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Publications

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Ning Tang[§], **Jing An**[§], Wei Deng, Yanqiang Gao, Zexiong Chen, Zhengguo Li, Metabolic and transcriptional regulatory mechanism associated with postharvest fruit ripening and senescence in cherry tomatoes, Postharvest Biology and Technology, *accepted,* June, 2020.

Eric de Castro Tobaruela[§], **Jing An**[§], Guojian Hu, Margot Zahm, Anis Djari, Mondher Bouzayen, Mohamed Zouine. Auxiscan: A tool for identification and functional annotation of auxin-related genes in plants, *in preparation*, 2020.

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Résumé

La tomate (*Solanum lycopersicum*) est généralement considérée comme une culture économiquement importante à l'échelle mondiale. En tant que fruit charnu, la tomate montre des caractéristiques de développement liées à la nouaison, à la croissance du fuit, et à la maturité du fruit qui font d'elle un modèle d'étude sur ces aspects. Parmi les étapes de la formation du fruit charnu, la nouaison est considérée comme une transition essentielle de la fleur au fruit, qui implique de nombreux changements physiologiques et structurels chez les plantes. Pendant ce processus, l'auxine en tant qu'hormone végétale est largement connue pour sa participation au déclenchement et à la coordination de ces changements. Les ARFs (Auxin Response Factor) sont des régulateurs transcriptionnels impliqués dans la signalisation de l'auxine et régulant les gènes de réponse à l'auxine en se fixant sur leur promoteur au niveau des éléments de réponse à l'auxine (AuxRE). Des travaux antérieurs basé sur l'analyse des profils d'expressions géniques des vingt-deux gènes *SlARF* caractérisées chez la tomate ont montré que le gène *SlARF8* présentait des niveaux d'expression élevés au cours de la transition fleur-fruit. L'objectif principal de ce projet de thèse est d'évaluer le rôle de *SlARF8* dans la nouaison par génétique inverse. Ainsi, deux gènes homologues *SlARF8A* et *SlARF8B* ont été identifiés et isolés. Des études de localisation subcellulaire ont révélées que les deux facteur de transcription SlARF8A/B étaient bien localisés dans le noyau des cellules. L'analyse d'expression par RT-qPCR a révélé que *SlARF8A* et *SlARF8B* présentaient des changements de niveaux d'expression avant et après la pollinisation et la fertilisation. Une augmentation notable du niveau de transcription de *SlARF8A* a lieu lors de la pollinisation, tandis que le niveau d'expression de *SlARF8B* diminue après la pollinisation. Les mutants knock-out de *SlARF8A* et *SlARF8B* ont été générés grâce à technologie d'édition du génome par CRISPR/Cas9, les mutants simples *slarf8a-cr*, *slarf8b-cr*, et le mutant double *slarf8a&b-cr* ont été obtenus et validés par séquençage. Les mutants *slarf8a-cr*, *slarf8b-cr* et *slarf8a&b-cr* présentaient des phénotypes pléiotropiques, notamment une taille de plante réduite et des fruits parthénocarpiques. L'analyse histologique a révélé que le développement du placenta, du péricarpe, du tissu loculaire et des ovules sont aussi très altérés dans ces mutants. Le profil d'expression global des gènes au cours du processus de la nouaison a été étudié par l'approche

ARN-Seq chez ces mutants. Parmi les catégories fonctionnelles significativement affectées par la perte de la fonction du gènes *SlARF8B* se trouve essentiellement des fonctions liées à l'auxine d'abord, puis à d'autres hormones connues pour leur rôle dans la nouaison comme la gibbérelline (GA). Une analyse *in silico* réalisée sur le promoteur des gènes différentiellements exprimés (DEGs) dans ces mutants a permis d'identifier chez certain DEGs le motif canonique de réponse à l'auxine AuxRE connu pour être le site de fixation des ARFs. De manière intéressante, parmi ces gènes se trouvent plusieurs SAURs et IAAs connus pour leur rôle dans le contrôle de la nouaison et faisant d'eux des cibles potentielles de SlARF8B.

En parallèles de ce travail et vue l'importance de l'identification des gènes liés à l'Auxine, l'outil informatique Auxiscan a été développé. Il combine différents processus informatiques et méthodes analytiques pour réaliser l'identification rapide et l'annotation fonctionnelle des gènes liés à l'auxines à partir d'une liste de séquences de gènes voir un génome entier. Cette identification est principalement basée sur la recherche des domaines fonctionnels et des cibles possibles des miARN et tasiARN habituellement connus pour réguler l'expression certains gènes liés à l'auxine. Cet outil permet aussi de réaliser des études phyllogénétiques intra et entre espèces végétales.

Dans l'ensemble, cette étude a fourni une caractérisation approfondie des gènes *SlARF8A* et *SlARF8B* et a permis de mieux comprendre leur fonction dans la régulation du processus de nouaison chez la tomate.

Abstract

Globally, tomato (*Solanum lycopersicum*) is generally considered to be an important economic crop. As a fleshy fruit, tomato displays developmental traits related to fruit set, fruit growth, and fruit repining. Among the developmental processes of making a fleshy fruit, the fruit set process is considered an essential flower to fruit transition, which involves many physiological and structural changes in plants. During this important process, the plant hormone auxin is widely known to participate in triggering and coordinating those changes. Auxin Response Factors (ARFs) are transcriptional regulators that involve the auxin signaling and regulating auxin-responsive genes by specifically binding with the Auxin Response Elements (AuxREs) located in their promoters. Based on the previous work of analyzing the expression patterns of twenty-two characterized *SlARF* genes in tomatoes, *SlARF8* was found to exhibit high expression levels in the fruit set transition. The main objective of this thesis is to gain more insight into the roles of *SlARF8* in fruit set by reverse genetics. Two homologue genes, *SlARF8A* and *SlARF8B*, were identified and isolated. The subcellular localization studies revealed that SlARF8A and SlARF8B localized in the nucleus of the cells. The expression pattern analysis by RT-qPCR revealed that *SlARF8A* and *SlARF8B* displayed different expression levels before and after pollination and fertilization. A notable increase in *SlARF8A* transcript was displayed upon flower pollination, while a decrease in *SlARF8B* expression level was shown after pollination. The knockout mutants of *SlARF8A* and *SlARF8B* were generated by performing the CRISPR/Cas9 genome editing system, with the single mutant *slarf8a-cr*, *slarf8b-cr*, and double mutant *slarf8a&b-cr* were obtained and validated by sequencing. The *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants displayed pleiotropic phenotypes, including dwarf plants, smaller and parthenocarpic fruits. Besides, histological analysis revealed the development of placenta, pericarp, locular tissue, and ovules were impaired in these mutants. The expression profiling at the genome level during fruit set process was studied by performing the RNA-Seq approach in these mutants. Among the significantly enriched functional categories in *slarf8b-cr*, many of which are related first to auxin, then with other hormones known for their role in fruit set, including gibberellin (GA). Besides, by analyzing the AuxRE motif in their promoters which is known to bind with ARFs, an *in silico* analysis was performed

on the promoters of differentially expressed genes (DEGs) in these mutants to identify the potential target genes. It is interesting to notice that among those DEGs, several SAURs and IAAs display high expression levels in fruit set process and be potential target genes of SlARF8B.

In parallel with this work and given the importance of identifying the auxin-related genes, a useful tool called Auxiscan was developed. This tool combines with different computer processes and analytical methods, which achieves the fast identification and functional annotation of auxin-related genes starting with a list of gene sequences or even an entire genome. The identification of auxin-related genes is mainly based on searching for conserved functional domains that characterizes each family. It also search for possible targets for miRNA and tasiRNA that are known to regulate some auxin-related genes.

Altogether, the present study provides a comprehensive characterization of the *SlARF8A* and *SlARF8B* genes and a better understanding of their functions in regulating the fruit set process in tomato.

Content

Acknowledgments……I

Publications……....II

Resume……..III

Abstract……IV

Abbreviations……..……1

Objective of the study…….....4

Chapter I……..6

1. The fruit initiation process……7

1.1 Tomato, a fruit crop with worldwide increasing importance……8

1.2 Tomato as a model plant for the fleshy fruit research……....9

1.3 The fruit set process and phytohormones……10

2. Auxin……10

2.1 Auxin, a crucial plant hormone……...10

2.2 Auxin metabolism and transport……11

2.2.1 Auxin biosynthesis pathways……..……11

2.2.2 Auxin transport……...13

2.3 Auxin signal transduction in plants: auxin perception, signaling, and transcriptional response……14

2.3.1 Auxin receptors: TIR1/AFB, the core of auxin signaling……...14

2.3.2 Aux/IAAs: the role in auxin response and transcriptional co-regulation……17

2.3.3 The roles of Auxin Response Factors……19

2.3.4 $SCF^{TIR1/AFBs}$-Aux/IAAs-ARFs model……..……...22

3. The crosstalk between auxin and other hormones in regulating the fruit set process…23

Review article **……..25**

Auxin and Ethylene Regulation of Fruit Set

Abstract……...25

Introduction……25

1. Ethylene and auxin regulate the formation of plant male and female organs...……...26

2. Ethylene and auxin regulate pollination-dependent fruit set…….....27

3. Ethylene and auxin roles in parthenocarpy……..……28

4. Conclusions and perspectives……29

References……...30

Chapter II Results and Discussion……...32

Introduction to ChapterII……..33

***Article 1.*……...34**

Knockout of ARF8 Using CRISPR/Cas9 in Tomato leads to Pleiotropic Vegetative Phenotypes and Parthenocarpic Fruits

Abstract……...34

Introduction……35

Results……37

Structure, localization and expression of tomato *SlARF8A* and *SlARF8B* genes……..…37

Generation of *SlARF8A* and *SlARF8B* mutants by the CRISPR/Cas9 gene-editing system..40

CRISPR/Cas9-mediated *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants exhibited parthenocarpy and vegetative phenotypes……42

Genome-wide transcriptomic profiling of *slarf8a-cr* and *slarf8b-cr* during fruit setting…..47

Discussion……54

SlARF8A/*B* and fruit development……54

Potential regulatory mechanism of *SlARF8A* and *SlARF8B* in the fruit set process…...……55

Materials and methods……...57

Supplemental figures and tables……60

References……...62

***Article 2*……....67**

Auxiscan: A tool for identification and functional annotation of auxin-related genes in plants

Abstract……...67

Introduction……68

Auxin biosynthesis……...69

Auxin conjugation and degradation……..71

Auxin transport……71

Auxin signaling……72

Post-transcriptional regulation……..72

Results and discussion……...……73

User operation and analytical options……...73

Unified data processing……75

Data visualization and report generation……..76
The versatility in Auxiscan application……78
Case study: standardization of ARFs annotations in Capsicum peppers……..78
Innovation, limitation, and perspectives……...80
Conclusion……...83
References…….....84
Chapter III General conclusion and perspectives……..90
Structural and functional features of *SlARF8A* and *SlARF8B*…….…….91
Molecular regulation of feedback-regulated genes by SlDELLA and SlARF8B/SlIAA9…93
Molecular regulation of downstream fruit development-related genes by *SlARF8*..……..93
Literature cited……..96

Abbreviations

1-MCP: 1-methylcyclopropene

4-Cl-IAA: 4-Chloroindole-3-acetic

AA: Amino acid

ABA: Abscisic acid

ABCB: ATP binding cassette subfamily B

ABP1: Auxin binding protein 1:

ACC: 1-aminocyclopropane-1-carboxylic acid

ACO: ACC oxidase

ACS: ACC synthase

AD: Activation domain

ARF8A: Auxin response factor 8A

ARF8B: Auxin response factor 8B

ARFs: Auxin response factors

ASK1: SKP1 homologous proteins:

At: Arabidopsis

Aux/IAAs: Auxin/Indole acetic acid factors

AUX1/LAX: AUXIN-RESISTANT 1/LIKE AUX1

AuxRE: Auxin response elements

bp: Base pair

cDNA: Complementary desoxyribonucleic acid

CTD: Carboxy-terminal dimerization

CTRs: Constitutive triple response

DBD: DNA binding domain

DEGs: Differently expressed genes

DPA: Days post anthesis

EAR: ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR–ASSOCIATED REPRESSOR

EIN2: ETHYLENE INSENSITIVE2 protein

ERFs: Ethylene response factors

FAO: Food and Agriculture Organization of the United Nations

GAs: Gibberellins

gDNA: Genomic desoxyribonucleic acid

GH3: Gretchen Hagen 3

GUS: β-Glucoronidase

IAA: Indole-3-acetic

IAM: Indole-3-acetamide

IAOx: Indole-3-acetaldoxime

IBA: Indole-3-butyric

IGP: Indole-3-glycerol phosphate

IND: Indole

IPA: Indole-3-propionic acid

JA: Jasmonate

LRR: Leucine-rich repeat

NLS: Nuclear Localization Signal

PAA: Phenylacetic acid

PCD: Programmed cell death

PCR: Polymerase Chain Reaction

PIN: PIN-FORMED

qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction

RD: Repression domain

RNA-Seq: Deep RNA Sequencing Analysis

ROPs: Rho-like GTPases:

SAM: S-Adenosyl-Methionine

SAUR: Small Auxin Up-regulated RNA

sgRNA: Synthetic guide ribonucleic acid

TAM: Tryptamine

TILLING: Targeting Induced Local Lesions in Genomes

TIR1/AFB: TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOX PROTEIN

TPLs: TOPLESS

Trp: Tryptophan

WT: Wild type

Y2H: Yeast two-hybrid

Objective of the study

&

Main components of the thesis

Fleshy fruits have common development and maturation steps, including fruit setting, fruit growth, maturation, and senescence. Fruit set usually initiates fruit development and depends on successful pollination and fertilization. After the fruit set process, the fruit enters the growth phase through cell division and cell expansion. Understanding the molecular and genetic basis of the flower to fruit transition process have a significant influence on fruit yield and quality characteristics.

The phytohormone auxin is widely known to play an important role in initiating the flower to fruit transition. In a widely accepted model, auxin is first perceived by Auxin Binding Protein 1 (ABP1) at the cell surface and TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) receptors intercellular, then auxin molecular is converted into a signal to induce the transcriptional control of auxin-responsive genes. Transcriptional regulators include the Auxin Response Factors (ARFs), the repressors Auxin/Indole Acetic Acid factors (Aux/IAAs), and the co-repressors TOPLESS (TPLs). They are considered as the main components in mediating auxin response.

This thesis's aim is to uncover the role of the Auxin Response Factors SlARF8A and SlARF8B in the tomato fruit initiation and development process. It includes three main chapters. The first chapter is dedicated to bibliographic reviews that consist of two topics. The first is a general introduction of the fruit initiation process that emphasizes the fruit set process, its importance for crop yield and quality, and the advantages of using tomato as a model plant for this thesis project. Further, it discusses the main roles of auxin, which includes the auxin biosynthesis pathways, auxin perception, and signaling pathways. The second topic is a review article about the role of auxin and ethylene crosstalk in regulating the fruit set process. It has widely been accepted about the role of auxin and gibberellin in regulating the fruit set process,

while other hormones, like ethylene, have barely been reviewed in regulating this process. In this review article, the role of ethylene and auxin in regulating fruit initiation and their crosstalk regulation are summarized. It discusses their roles in regulating the formation of male and female organs in plants, the pollination-dependent fruit set from pollen germination to ovary development, and the parthenocarpy in plants.

Chapter II is composed of two articles. The first article is dedicated to the characterization of two homologue genes, *SlARF8A* and *SlARF8B*, and addresses the important roles of each in tomato fruit initiation. Using reverse genetic approach, it describes the phenotypes of the *SlARF8A*, *SlARF8B* and *SlARF8A&B* knockout mutants (*slarf8a-cr*, *slarf8b-cr*, and *slarf8a&b*) generated by the CRISPR/cas9 genome editing system. Moreover, RNA-seq analysis in *slarf8a-cr* and *slarf8b-cr* mutants was performed to reveal the differently expressed genes and potential downstream regulated genes of *SlARF8A* and *SlARF8B*. This section is presented under the form of a published paper.

Moreover, the second article in chapter II will describe a useful tool for identification and functional annotation of auxin-related genes in plants, which is prepared as a research article.

Chapter III summarizes the main scientific outcomes of this thesis project, and the new prospects and insights were outlined by the present work. It mainly focuses on the potential regulation complex of SlARF8B, SlDELLA, and other ARFs in the fruit set process, the potential target genes of the process, and the perspectives of the plant hormone and ARFs to regulate parthenocarpic fruits.

Chapter I

Bibliographic review

1. The fruit initiation process.

1.1 Tomato: a fruit crop with worldwide increasing importance.

Tomato is a crop native to the Americas, and it is now widely distributed around the world at 45° N to 65° N. Until now, tomato was one of the top thirty crops in the world in terms of total yield, as well as an important economic crop. The annual yield of tomato is much higher than other important economic crops, including: peanuts, apples, coconuts, beets, and rye. According to the data from Food and Agriculture Organization of the United Nations (FAO), the production of tomatoes increased gradually in the past twenty years. Around 182 million tons of tomatoes were produced in 2018, an increase of 52 million tons compared to 2006. Among the worldwide tomato production, Asia contributed more than 111.69 million tons, accounting for 61% of world production (Table 1). The world tomato production distribution states that China, India, and the United States of America are the top three countries for tomato production (Figure1). With the increased demand for tomato consumption in both fresh market and canned products, the importance of increasing tomato production attracts more and more attention globally.

Table1. World tomato production (million tons)

Area	2006	2010	2014	2018
Africa	16.79	18.34	21.65	20.78
Americas	23.70	25.51	28.14	26.04
Asia	68.24	87.13	101.96	111.68
Europe	21.18	21.77	22.63	23.29
Oceania	0.55	0.56	0.41	0.46
Total	130.45	153.31	174.79	182.26

Data collected from FAO (http://www.fao.org/home/en/)

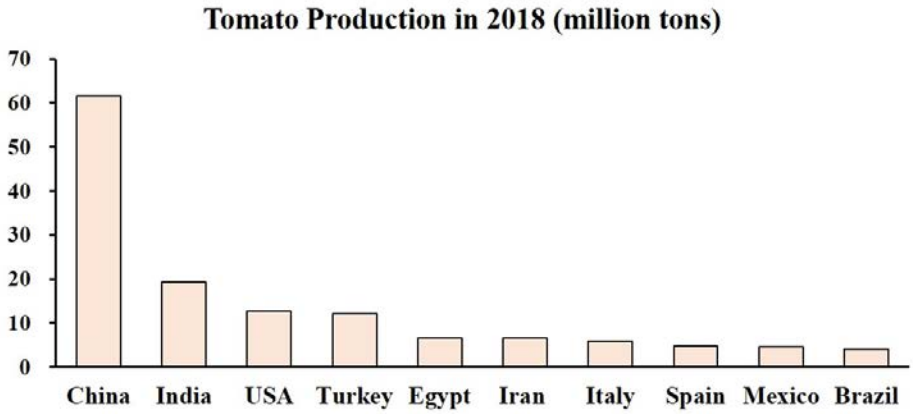

Figure 1. Top 10 countries of tomato production in global world in 2018. Data is collected from FAO (http://www.fao.org)

Tomato is preferred by consumers since its fruit is soft and juicy, with moderate sweetness, and the pulp and juice account for 95.9% of the total weight. Besides the preferred taste, tomatoes are a good source of Vitamin A, Vitamin C, mineral substance, and anti-oxidant compounds, including, for example, the lycopene. Vitamin A promotes growth and reproduction, maintaining bone, epithelial tissue, good vision, and normal secretion of mucosal epithelium, while Vitamin C is essential for the immune system and is a natural radical scavenger. Lycopene is a strong antioxidant in carotenoids, helping to protect body cells from oxidation and to prevent diseases like cancers and cardiovascular disease. Due to its good flavor and rich nutrition, tomato consumption has increased rapidly in recent years. Hence, the improvement of tomato production and quality became an important topic in scientific research.

1.2 Tomato as a model plant for the fleshy fruit research

The Solanaceae family is a very important type of plant for humans, for it provides various kinds of food and medicine, among which tomato (*Solanum lycopersicum*) has been widely used as a model plant for fleshy fruit research. Tomato has a relatively short lifespan of five to six months, and the development stages of tomato are remarkable and classic for research. In addition, compared with other Solanaceae plants, for example, eggplant, pepper, and potato, the tomato has a relatively small genome, high-density genetic maps, the high efficient and stable mutation resource. Besides, the genome sequencing of tomato has been completed by The International Solanaceae Genomics consortium in 2012 (Tomato_Genome_Consortium,

2012). Combined with the rapidly increased demand for tomato as a major crop, more and more efforts have been made on various aspects of tomato research.

1.3 The fruit set process and phytohormones

The fruit set process is defined as the transition period from flowers to fast-growing young fruits, and it is a critical period for flowering sexually reproductive plants. Fruit setting depends on successful pollination and fertilization (Gillaspy *et al.*, 1993). The tomato ovary is composed of two or more carpels and contains ovules in the ventricle. After successful pollination, the ovary develops into a fruit embryo, a process that begins with cell division and generally lasts ten to fourteen days. After six to seven weeks of flowering, fruit growth depends on cell expansion (Mapelli *et al.*, 1978; Bünger-Kibler & Bangerth, 1982). During this process, the carpel wall develops fruit peel, and the placenta develops into gel-like substances where the ovule is attached. At the later stage of cell expansion, the fruit reaches its final size and, finally, matures (Gillaspy *et al.*, 1993). It has been widely known that the fruit initiation process is regulated by plant growth substances, such as phytohormones, auxin and gibberellin have been found to induce fruit set independent of pollination and fertilization (Serrani *et al.*, 2008).

2. Auxin

2.1 Auxin, a crucial plant hormone

Auxin was known as the first discovered plant hormone. As early as 1880, Darwin discovered the small molecule compound auxin when he studied the phototropism of plants. According to previous studies, several molecules that showed auxin activity, including: indole-3-acetic (IAA), phenylacetic acid (PAA), indole-3-butyric (IBA), 4-Chloroindole-3-acetic (4-Cl-IAA), and indole-3-propionic acid (IPA), while the major form of auxin in plants is IAA (Schneider *et al.*, 1985; Epstein *et al.*, 1991; Ludwig-Müller & Epstein, 1994; Tam *et al.*, 2000; Zolman *et al.*, 2000; Ozga *et al.*, 2002; Woodward & Bartel, 2005). Auxin regulates many processes in higher plants, including: cell elongation, division and differentiation (Cleland *et al.*, 1991; Rayle & Cleland, 1992; Zhao *et al.*, 2002a; Petrášek *et al.*, 2002), organ formation

(Feng *et al.*, 2006; Gan *et al.*, 2019), apical dominance, root initiation (Laskowski *et al.*, 1995; Reed *et al.*, 1998), leaf morphology (Wang *et al.*, 2005a), fruit setting (Goetz *et al.*, 2006; de Jong *et al.*, 2009b; Du *et al.*, 2016), fruit development and maturation in higher plants(Sundberg & Østergaard, 2009). As such, auxin plays essential roles in regulating many essential processes in plants, and auxin is regulated at multiple levels: auxin biosynthesis, auxin transport, and auxin signal transduction.

2.2 Auxin metabolism and transport

2.2.1 Auxin biosynthesis pathways

As the major form of auxin in plants, IAA has two major routes for its biosynthesis: tryptophan (Trp)-dependent and Trp-independent pathways (Figure 2). For Trp-dependent auxin biosynthesis pathways, it has been revealed in Arabidopsis that tryptophan is an important precursor for auxin biosynthesis by functional identification of overproducing-IAA mutants (Bartel, 1997). There are several biosynthesis pathways contributing to Trp-dependent biosynthesis pathways (Figure 2). According to the intermediate difference in converting Trp to IAA, these pathways are identified as: the IPA pathway, the indole-3-acetaldoxime (IAOx) pathway, the indole-3-acetamide (IAM) pathway, and the tryptamine (TAM) pathway (reviewed in Mano & Nemoto, 2012). The IPA pathway is the first complete and universally conserved IAA biosynthesis pathway in plants (Figure 2). This pathway converts Trp to IAA in two consecutive chemical steps: firstly, Trp is metabolized into IPA by the TAA family of aminotransferases; secondly, IPA is catalyzed by the flavin-containing monooxygenases YUC family to produce IAA (Stepanova *et al.*, 2011; Mashiguchi *et al.*, 2011; Won *et al.*, 2011). Evidence has shown that TAA and YUC families play essential roles in IAA biosynthesis process in various plant species, such as Arabidopsis, maize, rice, and liverwort (Cheng *et al.*, 2007; Won *et al.*, 2011; Zhao, 2014a; Eklund *et al.*, 2015), indicating the crucial role of the IPA pathway in auxin biosynthesis. As for other pathways, they have not been as well defined as the IPA pathway until now. It has been studied in the IAOx pathway that cytochrome P450 monooxygenases CYP79B2 and CYP79B3 catalyze the conversion of Trp into IAOx, which is the first step of IAA and indole glucosinolate biosynthesis in Arabidopsis (Zhao *et al.*, 2002b;

Sugawara *et al.*, 2009). For IAM and TAM pathways which remains mostly unknown, it has only been known that these two components were supposed to be the IAA precursors that originated from Trp (Mano & Nemoto, 2012; Brumos *et al.*, 2014). For the Trp independent pathway, it was found that the endogenous IAA levels in tryptophan auxotrophic mutants and Wild Type show no obvious difference, implying that auxin synthesis may be independent of the tryptophan pathway (Wright *et al.*, 1991; Normanly *et al.*, 1993). In the Trp independent pathway, IAA is not converted from Trp, but mainly produced from Trp precursors, including indole (IND) and indole-3-glycerol phosphate (IGP); however, the molecular components of the Trp-independent pathway have not been identified (Wright *et al.*, 1991; Normanly *et al.*, 1993; Ouyang *et al.*, 2000).

