins; for example in *Arabidopsis* it contains 58 members (Millar and Heazlewood, 2003). Strikingly, the MCF member Brittle1 (BT1) plays an important role in starch metabolism. It was

first proposed to act as an ADP-glucose transporter in the maize endosperm (Sullivan *et al.*, 1991), thus being involved in substrate supply for starch synthesis during grain development. Similar transport proteins were later found in other cereals including barley (HvNST1), wheat (TaBT1) and rice (OsBT1) that are also able to transport ADP-Glucose and are located in the plastid (Patron *et al.*, 2004; Bowsher *et al.*, 2007; Kirchberger *et al.*, 2007; Li *et al.*, 2017). However, Solyc01g095510, the MCF member that was mutated in the P17C12 mutant investigated here, is not an orthologue of BT1, but of MTM1, which has been found in *Arabidopsis* (At4g27940.1, Manganese Tracking Factor For Mitochondrial SOD2) and in the yeast *Saccharomyces cerevisiae*. In *Arabidopsis*, it locates in the plastid according to the prediction of ePlant (http://bar.utoronto.ca/eplant_tomato/). In yeast, Mtm1p is essential for the posttranslational activation of a manganese-containing superoxide dismutase (SOD2) (Luk *et al.*, 2003). A complementation experiment performed with the yeast *mtm1* mutant showed that the *Arabidopsis* AtMTM1 protein is necessary for SOD2 activation (Su *et al.*, 2007).

Missense Mutation of the Gene Encoding a Phospholipid-transporting ATPase

The other candidate gene harbouring a missense mutation in the P17C12 mutant encodes a phospholipid-transporting ATPase (P-type ATPase), which is a superfamily of ATP driven biological pumps that share common features but transports different substrates. P-type ATPases can be divided into five subfamilies (P1 to P5) according to their transported substrate (Kühlbrandt, 2004). The P-type ATPases are involved in a wide range of fundamental cellular processes such as cellular signalling or micronutrient redistribution (Pedersen *et al.*, 2012). The mutated phospholipid-transporting ATPase (PTA, Solyc01g096930) found in P17C12 is an orthologue of the *Arabidopsis* ALA1 (At5g04930), a member of P4-ATPases that are also named flippases. *Arabidopsis ala1* mutants showed reduced growth only under chilling growth conditions (Gomès *et al.*, 2000). The KO or RNAi mutants of other members of P4 ATPases showed different phenotypes, including impaired defence against viruses (*ala2* KO) or pathogens (*ala3* KO), increased sensitivity to heat and heavy metals, altered male fertility (*ala4* KO, *ala6* KO, and *ala6*/7 double KO) or impaired stomatal regulation (*ala10* KO) (Davis *et al.*, 2020 and references therein). However, there is no report revealing a link between P4-ATPases and starch metabolism.

Part II-Results

SNPs in Non-coding Regions

For the other mutations, the SNPs located in non-coding sequences including those within introns, 5' UTR, 3' UTR and intergenic (upstream or downstream) regions might affect mRNA splicing, transcription or translation *etc.*, but the actual effects are difficult to predict, and need to be confirmed case-by-case.

SNPs Located within Introns

Intronic SNP can effect phenotypes mainly by creating mRNA alternative splicing, thus affecting the coding sequence and producing different proteins that may have altered localisation, stability or even function (Kelemen *et al.*, 2013). In plants, it is estimated that 40%–65% of the transcripts with introns are alternatively spliced, which suggests that alternative splicing plays an important role in many physiological processes (Zhang *et al.*, 2010; Marquez *et al.*, 2012; Shen *et al.*, 2014; Clark *et al.*, 2019). A well-known example is the Waxy (Wx) gene in rice encoding a granule bound starch synthase. A SNP (G to T) located at the 5' splice site of the first intron found in the Wxb allele was found to decrease the splicing efficiency and ultimately to lead to a decrease in amylose production compared to the Wxa allele (Cai *et al.*, 1998). There are four main types of alternative splicing events that are exon skipping (ES), alternative donor sites (AD), alternative acceptor sites (AA), and intron retention (IR), the last one (IR) is the main type in plant species (Wang and Brendel, 2006), whereas the exon skipping is the most common form of alternative splicing in human (Wang *et al.*, 2008).

SNPs Located at 5' UTR

Untranslated regions (UTRs) of mRNAs can play multiple roles in mRNA regulation, acting at levels such as stability, transport and translation efficiency, and also the functions and sub-localisation of the encoded protein (Srivastava *et al.*, 2018). Thus, UTRs are considered as important regulating modules functioning at both the mRNA and protein levels. For many eukaryotic mRNAs, a short open-reading frame (ORF) is present in the 5' UTR region; it is then named "upstream ORF" (uORF), the corresponding downstream mRNA then being named "major ORF" (mORF). Two general types of uORFs are found in plants: conserved peptide uORFs (CPuORFs) and sequence-independent uORFs (SIuORFs) (Merchante *et al.*, 2017). CPuORFs encode conserved peptides with an inhibitory function while SIuORFs have various sequences that

have been found located in conserved positions in the genome across related species. In *Arabidopsis*, ~35% of the genes contain at least one uORF (von Arnim *et al.*, 2014). For the function of uORF, a well demonstrated case is the uORFs belonging to SIRT (sucrose-induced repression of translation), and the corresponding major ORF is one member of basic leucine zipper (bZIP)-type transcription factor family genes, ATB2/AtbZIP11. In *Arabidopsis*, SIRT is highly conserved, located within the long 5' UTR of ATB2/AtbZIP11, which responds to sucrose, resulting in higher expression of SIRT, leading to reduced level of the AtbZIP11 protein (Wiese *et al.*, 2004). A new reverse genetics strategy took advantage of this discovery, in which sweeter tomato fruit was obtained by removing the depressors of SIbZIP1 and SIbZIP2 *via* creating lines free of SIRT responsive uORFs (Sagor *et al.*, 2016).

Among the SNP mutations found within the region found in P17C12 with the mapping-bysequencing strategy, there is one SNP located in the 5' UTR region of one CPuORF (Solyc01g105700), which Arabidopsis orthologue is uORF37 (At5g50010.UORF) that is located in the upstream of a bHLH transcript factor (At5g50010) named SACL2. In tomato, the downstream gene (Solyc01g105690) of the above mentioned CPuORF also encodes a basic helixloop-helix (bHLH) protein, which is the orthologue of the Arabidopsis SACL2. The full name of SACL2 is suppressor-of-acl5 (sac) like 2. Together with SACL1, SACL3 and SAC51, it belongs to the SAC family, which is associated with the loss-of-function mutant for ACAULIS 5 (acl5) that encodes spermine synthase. This mutant has a severe dwarf phenotype (Hanzawa *et al.*, 2000). SAC mutants were screened from M2 mutated seeds derived from seeds of the *acl5-1* mutant that were treated by EMS. A dominant mutant, sac51-d, in which the acl5 phenotype was almost completely restored, turned out to harbour a mutation in the uORF that provoked premature termination, thus leading to an increase in the transcript level of the corresponding mORF SAC51 (a bHLH transcription factor) (Imai et al., 2006). Among those 4 SAC family members, each single gene was mutagenized, but none showed an alteration in the phenotype. These mutants were then crossed, and double, triple and quadruple mutants obtained. Among these combinations, the sac51/sacl3 double mutant exhibited a slight but significant reduction in plant height. While SACL2 was KO in the background of sac51/sacl3, mutants exhibited a more severe phenotype, suggesting an additional effect of SACL2 on the phenotype (Cai *et al.*, 2016). Nevertheless, the function of SACL2 remains unknown.

Part II-Results

SNPs Located at 3' UTR

Three prime untranslated regions (3' UTRs) are also in the noncoding parts of mRNAs. According to the review of Mayr *et al* (2017), 3' UTRs are well known for regulating the degradation, translation, and localization of mRNAs, can also function like long noncoding or small RNAs, and even affect protein–protein interactions. These functions are usually achieved by the cooperation between RNA-binding proteins, effector proteins and 3' UTR cis-element. The 3' UTRs seem to be major regulators contributing to phenotypic diversity in higher plants.

In the region of interest of the P17C12 mutant, I found one SNP located in the 3' UTR of Rubredoxin (Solyc01g097910). Rubredoxins are found mostly in bacteria and anaerobic archae, and only in photosynthetic eukaryotes like plants and algae (Maiti et al., 2017). Calderon et al. (2013) found that photosystem II (PSII) was specifically decreased in Rubredoxin mutants in different species including the green alga Chlamydomonas reinhardtii, the cyanobacterium Synechocystis sp. PCC 6803, and Arabidopsis. Thus, Rubredoxin appears as universally required for photosystem II accumulation. Recently, two groups studied the roles of Rubredoxin in photosystem II assembly (García-Cerdán et al., 2019; Kiss et al., 2019). Kiss et al. (2019) used Synechocystis sp PCC 6803 to study the roles of Rubredoxin (or RubA) in PSII assembly. They found that a Rubredoxin-like domain is located on the cytoplasmic surface of PSII complexes, and that Rubredoxin plays an important role in early PSII biogenesis, by participating in the formation of the D1/D2 reaction centre complex. Lack of Rubredoxin also affected the accumulation of PSI, but in an indirect manner caused by the decrease in light-dependent chlorophyll biosynthesis, which was induced by PSII deficiency. In C. rheinhardtii, García-Cerdán et al. (2019) found that besides playing an important role in PSII assembly, Rubredoxin (or RBD1, as named in the article) is also involved in PSII repair. They proposed that during the *de novo* assembly of PSII complexes, Rubredoxin together with the cytochrome b559 protects PS complexes from photooxidative damage. In summary, those findings revealed the roles of Rubredoxin in the redox control of PSII biogenesis, supporting the fact that it is essential in photosynthetic eukaryotes.

There are two possible links between Rubredoxin and starch metabolism. As described above, Rubredoxin is involved in PSII and PSI accumulation in plants. In *Arabidopsis*, the homozygous KO Rubredoxin mutants were pale green, showed no detectable chlorophyll fluorescence and had an Fv/Fm value of 0, indicating a lack of PSII activity (Calderon *et al.*, 2013). Whereas starch synthesis is closely linked to photosynthetic carbon assimilation in the plastids of autotrophic organs such as leaves, it is possible that starch metabolism would be affected in Rubredoxin mutants. The other possible link is that enzymes involved in starch metabolism that are redox-regulated (*i.e.* AGPase and some degradation enzymes as mentioned in Chapter 1.2.2) could interact, directly or indirectly, with Rubredoxin.

SNPs Located at Intergenic (Upstream or Downstream) Regions

In the upstream region of genes, there are different regulators, such as promoters and enhancers. There are numerous examples of natural mutations in the promoter region that cause changes in the expression of the downstream genes and lead to phenotypic changes. Thus, in tomato a 3-bp InDel in the promoter of SI-ALMT9 (Al-Activated Malate Transporter 9) was found to provoke high fruit malate content. Further analysis suggested that this InDel impeded the binding of the WRKY transcription repressor to the promoter of SI-ALMT9, thereby alleviating the repression of SI-ALMT9 expression and increasing fruit malate accumulation (Ye *et al.*, 2017). Another recent case is an 8-bp InDel mutation of TFA9 (Tomato Fruit Ascorbate On Chromosome 9) that was found located in the promoter region of SIbHLH59, which promotes high ascorbate accumulation by directly binding to the promoter of structural genes involved in the D-mannose/L-galactose pathway (Ye *et al.*, 2019). In tomato, a causal mutation of the locule number was also found located 1,080 bp from the 3' end (downstream) of WUSCHEL, which encodes a homeodomain protein that regulates stem cell fate in *Arabidopsis* (Muños *et al.*, 2011).