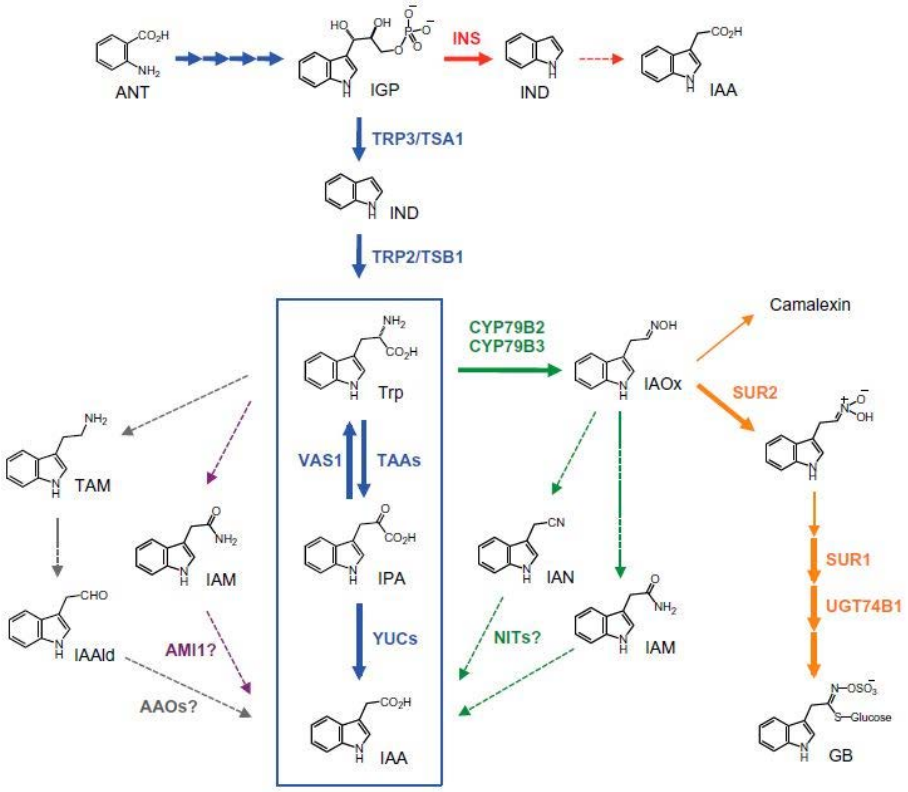

Figure 2. A schematic model of auxin biosynthesis pathways in Arabidopsis. The common IPA pathway was shown in blue, the IAOx pathway in green, the IAM pathway in purple, and the Trp-independent pathway in red. The enzymes involved in each step are in bold typeface. Enzyme reactions remain unclarified presents by dashed line with arrow heads (Kasahara, 2016).

2.2.2 Auxin transport

Since auxin biosynthesis is carried out in specific tissue in plants, it has to be transported to other various parts of the plant to exert its specific physiological functions. Auxin can be transported by diffusion (passive movement) and by auxin transporter (active movement) (Zazimalova *et al.*, 2010). The former is like other hormones, for auxin molecules can transport through the vascular system by mass flow. However, distinct from other hormones, auxin can be transported in both long and short distances, which is mostly polar transport and involves a cell-to-cell mechanism (Zazimalova *et al.*, 2010). This kind of active transport of auxin is based on the weak acid physical-chemical nature of auxin molecules. The anionic auxin molecules are unable to diffuse across the plasma membrane, thus they require the activity of transporters at the plasma membrane to exit the cells (Rubery & Sheldrake, 1974; Raven, 1975). Up to date, several auxin carrier families have been identified, such as PIN-FORMED (PIN), AUXIN-RESISTANT 1/LIKE AUX1 (AUX1/LAX) influx carriers, and ATP binding cassette subfamily B (ABCB) auxin efflux carriers (Swarup et al., 2004; Petrásek et al., 2006; Cho et al., 2007) . Among the auxin carriers, the PIN family was studied deeply. The PIN family was associated with polar auxin transport in the late 1990s (Gälweiler *et al.*, 1998). Some PIN family members are membrane protein and some are located in the endoplasmic reticulum. The polar auxin transport mostly depends on the directional localization of PINs, and the phosphorylation status of PINs is related with their polar localization (Wisniewska et al., 2006; Adamowski & Friml, 2015; Armengot et al., 2016). It has been shown that, in Arabidopsis, some *pin* mutants displayed severe defects in polar auxin transport (Okada *et al.*, 1991; Rashotte *et al.*, 2000). Furthermore, PIN proteins might involve in auxin transport by assisting the assembly of other efflux activity proteins, such as the ABCB carriers. It has been studied that, in Arabidopsis, *AtABCB1* and *AtABCB19* coordinate with *AtPIN1* in auxin long distance transport (Bandyopadhyay et al., 2007; Christie et al., 2011). PINs network plays important roles in polar auxin transport which is vital for initiating and maintaining plant polar development, apical dominance, tropic growth, vascular tissue differentiation, and organ development (Benková *et al.*, 2003; Billou *et al.*, 2005; Blancaflor *et al.*, 2003).

2.3 Auxin signal transduction in plants: auxin perception, signaling, and transcriptional response.

Auxin plays a crucial role in higher plants by participating in many plant development and physiological processes. How the auxin molecule is perceived, recognized, and finally regulated by downstream genes to achieve its physiological regulation has always been a hotspot for researchers. According to previous studies, a relatively clear understanding of the auxin signal transduction pathway has been illustrated (reviewed in Weijers & Wagner, 2016). Three types of proteins are considered as the core components involved in auxin signal transduction: TIR1/AFB proteins, the Aux/IAA transcriptional coregulators, and specific binding proteins ARFs.

2.3.1 Auxin receptors: TIR1/AFB, the core of auxin signaling

TIR1 is the first isolated and identified intracellular auxin receptor in Arabidopsis, and the signaling pathway with TIR1 as the receptor is also a relatively clear auxin signaling pathway studied (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005). The TIR1 protein belongs to the TIR1/AFB protein family, which is a subfamily of the F-box protein family. This gene family is divided into three evolutionary branches: TIR1/AFB1, AFB2/AFB3, and AFB4/AFB5. These proteins have a highly conserved F-box domain and a leucine-rich repeat (LRR) (Figure 3(a)). The TIR1/AFB family are nuclear proteins and have been confirmed to interact with SKP1 homologous proteins (ASK1) to form SCF protein complexes (Ruegger *et al.*, 1998; Gray *et al.*, 1999). The SCF protein complex is mainly composed of four subunits: SKP1, Cullin, RBX1, and F-box protein. The RING-H2 finger protein Cullin forms a dimer with RBX1 to catalyze multiubiquitin chain formation (Smalle & Vierstra, 2004). It has been known that ubiquitin conjugation to specific targets is a three-step process, including (1) being activated by the ubiquitin activating enzyme E1, (2) being passed to the ubiquitin conjugating enzyme E2, and (3) acted with the ubiquitin protein ligase E3 (reviewed in Voges *et al.*, 1999; Pickart, 2001). E3 ligase-mediated ubiquitination-proteolytic pathway has been proved to play an important role in regulating plant hormone signal transduction, and the most prominent E3 enzymes are SCF-type E3s (Santner & Estelle, 2009). Although Arabidopsis TIR1 and five other TIR1 homologous proteins AFB1-5 have been confirmed to be auxin receptors, the molecular mechanism of auxin sensing still needs further study. Tan et al., (Tan *et al.*, 2007)

analyzed the crystal structure of the TIR1-ASK1 complex, which showed a mushroom shape (Figure 4). It has been revealed that the LRR domain of TIR1-ASK1 complex contains the auxin binding pocket (Tan *et al.*, 2007). When auxin arrives and binds with the TIR1-ASK1 complex at the binding position, the Aux/IAA proteins were also adjacent with LRR of TIR1-ASK1 at the same auxin binding pocket, meaning that auxin stabilized the interaction between TIR1-ASK1 and Aux/IAA (reviewed in Weijers & Wagner, 2016; Leyser, 2018; Du *et al.*, 2020). At present, only the crystal structure of the short degradation determinants in SCF^{TIR1} and Aux/IAA is resolved (Tan *et al.*, 2007). The mechanism of how auxin triggers the ubiquitination degradation of Aux/IAA protein will be better revealed when the structure of SCF^{TIR1} and the full-length of Aux/IAA protein are fully resolved.

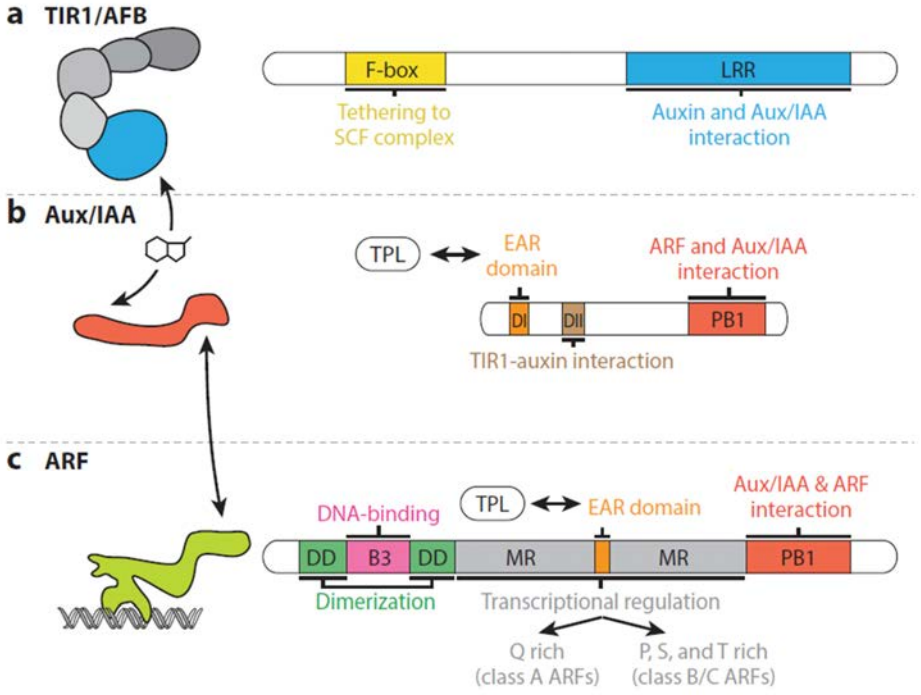

Figure 3. Domain architecture of core components of auxin response and signaling regulation. Structures and interaction domains of (a) TIR/AFB auxin receptors, (b) Aux/IAA transcriptional repressors, (c) ARF transcription factors. (Weijers & Wagner, 2016)

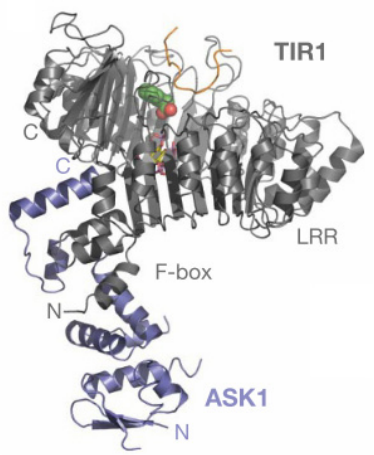

Figure 4. Crystal structure of the TIR1-ASK1 protein complex with auxin and the IAA7 peptide. The complex structure are shown as a ribbon diagram. TIR1, ASK1 and the IAA7 substrate peptide are coloured grey, blue and orange, respectively. (Tan *et al.*, 2007)

It has been found that TIR1/AFB auxin receptors regulate $SCF^{TIR1/AFB}$-dependent auxin responses in Arabidopsis. The *tir1* mutant was found to affect root growth, hypocotyl elongation, and lateral root formation (Ruegger *et al.*, 1998). Besides, the *tir1* mutant displayed weak sensitivity to exogenous auxin. The phenotype analysis of the multiple mutant *tir1/afb* confirmed that *TIR1* and *AFB2* play a major role in the auxin reaction (Parry *et al.*, 2009). Moreover, two *tir1* mutants *tir1D170E* and *tir1M473L* have been found to present developmental defect phenotypes and hypersensitive to exogenous auxin, which indicates that *TIR1* acts as a negative regulator in auxin signaling (Yu *et al.*, 2013).

Except for TIR1/AFBs, *ABP1* was also characterized as an auxin receptor. The earliest report on the auxin-binding activity of ABP1 was in the 1980s, and the ABP1 protein was originally isolated from maize; its crystal diffraction structure showed that it had an auxin-binding domain and was able to bind auxin (Löbler & Klämbt, 1985a,b; Jones, 1994). However, research and reviews pertaining to *ABP1* over the past twenty years have been complex and controversial. Since the homozygous mutants of Arabidopsis *abp1* are lethiferous, many methods have tried to obtain the lines that could interrupt the function of ABP1 (Chen *et al.*, 2001; Tzafrir *et al.*, 2004; Sassi *et al.*, 2014). Several lines were obtained, including using the cellular immunology method to achieve *ABP1* knockout lines, or inducing high expression of

single chain fragments of variable regions of ABP1 antibody mAb12 (Leblanc *et al.*, 1999; David *et al.*, 2007; Tromas *et al.*, 2009). Two strains, SS12S and SS12k, obtained by these methods are considered to be able to properly neutralize endogenous ABP1 in cell lines or plants; as such, they are widely used in the related research of *ABP1*. Later, the nonsense point mutation alleles of *abp1* were also obtained through the Targeting Induced Local Lesions in Genomes (TILLING) method in Arabidopsis (Henikoff *et al.*, 2004; Robert *et al.*, 2010; Xu *et al.*, 2010). All these *ABP1* mutant lines were used to study its function in later research, and show the auxin-related function. The loss of *ABP1* function was recovered to regulate cell division and reduce root development and early embryo lethality (Chen *et al.*, 2001; Braun *et al.*, 2008; Tromas *et al.*, 2009). Besides, *ABP1* is presumed to be an auxin receptor, the cell membrane was found to contain a complex of ABP and transmembrane receptor-like kinases on the plasma membrane, activating Rho-like GTPases (ROPs) in an auxin-dependent manner (Chen *et al.*, 2001, 2014; Xu *et al.*, 2010). However, these results have been brought into question by the new independent *abp1* lines obtained through CRISPR and T-DNA insertion methods, which showed no apparent features related to auxin (Gao *et al.*, 2015). These results arouse great concerns in the auxin-related area; hence, a genome-wide sequencing using *abp1* as the material was performed. It was found that, in addition to the mutation on *ABP1* site, there are a large number of other mutations, some of these mutations may be related to the aforementioned phenotypic differences (Enders *et al.*, 2015). Therefore, it is currently challenging to draw a definitive conclusion on the physiological function of *ABP1* and its role in auxin signaling, additional research is required.

2.3.2 Aux/IAAs: the role in auxin response and transcriptional co-regulation

Aux/IAAs is a short half-life protein commonly found in higher plants. It is located in the nucleus, is very easy to degrade and is rapidly induced by auxin (Wang & Estelle, 2014). Most Aux/IAA proteins contain four conserved protein interaction domains, including: (1) Domain I containing the ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR–ASSOCIATED REPRESSOR (EAR) module bound by the TPL co-repressor, (2) Domain II containing the binding site of auxin and TIR1/AFB auxin receptor, and (3, 4) Domain III and IV are the binding domains that interact with ARFs (Long *et al.*, 2006; Szemenyei *et al.*, 2008) (Figure 3(b)). As illustrated above, the effective binding of auxin to the TIR1/AFB auxin receptor requires a protein complex composed of TIR1/AFB and Aux/IAA protein, and the

interaction between the TIR1/AFB receptor and the domain II of Aux/IAA is auxin-dependent. There are twenty-nine Aux/IAAs in Arabidopsis, twenty-four members of which contain domain II that interacts with AFB proteins to sense auxin (Paponov *et al.*, 2008). Therefore, different AFB proteins have different binding abilities to interact with Aux/IAAs that have various half-lives range (Calderón Villalobos *et al.*, 2012). It has been confirmed that AFB2 has the higher binding capacity to the Aux/IAA protein IAA3 and IAA7 than other receptor members, indicating that TIR1/AFB has a different ability to interact with the same Aux/IAA protein (Parry *et al.*, 2009; Havens *et al.*, 2012). Moreover, the *IAA14* mutant displayed about ten to one hundred times lower affinity for auxin, since the point mutation in IAA14 protein increases its half-life, leading to delayed root emergence in Arabidopsis (Guseman *et al.*, 2015). Hence, the differences between TIR1/AFB-Aux/IAA proteins complex may play an essential role in determining the diversity and specificity of auxin responses.

Aux/IAA family genes play a crucial role in various aspects of the response to auxin in plants. To date, twenty-nine Aux/IAA family members have been identified in Arabidopsis, thirty-one Aux/IAA genes have been isolated from rice and maize, and twenty-five Aux/IAA genes have been identified in tomato (Jain *et al.*, 2006; Paponov *et al.*, 2008; Audran-Delalande *et al.*, 2012). In Arabidopsis, several Aux/IAA mutants were obtained by screening auxin-resistant mutants, including *IAA1/AXR5, IAA3/SHY2, IAA7/AXR2, IAA12/BDL, IAA14/SLR, IAA17/AXR3, IAA18/CRANE* and *IAA19/MSG* (Overvoorde *et al.*, 2005; Dreher *et al.*, 2006). The mutation region of these Aux/IAA mutants are all located in domain II, which prevents the interaction with $SCF^{TIR/AFBs}$ and leads to the auxin resistance phenotype. In tomato, the down-regulation lines of *SlIAA3* led to an alteration in several aspects, including apical dominance, auxin sensitivity, and apical hook. Moreover, the down-regulation lines of *SlIAA9* displayed simple leaves rather than compound ones, and the fruit initiation process could happen before pollination and fertilization, which led to parthenocarpic fruits (Wang *et al.*, 2005a). In addition, the down-regulation of *SlIAA17* induced the change of fruit shape (Ouellet *et al.*, 2001); *SlIAA15*-inhibited lines affected the axillary buds formation and trichome formation (Deng *et al.*, 2012). A recent study presented that the loss of function mutants *IAA6*, *IAA9*, and *IAA17* negatively regulate the adventitious root formation in Arabidopsis (Lakehal *et al.*, 2019). These results revealed that different members of the Aux / IAA family have a unique and vital function in plant development.

2.3.3 The roles of Auxin Response Factors

The primary mechanism by which changes in auxin levels translate into cellular responses is through transcriptional changes. A large number of genes rapidly change their expression in response to the supply of exogenous auxin, since the promoters of which contain AuxRE that bind with members of the ARF family (Guilfoyle & Hagen, 2007; Li *et al.*, 2016).

The ARF gene family is a modular transcription factor family that regulates the expression of auxin-responsive genes. A typical ARF protein consists of several conserved domains, including an amino-terminal DNA binding domain (DBD), a middle region that can be classified as an activation domain (AD) or repression domain (RD), and a carboxy-terminal dimerization domain (CTD) consisting of domain III and IV, which enables homodimerization between ARFs and regulates the interactions between Aux/IAA-ARFs proteins (Guilfoyle & Hagen, 2007; Piya *et al.*, 2014; Salehin *et al.*, 2015) (Figure 3(c)). The DBD of ARFs is a B3-type protein domain that can specifically bind with the conserved TGTC motif (TGTCTC/TGTCGG) of the AuxRE in the promoters of the auxin response genes in plants (Ulmasov *et al.*, 1995, 1997; Liao *et al.*, 2015). The transcriptional activation or repression regulation of ARFs is depended on the amino acid composition of the middle region, including the ADs in ARFs that are rich in glutamine, serine, and leucine residues, and the RDs that are rich in proline, serine, threonine, and glycine residues (Ulmasov *et al.*, 1999). The glutamine (Q)-rich ARFs were confirmed to act as activators, and the serine (S)-rich ARFs acted as repressors by protoplast transfection assays (Ulmasov *et al.*, 1999; Guilfoyle & Hagen, 2007). The homodimerization interactions between Aux/IAAs and ARFs are located on the domain III and IV of Aux/IAAs and the C-terminal of ARFs, and a PB1 sequence was located in both, which is important for the interactions between Aux/IAAs and ARFs (except for *ARF3* and *ARF17*) (Nanao *et al.*, 2014; Korasick *et al.*, 2014; Guilfoyle, 2015b).

Twenty-three members of the ARF family have been identified in Arabidopsis, and twenty-two ARFs were identified in tomato (Ulmasov *et al.*, 1997; Liscum & Reed, 2002; Zouine *et al.*, 2014) (Figure 5). In Arabidopsis, the expression of ARFs was expressed differently in different organs. For instance, *ARF1* expressed in flower in the developmental stage, *ARF2* in the seedling and developmental flower organs, *ARF3* and *ARF4* in the

developing reproductive and vegetative tissues, *ARF5* in the embryo and vascular tissues, *ARF6* in the flower organs before anthesis, *ARF7* in the roots, seedlings, and developing embryos, *ARF8* in the developing flowers, fruits, and seedlings, *ARF10* in the leaf margins, stems and flowers, *ARF16* in the root cap and vascular bundles in the leaves and roots, and *ARF19* in the seedlings, leaves, and lateral roots (reviewed in Guilfoyle & Hagen, 2007). Through forward and reverse genetics, ARFs family genes were also revealed to be involved in regulating different plant developmental processes, such as embryo morphology, lateral root formation, leaf structure and senescence, flower development, fruit initiation, and development. For instance, the *arf4/ettin* double mutant has defects in flower development, the number of stamens is reduced, and the anther morphology and pistil structure are changed (Hunter *et al.*, 2006). The *arf6/arf8* double mutant displayed defective flower development, as the flower is entirely sterile (Nagpal *et al.*, 2005); while the different *arf8* mutants were showed to regulate pollination and fertilization, including *arf8-1* displayed the longer hypocotyl, *arf8-3* was reported to obtain larger petals than the wild type, and the *arf8-4* mutant could initiate fruit development independent from pollination and fertilization (Tian *et al.*, 2004; Goetz *et al.*, 2007; Varaud *et al.*, 2011). Moreover, the *arf10/arf16* double mutants displayed root cap development defects and abnormal geotropism (Wang *et al.*, 2005b). In tomato, the functions of several ARFs have been identified. The Down-regulation of *SlARF2* in tomato resulted in a change in fruit color during the fruit break stage and displayed the delay of fruit ripening (Hao *et al.*, 2015). Besides, *SlARF7* RNAi lines induced parthenocarpic fruits (de Jong *et al.*, 2009b). These results indicate that ARFs play important roles in regulating tomato fruit development and maturation.

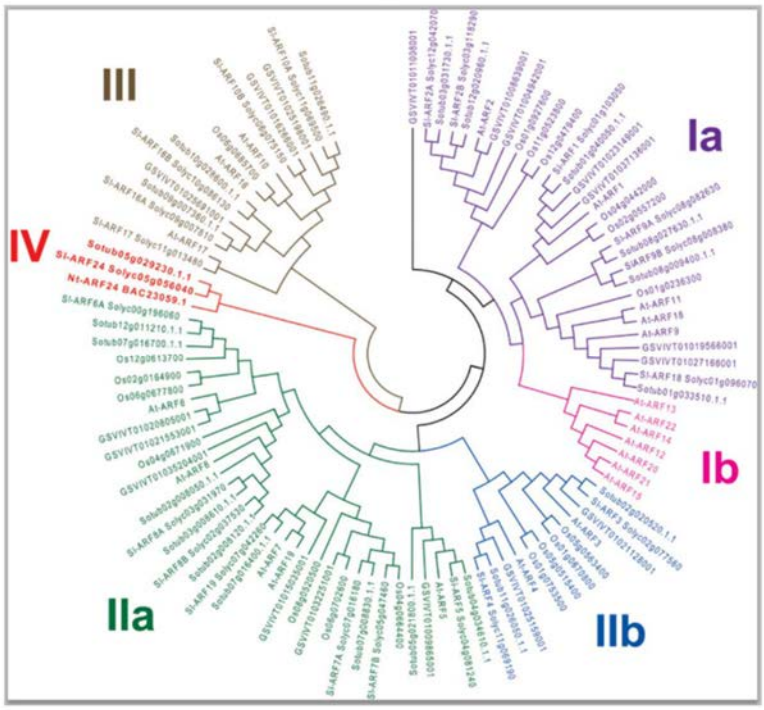

Figure 5. The identified ARF family and phylogenetic relationship between rice, potato, tomato, grape and Arabidopsis. All SlARFs contain a DBD (in brown color). Most of the SlARF proteins except *SlARF3, 10, 24, 16* and *17* contain a carboxy-terminal domain related to the domains III and IV found in the Aux/IAA proteins (in blue color).*SlARF5, 6A, 7, 8A, 8B, 19* contains a middle region that corresponds to the predicted activation domain (in green color) found in some AtARFs. The remaining SlARFs contains a predicted repression domain (in red color). (Zouine *et al.*, 2014)

Recently, the post-regulation of ARFs by microRNA (miRNA) has been found to play an important role in regulating plant development. The miRNA is a type of non-coding small RNA that has regulatory functions with about 20-24 nucleotides in length (Dugas & Bartel, 2004; Rogers & Chen, 2013). Through specifically binding with the mRNAs of target genes, miRNAs can cause the degradation of the mRNAs or inhibit the translation, which post-regulates the target genes. According to the previous study, miR160 inhibits the expression of *ARF10*, *ARF16*, and *ARF17*, which affect seed germination, embryo development, root growth, leaf morphology formation, and fruit development (Mallory *et al.*, 2005; Wang *et al.*, 2005b; Liu *et al.*, 2007; Hendelman *et al.*, 2012). Besides, miR167 is base complementary with *ARF6* and *ARF8*, and the over-expression of miR167 induced the inhibition of *ARF6* and *ARF8* expression, which displayed abnormal ovules and anthers (Wu *et al.*, 2006; Liu *et al.*, 2014). Moreover,

tasiRNA is complementary to *ARF3* and *ARF4* and regulates leaf polar development and floral organ development in Arabidopsis (Hunter *et al.*, 2006). Overall, miRNA plays a crucial regulatory role in the process of auxin signal transduction, which is essential for plant growth and development.