SNPs located in non-coding sequences, which include introns, 5' UTR, 3' UTR and intergenic (upstream or downstream) regions, might cause phenotypic changes through regulating mRNA splicing, transcription or translation *etc.* as described above. The evidences of those SNP effects are accumulating, but lacking systematic examination, especially in tomato, so that the actual effects are difficult to predict and have to be confirmed case-by-case. The candidate genes of the P17C12 mutant described before showed faint connections with already-known starch metabolism, which elevates the challenges of identifying the causal mutation. According to the SNP effect, genes with knock-out or missense mutation were first examined. Then SNPs found in non-coding 5' UTR and 3' UTR sequence were investigated, as such mutations are likely to impact gene

regulation as seen above. The other kinds of SNPs, *e.g.* intronic or intergenic, are still in the checking list.

Chapter3 Evaluation of Candidate Genes

3.1 Introduction

In chapter 2, we screened a large EMS tomato mutant population for alterations in fruit starch, and found several mutants accumulating more starch in fruit (shorted as starch-excess mutants). After confirming the starch-excess phenotype in fruits taken at different development stages, 3 mutants, P17C12, P21C7 and P21H6, were selected to perform a Mendelian inheritance analysis, by crossing the mutant with the WT and observing the phenotypes of the progenies (BC1F1). Among those three mutants, P17C12 showed a clear recessive and homozygous starch-excess phenotype, which can be considered as ideal for the next step, *i.e.* the mapping of the causal mutation (or in another word, genotyping). A BC1F2 population of about 200 plants was used to measure starch in the fruit pericarp at 20 and 30 DAP. In the 20 DAP fruit of the BC1F2 population we observed a clear segregation of the starch amount by using BC1F1, S2 and WT fruits as controls. This indicated that the phenotyping can be a valuable reference to select the individual plants to construct mutant-like bulk with 30–40 starch-excess plants and WT-like with plant containing normal starch amount for extracting DNA and then to perform the sequencing.

The analysis of sequencing data showed that at the end of chromosome 1, there was a narrow region with a significant difference of allelic frequencies between the two bulks. With the help of fine-mapping (details see methods of chapter 2), the region was further narrowed down and finally it showed to contain one knock-out and two missense mutations and also mutations in non-coding regions. After an intensive literature survey, we selected three mutations affected the corresponding proteins and two mutations in 5' UTR and 3' UTR which may affect mRNA functions. The details of those mutations are listed in **Table 6**.

The five candidate genes have distinctive functions, but little relation was found in the literature between the function/activities of these proteins and starch metabolism (see the discussion in Chapter 2). Thus, we decided to characterize all of them without *a priori*. To verify if a candidate mutation is the one related to the phenotype of interest, a valuable strategy is to mimic this mutation in a WT background to avoids the interference that could result from other EMS mutations. Thus,

in this chapter, we tested each candidate by using the CRISPR/Cas9 technique to create KO or to mimic mutations found in putative regulatory regions. We also overexpressed one candidate gene (Rubredoxin) in WT plants by using constructs with the constitutive CMV-35S promoter. Thereafter, the resulting transgenic plants were phenotyped, in particular by measuring fruit starch.

3.2 Materials and Methods

RNA Extraction and qRT-PCR Analysis

For each genotype, three plants were sampled as biological replicates. Total RNA was isolated from mature leaves at flowering and fruits at 12 and 20 DAP from WT, S2 and BC1F1 plants by using the TRIzolTM reagent (Life Technologies) following the manufacturer's instructions. After quality and quantity control of the RNA samples by gel electrophoresis and spectrophotometry (Nanodrop), 1 µg RNA of each sample was used to perform RQ1 RNase-free DNase (Promega) treatment. Reverse transcription was performed using a SuperScriptTM IV First-Strand Synthesis System Kit (Invitrogen). For each biological replicate, three technical replicates were analysed using a CFX96 real-time system (Bio-Rad) and GoTaq qPCR Master Mix (Promega). The qPCR mixture (20 µL in total) comprised 10 µL master mix, 250 µM (final concentration) of each primer, and 4 µL of the reverse transcription reaction diluted 20 times (~0.2 µg cDNA). The temperature program was as follows: initiated at 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, then completed with a melting curve analysis program. The primers of each candidate gene for qRT-PCR reaction were designed with the Primer3.0 software, listed in **Table 7**. The transcript levels of the genes were normalized with values obtained for the housekeeping genes SlActin2 and SleIF4A.

Gene name	Solyc	Forward/reverse primer	Sequence (5' to 3')
CPuORF	Solyc01g105700.3	FP	CAAACAAGATTTCGGGCCTC
		RP	GTATTCAGCCTGGCAATTCTC
bHLH146	Solyc01g105690.2	FP	ACGCTTGAAGATACAGATGAG
		RP	GTCCTTGCTCTTTATCCCAG

Table 7 List of primers for qRT-PCR

Rubredoxin	Solyc01g097910.2	FP TGACAGTAGGAGGCTTGAGG	
		RP	CGGAAGCTTATCAAACGGCA
eIF4A	Solyc12g095990.2	FP	AGTGGACGATTTGGAAGGAAG
		RP	GCTCCTCGATTACGACGTTG
Actin2	Solyc03g078400.2	FP	GGACTCTGGTGATGGTGTTAG
		RP	CCGTTCAGCAGTAGTGGTG

Construction of Plasmids for the CRISPR/Cas9 Approaches and Overexpression under CMV-35S and PEPC Promoters

CRISPR/Cas9 mutagenesis was performed as described by Bollier *et al.* (2018) with some modifications. Briefly, constructs were designed to create InDel mutations, with two sgRNAs alongside the Cas9 endonuclease gene. The guides of sgRNA target sequences were designed by using http://crispor.tefor.net/ at the coding sequence of each gene, and the distance between the 2 guides selected was from 60 to 100 bp. The sequences of all guides I used were listed in **Table 8**. All constructs were assembled using the Golden Gate cloning method (Weber *et al.*, 2011), and the plasmids used in cloning are listed in **Table 9**.

The forward primer contains the 20 nucleotides specific for the targeted gene (in red starting with a G) and an upstream BsaI site allowing cloning (Blue), and 23 nucleotides corresponding to the sgRNA to be fused by PCR amplification on the plasmid (green). Thus, in the case of using the *Arabidopsis* U6-26 promoter (pICH86966), the primer will have the following sequence:

TGTGGTCTCAATTGNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG.

The reverse primer is always the same and corresponds to the following sequence (BsaI site in blue): TGTGGTCTCAAGCGTAATGCCAACTTTGTAC.

At Level 1, the guide sequence attached with sgRNA was placed under the control of the *Arabidopsis* U6 promoter, and then the constructed plasmid would be used for Level 2 constructions to assemble with the modules to express the Cas9 protein, the neomycin
phosphotransferase (NPTII) reporter gene and the backbone of pICSL4723, which delivers the target sequences into the plant genome.

In order to generate the same mutation (C>T transition) in the 5' UTR of CPuORF of the P17C12 EMS mutant, a specific Cas9 was used. This is the D10A mutant nCas9^{At} fused with *Petromyzon marinus* cytidine deaminase and codon-optimization was made for *Arabidopsis* (thus nCas9^{At}-PmCDA1^{At}) (Shimatani *et al.*, 2017*b*). The plasmid was adapted for Golden Gate cloning.

The Golden Gate cloning method and similar construction procedures were also used to perform the overexpression of Rubredoxin in tomato under the CMV-35S promoter or under a fruit-specific expression promoter — the promoter of PEPC (phosphoenolpyruvate carboxylase 2, Solyc07g062530.2). For cloning the coding sequences of Rubredoxin and to avoid the possible recognition by the BsaI and BpiI restriction endonucleases, the coding sequences was changed to the synonymous codons by designing three pairs of primers and then assembling together. The corresponding primers are also listed in **Table 8**.

All plasmids were confirmed by Sanger sequencing (Eurofins Genomics Ltd, Germany) by covering the cloning regions.

Gene	Target region	Guide or primer	sequence (5' to 3')
RECQ4	Exon	Guide 1	ATTGAATAGCATGTTGCGTCCAGT
		Guide 2	ATTGCTGGCACAATGTCTTTGTGT
MCF	Exon	Guide 1	CAAGGGCTGTCTTCATGGAT
		Guide 2	GACAGGGCTATGTCCGCCGC
PTA	Exon	Guide 1	CATCTGGTGGGGGGGGTCTGTG
		Guide 2	CCCCGCGACCCACGTCTCAC
CPuORF	5' UTR	Guide 1	AACTAGCTTTTGGAGGAAAA
Rubredoxin	3' UTR	Guide 1	TAATTGTACATGGTTTAGAA
Rubredoxin	Exon	Guide 1	CTCCTGCTCGGATTCCGAAG

 Table 8 Sequences of the guides or primers used in Golden Gate cloning for CRISPR/Cas9

 mutagenesis or overexpression

		Guide 2	GTCGCACACCTCCAATCCTC
Rubredoxin	CDS	FP 1	ATGGCTTCAGCAACTAGTAGAG
		RP 1	CCTCTTGGGAGACATCAATAGATTTG
		FP 2	GAGGACAAACCCATCTCAGAACAAC
		RP 2	TCACCAGTAGCTTCATTGTACTTG
		FP 3	GTGACCCCTCTTATCCTATTCCTC
		RP 3	AAGCTTATTGAAGGAAATAACCAGAC

Table 9 Plasmids used for Golden Gate cloning

N Addgene	Type of using	Antibiotic selection	Name of vector				
46966	sgRNA	Kanamycin	pICH86966::AtU6p::sgRNA_PDS				
46968	U6 promoter	Spectinomycin	pICSL01009::AtU6p				
48002	Guide 1 acceptor (Level 1)	Ampicillin	pICH47751				
48003	Guide 2 acceptor (Level 1)	Ampicillin	pICH47761				
51144	Kana-plant	Ampicillin	pICSL11024(pICH47732::NOSp- NPTII-OCST)				
49771	Cas9	Ampicillin	pICH47742::2×35S-5'UTR- hCas9(STOP)-NOST				
91694	Cas9(CDA)	Kanamycin	pDicAID_nCas9-PmCDA (adapted for Golden Gate cloning)				
48018	Link for 1 guide	Spectinomycin	pICH41766				
48019	Link for 2 guides	Spectinomycin	pICH41780				
48015	Acceptor of level 2	Kanamycin	pICSL4723				
47998	LVL0 acceptor	Spectinomycin	pICH41308				
50267	35S promoter	Spectinomycin	pICH51266				
50337	CaMV terminator	Spectinomycin	pICH41414				
48001	acceptor for Level 1 of overexpression	Ampicillin	pICH47742				
48017	LVL2 end linker	Spectinomycin	pICH41744				

Transformation of Tomato

The different constructs were introduced into the Agrobacterium tumefaciens strain GV3101::pMP90 by electroporation. The agro-transformation of tomato cotyledons was performed as described by Bollier et al. (2018). Cotyledons were cut before the appearance of true leaves, and put upper side down on a Petri dish with KCMS medium (Knudson C and Murashige & Skoog medium), besides MS solution, which additionally contained thiamine (0.9 mg.L⁻¹), acetophenone (200 μ M), kinetin (100 μ g.L⁻¹) and 2,4-D (200 μ g.L⁻¹), and then placed in the dark at 25°C. After one- or two-days incubation, the cotyledons were transferred to a co-culture with the transformed Agrobacteria diluted with KCMS solution at OD between 0.05 and 0.08 under slow stirring in the dark during 30 min. The Agrobacteria was cultivated in LB medium and collected at OD between 0.6 and 1 (at log phase) before dilution and co-incubation. To prepare 50 mL co-incubation solution, additionally 100 µL acetophenone (100 mM) and 4.5 µL thiamine (0.9 $mg.L^{-1}$) were added to the KCMS solution. Cotyledons were put back to the same Petri dish for 2 days of co-incubation in the dark, and then transferred to the 2Z medium, which contains 250 mg.L⁻¹ timentin, 100 mg.L⁻¹ kanamycin and 2 mg.L⁻¹ zeatin, in the light. After 15 days, cotyledons were transferred to the 2Z medium additionally containing 150 mg.L⁻¹ timentin for inhibiting the growth of Agrobacteria and 150 mg.L⁻¹ kanamycin for selecting the transformants. This concentration of antibiotic was then maintained until stems would appear. When the meristematic stems were developed, they were cut without taking callus and transferred into selective rooting medium containing timentin (75 mg.L⁻¹), kanamycin (150 mg.L⁻¹) and IAA (1 mg.L⁻¹) for rooting. Once the roots had developed, plants were transferred into soil in a mini greenhouse until new leaves were obtained.

Identification and Selection of Transformants

To genotype each first-generation transgenic (named T0) line, leaf samples were collected from each individual to extract DNA as described above. For CRISPR/Cas9 mutants, two primers were designed for each target gene at the positions around 100 bp before the first guide sequence and around 100 bp after the second guide sequence. The CRISPR genotyping primers are listed in **Table 10**. The PCR products of each pair of primers were sequenced to identify potential mutations at each guide sequence region. Confirmed mutated T0 plants were kept to obtain seeds for the next

T1 generation. T1 plants of each line were selected on MS medium with 150 mg.L⁻¹ kanamycin, and then transferred to mini cabinet to be acclimated to natural environment. T1 plantlets were genotyped, as done for T0 generations and the "positive" T1 plants were finally transferred into the greenhouse. In the better cases, we expected to work with three homozygous plants in T1 for each line.

For overexpression mutants, as the plasmids contained the neomycin phosphotransferase (NPTII) gene for mutant selection, the specific primers corresponding to this gene were used to identify the transgenic plants for each generation by performing PCR on extracted leaf DNA as the template. These primers are also listed in **Table 10**.

Gene	Forward/Reverse primer	Sequence (5' to 3')	Size of PCR product (bp)
RECQ4	FP	CTGGACAATTTGGTGGCATT	487
	RP	AGTTCAGTCACCCCATGGTT	
MCF	FP	TTCAGCTTCTCCATGCAAAC	454
	RP	TCAAACAAACACCACTCCTC	
РТА	FP	TAGCCTTAGATTGTCTTGTG	340
	RP	GCAAGAAGGTCACAATAGAG	
CPuORF-5'UTR	FP	CTGCCTATCAACTGTAACCAG	362
	RP	GATTCCATTCTCATGCTTTGAG	
Rubredoxin-3'UTR	FP	GTGGATTGCTTTTGGGATTTGTG	307
	RP	CTTGAGCTCGATTCGTATTGTG	
Rubredoxin-KO	FP	TCACACTCCCACCATTTCAC	497
	RP	CACAAATCCCAAAAGCAATCCAC	
NPTII	FP	AGAGGCTATTCGGCTATGAC	730
	RP	AGAAGGCGATAGAAGGCGAT	

Table 10 Primers for selecting transformants

Phenotyping of Mutants

To measure starch in leaves, I sampled the fully expanded mature leaves between 3 and 4 pm, a period during which starch levels peak in mature tomato leaves (Bénard *et al.*, 2015). Lugol (KI-I₂) solution was prepared with 1 g of KI and 0.26 g of I₂ dissolved in 100 mL MilliQ water. First, leaves were decolorized in 95% alcohol at 100°C to get rid mainly of the chlorophyll and become white, rinsed twice with MilliQ water, and finally soaked in Lugol solution for 10 minutes.

To measure starch, fruits between 18 and 24 DAP, for which starch levels are relatively stable, were collected and pericarp tissue was sampled and snap frozen into liquid nitrogen. For some mutants, fruits between 12 and 14 DAP were also collected. It is worth mentioning that Micro-Tom fruits developed faster in the summer than in the winter, *i.e.* 42 days during summer against 52 days during winter between anthesis and red ripe stages. Thus, to make sure fruits were at the same stage of development, they were collected at 14/24 DAP in the winter and at 12/20 DAP in the summer.

Leaf chlorophyll was estimated with a Dualex[®] portable device, which is a clip sensor, according to the manufacturer's manual. Leaf photosynthesis was measured with a portable CIRAS-3 gas analyzer, according to the manufacturer manual. For each mature leaf, 3 to 5 technical measurement replicates were performed.

3.3 Results

According to the mutation effects of the five candidate genes, I clustered them into two groups, the first group included candidate mutations in coding region, and the second group included candidate mutations in non-coding regions. As explained before, I used different strategies to validate each candidate, and the results is presented here on a case-by-case basis.

Evaluation of the Candidates with KO/Missense Mutations: REQC4, MCF and PTA

According to the starch accumulation pattern found in Micro-Tom, fruits between 18 and 20 DAP have a relative stable and high level of starch (**Fig 14**). In consequence, this time-window was used to phenotype the mutants and transformants by measuring starch. In order to simplify the presentation of the results, the fruits harvested between 18 and 20 DAP will be designated by 18 DAP.

As a KO mutation clearly results in the suppression of the corresponding protein, RECQ4 was the first candidate characterized. Then, the missense mutations that corresponded to an amino acid change may affect the stability and/or the catalytic function of the protein, and possibly affect the phenotype. To verify the two missense candidate genes (MCF and PTA) in 17C12 mutant I therefore created KO or missense mutants by using CRISPR/Cas9 techniques.

RecQ helicase 4 (RECQ4)

In the P17C12 mutant, there was only one knock-out (KO) mutation in the identified region of chromosome 1. This KO is in the Solyc01g103960.3 gene named RECQ4 encoding the RecQ helicase 4. At the coding sequence position c.1096, the mutation corresponds to an A to T transition leading to the appearance of the stop codon "TGA" and a truncated protein translation. For the characterization of the RECQ4 mutation, I used two types of plants: a homozygous KO mutant of RECQ4 created by CRISPR/Cas9, and an EMS Micro-Tom mutant from the Bordeaux population that had been previously identified by TILLING (Mieulet *et al.*, 2018). This *recq4* tomato mutant was backcrossed several times with the WT to get rid of the very many mutations present and their

possible effects. Neither three homozygous T2 plants of one CRISPR/Cas9 KO mutant line nor EMS mutant plants (n=3 plants) displayed changes in the starch content in 18 DAP fruits compared to WT fruit (**Fig 18**). Consequently, we concluded that the KO mutation in the RECQ4 protein was not the causal mutation related to the starch- excess phenotype of the P17C12 mutant.





- A. The mutations of the EMS mutant P17C12, P79C8, and CRISPR KO mutants. The mutations are indicated by asterisks and shown in the genome sequences in the black box. For indicating different mutation types, [A] means an insertion of "A", and [A>T] means a transversion from "A" to "T".
- B. The mutations induced by CRISPR/Cas9 (grey cross), and the EMS treatment (red cross for P17C12, and black cross for P79C8) are positioned in the RECQ4 protein sequence. The EMS mutant was found with TILLING and backcrossed with WT for several generations to decrease the numbers of other mutations.
- C. Starch amount in fruits at 18 DAP was measured in WT as well as R1, R17 and R18 lines, which were T2 homozygous for the same mutation induced by CRISPR/Cas9. At least three individual plants were grown for each line and 3 fruits per individual were sampled and analysed. T-test was performed at P<0.05 by using WT as control, and there was no significant difference between mutants and WT.

Mitochondrial Carrier Protein Family (MCF)

The missense mutation found in this gene results is (c.127 G>A) leading to an aspartic acid (Asp) to asparagine (Asn) change. I succeeded with the CRISPR/Cas9 strategy in obtaining one missense (deletion of "LSSW" 4 amino acids) and two MCF-KO homozygous lines with 3 homozygous mutated plants per line in T1 generation (**Fig 19 B**). There was no difference found for starch in 18 DAP fruits of plants of all 3 lines compared to the WT (**Fig 19 C**), which suggested that MCF was not the responsible candidate protein associated with the starch-excessive phenotype of the P17C12 mutant.

A MCF Solyc01g095510





Fig 19 Mutagenesis of MCF by CRISPR/Cas9 (A, B) and starch amount (C) in fruits at 18 DAP in the mutants and WT

- A. The mutations are indicated by asterisks and shown in the genome sequences in the black box. For indicating different mutations, [T] means an insertion of "T", [G>A] means a transition from "G" to "A", and GC means a deletion of "GC".
- B. The mutations induced by CRISPR/Cas9 (grey cross) or the EMS treatment (red cross for P17C12) are positioned in the MCF protein sequence. The InDel mutations were induced by CRISPR/Cas9 via target the first exons of MCF (Solyc01g095510), resulting knock-out (KO, MCF3 and MCF12) or missense (MCF4) mutants.
- C. Starch amount in fruits at 18 DAP was measured in three transformed lines, which were T2 homozygous lines with different mutations. At least 3 individual plants were grown for each line and 3 fruits per individual were sampled and analysed. T-test was performed at P<0.05 by using WT as control, and there was no significant difference between mutants and WT.

Phospholipid-transporting ATPase (PTA)

The PTA gene (Solyc01g096930.3) encodes a phospholipid-transporting ATPase. In the P17C12 mutant, the mutation results in a transition of G to A at c.1354 leading to a change of glycine to serine that we hypothesized may affect the PTA function. I characterized one missense and two KO homozygous T1 lines in addition to the corresponding T2 progenies, with three plants per line (**Fig 20, A and B**). Phenotyping of these plants revealed that there was no significant difference in starch levels in 18 DAP fruits in comparison with the WT (**Fig 20, C**), which suggest that PTA is not related to the starch phenotype.

A PTA Solyc01g096930 PTA- Solyc01g096930: ...CATCTGGTGGGGGGTCTGTG...GTGAGACGTGGGTCGCGGGG... AATGAGGATTTGGGCCAAAT... PTA-P17C12:.....AATGAGGATTTG[G>A]GCCAAAT... PTA1-CRISPR:CATCTGGTGGGGGGGTCTGTG...GTGAGACGTGGGTCGCGGGG...... PTA4-CRISPR:GTGITJAGACGTGGGTCGCGGGG PTA6-CRISPR:.....CATCTGGTGGGGGGGTCTGTG...GTGAGACGTGGGTCGCGGGG 5' UTR Exon 3' UTR **B** PTA Protein 1172 PTA- Solyc01g096930: MENNSPVTDSIM......ASGGGSVREVNLGEFGSKPVRR......FQCRALNINEDLGQIKYVFS...... **X** PTA-P17C12: MENNSPVTDSIMFQCRALNINEDL[G>A]QIKYVFS... MENNSPVTDSIM......ASGGGSVREVNLGEFGSKPVRR..... XPTA1-CRISPR: XPTA4-CRISPR: MENNSPVTDSIM......ASGGGSVREVNLGEFGSKPV* **∦**PTA6-CRISPR: MENNSPVTDSIM......ASGGGCEGGEFR Phospholipid-translocating ATPase N-terminal haloacid dehalogenase-like hydrolase E1-E2 ATPase Phospholipid-translocating P-type ATPase C-terminal С (µmol.glucose.eq.g⁻¹FW) 100 Starch content 50 0 PTA1 PTA4 PTA6 ŴT Fruits at 18 DAP

Fig 20 Mutagenesis of PTA by CRISPR/Cas9 (A, B) and starch amount (C) in fruits at 18 DAP in the mutants and WT

- A. The mutations are indicated by asterisks and shown in the genome sequences in the black box. For indicating different mutations, [T] means an insertion of "T", [G>A] means a transition from "G" to "A", and GC means a deletion of "GC".
- B. The mutations induced by CRISPR/Cas9 (grey cross) or the EMS treatment (red cross for P17C12) are positioned in the PTA protein sequence. The InDel mutations were induced by CRISPR/Cas9 by targeting the first exons of PTA, resulting in knock-out (KO, PTA4 and PTA6) or missense (PTA1) mutants.
- C. Starch amount in fruits at 18 DAP was measured in three transformed lines, which were T2 homozygous lines with different mutations. At least 3 individual plants were grown for each

line and 3 fruits per individual were sampled and analysed. T-test was performed at P<0.05 by using WT as control, and there was no significant difference among mutants and WT.