2.3.4 $SCF^{TIR1/AFBs}$-Aux/IAAs-ARFs model

According to previous studies, the auxin signal transduction pathway mediated by TIR1/AFB has been illustrated. As shown in Figure 6, under low auxin levels, the Aux/IAA protein binds to the transcription inhibitor protein TPL, and then forms dimers with the ARF transcription factor to inhibit the regulation of downstream auxin-responsive genes (Guilfoyle & Hagen, 2007; Szemenyei *et al.*, 2008; Guilfoyle, 2015a; Leyser, 2018). Once the level of auxin increases, some auxin receptors, like *ABP1*, percepts auxin at the cell surface (Robert *et al.*, 2010; Xu *et al.*, 2010). TIR1/AFB proteins contain an F-box domain involves with the SCF E3 ubiquitin ligase to form a protein complex namely $SCF^{TIR1/AFB}$ (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005). When auxin arrives, it is recognized by the LRR domain of the $SCF^{TIR1/AFB}$ complex at the binding position, the Aux/IAA proteins were also adjacent with the LRR domain at the auxin binding pocket (Tan *et al.*, 2007). This auxin-induced $SCF^{TIR1/AFB}$-Aux/IAA complex results in the ubiquitination of the Aux/IAA protein and subsequent proteasome-mediated degradation (Long *et al.*, 2006; Szemenyei *et al.*, 2008). After the dimerization between Aux/IAAs and ARFs are released, the B3 DBD region of ARFs interacts with the AuxRE sequence that locates in the promoters of auxin-response genes, and the transcription of these downstream auxin-response genes are activated (Guilfoyle & Hagen, 2007; Li *et al.*, 2016).

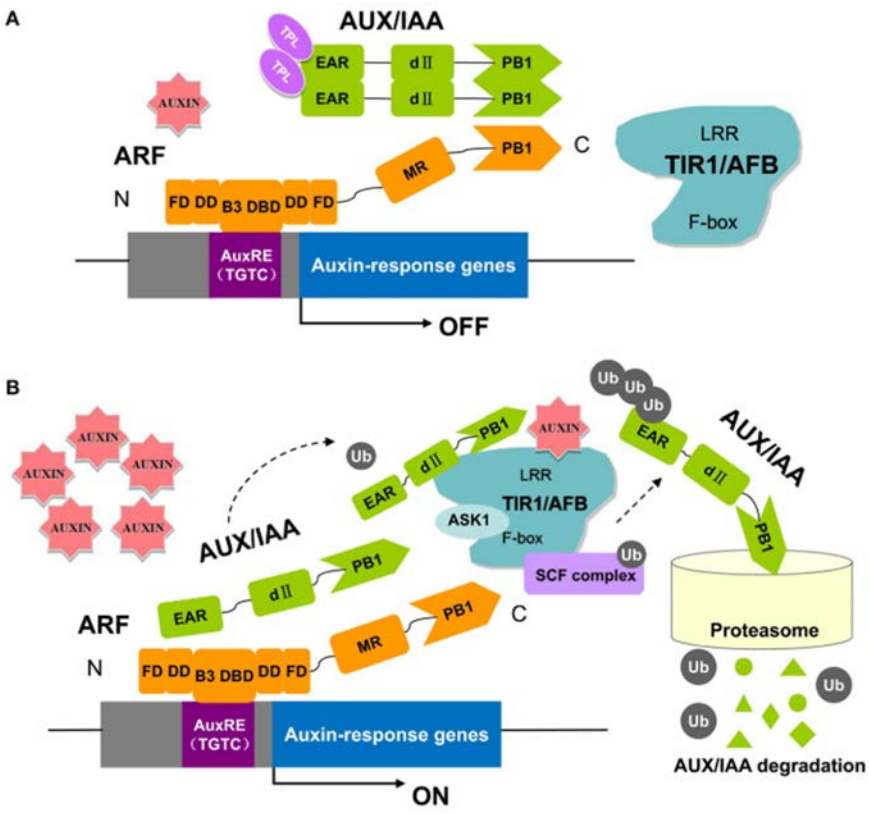

Figure 6. **The SCF$^{TIR1/AFBs}$-Aux/IAAs-ARFs model for transcription regulation by auxin.** At low auxin levels (A), the Aux/IAA proteins involve with ARFs to form a complex to repress the expression of auxin-inducible genes that have AuxRE in promoters, and Aux/IAAs also recruit TPL to the chromatin to stabilize the repressed stage; High levels of auxin (B) bring Aux/IAAs and SCF$^{TIR1/AFBs}$ together, inducing the ubiquitination and degradation of Aux/IAAs, which releases the repression at the AuxRE-containing promoters and the transcription of these genes. (Li *et al.*, 2016)

3. The crosstalk between auxin and other hormones in regulating the fruit set process

Considering the scarcity of reports in literature that review the role of auxin and ethylene crosstalk in regulating the fruit set process, a specific review of this topic was dedicated. This review covers the regulation mechanism of auxin in fruit initiation that are the main issues dealt with in Chapter II. It also addresses the important role of ethylene in regulating fruit initiation, and it especially talks about the crosstalk between auxin and ethylene in the fruit set process,

which provides new ideas towards designing strategies for a better manipulation of fruit initiation and, ultimately, yield. This review was accepted by Plant Science in December 2019.

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Review article

Auxin and ethylene regulation of fruit set

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ABSTRACT

With the forecasted fast increase in world population and global climate change, providing sufficient amounts of quality food becomes a major challenge for human society. Seed and fruit crop yield is determined by developmental processes including flower initiation, pollen fertility and fruit set. Fruit set is defined as the transition from flower to young fruit, a key step in the development of sexually reproducing higher plants. Plant hormones have important roles during flower pollination and fertilization, leading to fruit set. Moreover, it is well established that fruit set can be triggered by phytohormones like auxin and gibberellins (GAs), in the absence of fertilization, both hormones being commonly used to produce parthenocarpic fruits and to increase fruit yield. Additionally, a number of studies highlighted the role of ethylene in plant reproductive organ development. The present review integrates current knowledge on the roles of auxin and ethylene in different steps of the fruit set process with a specific emphasis on the interactions between the two hormones. A deeper understanding of the interplay between auxin and ethylene may provide new leads towards designing strategies for a better control of fruit initiation and ultimately yield.

1. Introduction

Fruit set is known as the initial step of fruit development in sexual reproduction of flowering plants, a process by which the flower turns into a fruit. The transition from flower to fruit corresponds to a developmental shift that is naturally triggered upon flower fertilization and leading to the activation of a high number of metabolic pathways and anatomical transformations that result in the change in organ identity. Fruit set relies on successful pollination of the stigma, followed by pollen germination and subsequent growth of the pollen tube towards the ovule [1]. Then, the fertilization of the ovule triggers the division and expansion of the cells surrounding the embryo [1], and it is widely accepted that the whole process is regulated by plant growth substances, such as phytohormones, with auxin playing a pivotal role in the regulation of this developmental shift [2,3].

Notably, the role of auxins in the development of fruit from unfertilized ovules, a process called parthenocarpy, has been largely documented, and it was proposed that auxin can serve as alternative signal replacing pollination and fertilization to initiate the fruit growth program. It was also shown that auxins interact with the GAs, and that both hormones stimulate cell division and expansion during the fruit set. In particular, auxin was reported to increase GA content, by upregulating GA biosynthesis genes [4]. Further insights into the molecular mechanisms involving the two hormones in the regulation of fruit initiation were provided by the discovery of SlARF7, an auxin response factor, which mediates a crosstalk between auxin and GA signaling [5]. Recent studies showed that abscisic acid also plays an important role in regulating tomato fruit set, further adding to the complexity of the network that regulates this process [1,4,5].

Ethylene has been recognized as an important hormone in several plant development processes and ethylene production increases in the flowers of many plant species after pollination [6], although it was also reported that ethylene biosynthesis starts to decrease once fruit set is completed [3]. However, our understanding of the function of ethylene during carpel development and fruit set in tomato is mostly limited to transcriptome profiling of tomato ovaries which revealed alterations in a high number of ethylene related genes involved in the flower-to-fruit transition [1,7]. The role of ethylene in pollination-independent fruit set has also been suggested based on treatments with the ethylene perception inhibitors, silver thiosulphate and 1-methylcyclopropene (1-MCP) or with the ethylene biosynthesis inhibitor, 2-aminoethoxyvinyl glycine, that leads to parthenocarpic fruit formation [8]. Taken together, these findings support the idea that ethylene plays an active role in the regulation of fruit set, although its precise contribution to this process and its mode of action remain to be further clarified.

The present review addresses various aspects of the regulation of the

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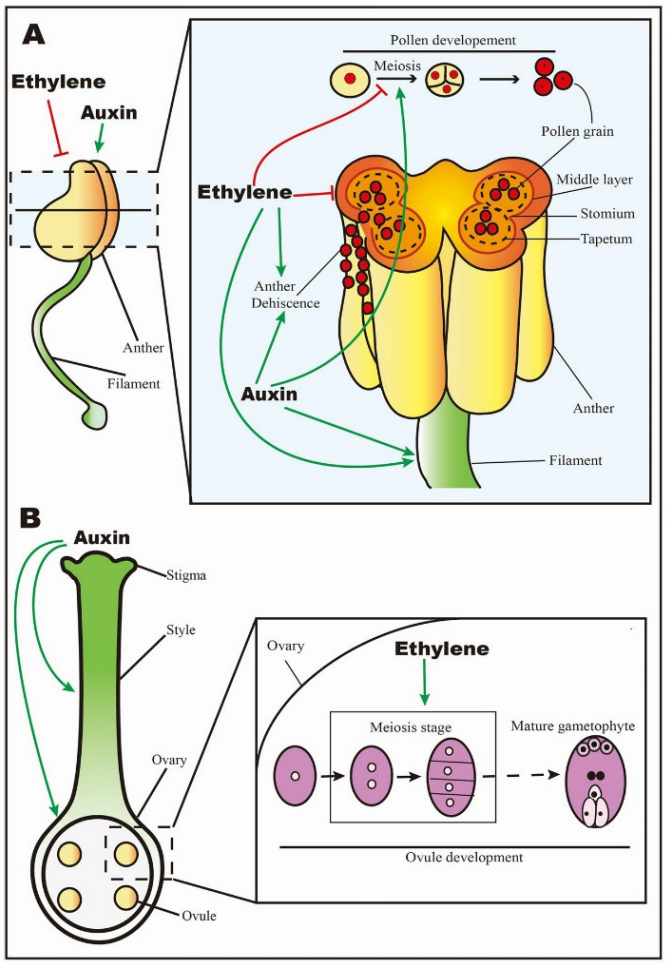

Fig. 1. Ethylene and auxin regulate the development of plant male and female organs.

Green lines stand for positive regulations. Red lines stand for negative regulations. ***(A) Ethylene and auxin regulate stamen formation and development.*** This panel illustrates ethylene and auxin roles detailed in the 2.1 paragraph. Briefly, ethylene and auxin participate in pollen formation and development, and play important roles at the meiosis stage; ethylene induces the programmed cell death (PCD) of the tapetum and middle layer, which leads to anther dehiscence and pollen release; auxin may induce anther dehiscence; ethylene and auxin may promote filament elongation. ***(B) Ethylene and auxin regulates pistil formation and development.*** This panel illustrates ethylene and auxin roles detailed in the 2.2 paragraph. Briefly, ethylene induces the early stage of ovule development; auxin synthesis in apical domain of gynoecium plays an important role in style and ovary formation.

fruit set by ethylene and auxin, and provides new leads towards unravelling the mechanisms underlying this hormonal crosstalk. For a better clarity, the roles of ethylene and auxin are considered at different stages of fruit set, including sexual organ formation, pollination, fertilization, and in parthenocarpic fruit formation.

1.1. Auxin biosynthesis, transport and signaling pathway

In higher plants, there are two major routes for indole acetic acid (IAA) biosynthesis: the tryptophan (Trp)-dependent and the Trp-independent pathways [9–11]. In the Trp-dependent pathways, IAA is biosynthesized from L-Trp by a two-step process: the first step corresponds to the transformation of Trp to indole-3-pyruvic acid (IPA) catalyzed by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA/TAR) family, then IPA is decarboxylated by YUCCA flavin monooxygenase enzymes to form IAA [10]. The Trp-independent pathway was discovered two decades ago. It assumes that IAA can be produced *de novo* without Trp [9]. In this pathway, the indole-3-glycerol phosphate (IGP) is converted to indole by indole synthase, which initiates IAA synthesis without Trp [11].

Auxin transport can be operated either by diffusion (passive movement) or by specific transporters (active movement) [12]. IAA is protonated in the apoplasmic compartment, crossing the plasma membrane to diffuse into the cell. Once in the cytosol, IAA is mainly deprotonated due to the higher pH, and the resulting charged molecule (IAA-) is membrane impermeable. Then transporters are required to help auxins to to cross the plasma membranes. Several auxin carrier families have been identified, such as AUXIN-RESISTANT 1/LIKE AUX1 (AUX1/LAX) influx carriers, PIN-FORMED (PIN) and ATP-BINDING CASSETTE (ABCB) auxin efflux carriers which mediate auxin distribution in and between cells [12].

Auxin is perceived by the cytoplasmic F-box-domain-containing proteins named TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) [13]. The pathway linking auxin perception to gene expression involves the ubiquitination of AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) proteins by the TIR1/AFB subunit of the SCFTIR1/AFB ubiquitin ligase and their degradation by the 26S proteasome. Then the Aux/IAA-mediated inhibition of AUXIN RESPONSE FACTORs (ARFs) is released, which allows ARFs to modulate the expression of their downstream target genes, known as auxin-responsive genes [14].

1.2. Ethylene biosynthesis and signaling pathways

The biosynthesis of ethylene includes three steps, the first leading from methionine to S-Adenosyl-Methionine (SAM) catalyzed by the S-adenosyl-methionine synthetase (SAM synthetase), the second transforming SAM into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and finally the production of ethylene from ACC by ACC oxidase (ACO). The pool of ACC is also regulated by conjugation via ACC acyl-transferases leading to the formation of ACC derivatives like malonyl-ACC, glutamyl-ACC, and jasmonyl-ACC, which affects the pools of free ACC and hence ethylene biosynthesis [15].

Ethylene is perceived by different ethylene receptor proteins (ETRs), located in the endoplasmic reticulum membrane. The number of ETRs varies among species with five receptors reported in Arabidopsis, ETR1, ETR2, EIN4, ERS1, and ERS2 [16] and seven ethylene receptors in the tomato, ETR1, ETR2, ETR3, ETR4, ETR5, ETR6, and ETR7, each having a distinct pattern of expression throughout development and in response to external stimuli [17]. The ETRs are negative regulators of ethylene responses and interact with other protein partners like CONSTITUTIVE TRIPLE RESPONSE (CTRs) protein kinases which are also negative regulators of the downstream signal. In the absence of ethylene, the ETR-CTR complex maintains the ETHYLENE INSENSITIVE2 protein (EIN2) in a phosphorylated state at its C-terminus domain which prevents its cleavage [18]. In the presence of ethylene, the protein complex (ETR-CTRs) is dephosphorylated and inactivated, thus causing the cleavage of the EIN2 C-terminus domain by proteases, and this C-terminus domain moves into the nucleus, promoting the accumulation of EIN3/EILs by preventing its degradation via EBF1 and EBF2 [16]. The EIN3/EIL3 proteins activate the transcription of ethylene response factors (ERFs), which in turn lead to expression of hundreds of ethylene responsive genes, through binding to GCC-boxes and other cis-acting elements [17,18].

Ethylene and auxin can interact synergistically or antagonistically to control a variety of plant development processes, such as fruit development, fruit ripening, root formation and hypocotyl elongation [19,20].

2. Ethylene and auxin regulate the formation of plant male and female organs

2.1. Male reproductive organs

In flowering plants, the stamen male reproductive organ is made of the filament and the anther where pollen is produced (Fig. 1A). The formation and development of normal stamens are essential for male fertility, and therefore for successful fruit set. A number of studies highlighted the expression dynamics of genes encoding various components of ethylene signaling and responses in male reproductive organs suggesting an active role for ethylene in pollen development [21,22]. For instance, in Arabidopsis, *AtEIN4* and *AtERS2* exhibit high expression at the transcript level in stamen, including pollen and tapetum cells. These latter form a specialized cell layer feeding the developing pollen grains. High expression of ethylene receptors in these tissues suggests that ethylene is important in these processes [21]. *AtERS2* gene shows a similar expression pattern to the ethylene receptor gene *Os-ERS1* in rice that is up-regulated during early pollen development, corresponding to meiotic and microspore stages [22]. In tomato, the ethylene production capacity of pollen grains is consistent with the expression of ethylene biosynthesis and signaling genes in these tissues [23]. Moreover, the application of high concentration of exogenous ethylene on male gametophyte induces the degradation of the generative cell at meiosis stage resulting in male sterility [24]. Regarding auxins, many studies showed that they are involved in pollen development. For example, IAA accumulates in anthers prior to pollination, specifically in pollen grains, tapetum, endothecium and epidermis cells of anthers [25]. Moreover, the repression of the *iaaL* gene encoding indoleacetic acid–lysine synthetase, which converts free IAA to its inactive form IAA-lysine, results in low free IAA content in anther and reduced pollen viability due to defective mitosis [25]. In tomato, the down-regulation of *SlPIN8*, a gene encoding an auxin-transporter specifically expressed in tomato pollen, resulted in up to 80 % abnormal pollen grains with extremely poor viability [26]. Altogether, these data suggest that ethylene and auxin play important roles in pollen development, and that high amount of ethylene may have detrimental effect on the process.

Additionally, several studies showed that ethylene has critical roles in pollen release. The process of anther dehiscence involves two main steps sequentially consisting of (i) degeneration of the middle layer and tapetum; (ii) breakage of the anther wall at the region of stomium between the two locules of each anther [27]. It has been reported, using transgenetic tabacco plants, that the mutant melon ethylene receptor gene *Cm-ERS1/H70A* exhibits delay in tapetum programmed cell death, resulting in the production of abnormal pollen [28]. In the Arabidopsis ethylene insensitive mutant *etr1-1*, the (i) stomium cells showed delayed degeneration and then (ii) anther dehiscence is delayed [29]. These two events have been related to ethylene production peaks in petunia [24]. Moreover, application of ethylene-perception inhibitors 2,5-norbornadiene and 1-MCP impaired anther dehiscence in petunia and tobacco, respectively, whereas ethylene treatment accelerated this process [29]. Similarly, in Petunia, the down-regulation lines of ethylene receptor gene *PhETR2* hastened stomium degeneration and anther dehiscence, making these process happened before anthesis, which is earlier than in wild type, indicating that PhETR2 regulates the timing of anther dehiscence [30]. This seems to indicate that through promoting anther dehiscence, ethylene promotes pollen release. It was shown that auxin also involves in anther dehiscence process, the quadruple mutant *tir1/ afb1/ afb2/ afb3* displaying auxin perception defects shows early anther dehiscence due to premature lignification of endothecium cell walls and precocious breakage of the stomium [31].

Regarding male sterility, ethylene and auxin also appear to be involved in the arrest of stamen development [29–36]. In Arabidopsis, it shows the stamen development arrest in *CsACO2* overexpressed lines, suggesting that the increase in ethylene production inhibits stamen development [32]. Also, down regulation of cucumber *ETR1* ethylene receptor gene induced abnormal stamen development leading to female flower only [33]. Moreover, it was shown in tobacco that ethylene perception is critical for filament elongation [34]. However, it was shown that auxin positively regulates stamen development, including filament elongation and pollen maturation [34–36]. Indeed, in the Arabidopsis *yuc2-yuc6* double mutant, the down regulation of auxin biosynthesis caused the failure of filament elongation and pollen maturation [35], similar observations were performed when auxin perception were altered [34]. When auxin transport is affected, in Arabidopsis *pin1-1* and *pin1-2* mutants, no stamen developed [36].

Finally, cross-talks between auxin and ethylene have been observed in *Gaillardia grandiflora* at the stamen level, as auxin promotes pollen tube elongation and ethylene production at the same stage. This auxin-induced ethylene has an important role in the later stigma opening stage [37]. Taken together, these findings support the idea that ethylene and auxin play important roles in male reproductive organs of higher plants.

2.2. Female reproductive organs

In female reproductive organ, the pistil (Fig. 1B) provides protection for the ovules, enables pollen capture and pollen tube guidance, and supports self- and inter-specific incompatibility. Following fertilization of the ovules, in true fruit, such as grape and tomato, the gynoecium develops into a fruit, which protects the developing seeds and ultimately facilitates mature seed dispersal; in false fruit, such as apple and pears, the fruit derives from other flower parts, after fertilization [38]. Gynoecium development involves the differentiation of specialized

functional modules, including the stigma which forms at the apex of pistil, and will capture and stimulate the pollen grain germination [39]. The style or pistil is located immediately below the stigma and contains transmitting tissues that conduct pollen tubes to the ovary, the basal structure containing the ovules [39]. The formation of normal and functional gynoecium is essential for female fertility, and hence for successful fruit set. The role of ethylene in female gametophyte development, and ultimately in promoting ovule fertilization, has been addressed in several studies [39,40]. In tobacco, the ethylene biosynthesis gene, *ACO*, is expressed during early stages of ovule development (meiosis stage), and its silencing results in the arrest of ovule development and failure to reach the maturity stage. Similar effect was obtained by the application of the ethylene biosynthesis inhibitor, silver thiosulfate [40]. Notably, upon exogenous ethylene treatment, the ovules recover their functionality and restore the guidance of pollen tubes to the ovule micropyle [40].

The role of auxin in female reproductive organ was revealed through the characterization of the *AGAMOUS*-clade of MADS-box genes showing that members of MADS-box B3 domain transcription factors *NGATHA* (*NGA1* to *NGA4*) play an essential role in style development; the loss-of-function of these four *NGA* genes results in complete loss of style and stigma development, and this phenotype is due to the failure of activating *YUCCA*-mediated auxin synthesis in apical domain of gynoecium [41]. These studies emphasize the role of auxin in apical-basal gynoecium pattern.

3. Ethylene and auxin regulate pollination-dependent fruit set

3.1. Pollen germination and tube growth

In plants, pollination refers to the release of the pollen from the anther and its deposition at the surface of the stigma (Fig. 2A). When the pollen grain rehydrates and germinates on the stigma, the pollen tube starts to grow inside the style. The pollen grain contains a nucleus and a generative cell, which divides into two sperm cells [42]. The pollen tube grows navigating through transmitting tissue in the style towards the ovule where it releases the sperm cells for double fertilization [43]. The reality and the nature of the pollination signal remains a matter of debate, even though auxin, or ACC, have been proposed as the acting molecular signals [44,45]. In petunia, the level of ACC increases 100 fold in mature pollen grain and in anthers one day before anthesis [46]. In tomato, it was shown that pollen grain has the capacity to produce and sense ethylene before germination, as suggested by the expression of several ethylene biosynthesis and signaling genes including *SlACS3*, *SlACS11*, *SlETR3* and *SlCTR2* [23]. Nevertheless, exogenous application of ACC in flowers cannot induce the post-pollination development [47]. When applying the synthetic auxin, NAA, to the stigma of orchids, the ethylene production was stimulated in flowers [45]. Although these findings support the idea that auxin and ethylene are important for pollination initiation, other factors might also contribute to the regulation of this process.

It was suggested that auxins regulate pollen germination [48,49]. In support to this hypothesis, IAA accumulates at high levels in the stigma when most pollen grains germinate [50] and, free IAA is high in the growing tip of the pollen tube where it was suggested to promote rapid pollen tube growth [49]. It has also been reported that IAA treatment results in straighter and more slender pollen tube growth which facilitates its elongation [51,52]. Notably, plasma membrane H^+ ATPase has been shown to interact with 14-3-3 proteins, an interaction that is important for pollen germination [53]. However, when the pollen tube enters the style, the amount of auxin decreases in the style tissues [50].

The growth and elongation of the pollen tube is essential for successful fertilization and subsequent fruit set since it carries two male gametes to the ovary and ovule over a long distance in the pistil (Fig. 2B). The transmission tissues in the style play an important role in providing nutrition and mechanical support for pollen tube growth and, after pollination, transmission tissues begin to be malformed and deteriorate, which could provide nutrition to surrounding tissues [51]. In addition, the cellular degeneration of transmission tissues in the style provides more space, allowing the penetration of the pollen tube in the stigma. This degeneration facilitates the passage of pollen tube in the style to reach the ovary, and these events are considered as a programmed cell death process [51]. Several studies have shown the important role of ethylene in pollen tube growth [6,54]. Both ACC and ethylene have a role in the degeneration of transmitting tissues, by promoting the shortening of the polyA tail of some RNAs specific to these transmitting tissues [51]. On one hand, the treatment with 1-MCP reduces pollen tube growth in petunia [6]. On the other hand, it was shown that ethylene promotes pollen tube growth by increasing the relative amount of F-actin, which is important for the polarized growth of this tube [55]. Upon pollination, the increase in ethylene production reaches a maximum in the stigma and is associated with enhanced expression of *ACS* and *ACO* genes in the ovary. By contrast, control emasculated flowers did not exhibit such a change in ethylene production [56]. In orchid gynoecium, *ACS* and *ACO* genes reach an expression peak 24–48 h after pollination, and then decline [47], and similar expression patterns for *ACS* or *ACO* were observed in petunia and carnation [57].

3.2. Changes to ovary development

The presence of auxin and ethylene is required to initiate changes in ovary development and to induce ovule differentiation (Fig. 2C). It has been found that following pollination with fresh pollen, enhanced ethylene synthesis and auxin transport are necessary to initiate the egg cell differentiation in maize ovules [58]. Before fertilization, auxin production is suppressed by FIS-PRC2 in the central cell, and this suppression is released upon fertilization. In *fis* mutants impaired in the FIS-PRC2 block system, auxin biosynthesis is induced before fertilization and initiates autonomous endosperm formation [59].

In flowering plants, when the pollen tube reaches the ovule, two male sperm cells are released to fuse with two female gametes, the egg cell and the central cell (Fig. 2D). The fused egg cell will develop into an embryo, and the fused central cell will give the endosperm, which provides nutrition and support to embryo [60]. The two synergids are responsible for pollen tube attraction, but only one pollen tube will fertilize the ovule [61]. Recent studies demonstrate that ethylene signal is critical to prevent the attraction of a second pollen tube and it was shown that egg cell fertilization induces the activation of EIN3- and EIN2-dependent ethylene response pathway necessary for the programmed cell death of the synergid cell [62]. In addition, the over-accumulation of EIN3 in synergid cells leads to the block of pollen tube attraction [63]. Auxin has been suggested to regulate maize endosperm proliferation. A study in the maize mutant *defective endosperm18 (de18)* showed that IAA biosynthesis is impaired and the total cell number is lower in the endosperm, and the level of endoreduplication of the endosperm is reduced [64].

4. Ethylene and auxin roles in parthenocarpy

Parthenocarpy corresponds to the ovary development in the absence of pollination and fertilization [65]. In this regard, it is considered as a desirable agronomic trait to improve fruit set in adverse climate conditions. In addition, the seedless fruits due to parthenocarpic development are preferred by consumers [65]. It has long been known that applying synthetic phytohormones like auxin and gibberellic acid (GA) to unpollinated flower buds induces parthenocarpic fruit set [66]. However, exogenous application of plant hormones to get seedless fruits is costly, thus genetic engineering strategies are promoted, aiming at altering hormone biosynthesis or signaling in order to obtain parthenocarpic fruits. Pioneering work, in this domain, targeted the up-regulation of the *iaaM* gene to promote the synthesis of indolacetamide,

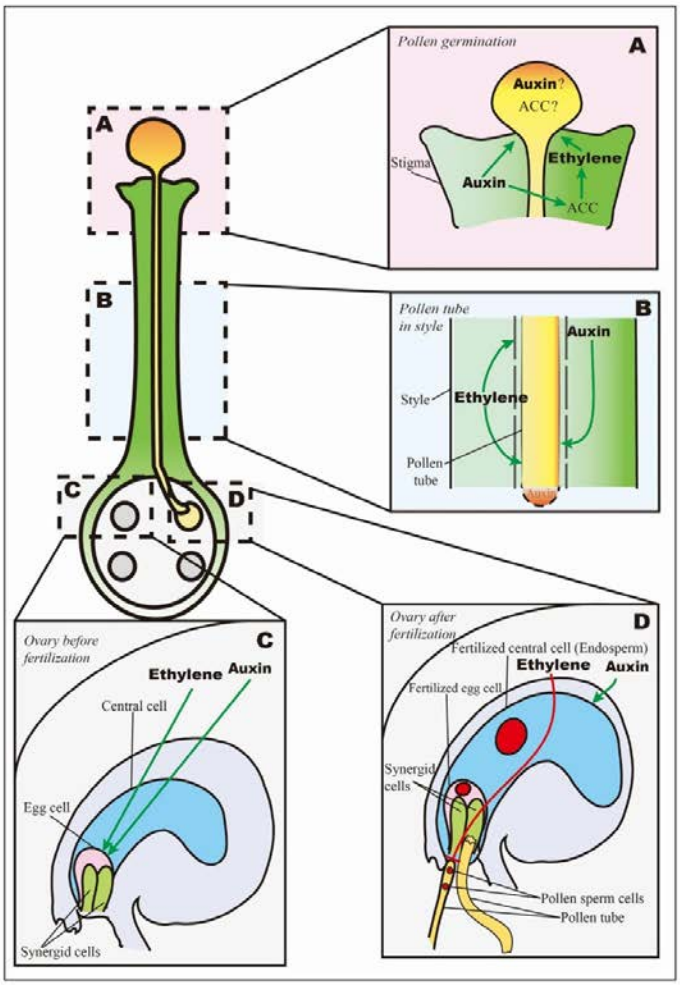

Fig. 2. Ethylene and auxin regulate plant pollination and fertilization processes.

Green lines stand for positive regulations. Red lines stand for negative regulations. ***(A) Pollen germination.*** This panel illustrates ethylene and auxin roles detailed in the 3.1 paragraph. Briefly, mature pollen grain contains ACC and auxin, which may induce pollen germination.
ethylene and auxin both induce pollen germination in stigma, and auxin stimulates ACC synthesis. ***(B) Pollen tube growth in style.*** This panel illustrates ethylene and auxin roles detailed in the 3.2 paragraph. Briefly, ethylene helps degradation of transmission tissues in the style, which facilitates pollen tube growth. Auxin accumulates in the tip of pollen tube, and this facilitates pollen tube growth. ***(C) Ovary before fertilization.*** This panel illustrates ethylene and auxin roles detailed in the 3.3 paragraph. Briefly, the increase of ethylene and auxin concentrations are necessary to initiate egg cells differentiation in ovules after pollination. ***(D) Ovary after fertilization.*** This panel illustrates ethylene and auxin roles detailed in the 3.4 paragraph. Briefly, ethylene promotes programmed cell death of synergid cell, which prevents second pollen tube attraction; auxin is important for endosperm development, especially in endoreduplication.

which can be converted to IAA [67]. The expression of this auxin biosynthesis gene, driven by the promoter of *DefH9*, a MADS-box gene expressed specifically in ovules, resulted in parthenocarpic fruit development in both tobacco and eggplant [67]. Thereafter, the same gene was used to obtain parthenocarpic fruit in several horticultural crops, like tomato and cucumber [68]. Another successful approach to produce parthenocarpic fruit dealt with the down-regulation of *SlIAA9*, encoding a negative regulator of auxin-dependent gene transcription [2]. Subsequently, the parthenocarpic trait was also achieved through up or down-regulation of members of auxin response factors in different species [69]. In Arabidopsis, the loss of function mutation of *AtARF8* produced dehiscent and parthenocarpic siliques [69]. In tomato, the down-regulation of *SlARF8* and *SlARF7* formed parthenocarpic fruit, and the over-expression of *SmARF8* in eggplant produced the same seedless trait [5,70,71]. Overall, auxin biosynthesis and signaling genes provide efficient targets to engineer parthenocarpic plants in a variety of species.

Most studies showed that ethylene acts as a negative regulator of parthenocarpy in plants [8]. For example, in Arabidopsis, ethylene is responsible for ovule senescence by preventing GA perception; hence, ethylene indirectly leads to the degradation of ovule tissues, thus reducing parthenocarpic fruit set [8]. On the other hand, preventing ethylene perception in emasculated flowers of tomato, either by using 1–MCP, or by using ethylene insensitive mutants (*etr1-1*), led to more parthenocarpic fruits [72]. Similarly, blocking ethylene synthesis, by using 2-aminoethoxyvinyl glycine, or blocking ethylene perception, by using silver thiosulphate, induced parthenocarpy in zucchini squash [73].

To date, there is no study focusing on the crosstalk of auxin and ethylene during parthenocarpic fruit set.

5. Conclusions and perspectives

A large set of data supports the notion that both ethylene and auxin play active roles in controlling the fruit set process, at different steps of the flower-to-fruit transition. But there is a need for research to detail the potential crosstalk between auxin and ethylene signals in the control of this transition. The two hormones impact all stages of stamen development, including elongation of stamen filament bringing anthers close to stigma, pollen maturation, and anther dehiscence to release

pollen on stigma. Proper completion of these events is critical to the formation and development of reproductive sexual organs and then to successful initiation of the fruit set process [74]. Ethylene and auxin are also involved in most events associated with the germination of pollen in the stigma, the growth of pollen tube through the transmission tissues in the style and finally the fertilization of the ovule. The use of mutant lines impaired in both auxin and ethylene responses will allow to better decipher the roles of auxin and ethylene in initiating and controlling the flower-to-fruit transition. By building on studies about crosstalk in other developmental processes, like root formation and hypocotyl elongation [20], future research will allow to further detail the dialogue engaged between the two hormones in the different phases of the fruit set. For example, it was shown that mutants altered in AUX1 and EIR1/AGR/PIN2 with impaired auxin transport, and those affected in TIR1 auxin receptor, exhibit ethylene insensitive root growth, which supports the idea of interdependence of both hormones [75,76]. More recently, it was reported that ERF109 binds directly to the promoters of *ASA1* and *YUC2* auxin biosynthesis genes, and ERF1 could bind with the promoter of *ASA1*, leading to auxin increase and ethylene-induced root growth inhibition [77,78]. Similarly, the expression of *Sl-IAA27* is regulated by direct binding of ERF.B3 to its promoter [79]. The regulation of *ACS* expression by ARFs, and of *TAA1* by EIN3 have been documented [80]. Finally, the impaired expression on *SlSAUR69* in the tomato results in altered auxin distribution and change in ethylene sensitivity [19]. All these examples should lead to new studies about potential ethylene and auxin crosstalks in fruit set. These studies should cover the parthenocarpic fruit formation, which is becoming an important trait, offering a mean to ensure yield stability in unfavorable environmental conditions, these latter reducing pollen viability and depressing flower fertilization. Additionally, the epigenetic control is an emerging theme in developmental biology and its contribution to the fruit set process needs to be unveiled. It is therefore important to investigate how epigenetic components impact the transcriptomic reprogramming underlying different steps of fruit set. Finally, while the present review emphasizes the role of ethylene and auxin in fruit set, input from gibberellins is also critical to control this process [4,5]. Thus it will be interesting to expand the crosstalk studies to such hormones.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter II

Results and Discussion

Introduction to Chapter II

Auxin is a crucial plant hormone for several development progresses, including cell elongation, division, and differentiation, organ formation, apical dominance, root initiation, leaf morphology, fruit setting, fruit development, and fruit maturation in higher plants (Cleland *et al.*, 1991; Laskowski *et al.*, 1995; Reed *et al.*, 1998; Petrášek *et al.*, 2002; Wang *et al.*, 2005a; Feng *et al.*, 2006; Goetz *et al.*, 2006; de Jong *et al.*, 2009a). ARFs are one of the important components for auxin signaling, which are widely known for their essential roles in regulating several plant development process. In tomato, twenty-two ARFs have been identified (Zouine *et al.*, 2014). Among all ARFs, *SlARF8A* and *SlARF8B* displayed the most prominent transcript accumulation during fruit set process (Zouine *et al.*, 2014). Several ARFs can be regulated by auxin, which imply their potential contribution to the mechanism underlying auxin signaling pathways. Besides, in Arabidopsis, the over-expression lines of *AtARF8* displayed early fruit set independent of pollination and fertilization (Goetz *et al.*, 2006). The roles of *SlARF8A* and *SlARF8B* in the fruit set process in fleshy fruit tomato need deeper research. To address the function of *SlARF8* homologs during fruit set process, the knockout lines of *SlARF8A* and *SlARF8B* by CRISPR/Cas9 genome editing system were generated respectively. In my thesis, Chapter II will describe the roles of *SlARF8A* and *SlARF8B* during the fruit set process through the following part: the identification, the expression pattern, the identification of mutants, the physiological molecular analyses of the *SlARF8A* and *SlARF8B* mutants, the RNA-Seq analysis of *SlARF8A* and *SlARF8B* mutants, and the potential mechanism underlying the regulation of *SlARF8* on the fruit set process. Besides, Chapter II will describe a useful tool for identification and functional annotation of auxin-related genes in plants, which is prepared as a research article.

Knockout of ARF8 Using CRISPR/Cas9 in Tomato leads to Pleiotropic Vegetative Phenotypes and Parthenocarpic Fruits

(Manuscript in preparation)

Abstract

Fruit set is defined as a transition from flower to young fruit, which is an essential step in the development stages in sexually reproducing higher plants. During this important process, the plant hormone auxin is widely known to participate in triggering and coordinating those changes. Auxin Response Factors (ARFs) are transcriptional regulators that involve the auxin signaling and regulating auxin-responsive genes by binding specifically with the Auxin Response Elements (AuxREs) located in their promoters. Two homologue genes, *SlARF8A* and *SlARF8B*, were identified as potential regulators of the fruits set based on their expression profile that changes dramatically during the transition from flower to young fruit. The subcellular localization studies validate that SlARF8A and SlARF8B proteins localized in the nucleus of the cells. The expression pattern analysis by RT-qPCR revealed that *SlARF8A* and *SlARF8B* displayed different expression levels before and after pollination and fertilization. A notable increase in *SlARF8A* transcript was displayed upon flower pollination, while a decrease in *SlARF8B* expression level was shown after pollination. The knockout mutants of *SlARF8A* and *SlARF8B* (*slarf8a-cr*, *slarf8b-cr*, and *slarf8a&b-cr*) were generated by performing the CRISPR/Cas9 genome editing approach. The *slarf8a-cr*, *slarf8b-cr*, and *slarf8a&b-cr* mutants displayed pleiotropic phenotypes, including dwarf plants, smaller and parthenocarpic fruits. Besides, histological analysis revealed the development of placenta, pericarp, locular tissue, and ovules were impaired in these mutants. The expression profiling of genes at the genome level during fruit set process was studied by performing the RNA-Seq approach. Among the significantly enriched functional categories in *slarf8b-cr*, many of which are related to auxin, then with other hormones known for their role in fruit set, including gibberellin (GA). Among

the DEGs, several *SAURs* and *IAAs* genes display high expression levels during the fruit set process and be potential target genes of *SlARF8B*. We hypothesized that *SlARF8A* and *SlARF8B* may participate in the regulation of early fruit set and development by mediating the crosstalk between auxin and GA hormones during tomato fruit development

Introduction

The fruit set process is known as the crucial step for fruit development, a transition stage by which the flower turns into a fruit via the sexual reproduction of flowering plants. Successful fruit set relies on the pollination on stigma, followed by pollen germination and the successful fertilization of ovules in the ovary (Gillaspy *et al.*, 1993). The initial transition from flowers to fruits is a developmental shift that leads to the activation of the large numbers of metabolic pathways and anatomical transformations in the organ identity changes (Vriezen *et al.*, 2008). It is widely known that plant hormones play vital roles in the fruit set process. GA and auxin can trigger a seedless fruit set without pollination or fertilization, which is known as parthenocarpy (Pandolfini, 2009). It has also been shown that auxin interacts with GA, leading to the biosynthesis of some auxin signaling pathway downstream GA genes, which could induce parthenocarpy (Serrani *et al.*, 2008).

The molecular mechanism of auxin mediates fruit set is still largely unknown. So far, some genes of the two important auxin responsive gene families Aux/IAA and ARFs have been found to play crucial roles in fruit set. Aux/IAA are early auxin responsive and regulated by auxin. Classic Aux/IAA proteins contain four conserved amino acid sequence motifs, including domains I, II, III and IV. Some of them lack one or more of these four domains are also included as this gene family (Reed, 2001). Domain III and IV of Aux/IAA proteins are shared with ARF proteins, which is recognized to involve in the interaction between Aux/IAAs and ARFs (Remington *et al.*, 2004). ARFs consist of an amino-terminal DNA binding domain (DBD), a middle region that functions as an activation domain (AD) rich in glutamine or a repression domain (RD) riches in praline and serine, and a carboxy-terminal dimerization domain (CTD) (Guilfoyle & Hagen, 2007). ARFs bind specifically with the conserved AuxRE which locate in the promoters of the auxin response genes and functions in combination with domain III and

IV of Aux/IAAs repressors, which represses ARFs by dimerization with them (Ulmasov *et al.*, 1999).

Twenty-three ARFs have been identified in Arabidopsis, of which *ARF5-8* and *19* are transcriptional activators and the rest are transcriptional repressors (Remington *et al.*, 2004). The important roles of many ARFs have been revealed up to now. *ARF2* regulates root formation in Arabidopsis (Lim *et al.*, 2010), and leaf senescence and flower organ senescence in tomato (Ren *et al.*, 2017). Inhibition of *AtARF2-4* expression may repress Arabidopsis lateral root growth (Marin *et al.*, 2010). *AtARF7* and *AtARF19* interact with *IAA14* could regulate lateral root formation (Marin *et al.*, 2010). Not only in plant growth and development, ARFs also play initial roles in reproductive development. *SlARF2* was found to be an essential component of fruit ripening regulatory mechanism (Hao *et al.*, 2015); *arf6* and *arf8* mutants were found to delay stamen development and fruit initiation (Nagpal *et al.*, 2005; Goetz *et al.*, 2006); By inducing the expression of miR167a to down regulate *SlARF6* and *SlARF8* in tomato could cause floral development defects and female sterility (Liu *et al.*, 2014); In addition, *SlARF7* could induce parthenocarpic fruit as a negative regulator of the fruit set process (de Jong *et al.*, 2009b).

As a model system, tomato has been widely used for fruit set and fruit ripening studies. Combined with the previous research, it will be essential to study functions of ARFs during the fruit set process to improve this important agronomic trait in tomato. A comprehensive set of twenty-two members of ARF was identified in tomato according to our previous study, and it has been found that *SlARF8A* and *SlARF8B* present different expression patterns in the fruit set process (Zouine *et al.*, 2014). To explore the function of *SlARF8A* and *SlARF8B* during fruit set, the CRISPR/Cas9 knock out mutants and overexpression transgenic lines of which was generated in tomato MicroTom cultivar respectively. *slarf8a*, *slarf8b* single mutants and *slarf8a&b* double mutants were observed to induce seedless fruits without pollination and fertilization after emasculation. The RNA-Seq transcriptomic profiling of *slarf8a* and *slarf8b* before and after pollination and the fertilization process was performed. According to this data, we hypothesized that *SlARF8A* and *SlARF8B* may participate in the regulation of early fruit set and development by mediating the crosstalk between IAA and GA hormones during tomato fruit development.

Results

Structure, localization and expression of tomato *SlARF8A* and *SlARF8B* genes.

Previous study has shown that the ARF8 transcription factor in tomato is encoded by two genes, *SlARF8A* (Solyc03g031970) and *SlARF8B* (Solyc02g037530) (Zouine *et al.*, 2014), which appeared to be highly similar (67.34% and 67.84% respectively) to the Arabidopsis thaliana *AtARF8* (At5g37020) protein sequence. The isolation of full-length cDNAs corresponding to *SlARF8A* (2796 bp) and *SlARF8B* (2786 bp) indicated that the deduced protein sizes are 845 and 843 amino acids, respectively. We show here that *SlARF8A* and *SlARF8B* displayed well-conserved genomic structure regarding the number and position of introns and exons (Figure 1). *SlARF8A* and *SlARF8B* belong to different chromosomes, *SlARF8A* locates at the top of chromosome 3, and *SlARF8B* locates at the middle of chromosome 2. It has been shown in the previous study that the protein sequence *SlARF8A* and *SlARF8B* both contain N-terminal B3-drived DNA binding domain, C-terminal domain III and IV, and the middle region. The middle region of SlARF8A and SlARF8B both enriched in glutamine, which has been identified as Q-rich activation domain (Zouine *et al.*, 2014). These findings correspond with the phylogenetic analysis in which *SlARF8A* and *SlARF8B* are clustered in activator subclade, which includes *ARF6*, *ARF5*, *ARF7*, and *ARF19* (Wu *et al.*, 2011; Zouine *et al.*, 2014).

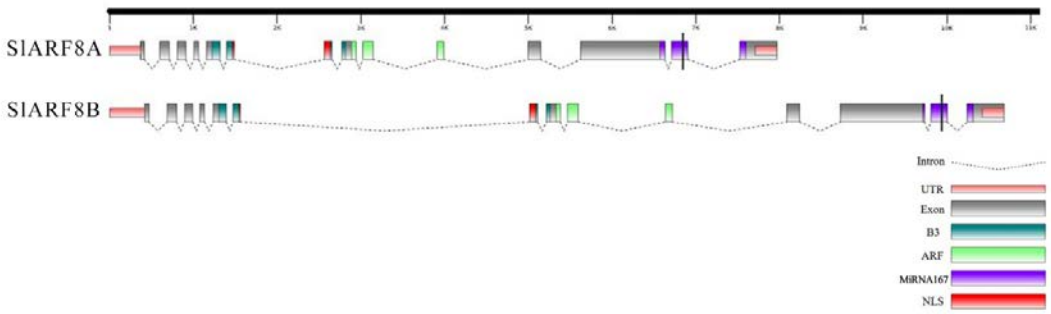


Figure 1 Transcription unit of SlARF8A and SlARF8B. The genomic sequences of *SlARF8A* and *SlARF8B* and their structural annotation were extracted from SGN database. The bioinformatics analysis was performed by Fancy gene V1.4 (http://bio.ifom-ieo-campus.it/fancygene). *SlARF8A* and *SlARF8B* display the same numbers of exons and introns, and they both interacted with MiRNA167.

Since nuclear localization of transcriptional factors is essential for their transcriptional activity, we first verify that SlARF8A/B are located in the nucleus. Bioinformatics studies indeed shown that the two proteins contain the predicted bipartite NLS nucleus targeting domain. The nuclear localization of the SlARF8A/B proteins was investigated by fusion to the green fluorescent protein (GFP) under the control of the 35S promoter of Cauliflower mosaic virus and transient expression in tobacco protoplasts. Fluorescence microscopy analysis associated with image overlay techniques demonstrated that control cells transformed with GFP alone displayed fluorescence throughout the cell (Figure 2), whereas the SlARF8A/B-GFP fusion proteins was exclusively localized to the nucleus, indicating that SlARF8A/B were able to fully direct the fusion protein to the nucleus (Figure 2). This nuclear targeting of the SlARF8A/B protein is consistent with a putative transcriptional regulatory function.

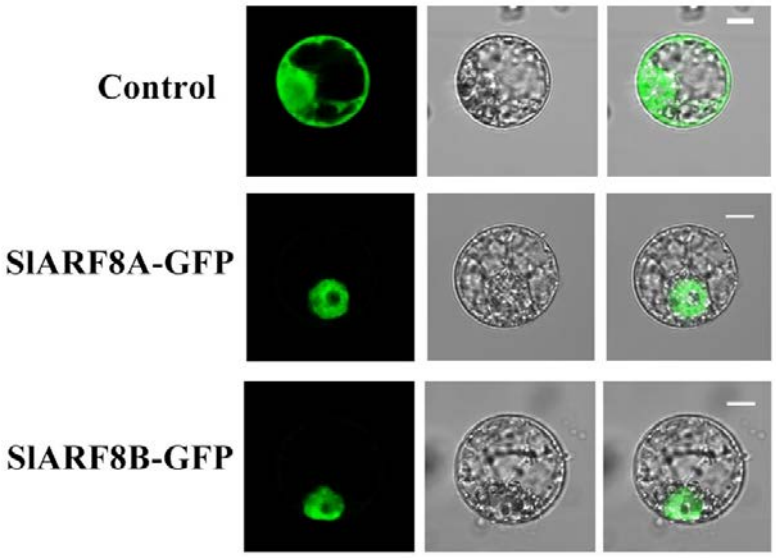


Figure 2. Nuclear localization of SlARF8A and SlARF8B proteins. Nuclear localization of SlARF8A and SlARF8B proteins fused with the GFP tag. Pro35S: SlARF8A/B-GFP and pro35S: GFP fusion proteins as control were transiently expressed in BY-2 tobacco protoplasts; the sub-cellular localization was analyzed by confocal laser scanning microscopy. The merged pictures of the green fluorescence channel (left panels) and the corresponding bright field (middle panels) are shown in the right panels. The scale bar indicates 10 μm.

The spatio-temporal expression of *SlARF8A* and *SlARF8B* in tomato were revealed by the GUS reporter gene driven by *SlARF8A* and *SlARF8B* promoter respectively (Figure 3). At the fruit set transition process from 0 DPA to 4 DPA, *SlARF8A* expression shows an increase in ovaries while *SlARF8B* shows a decrease. It is notable that both *ProSlARF8A::GUS* and *ProSlARF8B::GUS* lines present strong GUS signals in vascular tissue after pollination and fertilization, especially in the young fruit stage. The expression pattern of *ProSlARF8A::GUS* and *ProSlARF8A::GUS* is fully consistent with the expression pattern revealed by quantitative RT-PCR (Figure 4). *SlARF8A* and *SlARF8B* expression levels were assessed in all tomato organs, *SlARF8A* expressed highly in 8 days (8d) after pollination and fertilization, while *SlARF8B* expressed highly at 0 DPA and decreased dramatically in 8 DPA young fruit, and both *SlARF8A* and *SlARF8B* expressed low in red fruits.

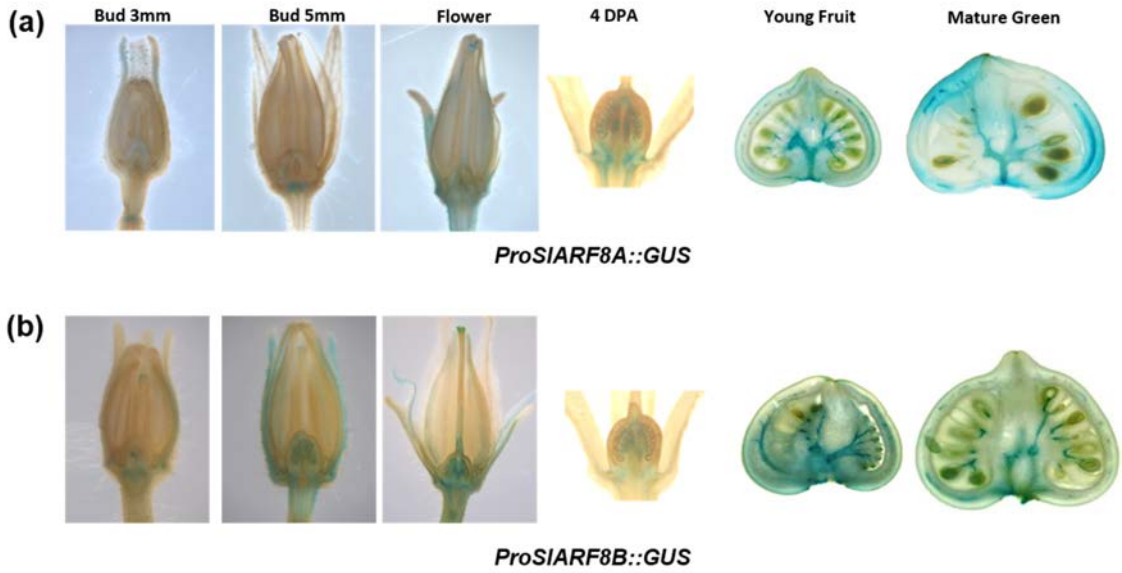


Figure 3. Expression patterns of *SlARF8A* and *SlARF8B* assessed by the GUS reporter gene. The vertical sections of tomato bud (3mm, 5mm), flower (0 DPA), 4 DPA young fruit, young fruit, and mature green fruit which carries the *ProARF8A::GUS* (a) and *ProARF8B::GUS* (b).