Evaluation of Candidates with Mutations in Non-coding-sequence Regions

Additional mutations within the promising chromosome 1 region predicted by mapping-bysequencing and fine-mapping were located in non-coding sequence regions, including upstream, 5' UTR, intron, 3' UTR and intergenic regions. The consequences of such mutations could be more sophisticated and indirect, and in order to be characterized, they required more complicated and specific strategies. Those mutations are listed in **Table 6**, and the possible effects are described in Chapter 2.4. Based on those hypotheses, I decided to begin with the mutations in the 5' UTR and 3' UTR, mainly because of the relatively well-known principle of such regulation mechanisms described in the literature. Firstly, we examined the transcript levels of those two candidates in leaves, and in 12 DAP and 18 DAP fruits, in order to predict their possible effects to thereafter define what strategies for their characterization.

Conserved Peptide Upstream Open Reading Frame (CPuORF)

According to the TEA (Tomato Expression Atlas, http://tea.solgenomics.net/) and ePlant (http://bar.utoronto.ca/eplant_tomato/) databases (**Fig 21, A**), CPuORF is weakly expressed in the leaves and fruits of the M82 and Heinz cultivars. I confirmed this result in Micro-Tom as the CPuORF transcript was hardly detectable and I also found that there was no difference of expression between the WT and the P17C12 mutant. I used the CRISPR/nCas9^{At}-PmCDA1^{At} (coupled with cytidine deaminase) strategy to target the 5' UTR region to mimic the possible effect of the SNP mutation found in the P17C12 mutant. However, due to limited efficiency of the CRISPR/nCas9^{At}-PmCDA1^{At} and to the unprecise mutation window (~3 bp in guide sequence (Shimatani *et al.*, 2017), I was not able to reproduce the same mutation (**Fig 21, B**).



B CPuORF Solyc01g105700



Fig 21 CPuORF transcript levels and mutations, and starch contents of CPuORF 5' UTR mutants *via* CRISPR/Cas9 mutagenesis

- A. Transcript levels of CPuORF of *S. lycopersicum* (cv. Heinz) whole plant, and in leaves and fruits of the wild species *S. pimpinellifolium*. The predicted subcellular localization of CPuORF is also showed on the left. The figure was obtained from the ePlant database (http://bar.utoronto.ca/eplant_tomato/).
- B. The InDel mutations were induced by CRISPR/Cas9 by targeting the 5' UTR of Solyc01g105700.3 (encoding a CPuORF), resulting in modifier mutations. For indicating different mutations, [T] means an insertion of "T", [G>A] means a transition from "G" to "A", and GC means a deletion of "GC".
- C. Starch amount in fruits at 18 DAP was measured in two transformed lines, which were T2 homozygous lines with different mutations. At least 3 individual plants were grown for each line and 3 fruits per individual were sampled and analysed. T-test was performed at P<0.05 by using WT as control, and there was no significant difference among mutants and WT.

Rubredoxin

The Rubredoxin gene is known to be highly expressed in the leaves and relatively lowly expressed in younger fruits in cv. Heinz tomato (**Fig 22, A**). I confirmed this result in Micro-Tom, in which Rubredoxin transcript levels were 70-times higher in leaves than in fruits (**Fig 22, B**). Remarkably, Rubredoxin appeared more strongly expressed in the fruits of the mutant (**Fig 22, C**). Thus, in mutant fruits at 12 DAP, the level of Rubredoxin transcript was ~4-times (P<0.01) higher and at 18 DAP it was ~2-times (P<0.01) higher than in the WT.





Fig 22 Rubredoxin transcript levels in different tomato organs and subcellular localisations in *S. lycopersicum* var. Heinz and *S. pimpinellifolium*, and in leaves and fruits of WT and S2 mutants of P17C12 in *S. lycopersicum* var. Micro-Tom

- A. Rubredoxin transcript levels in Heinz of whole plant and wild species *S.pimpinellifolium*, in leaves and fruits. The predicted subcellular localization of Rubredoxin is also shown on the left. The figure was obtained from the ePlant database (http://bar.utoronto.ca/eplant_tomato/).
- B. Rubredoxin transcript levels in leaves as well as in 12 and 18 DAP fruit in the Micro-Tom EMS mutant P17C12 and the WT. Significance was tested using a t-test.
- C. Rubredoxin transcript levels in 12 and 18 DAP fruit in the Micro-Tom EMS mutant of P17C12 and WT. Significance was tested using a t-test, and two asterisks indicate P<0.01.

Manipulation of the 3' UTR Region of the Rubredoxin Gene

To investigate whether a mutation in 3' UTR of Rubredoxin affected starch accumulation in fruits, I used more complex strategies. First, like for CPuORF, I used the CRISPR/Cas9 to directly target the region mutated in P17C12, expecting to introduce similar mutations. As described before, due to technical limitations, it was difficult to obtain mutants with the exact same mutations as in the original P17C12 mutant. In order to mimic the SNP in the 3' UTR region of the Rubredoxin gene, we designed one guide that covered the target region. This limited us to improve the mutagenesis efficiency by using multiple guides with carefully selected guide sequences. Among the first 50 transformants of the T0 generation, none of them showed mutations in the targeted region. We continued with several lines, which had lots of seeds to obtain the T1 generation. Fortunately, I obtained three T1 mutated lines, named R3'-15, R3'-25, and R3'-28 (R3' means that mutations were in 3' UTR), that displayed insertion or deletions close-by the original EMS SNP position in

P17C12 (**Fig 23, A**). Then, in the T2 generation, I could characterise 3 plants for each T1 line that were identified as homozygous mutants.

On those homozygous T2 plants cultured during winter 2019, I collected fruit samples at 14 and 24 DAP, in which I analysed the level of Rubredoxin transcript and starch content. Contrary to what I previously observed in the EMS P17C12 mutant (**Fig 22, C**), the mutations introduced by CRISPR/Cas9, did not cause any change in the expression of Rubredoxin (**Fig 23, B**). Meanwhile, neither 14 DAP nor 24 DAP fruits of those CRISPR/Cas9 mutants showed a significant difference compared to the WT. A first explanation for this result would be that the EMS SNP mutation of Rubredoxin is not the causal mutation of the starch excess phenotype of P17C12. A second explanation could be that the mutations obtained with CRISPR/Cas9 would not affect 3' UTR functions like the original EMS SNP mutation did. Finally, I could not conclude about the role of Rubredoxin using this strategy.

A Rubredoxin Solyc01g097910



Fig 23 Mutagenesis of 3' UTR of Rubredoxin by CRISPR/Cas9, and transcript level of Rubredoxin and starch amount in fruits at 14 DAP and 24 DAP in the mutants and WT

- A. The InDel mutations were induced by CRISPR/Cas9 by targeting the region harbouring the SNP of the P17C12 mutant found in the 3' UTR of Rubredoxin (Solyc01g097910), leading to three different lines, R3'-15, R3'-25 and R3'-28. For indicating different mutations, [T] means an insertion of "T", [G>A] means a transition from "G" to "A", and GC-means a deletion of "GC". In T2 homozygous generation, three individual plants were obtained to perform Rubredoxin transcript and starch analyses.
- B. Rubredoxin transcript levels were measured in selected mutated lines in 14 and 24 DAP fruit of CRISPR/Cas9 mutants and WT plants. T-test was performed by using WT as control, and there was no significant difference at P<0.05.
- C. Starch amount was examined in 14 and 24 DAP fruits of selected 3' UTR mutants of Rubredoxin and WT plants. T-test was performed by using WT as control, and there was no significant difference at P<0.05 except for fruits of R3-15 at 24 DAP.

In order to try to overcome this inconclusive result, I decided to use two other approaches that would affect the expression of Rubredoxin. The first one was to create homozygous Rubredoxin KO mutants. In this aim, I chose to target the two main pairs of cysteine motifs (XCXXCX) of Rubredoxin (Maiti *et al.*, 2017), in order to decrease or suppress the expression of Rubredoxin and investigate if it would affect starch metabolism.

The last strategy is based on the expression data showing that Rubredoxin was more expressed in the EMS mutant than in the WT. This strategy consisted to overexpress Rubredoxin under the CMV-35S and PEPC promoters to analyse if the increase in the Rubredoxin transcript would result in the increase of fruit starch content.

Knocking out Rubredoxin

To study the possible function of Rubredoxin, I also generated KO mutants *via* CRISPR/Cas9 by targeting the CDS. In T0 generation of Rub-KO mutants, 1 heterozygous mutant showed a strong phenotype compared to the control plant, which was transformed with the same plasmids and in the same procedures but not mutated. The mutant showed much slower growth and smaller size, with pale green and smaller leaves, giving few flowers, and failed to bear fruits, so it could not produce seeds (**Fig 24, A**). As the leaves of the mutant were pale green, I measured chlorophyll by Dualex with 3 to 5 fully expanded mature leaves. The results show that mutant leaves only contained half of the chlorophyll of control leaves (**Fig 24, B**). If the mutant had less chlorophyll, it may affect photosynthesis as well.

Therefore, I used a CIRAS-3 photosynthesis system to measure several parameters of photosynthesis, including CO₂ assimilation (A, related to net photosynthesis), transpiration (E), stomatal conductance (GS, related to stomata opening), and photosynthesis water use efficiency (WUE). Compared to control leaves, mutant leaves showed almost no transpiration (E=0), kept stomata almost closed (GS=0), had a very low water use efficiency (WUE=0), and a very low CO₂ assimilation rate. I then sampled fully expanded mature leaves between 3:00 and 4:00 PM, a period during which starch levels peak in mature leaves according to a previous study (Bénard *et al.*, 2015). KI-I₂ staining of those leaves (**Fig 24, C**) showed that mutant leaves accumulated much less starch compared to control leaves.



Fig 24 Phenotyping of Rub-KO mutants *via* measuring chlorophyll, photosynthesis abilities and starch content

- A. T0 heterozygous Rub-KO mutant and control plants.
- B. Examples of leaves and chlorophyll content of the mutant and control plants;
- C. Starch content evaluated by KI-I₂ staining, and the comparison of the photosynthesis capacities of the mutant and control plants. Abbreviations: A, assimilation of CO₂ (µmol CO₂ m⁻² s⁻¹), presented net photosynthesis; E, transpiration (mmol H₂O m⁻² s⁻¹); GS, Stomatal conductance (mmol H₂O m⁻² s⁻¹); WUE, photosynthetic water use efficiency (mmol CO₂ mol⁻¹ H₂O).

However, after performing Sanger sequencing, it was hard to distinguish the exact mutations by only sequencing the PCR product. I aimed to obtain homozygous in T1 generation, expecting to have materials and replications for further phenotyping. However, the T1 homozygous plants contained almost no chlorophyll, and could not grow beyond the cotyledon stage to give functional leaves. In addition, those plants gradually died at a very young stage, therefore no material for further analysis was available except for extracting DNA and genotyping to validate the mutations.

Overexpression of Rubredoxin

The last attempt for examining the possible connection between Rubredoxin and starch metabolism, was to overexpress Rubredoxin in WT plants to see if the up-regulated transcript led to higher accumulation of starch in IMG fruits, as we observed significant higher expressions of Rubredoxin in P17C12 EMS mutant. After selection of transformed T0 plants, I selected three T0 lines with 3 plants per line. Unfortunately, the comprehensive characterization of these plants was interrupted by the sanitary situation due to COVID-19. That is why I checked transcript levels of Rubredoxin in leaves after we sampled the fruits at 18 DAP. In the fruits at 18 DAP, there was no difference in starch content between all the transformed lines and the WT. Furthermore, Rubredoxin was overexpressed by nearly 100 times in the line 35S-1 while it was down regulated significantly in the line 35S-2 (P<0.01), being expressed less than 70% than in WT plants, and for the line 35S-3, one plant showed levels similar to those found in the line 35S-1, and two plants had normal expression of Rubredoxin. Those strong differences in expression among the different lines, even within the same line, are not astonishing since we know that it happens frequently with the 35S promoter, which being expressed very strongly can cause posttranscriptional gene silencing, thus leading to co-suppression of homologous genes in transformants (Napoli et al., 1990).