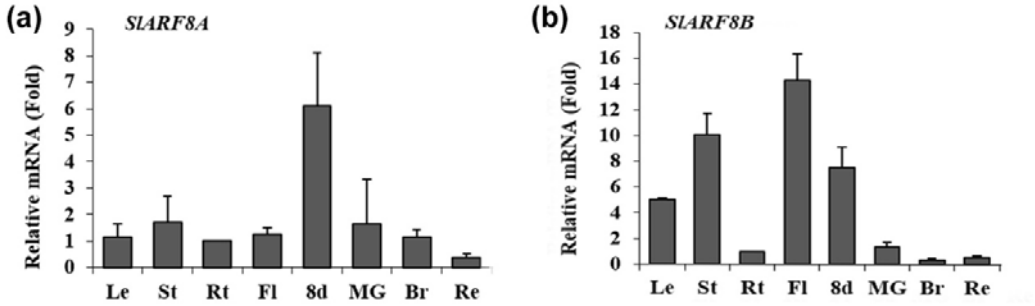


Figure 4. Expression patterns of *SlARF8A* and *SlARF8B* genes in different tomato tissues. Total RNA was extracted from leaf (Le), stem (St), root (Rt), flower (Fl), 8 DPA fruit (8d), fruit at mature green (MG), fruit at breaker (Br), and fruit at red (Re). The data are expressed as relative values based on the reference root expression set to 1.0 at each stage. Error bars represent + SE for three independent trials.

Generation of *SlARF8A* and *SlARF8B* mutants by the CRISPR/Cas9 gene-editing system.

To gain insights into the physiological significance of *SlARF8A* and *SlARF8B*, *slarf8* mutants were generated using CRISPR/Cas9 gene-editing technology. Due to the high sequence identity between *SlARF8A* and *SlARF8B*, two synthetic guide RNAs (sgRNAs) were

designed for *SlARF8A* and *SlARF8B* separately. To obtain *SlARF8A* and *SlARF8B* double mutants, the first sgRNA was designed in a region that is identical at the sequence level in both genes (Figure 5(a)). Following the transformation lines screening, six independent R_0 lines were generated and validated for the presence of the construct in their genome. All mutants displayed mutations in the targeted region and similar plant features. Then in R1 and R2 generations, more progeny lines were confirmed for the presence of mutations. Three Cas9 free and homozygous mutant lines that contains different mutations were selected in the following experiments. These three editing types can be classified as *SlARF8A* single mutation (*slarf8a-cr*), *SlARF8B* single mutation (*slarf8b-cr*) and *SlARF8A&B* double mutation (*slarf8a&b-cr*) (Figure 5(b)). It is interesting to notice that the editing rates of the two target sequences of each gene were 100% for sgRNA1 and 0% for sgRNA2, and the deletion/insertion mutations led to a frame shift mutation followed by an early stop codon leading to the expression of truncated SlARF8 proteins. The mutation sequence of these three editing types are shown in Figure 5 (b, c). For *slarf8a-cr*, it shows a deletion of 2bp on the sgRNA1 targeted region, leading to a 13 amino acid (AA) protein sequence compared with 845 AA in wild type (WT); for *slarf8b-cr*, there is a 1bp insertion on the sgRNA1 targeted region, leading to a 14AA protein sequence rather than 843 AA in WT; and, for *slarf8a&b-cr*, there is a 2bp deletion on *SlARF8A* and a 4bp deletion on *SlARF8B* at the same time, which contributes to a 13 AA SlARF8A protein and a 20 AA SlARF8B protein respectively. Even though there are no large fragment deletions found in these mutants, the extremely short and frame-shifted protein sequence compared with WT could lead to the defection of SlARF8A and SlARF8B function.

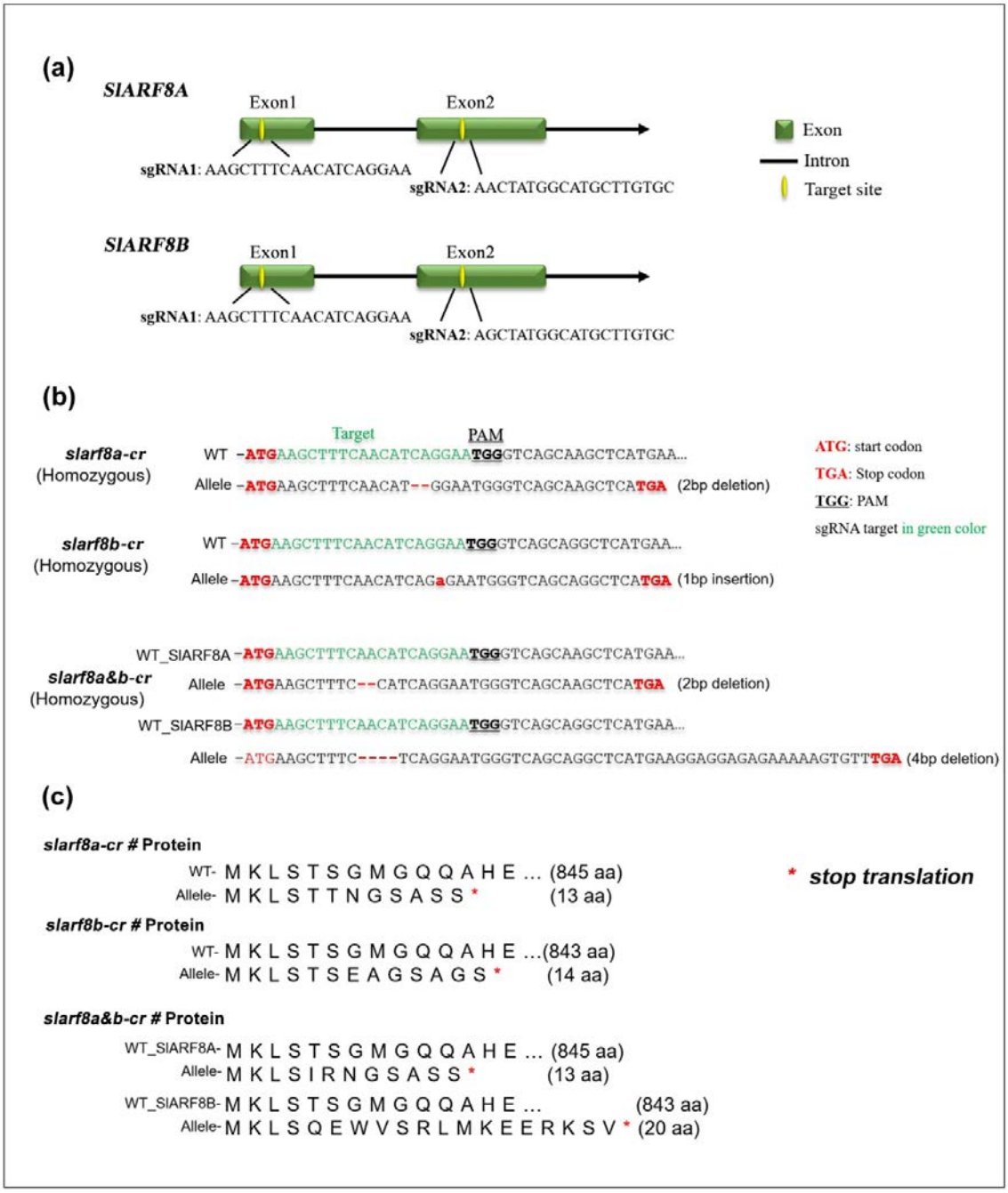


Figure 5. Mapping of CRISPR/Cas9 genome and protein editing on *SlARF8A* and *SlARF8B*. (a) The schematic illustration of the two CRISPR/Cas9 target sites in *SlARF8A* and *SlARF8B* genome sequence respectively. (b) Genome editing analysis of *slarf8a-cr*, *slarf8b-cr*, and *slarf8a&b-cr* mutants. (c) Translated protein editing analysis of *slarf8a-cr*, *slarf8b-cr*, and *slarf8a&b-cr* mutants.

CRISPR/Cas9-mediated *slarf8a-cr, slarf8b-cr,* and *slarf8a&b-cr* mutants exhibited parthenocarpy and vegetative defect.

All of the three types of *slarf8-cr* mutants displayed a heritable dwarf phenotype in each generation starting from R_0 mutant lines. The *slarf8a-cr, slarf8b-cr,* and *slarf8a&b-cr* mutants were used for further analysis, and evident differences in vegetative growth or fruit development were observed between these mutants and WT plants. The *slarf8a-cr, slarf8b-cr,* and *slarf8a&b-cr* mutants all showed dwarf plants compared with WT plants; *slarf8b-cr* presented the most severe dwarf plant phenotype, which reduced to a height of 16.5cm compared with the WT height of 34.5cm, while *slarf8a-cr* showed the slightest dwarf phenotype, reducing to a height of 27.5cm (Figure 6 (a, b)).

Moreover, fruit development was also dramatically affected in mutants compared with WT plants (Figure 6 (c, d)). It was observed that all *slarf8a-cr, slarf8b-cr,* and *slarf8a&b-cr* mutants harvested marked smaller fruit compared to WT. The quantification of weight and size mean (fruit height and diameter) of fruits is fully consistent with the observation. Notably, *slarf8a&b-cr* presented the most marked reduction in fruit weight and size compared with *slarf8a-cr* and *slarf8b-cr*, with the decreases of 65.3%, 32.2%, and 32.4% in weight, diameter and height respectively. Mutant *slarf8b-cr* displayed the decreases of 48.7%, 22.8% and 28.4% in weight, diameter and height respectively, while *slarf8a-cr* displayed the most slightly reduction. In addition, all *slarf8a-cr, slarf8b-cr*, and *slarf8a&b-cr* mutants produced seedless fruits. Overall, the knockout of *SlARF8A* and *SlARF8B* genes in tomato displayed not only vegetative phenotypes but also showed reproductive effects.

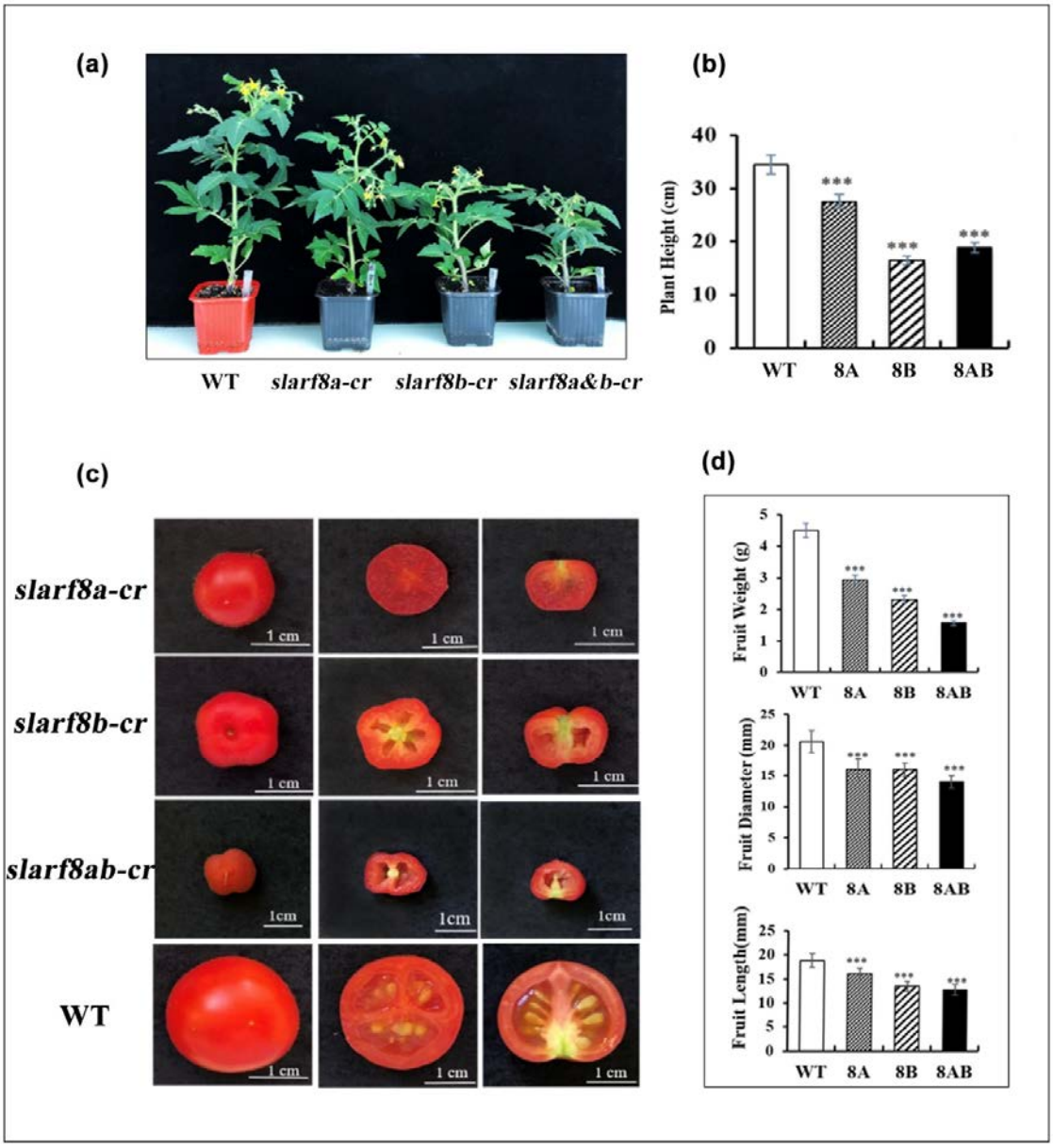


Figure 6. Mutants *slarf8a-cr, slarf8b-cr,* and *slarf8a&b-cr* exhibited parthenocarpy and vegetative phenotypes. (a)(b) Decreased plant height of *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants compared with WT lines. 30 plants were analyzed for each line. 8A, 8B, 8AB represents *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants respectively. (c) Comparison of the red fruits of *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants with WT in intact fruit, in cross-section and vertical section. (d) Decreased fruit size (diameter, weight, height) in *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants compared with WT. 8A, 8B, 8AB represents *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants respectively. Over 150 fruits were measured for each line of *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants. 40 fruits were measured in WT.

In WT plants, the successful fruit setting depends on successful pollination and fertilization, which requires viable pollen and ovary. To better understand the origin of seedless fruits in *slarf8-cr* mutants, *in vitro* pollen germination assay was carried out in mutants and WT plants (Figure 7). It was shown that there are no apparent differences in pollen germination between mutants and WT plants, thus excluding the possibility that seedless fruits arose from non-viable pollens in mutants. In addition, an emasculation assay was performed on three types of *slarf8-cr* mutants and WT lines (Table 1). It is obvious to observe that around 25% to 39% of emasculated flowers from all 3 types of *slarf8-cr* mutants remained attached to plants and developed into seedless (parthenocarpic) fruits, which indicates that *SlARF8A* and *SlARF8B* may act as a negative regulators of fruit set and development in WT plants.

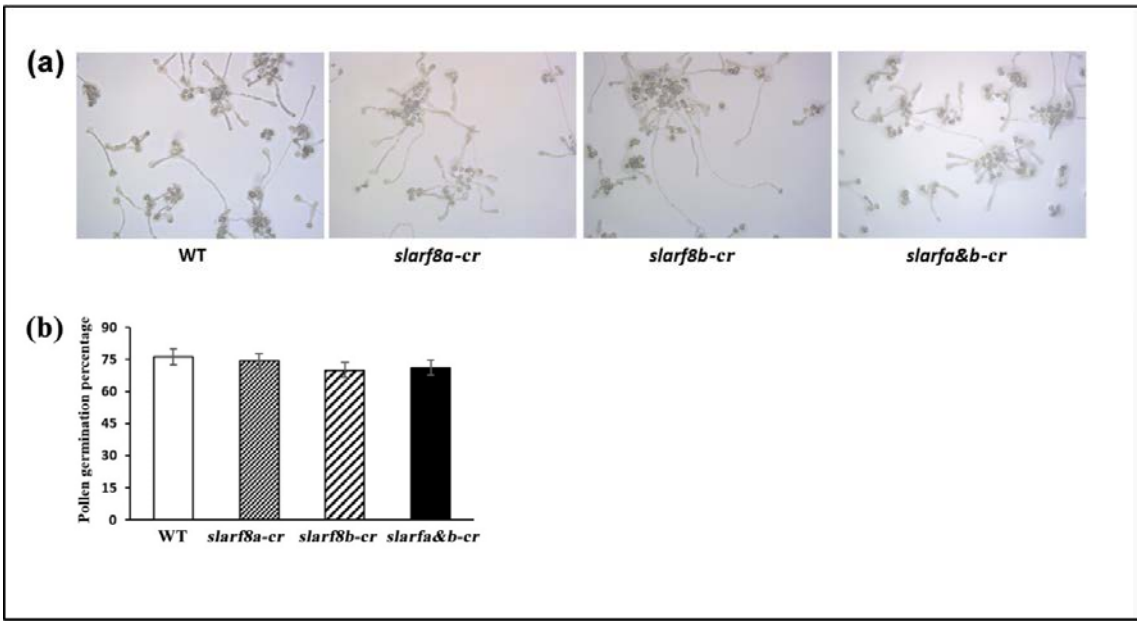


Figure 7. *In vitro* analysis of pollen germination in *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants and WT. (a) Germinated pollen of *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants and WT under microscopy. Pollens collected from 2DPA flowers and incubated in the germinating medium for 4 hours. (b) Statistical analysis of pollen germination percentage in WT compared with *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants. 15 flowers collected randomly in 3 Petri dishes each line, 20 pictures were taken randomly for every Petri dish.

Table1. Emasculation assay of *slarf8-cr* mutants[a]

Code	Fruit set/Emasculation	Fruit set (%)
WT	0/15	0
slarf8a-cr	6/25	24
slarf8b-cr	8/20	40
slarf8a&b-cr	7/21	33

a. Flowers of *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants and WT were emasculated 1-2 days before anthesis to assess the ability of fruit setting in the absence of pollination. The results represent two independent trials.

Histological observations on pollinated ovaries sections stained with toluidine blue revealed major changes in the pericarp and locular tissue (Figure 8). In the inner part of both *slarf8a-cr* and *slarf8b-cr* fruits, a significant increase of pericarp area was observed. In addition, the locular tissue all decreased in these two types of mutants. It is interesting to observe that, on the one hand, the reduction of locular tissue in *slarf8a-cr* was restricted to the increase of both pericarp and placenta tissue, while for *slarf8b-cr* the decreased locular tissue did not correspond to the increased placenta tissue. On the other hand, the placenta tissue has a marked reduction compared with both WT and *slarf8a-cr.* Moreover, these features correspond to the observation of red fruits showed in Figure 6. It is interesting to notice that all *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* mutants have much smaller fruits in the ripening stage, while *slarf8a-cr* presented more “juicy” red fruits with normal locular gel, *slarf8b-cr* and *slarf8a&b-cr* displayed almost no locular gel in their red fruits.

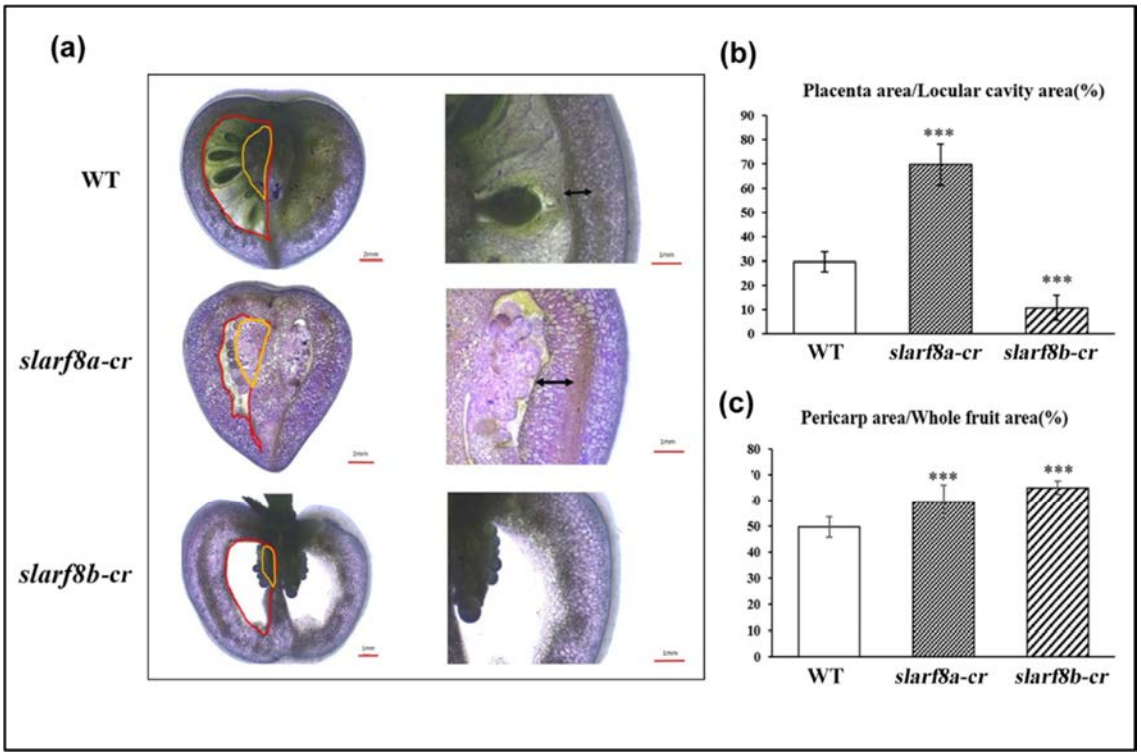


Figure 8. Dramatic modifications in young fruits triggered by *slarf8-cr* mutants (a) Histological observations of fruit in *slarf8a-cr*, *slarf8b-cr,* and WT at the DPA15 stage. Sections were stained with toluidine blue**.** (b)(c) Relative proportions of DPA20 fruit inner tissue of WT, *slarf8a-cr,* and *slarf8b-cr* mutants. (b) Placenta area compared with the locular cavity area. (c) Pericarp area compared with the whole fruit area. A total of 20 fruits were used for each line measurement. ***P<0.001.

In WT, seedless fruits can be produces naturally. We observed that seeded fruit percentage decreased from 67.85% in WT to 41.25% in *slarf8a-cr*, 28.75% in *slarf8b-cr* and 5.44% in *slarf8a&b-cr* under nature pollination conditions (Table 2). Moreover, the shapes of the seed changed in mutants, and the seeds presented smaller sizes and irregular shapes compared with WT (Figure 9).

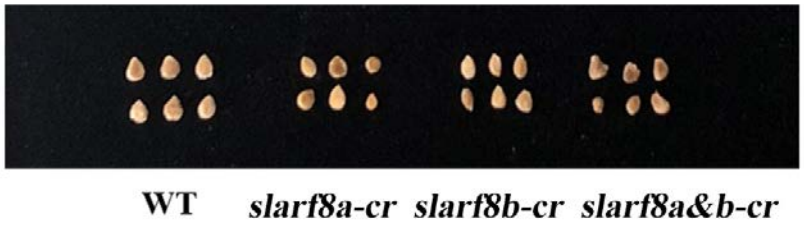


Figure 9. Morphological characters of mutants seeds. The *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* presented irregular shapes of seeds compared with WT.

Table2. Seed characteristics in *slarf8-cr* mutants

Seeds	WT[a]	*slarf8a-cr*[b]	*slarf8b-cr*[b]	*slarf8a&b-cr*[b]
Fruit with seeds (%)	68.75	41.25	28.75	5.44
Average seed number per fruit	11.01	3.45	2.11	0

a. 80 fruits were collected randomly from 5 WT plants.
b. Over 80 fruits were collected randomly from 5 plants in *slarf8a-cr, slarf8b-cr, and slarf8a&b-cr* respectively.

Genome-wide transcriptomic profiling of *slarf8a-cr* and *slarf8b-cr during* fruit setting.

Due to the significant physiological and histological changes that were observed during the fruit set process in *slarf8a*, *slarf8b* and *slarf8a&b* mutants, genome-wide transcriptomic profiling was performed in mutants and WT flowers at anthesis stage (0DPA) and young fruits harvested four days after pollination (4DPA) (Supplementary Figure2). Total RNA samples were prepared from three independent biological replicates and used to performe deep RNA sequencing. Around 30 million of reads have been generated for each repeat; at least 89% were mapped to the latest MicroTom tomato genome sequence (http://tomatogenome.gbfwebtools.fr/) and the level of gene expression was calculated for each annotated tomato gene (as indicated in Materials and Methods).

The whole gene expression data in the different samples were subjected to a principal component analysis to assess transcriptome similarities between the biological repeats and in the two conditions (Figure 10 (a)). The results revealed two discrete groupings: one group consisting of the samples of 0DPA ovaries, and the other consisting of the samples of 4DPA fruits. In addition, each three biological replicates clustered together indicated that the

experiment was performed correctly. It was shown that at 0DPA stage, the variance between *slarf8b-cr* and WT on PC1 is larger than *slarf8a-cr* and WT; while it shows a different pattern in stage 4DPA, when the variance on PC2 between *slarf8a-cr* and WT is dramatically larger than *slarf8b-cr* and WT, which indicates a possible different effect of *SlARF8A* and *SlARF8B* loss of function on gene expression.

To identify DEGs between WT and *slarf8-cr* in flowers and young fruits, the expression of each gene within the library of each developmental stage of WT was compared to that of *slarf8-cr* mutants (pairwise comparisons) and then filtered using |log2 (fold change)| >1 and adjusted P-value < 0.05. It is shown in Figure 10(c) that, in 0DPA_ *slarf8a-cr* versus 0DPA_WT (0DPA_ *slarf8a-cr* vs 0DPA_WT), the DEGs number is only 128 DEGs, while, in 4DPA_ *slarf8a-cr* vs 4DPA_WT, the DEGs number increased to 16231. Meanwhile, the numbers of DEGs in 0DPA_ *slarf8b-cr* vs 0DPA_WT and 4DPA_ *slarf8b-cr* vs 4DPA_WT is 4457 and 4326 respectively. Moreover, there is more up-regulated DEGs than down-regulated DEGs in each of the above comparisons. In addition, we compared the sets of DEGs among the comparison groups. Due to the less DEGs in 0DPA_ *slarf8a-cr* vs 0DPA_WT comparison, the common differentially expressed genes of all the groups is 40 (Figure 10(b)).

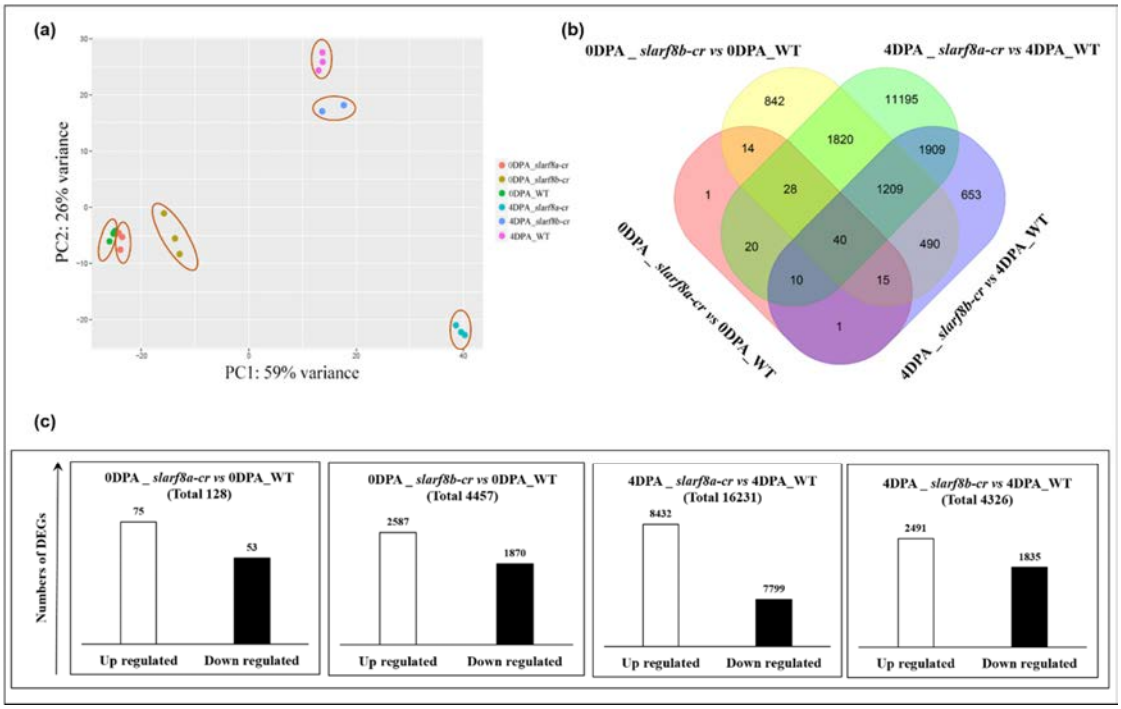


Figure 10. RNA-Seq profiling of 0DPA ovary and 4DPA fruits in *slarf8-cr* and WT. (a) Principal component analysis and cluster analysis based on all expressed genes in WT and *slarf8-cr* mutants**.** The projection of axes 1 and 2 that held 85% of the inertia showed six distinct groups of experiments. (b)Venn diagram of DEGs and the numbers of up and down-regulated DEGs in different experiments. (c) The numbers of up and down-regulated DEGs in different experiments.

In order to identify specific functions impacted by *SlARF8*, DEGs were associated with their respective MAPMAN gene annotation category and the overview of categories enriched in different experiments was presented in Figure 11(a). The functional categories displaying the highest over-representation were determined using a Wilcoxon rank sum test on the MapMan BiNs for the *slarf8a-cr* versus WT and *slarf8b-cr* versus WT experiments (Figure 11(b)). When comparing WT and *slarf8b* 0DPA or 4DPA, 'Vesicle trafficking' and 'Chromatin organization' categories were over-represented for both stages (Figure 11(b)). Since *slarf8a-cr* and *slarf8b-cr* plants exhibited a thicker pericarp and abnormal placenta tissue, we focused on the analysis on DEGs in the cell wall organization category. For 0DPA_*slarf8b-cr* vs 0DPA_WT, 147 genes (24%) were differentially expressed out of the 589 annotated cell wall organization genes, and 66% were down-regulated. Among these genes, many of which

encoding proteins in cell wall metabolism were down-regulated in *slarf8b-cr* 0DPA ovaries, which includes alpha-class expansin family and galactosyltransferase related genes. Also, genes related with pectin metabolism were differentially expressed, such as pectin methylesterase, pectate lyase and pectin acetylesterase. Further, genes involved in cutin and suberin formation, cellulose synthase were expressed differently compared with WT. In the 4DPA_*slarf8a-cr* vs 4DPA_ WT experiment, more cell wall related genes were differentially expressed among 332 genes (56% out of 589 annotated cell wall genes), while most were up-regulated, which shows the different pattern compared to *slarf8b-cr*. Besides, the cell cycle organization category was enriched in *slarf8a-cr* vs WT at 4DPA, which means that at 4DPA, many genes related with cell division were differential expressed in *slarf8a-cr* mutants, so *SlARF8A* may play an important role in cell divison after pollination and fertilization in tomato.

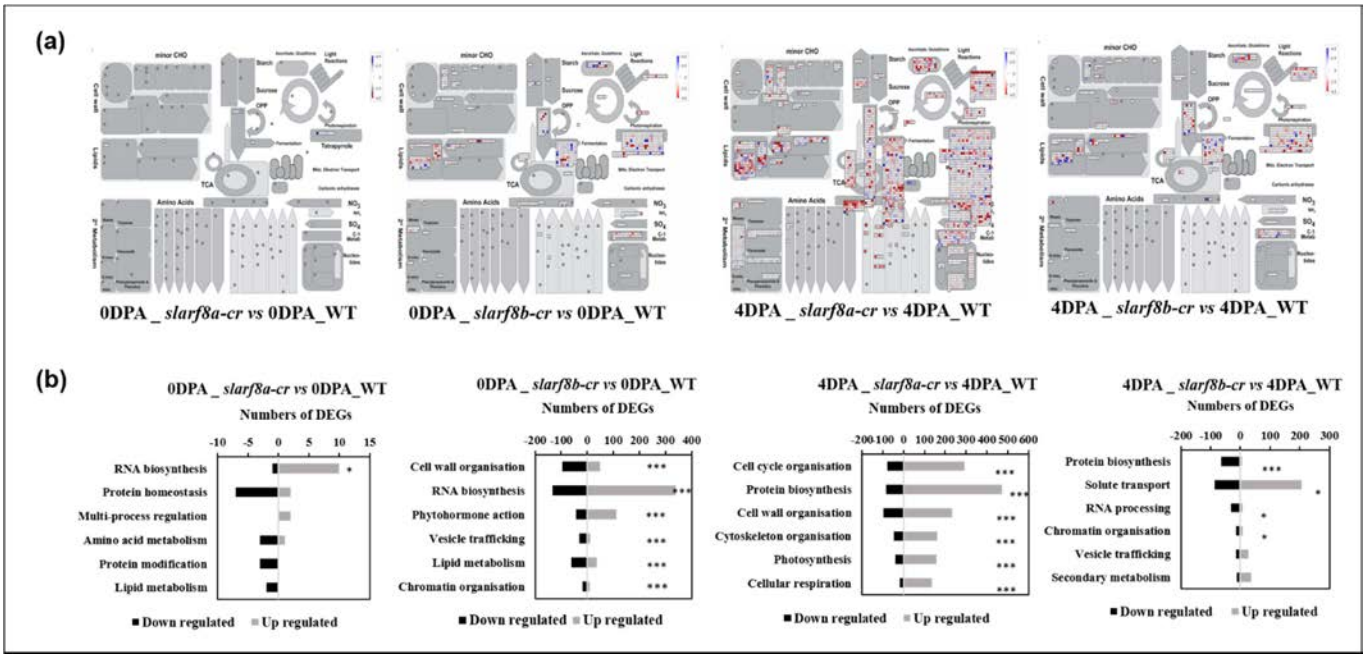


Figure 11. Mapman functional categories enrichment analysis of DEGs. (a) Overview of functional categories enriched in different experiments. (b) The top six categories enriched in different experiments. P-values shown by stars.* represents P-value <0.05, ** represents P-value <0.01, *** represents P-value <0.001. The black bar represents down-regulated genes, while, the white bar represents up-regulated genes.

Regarding that *SlARF8A* and *SlARF8B* genes are transcription factors related to auxin signaling, we focused on the DEGs with functional categories that are belong to phytohormone and found that among the DEGs, several are linked to GA, auxin, and cytokinin. It is interesting to notice that almost all the DEGs involved in the metabolism of these three phytohormones are up-regulated, including the GA biosynthesis gene *GA20OXs* and *GA3OXs*, the auxin biosynthesis gene *YUCs* and transport genes *PINs*, bioactive cytokinin synthesis enzyme LOGs and cytokinin oxidase CKXs. For ethylene metabolism, *ACS2* was down-regulated while all *ACOs* in DEGs were up-regulated, except *ACO2* and *ACO4*. It is interesting to observe that the MAPMAN vesicle trafficking bin is enriched in both 0DPA_*slarf8b-cr* vs 0DPA_WT and 4DPA_*slarf8b-cr* vs 4DPA_WT experiments, while the expression pattern is contrary. In 0DPA *slarf8b-cr* vs 0DPA_WT, 71% DEGs were down-regulated and this ratio reached 88% when considering only DEGs with high expression and a marked difference (|log2fold|>1); however in 4DPA *slarf8b-cr* versus WT, 66% of DEGs were up-regulated.

A promoter analysis of the down-regulated and identified DEGs (|log2fold|>1) in *slarf8b-cr* vs WT at 0DPA and 4DPA was performed, which aims to find the potential target genes of *SlARF8B* (Table3). By searching the AuxRE elements in the 2000bp region before the translation start codon (ATG), several potential binding positions were found in 55% of the DEGs. The list included the hormone related genes: *ACS*, cytokinin related genes *CKX*, *ARR*, *SAURs*, *IAAs*, *TSB*, *YUC* and *TAA* (Table3).

Table 3. List of down-regulated DEGs in *slarf8b-cr* vs WT and their potential binding positions in promoter. (|Log2fold|>1, p-value<0.05)

Sly Number[a]	Solyc Number	Log2Fold	Binding Positions in Promoter[b]	Gene Name
Down-regulated and identified DEGs in 0DPA_*slarf8b-cr* vs 0DPA_WT (|Log2fold|>1, p-value<0.05)				
Sly10g0162061	Solyc10g005320	-1.522105548	-1646	*TSB-like6*
Sly03g0210461	Solyc03g093790	-1.184624524	N/A	*SlCycU4*
Sly12g0049131	Solyc12g005580	-3.496118978	-414/-1372/-1844	*Syntaxin124*
Sly06g0375261	Solyc06g072680	-1.971073229	-861	*HVA22E*
Sly10g0179341	Solyc10g054770	-6.727697197	-168	*SAUR81*
Sly04g0244921	Solyc04g052980	-3.536266402	-302	*SAUR*
Sly06g0377801	Solyc06g074950	-2.730165548	-1560/-1587	*SAUR62*
Sly08g0280591	Solyc08g078180	-2.084961303	N/A	*SlERF.A1*
Sly01g0030361	Solyc01g095080	-3.685249478	N/A	*ACS2*
Sly09g0098461	Solyc09g075860	-1.010257422	-1628	*LOX5-like7*
Sly09g0102051	Solyc09g091030	-3.045213465	N/A	*BAM7-like*
Sly06g0369751	Solyc06g066020	-1.416415691	N/A	*SlAux/IAA36*
Sly02g0332491	Solyc02g076670	-3.097449317	-544	*ARR18-like*
Sly09g0101561	Solyc09g090630	-1.046988367	-992/-1514	*SlSDG12*
Sly05g0150661	Solyc05g031600	-2.750836466	-1960/-2023	*TAA1-like3*
Down-regulated and identified DEGs in 4DPA_*slarf8b-cr* vs 4DPA_WT (|Log2fold|>1, p-value<0.05)				
Sly03g0220501	Solyc03g115770	-1.054348921	N/A	*ARR1-like2*
Sly05g0134911	Solyc05g006420	-1.263842349	N/A	*ARR5-like4*
Sly10g0184211	Solyc10g079700	-1.179182114	-1015	*ARR8*
Sly10g0184111	Solyc10g079600	-1.614754551	-140/-369	*ARR9*
Sly12g0051891	Solyc12g008900	-1.706139807	N/A	*CKX3*
Sly01g0026241	Solyc01g088160	-1.59877318	-169	*CKX6-like*
Sly01g0043191	Solyc01g107400	-1.019298014	N/A	*Conjugation*
Sly04g0230751	Solyc04g008110	-1.034980648	-292	*HK4*
Sly05g0138201	Solyc05g009410	-2.399249277	N/A	*IPT1-like2*
Sly10g0187841	Solyc10g084010	-4.350443663	N/A	*SAUR*
Sly05g0149471	Solyc05g025920	-2.343774413	N/A	*SAUR*
Sly08g0281591	Solyc08g079150	-1.056545477	-1787	*SAUR*
Sly03g0207951	Solyc03g082510	-1.20348251	N/A	*SAUR35*
Sly03g0207961	Solyc03g082520	-3.794878192	N/A	*SAUR36*
Sly01g0046515	Solyc01g110580	-1.216956096	-172	*SAUR5*
Sly04g0257361	Solyc04g081250	-1.518209519	N/A	*SAUR51*
Sly12g0052351	Solyc12g009280	-2.392573225	N/A	*SAUR98*
Sly01g0021881	Solyc01g079790	-1.06942241	-499	*SlAGPase-L3*
Sly06g0354661	Solyc06g008590	-3.021740736	-488/-1548	*SlAux/IAA17*
Sly06g0364111	Solyc06g053840	-1.047646132	-1753	*SlAux/IAA4*
Sly03g0193761	Solyc03g007460	-1.305400826	-109/-1643	*SlERF.G3*
Sly06g0370381	Solyc06g066540	-2.782206714	N/A	*SlERF.H7*
Sly01g0001761	Solyc01g006680	-1.207212029	-1732	*SlJMJ24*

Sly12g0049131	Solyc12g005580	-2.450020451	-414/-1372/-1844	*Syntaxin124*
Sly05g0150661	Solyc05g031600	-3.684633389	-1960	*TAA1-like3*
Sly09g0102131	Solyc09g091090	-1.324194903	-653/-808	*YUC-like3*

a. The corresponding number of genes in Mirotom reference genome used in this study (http://tomatogenome.gbfwebtools.fr/). b. The number represents the possible binding positions of the listed DEGs, which considers the 2000bp sequence region before the start codon (ATG) as promoter sequence of each gene. The position of start codon be considered as 0. The positions vary from one to 3 for listed DEGs.

Discussion

Previous work in Arabidopsis showed that *AtARF8* might be involved in the regulatory complex which negatively regulates the fruit set process (Wu *et al.*, 2006; Goetz *et al.*, 2006, 2007). In order to explore further about fleshy fruit initiation, reverse genetics through CRISPR/Cas9 genome editing approach was performed to knockout *SlARF8* genes in tomato. We found that *SlARF8A, SlARF8B and SlARF8A&B* knockout mutants showed seedless fruits, dwarf plants, and altered placenta tissue. The genome-wide transcriptomic profiling of *slarf8a-cr* and *slarf8b-cr* mutants allow us to gain insight of effect of loss of function of these genes on global tomato gene expression, which can provide us an identification of the potential target genes of *SlARF8A* and *SlARF8B*. Our results showed that *SlARF8A* and *SlARF8B* may control the expression of different set of genes, thus they may play different and important roles in the tomato fruit initiation process.

SlARF8A/B and fruit development

So far, SlARF8 has been identified as the homolog of Arabidopsis FRUIT WITHOUT *FERTILIZATION (FWF)/ARF8* (Goetz et al., 2007). The *fwf/arf8* mutants that do not produce functional FWF/ARF8 proteins form parthenocarpic siliques (Goetz et al., 2006), indicating that fruit set is not only regulated by positive growth factors but also by negative regulators. Silencing of *SlARF7*, another auxin response factor, leads to parthenocarpic fruit growth, suggesting that *SlARF7* also acts as a negative regulator of fruit set (De Jong et al. 2008). *SlARF7* belongs to the same subclade of *SlARF8A* and *SlARF8B*, and its transcript levels decrease after pollination, similar to *FWF/ARF8* (De Jong et al. 2008). In addition, both genes

are expressed in the vascular tissues of the funiculi, suggesting that *SlARF7* is a functional equivalent of *FWF/ARF8*.

In this study, we showed that *SlARF8B* transcripts accumulation was higher than *SlARF8A* at anthesis stage and decreased after pollination and fertilization like *SlARF7* and *FWF/ARF8*. In contrary, we found that the tomato *SlARF8A* transcripts displayed a notable increase after pollination and fertilization, suggesting that *SlARF8A* and *SlARF8B* might function differently during fruit set although *SlARF8A* or/and *SlARF8B* knockout mutants presented parthenocarpic fruits. Previous research demonstrated that the parthenocarpy feature of Arabidopsis *arf8* mutants is not produced if floral whorls surrounding the carpel are not removed, which indicates that emasculation removes the parthenocarpy inhibition signals from these tissues. Moreover, some studies showed that pollen is the inhibition signals for parthenocarpy (Vivian-Smith *et al.*, 2001; Goetz *et al.*, 2007). However, in our study, the parthenocarpy features do not exhibit a noticeable difference before and after emasculation, and the parthenocarpic trait is observed steadily in progeny like what was observed in *SlARF7*-RNAi down regulated lines (De Jong et al. 2008).

Furthermore, *slarf8a-cr* and *slarf8b-cr* both exhibited an increased pericarp area and undeveloped ovules, accompanied by altered morphological characteristics of the placenta area compared to WT fruits. We found that the placenta area is increased in *slarf8a-cr* and decreased in *slarf8b-cr* with no locular gel in both kind of mutant fruits. The reduced placenta and the absence of locular gel traits in *slarf8b-cr* are similar in *SlARF7* RNAi lines, which has very few locular gel around the placenta (de Jong *et al.*, 2009b). Previous studies demonstrated that mucilage around the placenta provides the proper physical medium for pollen tube growth, meaning the absence of locular gel may hinder the pollen tube reaching to the ovary at the pollination stage (Webb & Williams, 1988; Genetic *et al.*, 1994). In contrast, the placenta and pericarp area both increased in *slarf8a-cr.* These morphological changes make the space for locular gel growth limited. So far, down regulation of the class D MADS- box gene *SlMBP3* showed fleshy placental tissues with no locular gel and defective seeds, and the down regulation lines of another class D MADS- box gene *TAG1* in tomato also presented no locular gel, which was confirmed that *SlMBP3* and *TAG1* play influential roles in regulating placenta

development (Garceau *et al.*, 2017; Zhang *et al.*, 2019). Therefore, we supposed that *SlARF8A* might be regulated by the class D MADS- box gene *SlMBP3* and *TAG1*.

Potential regulatory mechanism of *SlARF8A* and *SlARF8B* in fruit set process

Our data suggest that *SlARF8A* and *SlARF8B* repress fruit development in tomato. Previous studies have shown that this process is regulated by activating the genes that repress fruit initiation directly or by heterodimerizing with AUX/IAA proteins to repress fruit initiation (Guilfoyle *et al.*, 1998; Ulmasov *et al.*, 1999; Liscum & Reed, 2002; Guilfoyle & Hagen, 2007). Moreover, it has been demonstrated that the silencing of *SlAux/IAA9* led to parthenocarpic fruits in tomato (Wang *et al.*, 2005a, 2009). The ARF8 and AUX/IAA9 complex could bind with the promoters of auxin responsive genes which plays essential roles in regulating fruit initiation, then in Wild type an auxin burst caused by pollination and fertilization induce the degradation of this ARF8 and AUX/IAA9 complex, then unknown factors activate the expression of those fruit initiation genes (O'Neill, 1997; Wang *et al.*, 2005a; Goetz *et al.*, 2006, 2007; Du *et al.*, 2016). Combined with our results, the early stop codon on the mutation region of *slarf8a-cr* and *slarf8b-cr* lead to very small truncated SlARF8A and SlARF8B proteins free of AUX/IAA dimerization domain. Thus we assumed that when no SlARF8A or SlARF8B protein could interact with AUX/IAA9 to form a repression complex, the expression of fruit initiation genes can be activated in the absence of pollination and fertilization, leading to seedless fruits and fruit development.

Previous studies showed that both auxin and GA play important roles in fruit initiation and subsequent growth, auxin promotes cell division and GA promotes later cell expansion (Srivastava & Handa, 2005; Serrani *et al.*, 2007; Ruan *et al.*, 2012). Auxin was reported to increase GA level by upregulating GA biosynthesis genes, and further studies reported that the GA and auxin regulate fruit initiation by the crosstalk between SlDELLA and SlARF7/SlIAA9 (Dorcey *et al.*, 2009; Hu *et al.*, 2018). GA response pathway is negatively regulated by DELLA proteins, moreover, GA receptor GID1 induces the GID1-DELLA interaction which leads to the degradation of DELLAs thus releasing growth repression imposed by DELLAs (Peng *et al.*, 1997; Ueguchi-Tanaka *et al.*, 2005; Murase *et al.*, 2008; Davière & Achard, 2016; Hu *et al.*, 2018; Tomlinson *et al.*, 2019).

Our global transcriptomic study using RNA-Seq approach showed that in the up-regulated DEGs lists of *slarf8b-cr*, *GID1* and GA biosynthesis gene *GA2OX2-like* contain the putative AuxRE elements. It has been shown that SlARF7/SlIAA9 and SlDELLA adversely regulate the expression of GA and auxin feedback-regulated genes, including *GA20ox1* and *GA3ox1* and auxin deactivation gene *GH3.2* (Serrani *et al.*, 2008; Kumar *et al.*, 2012). Those GA and auxin metabolism feedback-regulated genes are supposed to be repressed by the SlARF7/SlIAA9 protein complex but are induced by SlDELLA. The binding of SlDELLA to SlARF7 may remove the inhibition of SlARF7/SlIAA9 protein complex, activating the transcription of these genes (Hu *et al.*, 2018). This regulation model was supposed to be suitable for SlARF8B, since the data displayed the up-regulation of GA and auxin feedback-regulated genes *GA20OXs*, *GA3OXs*, and *GH3.4* in *slarf8b-cr*. Besides, the SlARF8B interacts with SlIAA9, and SlDELLA involves with SlARF8B have been confirmed by Y2H assay (Hu *et al.*, 2018). Therefore, the emerging question from this findings is to know whether SlARF8B could interact with SlIAA9 and SlDELLA to regulate GA and auxin regulated genes, which may elucidate the parthenocarpic fruit formation mechanism in ARFs mutants, and gain insight of a possible crosstalk between auxin and GA signaling through these genes to control the fruit set process.

Materials and methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum cv* MicroTom) seeds were air-dried and sterilized, then sown on 50% Murashige and Skoog (MS) medium (pH 6) with 0.8% (w/v) agar. After 2 weeks, the tomato seedlings were transferred to soil under conditions in a culture chamber: 14 h day/10 h night cycle, 25/20 °C day/night temperature, 80% relative humidity, 250 µmol $m^{-2}s^{-1}$ intense luminosity.

Vector construction and plant transformation

For CRISPR/Cas9 construct, the sgRNAs were designed by using the CRISPR-P website tool (http://cbi.hzau.edu.cn/crispr/) and are listed in Supplementary Table1. The four sgRNAs were cloned into pAGM4723 final vector by golden gate ligation method.

All constructs were confirmed by sequencing before introduction into the C58 Agrobacterium strain. Tomato seedlings were used for the next step plant transformation according to Hao *et al.*(2015).

Subcellular localization

The full length cDNAs of *SlARF8A* and *SlARF8B* gene were fused with green fluorescent protein (GFP) and cloned into the pGreen vector backbone under the 35S CaMV promoter. Protoplasts were obtained from suspension cultured tobacco BY-2 cells and were transfected according to previous described methods (Leclercq *et al.*, 2005), the confocal fluorescence images were obtained under a confocal laser scanning microscopy.

Genome DNA extraction and mutation analysis

Total genomic DNA was extracted from liquid nitrogen frozen fresh leaves of tomato by using a ReliaPrep™ gDNA Tissue Miniprep System (Promega) and used as the templates for amplifying the designed mutation region sequence using primers designed flanking the target sites. The PCR product was purified by performing the Wizard® SV Gel and PCR Clean-Up System kit (Promega) and then sequenced to identify mutations. The primers used for PCR are listed in Supplementary Table1.