Fig 25 Fruit starch content and leaf Rubredoxin transcript levels in tomato cv. Micro-Tom plants transformed with Rubredoxin under the control of the 35S promoter

- A. Starch contents in fruits at 18 DAP of transformants overexpressing Rubredoxin under the control of the 35S promoter and WT plants. Three transformed lines and three individuals per line were used to measure starch. There was no significant difference between transformants and WT plants at P<0.05 (with t test).
- B. Transcript levels of Rubredoxin in mature leaves of the same transformants of Rubredoxin for measuring starch content in fruits, and WT plants.

3.4 Discussion

The homozygous KO or missense mutants of RECQ4, MCF and PTA did not show any phenotype associated with starch metabolism. Moreover, under optimal growth conditions, they also showed no obvious phenotype for growth or morphology, whatever the organ. A recent study (Maagd *et al.*, 2020*b*) used CRISPR/Cas9 to create a RECQ4 homozygous KO mutant of *S. lycopersicum* (cv. Moneymaker) and *S. pimpinellifolium*, then through crossing they directly created an interspecific F1 hybrid without RECQ4. Compared with the wild-type F1 hybrid, the F1 *recq4* mutant had a significant increase in crossovers, but no other phenotypes were found.

As mentioned above, MCF is encoded by Solyc01g095510, an orthologue of AtMTM1 (At4g27940) in *Arabidopsis*. AtMTM1 may also play an important role in activation of MnSOD1 in *Arabidopsis* (Su *et al.*, 2007). However, there is no report for MTM1 mutated plants or further investigations of MTM1 function in plants. As our results based on simple observations showed, mutating MCF (Solyc01g095510) in tomato had no effect on plant growth and organ morphology.

The transformants obtained for PTA (Solyc01g096930) showed no phenotype related to starch, and also not for plant growth under optimal growth conditions. In *Arabidopsis*, the orthologue of this PTA is ALA1 (At5g04930), a member of P4-ATPases (also named flippase). Under normal growth conditions, *Arabidopsis ala1* mutants also showed no growth phenotype. However, under chilling stress, the lack of ALA1 resulted in reduced growth at the whole plant level (Gomès *et al.*, 2000).

At this stage of my work, I decided to not further investigate the mutants generated for RECQ4, MCF and PTA.

The mutation found within the CPuORF gene (Solyc01g105700) in P17C12 is located in the 5' UTR region, which may change the transcript level of the downstream mORF to cause phenotypic changes. Therefore, we firstly checked the transcript levels of CPuORF and mORF (bHLH145); they were both very low and thus close to background noise in the examined tissues including leaves and fruits of 12 and 20 DAP. Thus, I could not rule out that there was no difference between the P17C12 mutant and the WT. Since the expression did not give us extra clues for the effect of

mutations in 5' UTR, I tried to mimic the mutation by using a specially developed CRISPR/Cas9 technique consisting in a nickase Cas9 equipped with cytidine deaminase, which was supposed to change A to T and thus mimic the SNP detected in the 5' UTR region of CPuORF. Performing this technique requires enormous lab work and also some luck, because the efficiency of this specifically developed method is much lower than for usual CRISPR/Cas9 techniques (Shimatani et al., 2017a). Another difficulty was that the promoter we used for Cas9 is a U6 promoter, which belongs to Type 3 Pol III promoters and is widely used for the expression of small RNAs like guide RNA in CRISPR. It was found that the U6 promoter uses a T-stretch as termination signal, when there is "TTTT" (T4), the termination efficiency reaches more than 70% (Gao et al., 2018), which was the case for the guide sequence (AACTAGCTTTTGGAGGAAAA) of CPuORF. We obtained over 50 T0 transformants but only 2 lines showed possible mutations according to Sanger sequencing. We further analysed the T1 generation for these 2 lines. Unfortunately, the mutations were not A/T transitions, but InDel mutations (Fig 21, B), which were relatively close to the EMS SNP. However, those two CPuORF mutant lines showed no alteration in starch amount either. The Arabidopsis orthologue of CPuORF (Solyc01g105700), CPuORF37 (At5g50010.UORF), has been associated to the metabolism of the polyamine spermine. None of the KO mutants of the single member of this gene family have shown phenotypic changes (Cai *et al.*, 2016).

The case of CPuORF reveals how difficult it can be to identify a mutation located in a non-coding region. Firstly, there is only limited information available for those non-coding regions and the corresponding genes. Secondly, we found numerous of such mutations in the EMS mutant, which multiplies the challenges mentioned before. Thirdly, even if large progress has been achieved regarding bioengineering, we still lack highly efficient single-base-editing tools that would work for every possible SNP. So, because we were not able to exactly mimic the mutation found within this 5' UTR region of the P17C12 mutant, I could not conclude for its involvement in the starch phenotype.

The last candidate mutation was located within the 3' UTR of the Rubredoxin gene. It is well known that 3' UTR regions can regulate the degradation, translation and localization of mRNA (Mayr, 2017). Thus, to examine the possible effect of the SNP found in this region in P17C12, I first measured the transcript levels of Rubredoxin in the leaves and fruits of the BC1F1 and S2 plants of P17C12, and WT plants. Interestingly, Rubredoxin appeared up-regulated in the 12 and

20 DAP fruit of BC1F1 and S2 mutants, suggesting that this SNP may have a positive effect on the transcript levels. In consequence, in addition to my attempts to create KO mutants and mutants with possible InDel mutations near the SNP position, I generated plants overexpressing Rubredoxin under 35S and PEPC promoters.

We firstly observed interesting phenotypes in a heterozygous KO Rubredoxin mutant. The leaves were pale green, with highly reduced photosynthetic capacity and reduced amount of chlorophyll, and, not surprisingly, very little starch in the leaves (**Fig 24**). I then tried to obtain homozygous mutants, but the latter turned out to be unable to grow through the cotyledon stage, with the two cotyledons totally bleached and looking like "burned". Those phenotypes were consistent with previous observations on *Arabidopsis* mutants lacking Rubredoxin (Calderon *et al.*, 2013). Therefore, my results confirmed that the lack of Rubredoxin, for which there is only one copy in the tomato genome, is lethal for phototrophic plants. Recent studies on phototrophic bacteria and alga revealed the roles of Rubredoxin in the protection and repair of PSII from photooxidative damage during the PSII complex assembly (García-Cerdán *et al.*, 2019; Kiss *et al.*, 2019). The lethal effect in higher plants may be due to the lack of protection against ROS by Rubredoxin at the beginning of PSII biogenesis, when the very young plant becomes autotrophic. As all homozygous KO mutants of Rubredoxin stopped to grow and died at the cotyledon stage, we could not collect enough material to be able to perform more investigations on those mutants.

Because results obtained with the Rubredoxin KO mutants were promising (nearly starchless leaves phenotype), I put more efforts into testing this candidate gene. However, the normal phenotypes of 3' UTR mutated plants and overexpressing mutants invalidate the hypothesis that increased Rubredoxin would activate starch accumulation.

There are two possible explanations for those phenotypes of 3' UTR mutants of Rubredoxin. The first one is that the InDel mutations did not mimic the exact SNP mutation, thus, they might not have the same effect as the SNP mutation found in P17C12. The second one is that the SNP mutation may have no effect on the starch phenotype. The up-regulated mRNA could be a consequence of other mutations. For example it is known that one mutation can cause mRNA level to fluctuate on a large scale, as observed for *slgpat6* (for glycerol-3-phosphate acyltransferase 6) mutant and a series of ripe-related mutants (Petit *et al.*, 2016; Li *et al.*, 2018).

I obtained only one overexpressor of Rubredoxin under the control of the fruit specific PEPC promoter and thus not enough replication, so I did not show the data, as it would not be statistically valid. Then, the three 35S-overexpressor lines that were selected for phenotyping showed no starch-excess phenotype either, although transcripts encoding Rubredoxin were increased by up to 100-times. Finally, those results suggested that up-regulating Rubredoxin expression does not lead to increased starch accumulation during fruit growth.

To conclude, more efforts will be needed to isolate the causal mutation responsible for starchexcess in P17C12 fruit. Possible strategies for better fine-mapping or examination of new candidate genes will be discussed in Chapter 5.

Chapter4 Generating Starch-related

Mutant by Knocking out Alpha- and Beta-

amylase

4.1 Introduction

Among the enzymes participating in starch degradation in leaves, β-amylases (BAMs) present the highest diversity regarding sub-cellular localization, catalytic properties and functions (reviewed by Monroe and Storm, 2018). In *Arabidopsis*, there are nine gene members encoding BAM, named BAM1–9. Among 9 members, BAM1–4, BAM6 and BAM9 are located in the plastid, BAM5 in the cytosol and the remaining two (BAM7 and 8) in the nucleus. Some of the members (BAM1– 3, BAM5 and BAM6) are catalytically active *in vivo*, but others such as BAM4 and BAM7–9 show no catalytic activity *in vivo*, suggesting that the members of the BAM family may have distinctive roles in starch metabolism.

It was suggested that BAM3 is the predominant enzyme involved in starch degradation in leaf mesophyll cells (Monroe, 2020). BAM1 was also found to play an important role, especially in guard cell starch degradation (Thalmann *et al.*, 2016). In contrast, knocking out the other 3 BAMs, *i.e.* BAM2, BAM5, and BAM6, did not impact starch degradation or plant growth under optimal growth conditions (Monroe, 2020). The fact that BAM5 seems to be only a minor player in starch degradation is striking, especially when considering that BAM5 shows a 5 to 20 times higher affinity for soluble starch than other members (Monroe *et al.*, 2017) and is responsible for up to 80% β -amylase activity in extracts of *Arabidopsis* leaves (Lin *et al.*, 1988).

Compared to BAMs, α -amylase (AMY) seems to play less important roles in leaf starch degradation, as the plastid-localized α -amylase (AMY3) KO mutants showed no starch-excess phenotypes in *Arabidopsis* (Yu *et al.*, 2005). However, reducing activity of chloroplastic α -amylase in rice plants resulted in increased starch levels in leaves (Asatsuma *et al.*, 2005), implying

that the impact of the chloroplastic α -amylase on starch degradation may vary from species to species. Furthermore, it has been found that the genes encoding AMY3 and BAM1 both contain ABA-responsive element (ABRE) motifs in the promoter region, and the transcript levels of AMY3 and BAM1 are both up-regulated by ABA, and responding to osmotic stress (Thalmann *et al.*, 2016). In endosperms, it is widely accepted that AMY plays a central role to initiate the attack of the starch granule and the hydrolysis of α -1,4 bonds to produce linear or branched oligosaccharides (Zeeman *et al.*, 2010).

In fruit, BAM3, BAM9 and also AMY3 are mentioned to participate in starch degradation in kiwifruit and banana (Nardozza *et al.*, 2013; Hu *et al.*, 2016; Peroni-Okita *et al.*, 2010; do Nascimento *et al.*, 2006). This assumption was based on correlations found between transcripts and starch levels in different development stages, treatments, cultivars and/or species, while the *in vivo* roles (BAM9 is a non-catalytic protein) still need to be investigated.

In this chapter I focused on the two BAM and AMY families in tomato fruit, as they play important and diversified roles in starch degradation. I first mined and named all the members of those two families based on the phylogenetic analysis. Then, I analysed the transcript levels of those genes in public databases, as well as their subcellular localizations to select the candidate genes in order to generate KO mutant plants *via* the CRISPR/Cas9 techniques. Finally, I selected four genes in tomato fruit, namely AMY3.2 (Solyc05g007070), BAM1.2 (Solyc09g091030), BAM3.2 (Solyc08g077530) and BAM9 (Solyc01g067660). Besides, our preliminary phenotyping results showed that BAM9 may play important roles in the reproduction process in tomato.

4.2 Materials and Methods

Phylogenetic Analysis of BAM and AMY Family Members

Family members of BAM and AMY of tomato were identified by Moustakas (2013) with comprehensive bioinformatic methods. He also performed a phylogenetic tree analysis, with three species, *Arabidopsis*, tomato and potato. In order to compare only tomato and *Arabidopsis*, I defined a new phylogenetic analysis with genes identified as encoding BAM and AMY protein sequences (gene ID are listed in **Table 3** in Chapter 1.2). Multiple sequence alignments of full-

length protein sequences were executed by using the MEGA X software with default parameters (Kumar *et al.*, 2018). The Neighbor-Joining phylogenetic tree was constructed using protein sequences with the Poisson correlation model, with 1000 bootstrap replications (Zuckerkandl and Pauling, 1965; Saitou and Nei, 1987), and all positions containing gaps and missing data were eliminated (complete deletion option).