Total RNA extraction and RT-PCR

Total RNA was extracted from roots, leaves, stems, flowers and seedlings by using a Plant RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and total RNA was extracted from tomato fruits according to Hao *et al.*(2015). The extracted RNA were then treated with DNaseI, and then 1ug of total RNA was reversed to cDNA by using an Omniscript Reverse Transcription kit (Qiagen). RT-PCR was performed in a 10 μl volume reaction applying the SYBR Green PCR Master Mix on an ABI PRISM 7900HT sequence detection

system (Applied Biosystems). *Sl-ACTIN* (Solyc11g005330) was used as a reference gene. Primers used for RT-PCR are listed in Supplementary Table1.

Histological observations

To observe the *SlARF8A* and *SlARF8B* promoter-GUS fused transgenic lines, GUS staining solution (100uM sodium phosphate buffer, 10uM EDTA, 0.1% Triton, 0.3mg/ml X-Gluc, pH 7.2) was used to treat different organs for 4-12 hours under 37 ℃, then samples were washed by 35%, 50%, 75% and 95% ethyl alcohol solution several times under 60 ℃. All samples were observed under an Axio Zoom V16 microscope.

To observe the fruit anatomy, fruits of different stages were hand-cut and stained by 0.01% toluidine blue O for 30s to 60s, and then rinsed by distilled water. All samples were observed under an Axio Zoom V16 microscope.

Pollen germination assay

Pollen was collected from 5 flowers in per plant at stage 1-2 DPA of mutants and WT and put on one pollen germination culture medium, and this step was repeated for 3 times (Firon *et al.*, 2012). The culture dishes were covered with silver paper and put in the culture room 14-h-day/10-h-night cycle, 25/20°C day/night temperature for 4 hours. Then the samples were observed under microscopy, 10 pictures were taken randomly from one culture dish.

Flower emasculation and cross fertilization assay

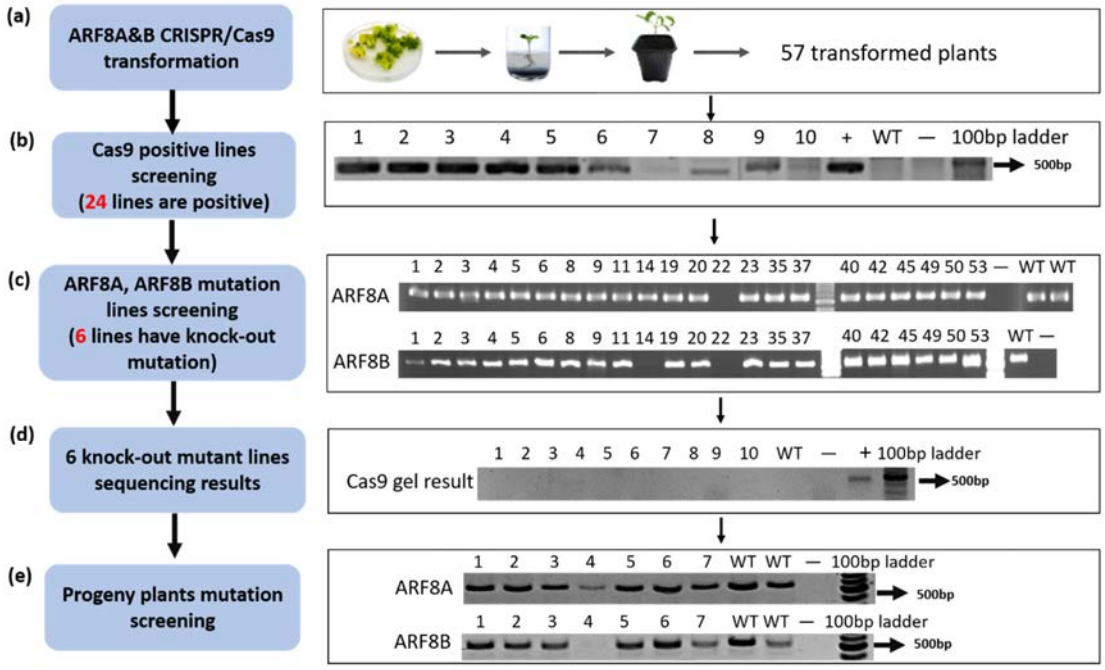
Flower anthers were removed before anther dehiscence to avoid accidental self-pollination. Cross-pollination was performed on emasculated flowers before anthesis. 10 flowers per plant were kept for both emasculation assay and cross fertilization assay.

RNA-Seq analysis and data processing

The DNase I treated total RNA samples were analyzed by Agilent RNA 6000 Nano Assay, which RIN value above 8 was selected for further sequencing. Raw paired-end RNA-seq sequences in FASTQ format were analyzed as follows. First, low quality reads were removed using the FASTX toolkit version 0.0.13 (http://hannon- lab.cshl.edu/fastx_toolkit/). Trimmed

reads were then mapped to the *S. lycopersicum* reference genome and gene annotation (ITAG2.4) using TopHat-2.0.14 (Trapnell *et al.*, 2009) calling bowtie 2.1.0 (Langmead & Salzberg, 2012). The differential expression analysis has been carried out with the DESeq2 R package (https://www.r-project.org) with default settings (Love *et al.*, 2014). The normalization method used by default (LRE) agrees with the assumption that <50% of genes are up-regulated and <50% of genes are down-regulated between two given conditions (Maza *et al.*, 2013; Maza, 2016). The false discovery rate (FDR) is controlled by the Benjamini–Hochberg method; genes were declared as differentially expressed genes (DEGs) if the adjusted P-value was <0.05. All statistical analyses have been performed with the R software. Expression data were visualized by using the MapMan software.

Supplemental figures and tables



Supplemental Figure1. The process of stable CRISPR/Cas9 mutation screening. (a) The transformation of *SlARF8A&B* CRISPR/Cas9 constructs in tomato seedlings. (b) PCR results of positive Cas9 screening in R0. (c) PCR results of the amplified mutation regions of *SlARF8A* and *SlARF8B* in R0. (d) The PCR results of negative Cas9 screening in the progeny plants of R0. (e) PCR results of the amplified mutation regions of *SlARF8A* and *SlARF8B* in the progeny plants of R0. "-" represents the empty control using ddH20 as template, "+" represents the positive control using vector contains cas9, "WT" represents the control using wild type as template, the ladder used is 100bp.

Supplemental Table 1. Primer list

Primer Name	Forward Primer	Reverse Primer
SlARF8A-sgRNA1	TGTGGTCTCAATTGAAGCTTTCAACATCAGGAAGTTTTAGAGCTAGAAATAGCAAG	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
SlARF8A-sgRNA2	TGTGGTCTCAATTGAACTATGGCATGCTTGTGCGTTTTAGAGCTAGAAATAGCAAG	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
SlARF8B-sgRNA1	TGTGGTCTCAATTGAAGCTTTCAACATCAGGAAGTTTTAGAGCTAGAAATAGCAAG	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
SlARF8B-sgRNA2	TGTGGTCTCAATTGAGCTATGGCATGCTTGTGCGTTTTAGAGCTAGAAATAGCAAG	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
Cas9	AGACACGCCAGATCACCAAG	TCGATAAGTGGTCGCTTCCG
SlARF8A_mutation	TTGGGGTTGTTAATGGAGGTCTT	AGTTGTCGCCGCAACCTACA
SlARF8B_mutation	TGGAGAAAAGGTGGGGTATCTTG	CTCGACTTCCGACAGTTGGT
ARF8A	ATGAAGCTTTCAACATCAGGAATG	TCAGTAATCAAGTGA TCCTATAG
ARF8B	ATGAAGCTTTCAACATCAGGAATGGGTCAG	TCAGTACTCCAGCGATCCAAGAGA
pro_ARF8A	AAAAAGCAGGCTTCTTTACCATGTCCCTACCCTCT	AAGAAAGCTGGGTCCTTTCTCCAAGACCTCCATT
pro_ARF8B	AAAAAGCAGGCTTCTTAACTAACAAACCAACACAGCCAAC	AAGAAAGCTGGGTCTTTATTTAAAAACCCACTTCCTCTTGA
SlARF8A_GFP	AAAAAGCAGGCTTCATGAAGCTTTCAACATCAGGAATG	AAGAAAGCTGGGTCGTAATCAAGTGATCCTATAG
SlARF8B_GFP	AAAAAGCAGGCTTCATGAAGCTTTCAACATCAGGAATGGGTCAG	AAGAAAGCTGGGTCGACGAAAGCTAAAGAAGCCAGG

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Introduction to Article two:

Auxiscan: A tool for identification and functional annotation of auxin-related genes in plants

Considering the importance of auxin-related genes in plant development, a useful web-tool Auxiscan was developed by the GBF bioinformatics team. Auxiscan was developed to fast identify and functional annotate auxin-related genes in various plant by uploading single or a set of protein sequences. In addition, Auxiscan allows to validate the presence of miRNA and tasiRNA target sites on specific mRNA sequences of some auxin-related genes known to be regulated by these siRNA. Moreover, Auxiscan can be used to provide phylogenic trees of auxin-related genes, which also provides new ideas towards the consensus nomenclature for all ARF genes across plant species. Regarding the deep bibliography I performed on auxin related genes, I participated in this work by contributing to define the pipeline steps that needs biological knowledge and conserved domains in auxin related families that allows their *in silico* identification. I also run the pipeline several times and was responsible for the paper writing work. This chapter part is presented under the form of a published paper. This article will introduce the design concept, data analysis pipeline, and the instructions for use of Auxiscan.

Auxiscan: A tool for identification and functional annotation of auxin-related genes in plants

(Manuscript in preparation)

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RUNNING TITLE: Web-tool for identification, functional annotation and analysis of auxin related genes.

KEYWORDS: Auxin, gene annotation, web-tool.

Abstract

According to the molecular mechanisms underlying the auxin role in the hormonal regulation of the entire plant life are known, the researches about the genes related to auxin homeostasis (biosynthesis, conjugation, and degradation), transport and signaling are increasing. In this context, Auxiscan was developed as an automated tool that combines

different processes, analytical methods, and pipelines to allow the fast identification and functional protein annotation of auxin-related genes. In addition to the identification of functional protein domain signatures that characterize each gene family, the presence of miRNA and tasiRNA target sites on specific mRNA sequences known to regulate the expression of some auxin related genes, this tool may also be used to infer evolutionary relationships and present similarities inside gene families and between different species.
(Web link to the platform: http://auxiscan.gbfwebtools.fr/).

Introduction

Auxin plays essential roles during the entire life of a plant. Indeed, this small organic acid impacts cell division, cell elongation, and cell differentiation, and has a great impact on the final shape and functions of cells and tissues in all higher plants. The first study of auxin comes from Darwin who found that coleoptiles bend towards a light source. This chemical signal termed auxin was identified as Indole-3-acetic acid (IAA), indole-3-butyric (IBA), indole-3-propionic acid (IPA), and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Schneider et al., 1985; Epstein et al., 1991; Ludwig-Müller & Epstein, 1994; Tam et al., 2000). While the major form of auxin in plants is IAA, and it is regulated at multiple levels: auxin homeostasis (biosynthesis, conjugation, and degradation), auxin polar transport and auxin signal transduction (Zolman et al., 2000; Ozga et al., 2002; Woodward & Bartel, 2005). These processes are schematically summarized in Figure 1.

Auxin biosynthesis

There are two major routes for IAA biosynthesis: tryptophan (Trp)-dependent and Trp-independent pathways (Mano & Nemoto, 2012). For Trp-dependent auxin biosynthesis pathways, there are several biosynthesis pathways that contribute to IAA levels, including the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetaldoxime (IAOx) pathway, the indole-3-acetamide (IAM) pathway, and the tryptamine (TAM) pathway (Mano & Nemoto, 2012). The IPA pathway is the first complete and universally conserved IAA biosynthesis pathway in plants (Korasick *et al.*, 2013; Brumos *et al.*, 2014; Zhao, 2014b). Several researchers have

independently demonstrated that TAA and YUCCA flavin-containing monooxygenase families produce IAA from Trp via IPA in two-step reactions in *Arabidopsis thaliana* (Stepanova *et al.*, 2011; Mashiguchi *et al.*, 2011; Won *et al.*, 2011). Evidence shows that TAA and YUCCA families play a crucial role in IAA biosynthesis in various plant species, including Arabidopsis, maize, rice, and liverwort (Korasick *et al.*, 2013; Zhao, 2014b; Eklund *et al.*, 2015). In addition, several lines of evidence indicate that, in the IAOx pathway, cytochrome P450 monooxygenases CYP79B2 and CYP79B3 catalyze the conversion of Trp into IAOx in the first step of both IAA and indole glucosinolate (IG) biosynthesis in Arabidopsis, although the pathway leading to IAA is yet to be fully understood (Zhao *et al.*, 2002b; Sugawara *et al.*, 2009). For the Trp independent pathway, the previous studies suggest that IAA can be produced de novo without Trp as an intermediate (Wright *et al.*, 1991; Normanly *et al.*, 1993). In the Trp independent pathway, horismate converts to Trp via several enzymatic reactions (Radwanski & Last, 1995), and IAA is mainly produced from Trp precursors. For example, indole (IND) and indole-3-glycerol phosphate (IGP), but not from Trp (Bohlmann *et al.*, 1995; Zhao & Last, 1996; Kriechbaumer *et al.*, 2008; Kasahara, 2016). Former research shows that the Trp independent pathway is not a major route for IAA biosynthesis, but they reveal their important role for a cytosolic indole synthase in this pathway (Kasahara, 2016).

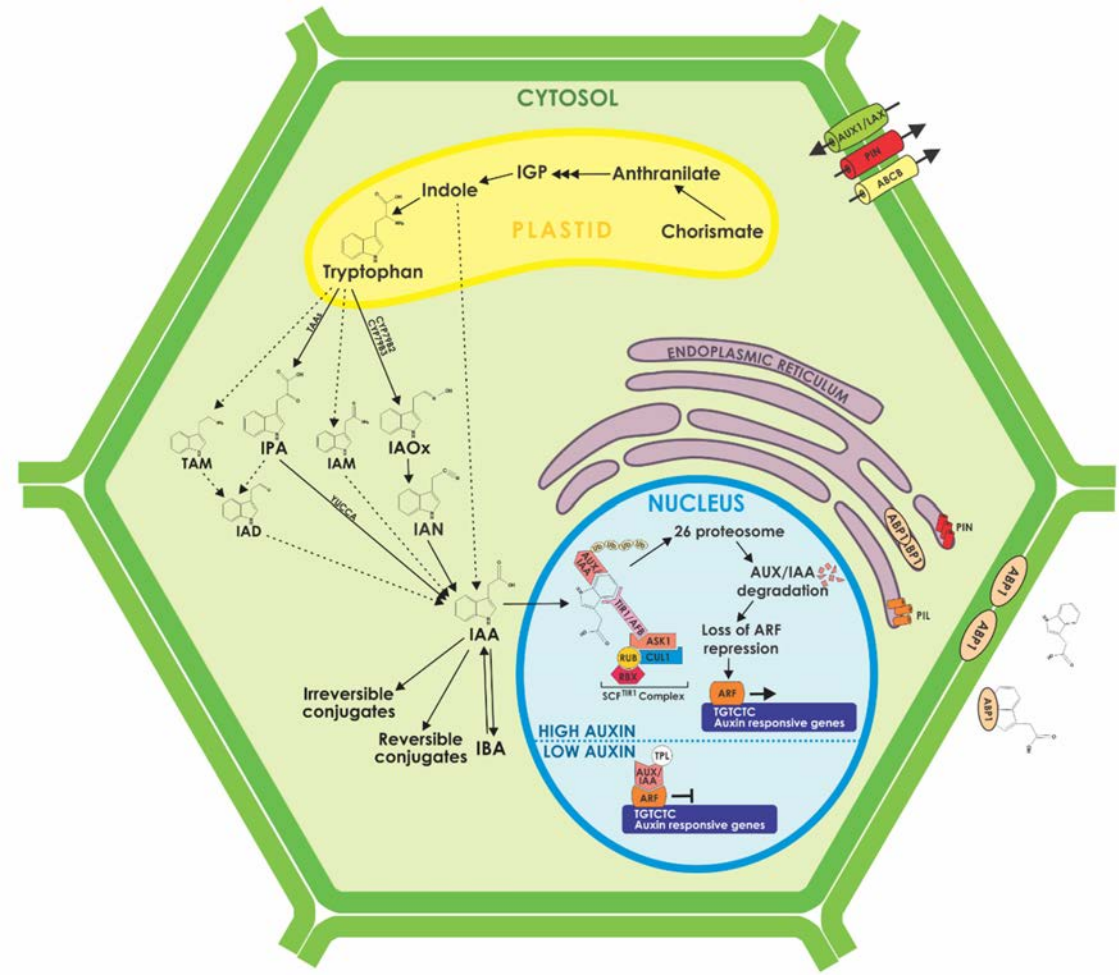


Figure 1. Schematic view of auxin homeostasis (biosynthesis, conjugation and degradation), transport, and signaling. The biosynthesis of IAA precursors takes place in plastids and the Trp, major IAA precursor, is generated via the Chorismate pathway. The subsequent Trp-dependent IAA biosynthesis pathways are located in the cytosol. One Trp-independent IAA biosynthesis pathway and four putative Trp-dependent pathways are shown: IAOx, IAM, IPA, and TAM pathways. The known pathways are shown as complete arrows and the unknown are shown as dashed-arrow. IAA can be reversible or irreversible conjugated and some IAA conjugates can be regarded as storage forms IBA that can be hydrolyzed to form free IAA. TIR1/AFB auxin receptor is an F-box protein that forms an SCF E3 ubiquitin ligase complex between SKP (ASK1) and cullin1 (CUL1). $SCF^{TIR1/AFB}$ catalyzes the ubiquitination of auxin/IAA proteins (Aux/IAAs) in the presence of auxin. The activity of the auxin response factor (ARF) is blocked by Aux/IAA bound to TOPLESS (TPL). Auxin is bound in the small cavity formed between TIR1 and Aux/IAA. The auxin-induced degradation of Aux/IAA repressors recovers the ARF activity and activates the transcription of auxin-responsive genes (auxin response element, AuxRE: TGTCTC). Auxin-binding protein 1 (ABP1) is an Endoplasmic Reticulum (ER)-localized protein, but small amounts of functional ABP1 protein

act at the plasma membrane as an auxin receptor. Scheme view of ABCBs and PINs as efflux auxin carrier proteins and AUX1/LAX as influx auxin carrier protein. ER marks endoplasmic reticulum which PIN, PIN like (PILs) and ABP1 seems to be placed on its cell membrane.

Auxin conjugation and degradation

The regulation of *de novo* synthesed auxin levels is one important homeostatic mechanism operating in plant cells, and the levels of IAA can also be attenuated by conjugation and by degradation (Normanly, 2010; Ruiz Rosquete *et al.*, 2012). Free IAA levels can be adjusted via conversion into IBA or conjugation to amides or esters (Ruiz Rosquete *et al.*, 2012), and the IAA conjugates are regarded as either reversible or irreversible storage compounds. Auxin conjugates are generally considered as temporary storage of inactive IAA, releasing the free active hormone upon hydrolysis (Fluck *et al.*, 2000).

Auxin transport

Auxin can be transported by diffusion (passive movement) and by auxin transporter (active movement) (Zazimalova *et al.*, 2010). IAA is largely protonated at apoplast and can pass through the plasma membrane via diffusion into the cell. Once in the cytosol, it is mainly deprotonated due to the higher pH, and the resulting charged molecule (IAA-) is membrane impermeable. This concept is based on the chemiosmotic polar diffusion model (Rubery & Sheldrake, 1974; Raven, 1975; Goldsmith, 1977). As anionic auxins cannot diffuse across the plasma membrane, they thus require the activity of transporters at the plasma membrane to exit the cells. Up to date several auxin carrier families have been identified, including PIN, AUX1/LAX influx carriers, and ABCB auxin efflux carriers that mediate auxin distribution (Zazimalova *et al.*, 2010).

Auxin signaling

Auxin signaling regulates cell responses to the different auxin levels that are formed by a combination of auxin metabolism and transport. Cell responses to auxin can be controlled transcriptionally and non-transcriptionally (Hayashi, 2012). Transcriptionally, auxin is perceived by the TIR1/AFB nuclear receptors, which are cytoplasmic F-box-domain-

containing proteins (Dharmasiri *et al.*, 2005; Kepinski & Leyser, 2005). One important pathway linking auxin perception to gene expression is now well-established. The Aux/IAA proteins was ubiquitinated by the TIR1/AFB subunit of the $SCF^{TIR1/AFB}$ ubiquitin ligase and the degradation of Aux/IAA proteins by the 26S proteasome, which then releases the Aux/IAA-mediated inhibition of ARFs and allows these transcription factors to modulate the expression of their target genes (Chapman & Estelle, 2009). Auxin signaling machinery that is involved in non-transcriptional regulation is not as well characterized. Knowledge of the signaling components and the physiological events involved in this rapid auxin response is limited. Auxin-binding protein 1 (ABP1) is one of the receptor candidates implicated in non-transcriptional auxin signaling (Hayashi, 2012).

Post-transcriptional regulation

Although much is known about auxin regulation, the post-transcriptional regulation of gene expression is relatively less known. Recent studies have evidenced the importance of gene regulation involving several classes of small RNAs, mainly represented by endogenous RNAs with 21–25 nucleotides in length in a mature form known as microRNAs (miRNAs) (Ambros *et al.*, 2003; Dugas & Bartel, 2004). Post-transcriptional miRNA regulation may involve repression of the translation process, degradation of mRNA or gene silencing, either by target complementarity or by signaling DNA modifications in specific regions of genome (Bartel, 2004; Axtell, 2013).

In plants, important biological functions are mediated by miRNAs, including the regulation of auxin-related genes by translation inhibition or mRNA degradation (Mallory & Vaucheret, 2006; Axtell, 2013; Song *et al.*, 2019). Among these genes, *ARF6* and *ARF8* are targeted by miRNA167 (Ambros *et al.*, 2003; Ru, 2006; Wu *et al.*, 2006), while *ARF10*, *ARF16* and *ARF17* are targeted by miRNA160 (Mallory *et al.*, 2005; Wang *et al.*, 2005a; Liu *et al.*, 2007). Furthermore, miRNA390 and other plant-specific class of endogenous small RNAs known as tasiRNAs (trans-acting siRNA) repressed *ARF2*, *ARF3*, and *ARF4* translation (Fahlgren *et al.*, 2006; Hunter *et al.*, 2006; Marin *et al.*, 2010). Additionally, miRNA393 is expressed in a pattern complementary to auxin receptors and is shown to regulate TIR1/AFB

expression, which may play an important role in auxin response (Navarro *et al.*, 2006; Parry *et al.*, 2009).

According to former studies, the plant hormone auxin plays important roles in plant development. However, most of the research concerning auxin homeostasis, transport and signaling pathways are in the model plant Arabidopsis, for the research of the related pathways and genes in other species has many difficulties. To pursue this aim, we introduce Auxiscan as a powerful tool that can automatically identify, annotate, and provide phylogenic trees of auxin-related genes. Providing phylogenic trees enables users to find the best homolog genes, name them, and study their evolution. In addition, this tool is able to identify the auxin-related miRNAs and tasiRNAs targets on specific genes known in the literature to be subjected to such regulation and thus providing an evidence for gene naming curation as well.

Results and discussion

Auxiscan is written in Perl (version 5.10.1) and the web interface is implemented using Symfony framework (version 3.4.6). Auxiscan platform has been successfully tested on Mozilla Firefox, Safari, Google-Chrome, Opera, and Internet Explorer. A diagram illustrating Auxiscan's workflow is shown in Figure 2.

A typical Auxiscan analysis consists of six steps: data upload, analytical information description, optional analytical selections, data processing and report generation. Users are guided through these steps by Auxiscan's intuitive interface. Detailed descriptions, help files, examples and helpful hints are available on Auxiscan's website at the 'Help & Documentation' session.