Plasmid Constructions and Tomato Transformation

The same set of plasmids and manipulation procedures for plasmid construction that are described in the Materials and Methods section of Chapter 3.2 were used. The specific target sequences (guide sequences) are listed in **Table 11**. I used cotyledons of WT Micro-Tom to do the transformation by following the same protocol as described in Chapter 3.2.

Gene	Guide	Sequence (5' to 3')
AMY3.2	G1	CGGAGATAATGGCCGACGAG
	G2	TTCTCTCGGAGGCCACTCTC
BAM1.2	G1	GATAAGGCACCGATCTGGTG
	G2	TTACAGGCGTTGAAAAGCGC
BAM3.2	G1	TAGCCGACATGATGATGGCT
	G2	CTTGACACCATGACTATGGG
BAM9	G1	ATAGAACTCCCCGTCTGGTG
	G2	ACTGATGTTCCTGTCCTCGA

Table 11 The guide sequences selected for CRISPR/Cas9 mutagenesis of AMY/BAMs

Genotyping and Phenotyping of Mutants

To select mutated KO mutants, the same procedures as described in Chapter 3.2 were used, and the specific primers used are listed in **Table 12.** The phenotyping of the obtained mutants, which included the determination of starch and hexoses, was as described in Chapters 2 and 3.

Gene	Forward/Reverse primer	Sequence (5' to 3')	Size of PCR product (bp)
SIAMY3.2	FP	TCGAACCGCAAAAACCGAAT	706
	RP	AGAATACACAACGCCCAATC	
SIBAM1.2	FP	CCACAATTTCAACTCCTCCTTC	850
	RP	CATGTTTGATTTGACGGTATTC	
SIBAM3.2	FP	ATCAGCACAAATCATCACACAT	595
	RP	CCTCCACACTGATGAAAAGAC	
SIBAM9	FP	ATTCTCTTGCAGAGTGATGGTG	533
	RP	CATAATGCCCAAAAGCTTGAAAG	

Table 12 The primers for PCR

Pollen Germination

Solution for *in vitro* **pollen germination**: The pollen germination medium contained necessary nutrients listed in **Table 13**. The pH of the solution was adjusted to 7 with 0.1 M KOH, and autoclaved at 120°C for 20 min for sterilization.

Composition	Final concentration					
Sucrose	180 g/L					
Boric Acid	100 mg/L					
CaCl ₂	1 mM					
$Ca(NO_3)_2$	1 mM					
MgSO ₄	1 mM					
Tris-HCL	1 mg/L					

Table 13 Pollen germination solution

Sample harvesting and observation: To harvest the pollen, we selected fully opened flowers at anthesis stage. The anthers were collected and split with tweezers and put into the germination solution directly, which was then vortex to release the pollen. After overnight incubation at room temperature, the pollen suspension was analysed using a stereomicroscope. In order to count the rate of germinated pollen, I usually took at least three pictures per sample and used the ImageJ software to evaluate the proportion of germinated pollen.

4.3 Results

Identification of AMY and BAM Orthologues in Tomato

The orthologues of AMY and BAM in tomato were searched by using the corresponding *Arabidopsis* sequences as seeds (results are listed in **Table 3**). As the previous phylogenetic trees of AMY and BAM were made by Moustakas (2013) with *Arabidopsis*, tomato and also potato with a different naming system (like BAM can be named as BMY and given a different number), I constructed a phylogenetic tree based on the Neighbor-joining method using MEGA 7.0 with AMY and BAM family members only from *Arabidopsis* and tomato, to further confirm their phylogenetic relationships. In *Arabidopsis*, AMY and BAM consist of 3 and 9 family members, respectively. In tomato, there are 5 AMY members; it is likely that both AMY1 and AMY3 are duplicated copies, and 8 BAM members, which also showed duplicates, *i.e.* for BAM1 and BAM3. However, for the subcluster that contains *Arabidopsis* BAM4 and BAM9, there was only one tomato gene close to BAM9. A similar result was found for the subcluster containing *Arabidopsis* BAM5 and BAM6, in which only the tomato member BAM5 was found. Those corresponding genes found in tomato were named based on the results of the clustering analysis used to build the phylogenetic tree and the position on the chromosome.



Fig 26 Evolutionary relationships of α-amylase and β-amylase in Arabidopsis and tomato

- A. Phylogenetic tree of *Arabidopsis* and tomato AMY. The optimal tree with the sum of branch length = 1.84710964 is shown. This analysis involved 8 amino acid sequences. There were a total of 385 positions in the final dataset.
- B. Phylogenetic tree of *Arabidopsis* and tomato BAM. The optimal tree with the sum of branch length = 4.31328901 is shown. This analysis involved 17 amino acid sequences. There were a total of 379 positions in the final dataset.

The tree is drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

To be able to predict which genes are possibly involved in fruit starch degradation in tomato, we retrieved the transcript levels and putative sublocations of AMY and BAM members from the online database Tomato eFP Browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi) and eplant (http://bar.utoronto.ca/eplant_tomato/), as shown in Table 14. When comparing those members, two genes, AMY3.2 and BAM9 appeared striking for their high levels of expression which significantly increase along with starch degradation during fruit development, especially considering that BAM9 was reported as being non-catalytic and with unknown function in starch degradation in *Arabidopsis* (Steidle, 2010). There were two other members, BAM1.2 and BAM3.2 that were interesting too, as they also showed relatively high levels of expression in fruit that slightly increased during fruit development towards ripening. As described before, BAM3 and BAM1 are the two most important BAM members involved in starch degradation in the leaf. They may also play important roles in fruits. Thus, based on their plastid subcellular localization, the association of their transcript levels with starch degradation, and their potential involvement in starch degradation in leaf, I selected AMY3.2, BAM1.2, BAM3.2 and BAM9, *i.e.* 4 out of 13 AMY/BAM members, for further investigations.

Coding gene				S. lycopersicum cv Heinz						S. pimpinellifolium						
Name	Solyc	Subcellullar location	Leaves	Root	Unopened Flower Bud	Fully Opened Flower	1cm Fruit	2cm Fruit	3cm Fruit	Mature Green Fruit	Breaker Fruit	Breaker Fruit + 10	Leaf	Immature Green Fruit	Breaker Fruit	Breaker + 5 Fruit
AMY1.1	Solyc03g095710	extracelluar	5.51	6.54	13.03	13	2.81	5.95	8.81	17.36	29.67	24.17	4.25	30.04	33.27	22.45
AMY1.2	Solyc04g078930	plastid	0.69	5.67	0.72	0.36	0.28	0.43	0.23	0.24	0.52	0.22	0.05	0.47	0.57	0.3
AMY2	Solyc04g082090	cytosol	7.75	18.43	15.68	12.5	14.3	17.05	14.89	20.72	19.7	20.48	5.12	20.39	42.04	44.01
AMY3.1	Solyc02g066950	cytosol	39.13	13.48	11.74	12.42	18.13	20.5	12.48	7.11	2.16	1.12	52.12	13.67	11.32	5.09
AMY3.2	Solyc05g007070	plastid	44.48	16.23	11.81	33.7	20.07	34.44	65.14	135.41	347.97	241.49	72.09	256.52	351	164.96
BAM1.1	Solyc08g082810	plastid	7.83	8.06	9.02	7.83	8.86	8.72	9.78	6.77	7.57	13.73	5.88	11.48	10.03	11.95
BAM1.2	Solyc09g091030	plastid	23.68	8.71	61.76	167.13	32.18	27.82	15.43	22.71	23.22	56.37	13.83	13.92	18.1	23.78
BAM3.1	Solyc08g007130	Unknown	2.34	2.21	4.9	73.56	4.01	6.97	4.43	1.26	0.15	0.56	2.16	0.18	0.13	0.11
BAM3.2	Solyc08g077530	plastid	40.96	3.39	6.93	19.36	23.95	64.45	80.7	71.51	62.52	23.01	8.24	26.26	15.82	24.75
BAM5	Solyc07g052690	plastid	26.08	1.77	12.19	19.57	2.49	1.45	0.98	9.83	11.95	15.56	29.07	11.88	12.95	12.17
BAM7	Solyc01g094580	nucleus	8.46	16.72	11.95	11.91	12.83	11.73	18.01	11.74	10.44	7.34	7.65	14.45	12.29	8.38
BAM8	Solyc08g005780	nucleus	6.13	5.87	8.01	6.68	14.01	15.71	13.02	14.09	6.05	5.83	5.42	7.7	6.78	4.24
BAM9	Solyc01g067660	plastid	21.64	19.14	39.18	181.62	67.66	188.06	364.32	224.14	415.51	547.63	6.98	232.36	349.51	1002.2

Table 14 transcript levels of AMY and BAM family in different tissues of tomato

The information of Solyc number, the putative subcellular location and transcript levels of tomato were extracted mainly in eFP (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi) and ePlant (http://bar.utoronto.ca/eplant_tomato/) database, and listed in **Table 14**.

Generation of Mutants for Putative Fruit-specific Amylases (AMY3.2, BAM1.2, BAM3.2 and BAM9)

I used CRISPR/Cas9 to generate mutants for each of the four selected candidate gene, i.e. AMY3.2, BAM1.2, BAM3.2 and BAM9. I designed 2 single-guide RNAs targeted to the exon 1 or exon 2, aiming to cause InDel mutations that lead to in-frame premature stop codons and truncated proteins. The putative mutants were screened firstly by PCR amplification of the targeted regions, and the PCR fragments were Sanger sequenced to identify the InDel mutations. For each candidate gene, we identified three independent transformants with mutations (from **Fig 27** to **30**). Before the COVID-19 lockdown, I had only obtained the T1 generation. In this generation, there were homozygous KO mutants only for AMY3.2 and BAM9.

For AMY3.2, there were two lines with homozygous KO/missense individual plants, and one line with a heterozygous mutation. It is necessary to mention that for the transgenic line AMY3.2-3, there were two types of mutations: plant 3 was a homozygous KO, though plants 5 and 8 were missense mutants that only missed "RPL" (arginine, proline and leucine), 3 amino acids at the beginning of the protein, which may not affect the protein function. For BAM9, luckily, we obtained 3 homozygous KO lines, and at least 3 individual plants per line. All of them had mutations with single nucleotide insertion or deletion, which led to premature stop codons and thus truncated proteins. For BAM1.2 and BAM3.2, we only produced heterozygous mutants with various types of mutations (**Fig 28** and **29**).

Considering that in *Arabidopsis*, BAM mutations are usually recessive and also that there is functional redundancy between these AMY and BAM gene families (reviewed by Monroe and Storm, 2018), I decided to start with the homozygous mutant lines of AMY3.2 and BAM9 for detailed phenotyping. For BAM1.2 and BAM3.2, I attempted to select homozygous KO mutants in the T2 generation.

A AMY3.2 Solyc05g007070



Fig 27 Mutations in T1 generation of CRISPR/Cas9 mutants for AMY3.2

- A. Mutations of 3 transgenic lines are shown for AMY3.2 (Solyc05g007070). The mutations generated by CRISPR/Cas9 were represented by black asterisks indicating the mutated positions, and the exact insertions or deletions were also showed in black box. For example, [C] means an insertion of "C"; C means a deletion of "C", and [T>C] means a transition from "T" to "C". The sequences of two guides are also shown in the black box. For the AMY3.2-3 line there were two types of mutations, which are shown in the black box.
- B. The mutated protein sequences are indicated by a grey cross and the exact mutated sequences are shown in the black box. The stop codon is shown by red asterisks, and **M** is the methionine for the start of translation. RPL means the deletion of three amino acids "RPL".



Fig 28 Mutations in T1 generation of CRISPR/Cas9 mutants for BAM1.2

- A. Mutations of 3 transgenic lines are shown for BAM1.2 (Solyc09g091030). The mutations generated by CRISPR/Cas9 were represented by black asterisks indicating the mutated positions, and the exact insertions or deletions were also showed in black box. For example, [C] means an insertion of "C"; C means a deletion of "C". The start position of translation is indicated as "ATG", and two guide sequences are shown in the black box. For the two BAM1.2-1 and -3 lines, there was chaos in the Sanger sequencing results, so that we could not figure out the exact mutations for those two lines.
- B. The mutated protein sequences are indicated by a grey cross and the exact mutated sequences are shown in the black box. The stop codon is shown a by red asterisk, and **M** is the methionine for the start of translation.
A BAM3.2 Solyc08g077530



Glycosyl hydrolase family 14

Fig 29 Mutations in T1 generation of CRISPR/Cas9 mutants for BAM3.2

- A. Mutations of 3 transgenic lines are shown for BAM3.2 (Solyc08g077530). The mutations generated by CRISPR/Cas9 are represented by black asterisks indicating the mutated positions, and the exact insertions or deletions are shown in the black box. For example, [C] means an insertion of "C"; C means a deletion of "C". The start position of the translation is indicated as "ATG", and two guide sequences are shown in the black box.
- B. The mutated protein sequences are indicated by a grey cross and the exact mutated sequences are shown in the black box. The stop codon is shown by a red asterisk, and \mathbf{M} is the methionine for the start of translation.

A BAM9 Solyc01g067660



Glycosyl hydrolase family 14

Fig 30 Mutations in T1 generation of CRISPR/Cas9 mutants for BAM9

- A. Mutations of 3 transgenic lines are shown for BAM9 (Solyc01g067660). The mutations generated by CRISPR/Cas9 are represented by asterisks indicating the mutated positions, and the exact insertions or deletions are shown in the black box. For example, [A] means a insertion of "A"; G means a deletion of "G". For the BAM9-10 line, there were 2 types of mutations found in the T1 generation, plant 6 and 11 had an insertion of "G", and plant 1, 3 and 8 had a deletion of "G". The start position of translation is indicated as "ATG", and two guide sequences are also shown in the black box.
- B. The mutated protein sequences are indicated by a grey cross and the exact mutated sequences are shown in the black box. The stop codon is shown by a red asterisk, and **M** is the methionine for the start of translation.

Phenotyping of AMY3.2 and BAM9 Homozygous Mutants

Involvement of AMY3.2 in Fruit Starch Metabolism

The first phenotyping was mainly performed on fruit samples collected at three developmental stages, *i.e.* 20 and 35 DAP and red ripe. For starch metabolism, those three stages are the most representative, since starch is most abundant at 20 DAP when expressed on a fresh weight basis. After 20 DAP starch rapidly decreases to become 4 times lower at 35 DAP, and at the red ripe stage it is almost totally degraded. Under our growth condition, the mutants grew normally, like the WT plants. However, in the two homozygous mutant lines of AMY3.2, starch amounts were significantly lower than in the WT at 20 DAP. In contrast, there was no obvious difference found for starch at 35 DAP and at ripeness. For hexoses, there was no significant change between mutants and WT at the red ripe stage (**Fig 31, B**).



Fig 31 Starch and hexoses contents in AMY3.2 homozygous mutants

- A. Amount of starch in fruits at 20 and 35 DAP of AMY3.2 homozygous mutants. Starch content is presented as means \pm SD (n=5 for the WT; n=3 for the AMY3.2 mutant lines).
- B. Fructose and glucose contents in red ripe fruits of AMY homozygous mutants. Hexose content is presented as means \pm SD (n=5 for the WT; n=3 for AMY3.2 mutant lines).

The Significance was tested using a t-test, and one or two asterisks indicate P<0.05 or P<0.01, respectively.

Part II-Results

Impaired Pollen Germination in BAM9 Mutants

BAM9 mutants showed more diversified phenotypes. Firstly, at the flowering stage, in two individuals KO plants BAM9-2_5 and BAM9-8_6 the fertility was impaired as shown by the total absence of germination for the pollen of these two mutants (**Fig 32**). As a consequence, these plants did not produce enough fruit for analysing starch content at 20 and 35 DAP and at red ripe stages.



Fig 32 Rate of pollen germination in two BAM9 KO individual plants

Pollen was taken from fully opened flowers and submitted to a germination test. For the WT, pollen was taken from 3 individual plants, and 3 flowers each. For BAM9-2_5, and BAM9-8_6 KO mutants, pollen was taken from 1 individual per line, and 3 flowers each. Data is expressed as % of germination (mean \pm SD).

Involvement of BAM9 in Starch Remobilisation in Ripening Fruit

The other individual BAM9 KO mutants produced more fruits than BAM9-2_5 and BAM9-8_6 lines, thus I could sample enough fruits for analysing starch or hexose contents at 3 stages. Starch levels measured at 20 and 35 DAP were very fluctuating, varying from 22 to 154

 μ mol.eq.glucose.g⁻¹ FW at 24 DAP, and from 11 to 149 μ mol.eq.glucose.g⁻¹ FW at 35 DAP (**Fig 33, A**). Thus, some mutants had higher starch levels than the WT and others had less starch. In the WT and as expected starch was highest at 20 DAP and strongly decreased at 35 DAP. However, in a majority of mutant plants (5 out of 9) starch was unchanged or even increased between 20 and 35 DAP (*e.g.* in lines BAM9-2_8, 8_7, 10_6, 10_8 and 10_11). Besides, in the BAM9-10_11 mutant, starch was much higher than in the WT, whereas in BAM9-2_1, 8_5 and 10_3 mutants the starch content was similar to that of the WT.

Lack of BAM9 Leads to Increased Glucose in Red Ripe Fruit

I next focused on hexoses (fructose and glucose) amount in red ripe fruits, since starch degradation is often assumed to contribute to hexose accumulation during ripening (Schaffer and Petreikov, 1997). Because hexose contents in those plants were much more stable and repeatable for individuals of each transgenic line, I used each individual as biological replicate. Results showed no differences for fructose among all mutants and WT, but for glucose, in all the three lines studied glucose content was significantly increased by 1.4 and 1.6 times (P<0.01, **Fig 33, B**) in comparison with the WT.



Fig 33 Starch and hexoses contents in BAM9 homozygous KO mutants

- A. Amount of starch in fruits at 20 DAP and 30 DAP of BAM9 homozygous KO mutants. Data are presented as means ± SD (5 individuals for the WT; for each individual of BAM9 mutant line, 3 technical replicates were used to calculate the SD).
- B. Fructose and glucose contents in red ripe fruits of BAM9 homozygous KO mutants. Data are presented as means \pm SD (n=5 for the WT; n=3 for each line). The Significance was tested using a t-test, and one or two asterisks indicate P<0.05 or P<0.01, respectively.

Part II-Results

4.4 Discussion

Following the integration of genomic and transcriptional data available online, I selected AMY3.2, BAM1.2, BAM3.3 and BAM9 as candidate proteins potentially involved in the degradation and/or regulation of starch turnover in tomato fruit. Although homologues of these genes have been proposed to be involved in starch degradation at ripening in kiwi and banana, based on correlative evidence (do Nascimento *et al.*, 2006; Nardozza *et al.*, 2013; Hu *et al.*, 2016; Xiao *et al.*, 2018), they have not been tested *in vivo*. Taking advantage of the tools available for tomato, I used CRISPR/Cas9 to generate KO mutants for these four genes.

In the two homozygous lines of AMY3.2, starch content was significantly lower in fruits at 20 DAP than WT. It is tempting to speculate that it may play a role in starch degradation. However, in the T1 generation, I could only characterize two lines of homozygous mutants, and considering that starch amounts show large variations along fruit growth and are affected by culture conditions (described in Chapter 2 Results), more mutants will be required to confirm these data with the T2 generation. Contrarily, Arabidopsis mutant lacking AMY3 displayed no change in leaf starch turnover (Yu et al., 2005). However, knocking out AMY3 in other mutant backgrounds, such as KO mutants for the four DBEs or for ISA3 and LDA genes, resulted in alterations of starch accumulation. These results suggest that there is a functional redundancy of starch related enzymes in leaf, and that AMY3 also participates in starch degradation. Thalmann et al. (2016) also reported that AMY3 together with BAM1 are involved in stress-induced starch degradation. Loss of AMY3 and BAM1 activities impaired root growth of Arabidopsis, and those double mutants were hypersensitive to osmotic stress (Thalmann et al., 2016). However, loss of BAM1 and AMY3 alone or together does not affect starch metabolism in mesophyll cells under normal conditions (Horrer et al., 2016; Thalmann et al., 2016), indicating that they have tissue-specific functions and respond to certain abiotic stress signals.

For BAM9 KO mutants, we observed more diversified phenotypes. In two individual lines, a drastic reduction of pollen germination rate suggested that BAM9 is essential for pollen viability. A series of studies on the effect of heat stress in tomato suggested that starch plays important roles in pollen viability and germination. Under optimal temperature conditions (28/22°C, day/night temperature), the maximum of starch amount in the pollen grains was observed at 3 days before

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anthesis, then starch content decreased towards anthesis. In contrast, hexose amounts gradually increased during flower development, reaching their maximum at anthesis. However, continuous exposure of the plants to high temperatures (32/26°C) prevented this transient increase of starch and led to decreased soluble sugar levels in the cell wall of anthers and pollen grains (Pressman *et al.*, 2002). It was observed that heat stress did not significantly reduce the flower or pollen grain numbers, but caused significant decreases of pollen viability and the number of released pollen grains, thus markedly decreasing fruit set (Sato *et al.*, 2006). Those results indicate that in tomato the failure of fruit set under high temperature is possibly due to the alteration of sugar metabolism during flower development, mainly at the anther level. A similar scenario was observed in maize, in which heat stress reduced pollen starch content, sugar related enzymatic activities, decreased pollen germination rate, and consequently reduced fertility. The integration of metabolic, lipidomic, and transcriptomic data suggested that, in maize, heat stress strongly affects central metabolic pathways during pollen development, which impairs its germination capacity and can result in severe yield losses (Begcy *et al.*, 2019).

There was one attempt to obtain mutants lacking GWD (Glucan water dikinase), an enzyme which controls the phosphate content of starch in tomato, but it caused sterility in the heterozygous KO mutant, making the selection homozygous mutant impossible (Nashilevitz *et al.*, 2009). In the GWD heterozygous mutant, starch content was higher in the pollen at anthesis and pollen germination rate was reduced. However, in the BAM9-mutant obtained here, the pollen did not show such a starch-excess phenotype. It is thus tempting to hypothesise that BAM9 may affect starch degradation in flowers, thus impacting the availability of carbon and/or energy during anther development, thus impairing pollen viability, and ultimately, fruit set. It will be interesting to confirm this result.

The large fluctuations found for starch between individual *bam9* mutants in fruits at 20 and 35 DAP were unexpected. Was it a consequence of the growth conditions? Probably not, because in AMY3.2 mutants and WT the starch content was much more reproducible. Was it a consequence of poor fertility? Even though we had enough fruit set for sampling, the number of seeds per fruit may have varied. Unfortunately, we did not pay special attention to seed numbers during sampling, which must be considered in T2 phenotyping. Another possible explanation for this fluctuation in starch content could be that the mutation impacts the expression and/or activity of other starch-

related genes, thus leading to pleiotropic effects that would partially alleviate the expected starchexcess phenotype. However, when we compared the relative starch content at two stages of fruit development within a given plant, there was an obvious trend indicating that BAM9 is involved in starch degradation. Thus, when expressing starch measured at 35 DAP as a percentage of starch measured at 20 DAP, we found that in 6 out of 9 transgenic plants, there was still more starch stored than in the WT (>30%). However, I could not conclude about the starch-excess phenotype at 35DAP in the BAM9, but this result demonstrates that to be confirmed (or not), more individual plants for T2 generation are needed to phenotype fruits at those two stages.

Another promising result we observed in red ripe fruits was a significant increase in the glucose content, in all three BAM9 KO lines, whereas fructose and starch were unchanged. Glucose and fructose contents are usually very reproducible in red ripe tomato fruit, whatever the position on the truss, the position of the truss, the growth conditions or even the growth location (Biais *et al.*, 2014). Moreover, for fruits grown under high salinity, both glucose and fructose increased in parallel (Yin *et al.*, 2010). Therefore, in transgenic tomato with down-regulated fumarase, starch content increased by two fold, besides in some lines, glucose and fructose contents were also increased together (Centeno *et al.*, 2011). Thus, the specific and relatively large increase in glucose but not in fructose found in the BAM9 KO red ripe fruits can be considered as striking.

There is little information about the possible functions of BAM9 in starch metabolism. In potato, StBAM9 and also StBAM1 were silenced to study their role during cold-induced sweetening. However, the silencing of the BAM9 gene did not result in a decrease of β -amylase activity, thus confirming that BAM9 is a non-catalytic protein (Hou *et al.*, 2017). Nevertheless, silencing StBAM9 and StBAM1 in potato resulted in elevated starch levels in tubers. This gain was enhanced for silenced lines for the two genes, implying that a functional redundancy may exist. More recently, it was shown that StBAM9 interacts with StBAM1 during the starch granules fate, suggesting that BAM9 may facilitate starch degradation by recruiting StBAM1 to the starch granule (Hou *et al.*, 2017).

One hypothesis to explain the increase in glucose observed in the BAM9 mutants is that delayed starch degradation during ripening could result in less sugar used in processes such as the climacteric respiration, which dissipates energy (Colombié *et al.*, 2017). This hypothesis assumes

that starch would be ultimately degraded into glucose in red ripe fruits. However, we still lack proof to validate this hypothesis. Firstly, more reproducible data for starch content need to be obtained from more BAM9 mutants. Next, some direct observations of the starch granule could be performed during the ripening stage (like 35 DAP), as BAM9 was assumed to act on the starch granule (Hou *et al.*, 2017), lacking of BAM9 may result the changes of morphology of starch.

All those promising results obtained with the T1 generation of BAM9-KO mutants encouraged us to include more individual plants in the T2 generation, and examine pollen germination as well as BAM enzyme activities for all transformants. Besides, as we also generated mutants of BAM1.2 and BAM3.2, which are the two main players of starch degradation in *Arabidopsis* leaves, it will be important to phenotype them too. It is worthy to mention that large amounts of starch are completely degraded when fruits reach the red ripe stage, indicating that a very efficient degradation machinery exists for fruit starch fate. The extinction of a single starch-related enzyme may slow down the degradation process without totally stopping it. Thus, it would be interesting to generate double or triple mutants by crossing the BAM9 with BAM1.2 and BAM3.2 mutants, to analyse the possible interactions between these three proteins, and the possible functional redundancy between them. However, we also found that pollen viability is very sensitive to alterations in starch metabolism. Thus, such multiple mutants might be sterile, and thus very difficult to study. To overcome this undesired effect on pollen fertility, silencing BAMs in a fruit-specific manner would be feasible.

Summary and Outlook

Part III-Summary and Outlook

Chapter5 Summary and Outlook

Forward genetics have proven powerful for discovering novel mechanisms in plants. To our knowledge, this the first report of an attempt to screen for fruit starch mutants in tomato and identify the causal mutations. The fact that screening Arabidopsis mutants for leaf starch phenotypes has been extremely successful (Caspar et al., 1985; Zeeman et al., 1998) motivated my project exploiting the Micro-Tom EMS population developed in Bordeaux (Just et al., 2013). However, we found that compared to starch accumulation in leaves of *Arabidopsis*, starch levels found in tomato fruits present large fluctuations, which largely depend on the development stage, but also the season (temperature and irradiance) as well as sink-source relationships. To reduce the variation and obtain as reproducible as possible starch measurements, and thus avoid falsepositives or -negatives, I established a protocol with a range of extra controls including the marking of the pollination date combined to precise sampling dates of the fruit to obtain material at precise developmental stages, the thinning of flowers and fruits to decrease the influence of source-sink relationships, and always including enough WT individuals as controls in the experiments. By following the pipeline proposed by Garcia et al. (2016), I was able to confirm the starch-excess mutant P17C12 as homozygous mutant with a recessive phenotype, and I subsequently sequenced and mapped the causal region on chromosome 1. The whole procedure and results are summarized in **Fig 34**.

Our results also show that thanks to the combination of next-generation sequencing and robotic high throughout biochemical phenotyping, the screening and mapping procedure was largely shorted, so that within 2 years we had identified the region of interest. However, we then encountered other challenges, especially when considering the difficulties we faced in predicting and identifying the possible effect of SNP located in non-coding sequence regions.



Fig 34 Summary of results we obtained by using forward and reverse genetics to study starch metabolism

The arrows indicate the next steps for continuing. The key steps of the whole project were indicated in dark blue background, and the main outputs (also the inputs for the next steps) were indicated in purple background. The steps in dashed boxes were the goals for our research.

After excluding the possible candidate mutations according to the SNP effect (KO, missense or modifications in 5' UTR and 3' UTR), how to find the causal mutation in P17C12? By having used the controls mentioned above, I am confident about the phenotyping, the bulk construction and the sequencing procedures that have been used. However, it appeared that identifying the causal gene was much more difficult that initially foreseen. In particular, the most obvious candidates, which include knockout and missense mutations, were not validated. A previous case of a causal mutation for locule numbers (the corresponding mutation was named lc) that turned

out to be an intergenic mutation may give us some clues for finding the causal SNP in a non-coding region (Muños *et al.*, 2011). A large F2 population was used to identify the genomic region of lc and a final sequence analysis of the region restricted lc to a 1,608-bp region including 14 polymorphic sites (13 SNPs and a 1 bp InDel). This region was located in a non-coding region between the WUSCHEL gene and a gene encoding a WD40-repeat protein. Functional studies of this 1608-bp region (mainly examined the possible transcript and transcript levels) revealed that there were no differences in the expression of these two genes in sub-near-isogenic lines. Then, quantitative RT-PCR assays conducted by using several primer pairs inside and surrounding the locus revealed no expression of this region. Thus, instead of using transformation to try to validate the mutations, the researchers used a combination of fine mapping and association mapping and phenotyping with 9,456 F2 individuals to successfully identify the two SNPs responsible for lc.

In our case, we probably need more BC1F2 plants derived from P17C12 to obtain a fine mapping with higher resolution to be able to further narrow down the possible regions. The BC1F2 population of 200 plants that we used was perhaps too small compared to previous approaches used to map causal mutations. For example, 343 BC1F2 plants were used to map the mutations responsible for the glossy phenotype of fruits (Petit et al., 2016) and 440 to map a mutant with excessive ascorbate in the fruit (Bournonville, 2015). The needed size of the F2 population strongly depends on the accuracy of the phenotyping but also on the rate of recombination. In our case, thanks to the KO mutation of RECQ4, we have a very high recombination rate, which enabled to narrow down the possible candidate regions to a 14 cM region with only 1 KO, 2 missense and numerous mutations in non-coding sequence regions. However, since we found that the most possible candidates (according to the mutation effect) were not the causal ones, other mutations present in non-coding sequences need to be investigated. Examining those candidates will be more difficult, because mimicking the exact EMS SNP mutations would request the generation of a very large number of mutants, according to the results we obtained when we tried to mimic the SNP in the 5' UTR region of CPuORF. Another concern will be about the assembly of chromosome 1. Chromosome 1 is the longest chromosome with large physical and genetic distances (Hosmani et al., 2019). When comparing the end of chromosome 1 as described by SL3.0 and by the recently released SL4.0 two genome-assembly versions we saw obvious differences between the respective sequences, such as ch01G91491515A predicted as a downstream gene variant of

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Solyc01g102950.3, but actually located in an intron of Solyc01g102970.2. Additionally, we mapped the sequences to the genome of cv. Heinz, not directly to the Micro-Tom genome; it may produce some bias too. Thus, it will also be necessary to remap the sequencing results according to SL4.0 or Micro-Tom genome and to re-analyse the SNP effect.

Reverse genetics, as a complementary strategy for studying starch metabolism in tomato fruit, gave us some promising results, for AMY3.2 and BAM9. Thus the KO homozygous mutants obtained for this gene showed an impaired degradation of starch during ripening as well as an increase in glucose content in the ripe fruit. However, as suggested by our results, knocking out genes involved in starch metabolism may also affect other reproductive stages. Thus, we have seen that the pollen of two individuals BAM9 KO mutants was unable to germinate, implying that starch metabolism is essential for pollen viability. A similar scenario was previously found in a heterozygous GWD (Glucan, Water Dikinase) mutant (Nashilevitz *et al.*, 2009). We decided to continue the study of the KO mutants obtained for AMY3.2, BAM1.2, BAM3.2 and BAM9 by phenotyping the T2 generations, of both the pollen and the fruit.

Moreover, starch mutants can be used for studying the diversified physiological roles of starch in fruit development. In our group, a constraint-based modelling approach was used to estimate metabolic fluxes along fruit development (Colombié *et al.* 2017). By investigating the energetic metabolism of the fruit pericarp, they associated the metabolism of starch (and to a lesser extent of the cell wall) to the respiration climacteric. According to the model output, it was proposed that in the ripening tomato fruit, excessive energy resulting from the massive degradation of starch and cell wall degradation is dissipated *via* the respiration climacteric. We would like to further confirm this hypothesis with mutants that accumulate abnormal amount of starch. The mutants obtained here could be used to verify the hypothesis generated by the model. Additionally, the KO mutants of BAMs of tomato can be used to study the functions of the orthologues from other species, such as kiwifruit, banana and apple, as for those fruits *in vivo* manipulations are more difficult and time-consuming.

According to the knowledge and experience I obtained from forward genetics for screening starch mutants in fruits, and reverse genetics for generating starch-related mutants *via* target AMY/BAMs by using CRISPR/Cas9 techniques, I would like to propose an alternative way to screen mutants

related to starch degradation or accumulation, which would focus more on glucose content in red ripe fruit, other than measuring starch directly in immature green, mature green or breaker fruits. One reason is that starch content shows remarkable variations during fruit growth, affected significantly by sink/source relationship and environmental conditions, thus it asked more effort for controlling fruit growth and sampling. Even so, we still obtained results with big fluctuations. Therefore, in order to overcome this, it required to repeat in a new generation (like for BAM3.2 and BAM9) and include more individuals. All those procedures actually limited us to screen more mutants and impeded us to confirm a starch-related mutant quickly and robustly.

Secondly, big variations of starch seem have no impact on the repeatability and stability of hexose contents. A striking case was showed in Chapter 4 for BAM9 KO mutant, regardless how fluctuating results we obtained for starch content at 20 DAP and 35 DAP, I observed repeatable results for fructose, and a significant difference for the glucose content in red ripe fruits of mutants and WT (**Fig 33, B**). Those results indicated that measuring glucose content instead of starch content may overcome the fluctuations of starch content. And for starch-excess mutant P17C12, higher glucose contents were also observed in S1 or S2 mutants than BC1F1 and WT red ripe fruits (**Fig 11, D**).

Thirdly, the measurements on glucose could provide us more repeatable phenotyping results, as previously showed by our group, hexose and sucrose are much more stable than starch in different growth localization and condition (Biais *et al.*, 2014). But the contents of hexose could reflect over-accumulation of starch in fruits. Schaffer and Petreikov (1997) proposed that the sugar content could be affected in a genotype with continually active starch accumulation for a long period and that reaches a transient maximum of 3% of the FW as starch in the fruit. Thus, if we aimed for screening for mutants accumulating more starch for a long period in fruits, contents of sugars (especially for glucose) in red ripe fruits are valuable traits to indicate the potential accumulations of starch in previous stages.

So for a big BC1F2 population of P17C12 of fine-mapping, I would like to propose to sample fruits at 20 DAP and red ripe stages, and take the contents of glucose as an important trait related to starch accumulation in fruits during growth.

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