AuxiScan
A tool for Automatic Functional Annotation of Auxin-related genes in Plants
Home
Tool
Results
Help & Documentation
Publication
STEP 01 DATA UPLOAD
STEP 1 - Upload your protein sequences
Upload the multi-FASTA file containing the proteome. CARREFULL : This file must be unzipped and less than 10MB
Escolher arquivo
Nenhum arquivo selecionado
STEP 02 ANALYSIS INFORMATION
STEP 2 - Enter your analysis name and email
Analysis name:
Email:
STEP 03 CHOICE OF GENES
STEP 3 - Select family genes you want to look for
Biosynthesis
AMI1
NIT
TAA
TSB
YUC
Perception
ABP1
TIR
Regulation
ARF
AUX/IAA
TOPLESS
Response & Homeostasis
GH3
SAUR
Transport
AUX/LAX
PIN
Select/Unselect all
STEP 04 ANALYTICAL OPTIONS
STEP 4 (optional) - Look for miRNA targets
Upload the multi-FASTA file containing cDNAs CARREFULL : This file must be unzipped and less than 10MB
Escolher arquivo
Nenhum arquivo selecionado
STEP 5 (optional) - Build phylogenetic tree(s)
Do you want phylogenetic analysis? CARREFULL : Phylogeny analysis could take several hours. We recommand you to begin with simple analyses.
Yes using fast likelihood-based method
Yes using bootstrap
No
Submit
INRA
SCIENCE & IMPACT
GBF
INP
ENSAT

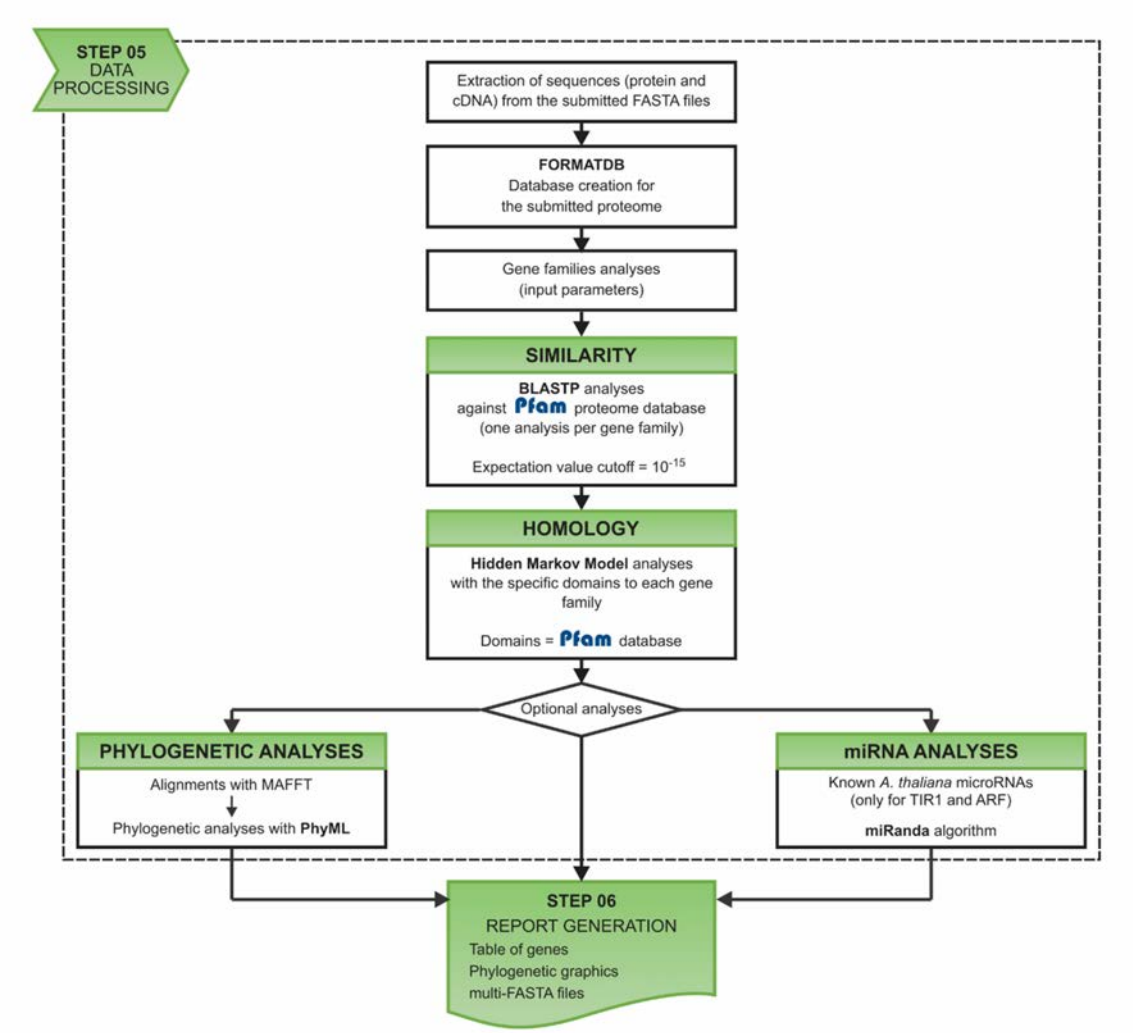


Figure 2. Diagram showing Auxiscan web interface and the data processing. The green arrows highlight the steps (STEP 01 to STEP 06) among data file submission and analytical options chosen. The bordered box shows the data processing step and the green box highlights the report generation step. Data processing includes: extraction of protein and cDNA sequences, database creation for the submitted proteome, gene families analyses, BLASTP analyses against Pfam proteome database, Hidden Markov model analyses with the specific domains for each gene family, optional analyses for miRNA and tasiRNA targets, and phylogenetic tree. At the end, the results are visualized in a table and phylogenic graphic. Multi-FASTA files are available for download.

User operation and analytical options

Auxiscan pipelines begin with the upload of the proteome data file (Figure 2 – Step 1), the description of the analysis and the information to receive the results (Figure 2 – Step 2), and to select target auxin-related genes by function or by family or generally (Figure 2 – Step 3). The separation by function allows users to analyze genes related to auxin biosynthesis, perception, regulation, response, homeostasis and transport specifically, while the separation by family shows the identification and functional annotation of specific gene families, such as, ARF, AUX/IAA, and TOPLESS families of genes related to auxin regulation.

The input file can be a multifasta protein sequence list that driven from the whole genome annotation. Auxiscan will select the potential genes that belongs to the desired gene families respectively among the Auxiscan web form. In addition, the users can also unload a single sequence or a small set of sequences as input file to analyse.

Auxiscan also allows as optional analyses looking for miRNA and tasiRNA possible target sites in specific mRNA sequences known in the literature to be subjected to such a regulation. The analyses of miRNA and tasiRNA targets require upload of cDNA data file and the selection of miRanda (version 3.3a) cutoff score (Enright *et al.*, 2003) (standard value = 160). Auxiscan also allows the construction of phylogenetic trees after multi-alignment of protein sequences of each identified gene family (Figure 2 – Step 4). Fast likelihood-based method (Anisimova & Gascuel, 2006) and bootstrap method (Felsenstein, 1985), with a changeable score value (50 is the standard proposed value), are available for phylogenetic analyses.

Data processing pipeline

Submitted data files and selected information are combined and used during the data analyses (Figure 2 – Step 5). At first, the uploaded sequence data is converted in an individual proteomic database. BLASTP analyses (Altschul *et al.*, 1990, 1997) are conducted to find the protein sequence similarity comparing submitted sequences to the desired *Arabidopsis Thaliana* (*A. thaliana*) auxin related gene families (using expected cutoff value of 10^{-15} as threshold). The reference *A. thaliana* database was chosen thanks to its high quality gene annotation characterized by the highest identified BUSCO score in a plant genome untill now (Waterhouse *et al.*, 2018). Regarding that each auxin related gene family is characterized by

the presence of one or several specific domains in the protein sequence of its members, Hidden Markov model (version 3.0) (Baum & Petrie, 1966) analyses using the PFAM database (Finn *et al.*, 2016) as reference were used to validate the presence of these domains in each gene family homolog. This step allows to remove possible false positives that can be introduced by the BLASTP similarity search step.

If requested, miRNA and tasiRNA analyses can be conducted using miRanda algorithm to validate the presence of auxin related miRNA (miRNA160, miRAN167, miRNA393) and tasiRNA (tas3) target sites in specific auxin related members of some gene families like ARFs (miRNA160 , miRAN167, tas3) and TIRs (miRNA393) (Rhoades *et al.*, 2002; Sattar *et al.*, 2016). Considering binding energy of the duplex structure, evolutionary conservation of the whole target site, the miRanda score is used to determine the cutoff value (cutoff ≥ 160, energy cutoff ≤ −20 kcal/mol, gap opening = −9.0 and gap extension = −4.0) in the comparisons of miRNA and tasiRNA sequence complementarity sites in the submitted cDNA sequences.

Using MAFFT multi-alignment tool (version 7.031b) (Kuraku *et al.*, 2013), phylogenetic analyses are completed by aligning the identified auxin related protein sequences of each family with *A. thaliana*, *Oryza sativa*, *Physcomitrella patens*, *Selaginella moellendorffii,* and *S. lycopersicum* ones chosen as reference. Lastly, alignment data is converted to standard Phylip file format and submitted to heuristic analyses with PhyML (version 3.0) (Guindon *et al.*, 2005) using bootstrap or fast likelihood-based estimative methods, inferring evolutionary relationships and the building of phylogenetic trees. Auxiscan platform allows users to choose the desired estimative and bootstrap methods to tune phylogenetic analysis.

Therefore, data processing conclusions may vary depending on the optional analyzes chosen and the number of analyzes performed simultaneously. Overall, simple analyzes with identification and functional annotation of auxin genes (with miRNA and tasiRNA targets) run fast, while phylogenetic trees construction may take more time due to multiple sequence aligning analyses.

Data visualization and report generation

When the data processing is completed, users can access to the analysis report through the 'Results' table. It contains a detailed description of all the results in tabular format grouped by gene family (Figure 3). Result data files are stored on the server in a temporary folder for seven days. The URL link sent via user email allows access to the analyses' results session.

At first, a table is presented with all the results classified by pathway, gene family, protein ID, *A. thaliana* ID, symbol, domains, query length, *A. thaliana* length, and miRNA and tasiRNA possible targets. Results can be sorted using any of these classifications and filtered by searching for specific terms. If requested, phylogenetic trees graphics are presented by family in the same session. Protein FASTA files of each gene family can be downloadable. Tables are available in a CSV, XLS, or PDF file format, and graphics (phylogenetic results) in a SVG, NWK, or Phylip file format for eventual further analysis by the user with other software.

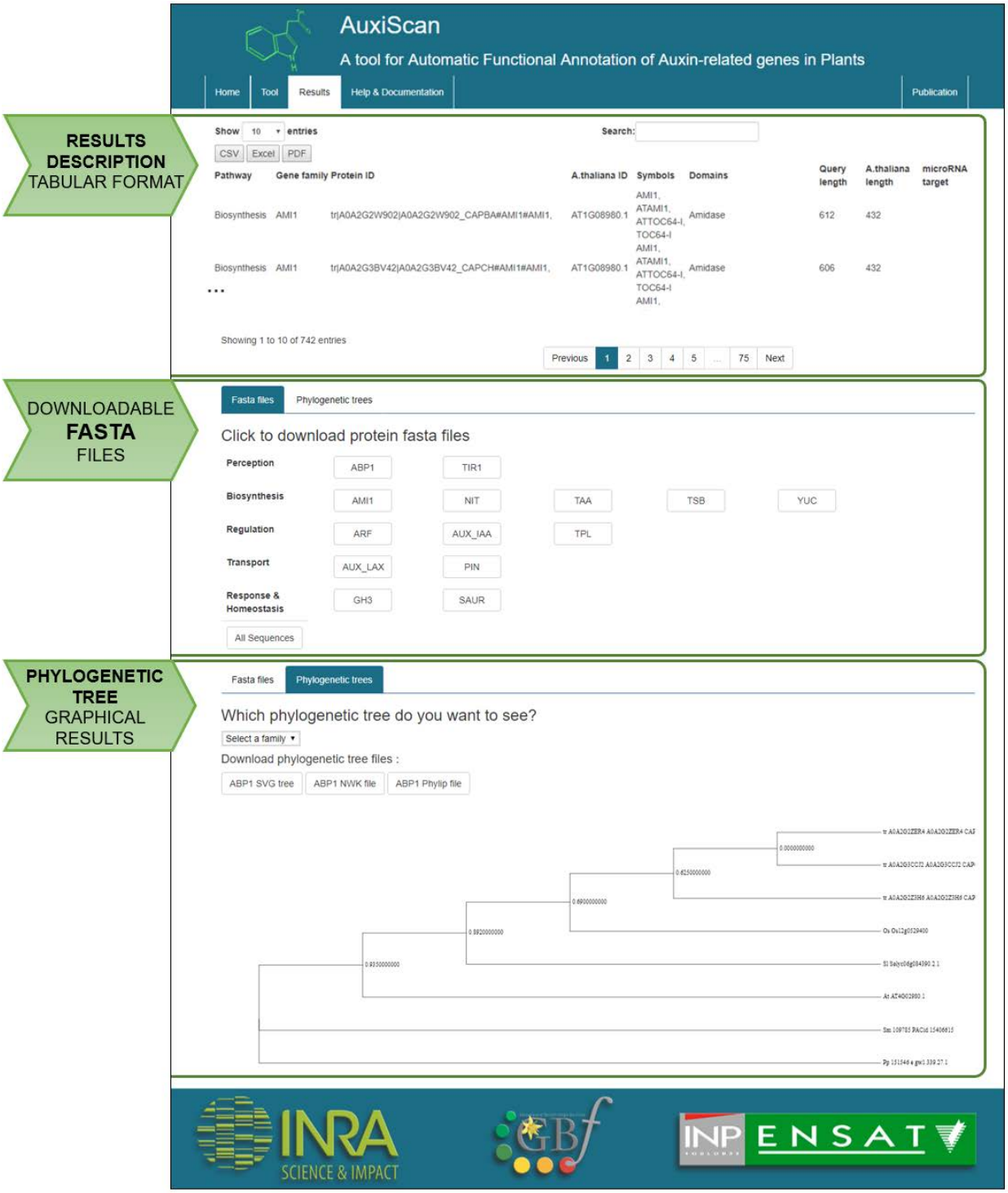


Figure 3. Auxiscan web interface for 'Results' session. The green arrows highlight the result options: results description in tabular format, protein FASTA files downloadable in the CSV, XLS, or PDF file format, and phylogenetic tree graphical results separated by gene family and downloadable in the SVG, NWK or Phylip file format.

The versatility in Auxiscan application

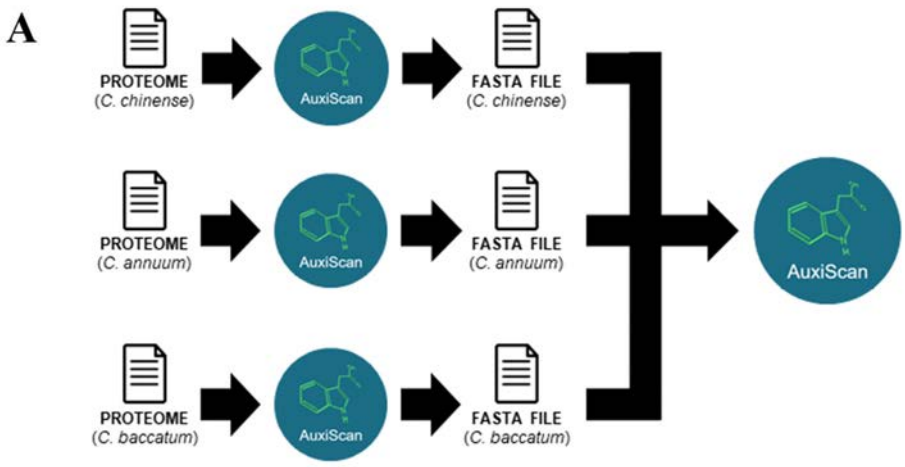
The Auxiscan's single web interface combines several functional modules that enable its use from simple individual description analyses to more complex comparisons between different plant species. Foremost, the tool may be used to identify and functionally annotate the auxin-related genes of plants. Besides, the tool could be used to validate the presence of specific functional domains, identify possible miRNA and tasiRNA targets, and infer evolutionary relationships using similar values calculated between protein sequences of different species and inside families of genes. At the same time, Auxiscan may be simply used to filter sequences related to auxin genes in huge proteome multi-FASTA files. For primary auxin genes identification and functional annotation, secondary analyses become possible with assembling or disassembling multi-FASTA data files. For example, after previous analyzes in Auxiscan or other tools. In this context, the following case study (Figure 4) is presented to exemplify a deeper use of Auxiscan.

Case study: standardization of ARFs annotations in Capsicum peppers

In addition to the identification and functional annotation of auxin-related genes, the assembling or disassembling of proteomic data before subsequent analyzes enable the use of Auxiscan as a powerful tool to identify, compare and standardize auxin genes between different species or cultivars of the same species. The following case study aims to compare, and standardize the annotation of ARFs among reference proteomes of three *Capsicum* (pepper) obtained from UniProt database (The Uniprot Consortium, 2017): *Capsicum chinense*, *Capsicum annuum*, and *Capsicum baccatum.* At first, the proteome of each species is individually analyzed using Auxiscan. The multi-FASTA files referent to the ARF gene family are downloaded and then converted into a single multi-FASTA file, with the protein sequences present in the three species. This secondary data file is analyzed again by Auxiscan, with optional phylogenetic analyses included using the Fast likelihood-based method (Anisimova and Gascuel, 2006) (Figure 4A).

In addition to the identification and description of functional domains of the ARFs, this approach also allows one to infer evolutionary relationships and, evidence similarities and differences inside the ARF family and among species. As shown in the phylogenetic tree, all

the ARFs are present in three species, except for a member annotated as *ARF8* that is present only in *C. annuum* and *C. baccatum* (Figure 4B). Comparing the ARF protein nomenclatures of the three species with *A. thaliana* and *S. Lycopersicum*, there is a lack of consensus in ARF genes nomenclature. As shown in Figure 4C, three 'sub-groups' are displayed as the *ARF9* in peppers species. It is clear to show that two pepper *ARF9* 'sub-groups' have the same phylogenetic distances (in terms of branch arrangement) and evolutionary relationship with tomato *SlARF9A* (Solyc08g082630) and *SlARF9B* (Solyc08g008380), while a third pepper ARF9 'sub-group' presents a different phylogenetic distance and is evolutionary relationship to *SlARF18* (Solyc01g096070). In Figure 4D, three ARF 'sub-groups' in peppers species are generically described as *ARF19*, but only one is evolutionary related to *SlARF19* of tomato (Solyc07g042260) and *Arabidopsis* (At1g19220). The other two *ARF19* 'sub-groups' have the same phylogenetic distances and evolutionary relationship with the tomato *SlARF7A* (Solyc07g016180) and *SlARF7B* (Solyc05g047460). As a conclusion, Auxiscan can not only present the results of the identification and functional annotation of ARF family, but also identify differences among different capsicum species. Moreover, Auxiscan evidences the necessity of renaming the ARF family members in peppers, in order to provide consensus nomenclature for all ARF genes across plant species.



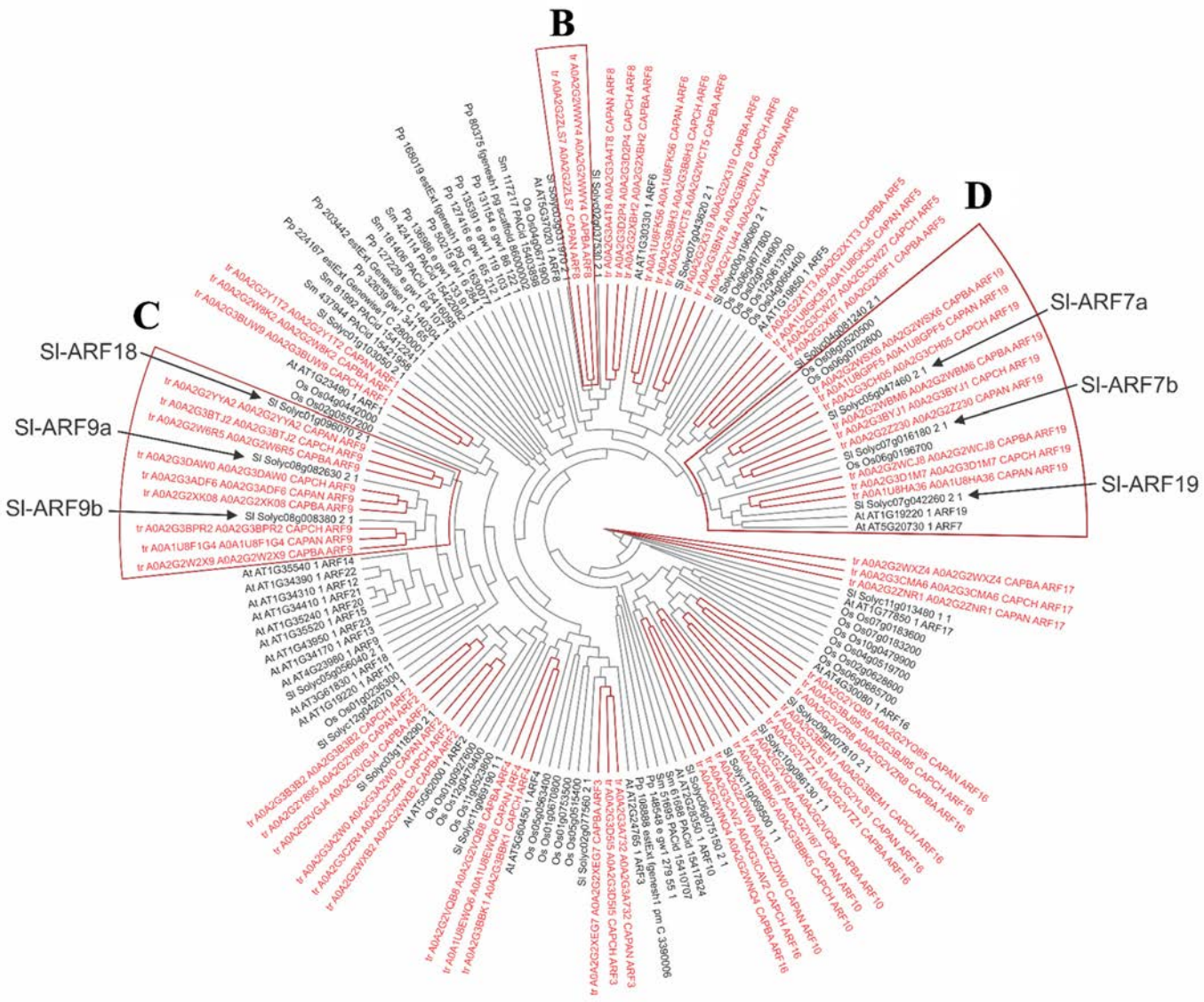


Figure 4. Case study workflow and phylogenetic tree of auxin response factors (ARF) genes in various plant species. Three Capsicum pepper species (in red color) and *Arabidopsis thaliana*, *Oryza sativa*, *Physcomitrella patens*, *Selaginella moellendorffii* and *Solanum lycopersicum* proteomic databases (in black color). A: Case study workflow; B, C and D: different cases observed when ARF families were compared among different species. At: *Arabidopsis thaliana*; CAPAN: *Capsicum annuum*; CAPCH: *Capicum chinense*; CAPBA: *Capsicum baccatum*; Os: *Oryza sativa*; Pp: *Physcomitrella patens*; Sm: *Selaginella moellendorffii*; Sl: *Solanum lycopersicum*.

Innovation, limitation, and perspectives

The bioinformatics and statistics pipelines used in Auxiscan allow the identification and functional annotation of auxin-related genes in various species. Auxiscan has intuitive and user-friendly web interface, allowing the fast identification of auxin genes, their domains, and the possible miRNA and tasiRNA targets. Furthermore, Auxiscan may infer evolutionary relationships and evidence similarities between the proteome of the studied species by phylogenetic analyses. Although the current version is only applicable to auxin-related research, the pipeline developed in this project allows the tool to be easily updated to include the genes related to ethylene, abscisic acid or other hormones. Besides, the long time needed for phylogenetic analyses still need to be worked and overcomed.

Conclusion

The growing interest in the role of auxin in plant development and fruit ripening has increased the need to study genes related to the synthesis, regulation, and perception pathways of this hormone. In this context, the Auxiscan program was developed as a new tool in auxin-related genes' research, helping researchers to quickly and easily identify these elements by only using proteomics data. Auxiscan appears as a powerful tool to guide and optimize studies of the roles of auxin in the plant hormonal regulation.

Material and methods

Proteome selection

Reference proteomes of three *Capsicum* spp. peppers were obtained from UniProt database (The UniProt Consortium, 2017): *Capicum chinense* cv. PI159236 (Proteome ID: UP000224522); *Capsicum annuum* cv. CM334 (Proteome ID: UP000222542); and *Capsicum baccatum* cv. PBC81 (Proteome ID: UP000224567).

Phylogenetic tree graphical display

Phylogenetic trees were generated by Auxiscan based on multiple alignment results for ARF protein sequences using the Fast likelihood-based method (Anisimova and Gascuel, 2006). Dendrogram visualization was performed using NCBI Tree Viewer tool (https://www.ncbi.nlm.nih.gov/tools/treeviewer/).

Availability and requirements

Project name: Auxiscan;

Project home page: http://auxiscan.gbfwebtools.fr/;

Operating system: genotoul platform;

Programming language: Perl (version 5.10.1);

Requirements for webversion: Browser with JavaScript support;

Any restrictions to use by non-academics: No.

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Chapter III

General conclusion and perspectives

Auxin signaling control of gene expression related with fruit set: future perspectives

In angiosperm plants, fruit set refers to the transition step from the flower organ to a fruit, which is a key developmental process that normally arises after successful pollination and fertilization. This fruit set process is a complex developmental event that involves dramatic physiological, structural, and molecular changes. It has been widely known that the phytohormone auxin plays a vital role in the fruit set process. Up to date, the research to elucidate the molecular mechanism underlying the role of auxin in this process has made significant progress in the model plant Arabidopsis. ARFs are important transcriptional factors involved in the auxin signaling network, regulating early auxin-responsive genes by binding to the AuxREs in the promoters of these genes, such as Aux/IAAs, GH3s, and SAURs. In this thesis project, we performed the structural and functional characterization of two homologues tomato genes SlARF8A and SlARF8B, which displayed new clues different from their orthologs in Arabidopsis. The investigations allow to identify the potential target genes of SlARF8A and SlARF8B in tomato, and opens new avenues towards a better understanding of hormone cross-talk in the fruit set process. In this chapter, I proposed the future developments of the topic related to the multihormones-regulated fruit set process, and the potential of the parthenocarpy trait in fleshy fruit to provide more insight into plant development, signaling, and high-value agricultural products.

Structural and functional features of SlARF8A and SlARF8B

Previous studies showed that the AtARF8 gene regulates hypocotyl growth, petal development, and fruit initiation in Arabidopsis (Nagpal *et al.*, 2005; Goetz *et al.*, 2006; Varaud *et al.*, 2011). Besides, the expression level of AtARF8 in Arabidopsis displayed an increase from anthesis to 1 DPA, and decreased from 2 DPA. Expression analysis performed by RT-qPCR revealed an increase of SlARF8A transcript levels in ovary within 4 days post pollination and fertilization, while SlARF8B transcript levels showed the highest level in the anthesis stage, and decreased after pollination and fertilization. This suggests that SlARF8A in tomato

displayed a similar expression pattern to *AtARF8* in Arabidopsis, and that *SlARF8B* is potentially to show a distinct functional specialization compared with *SlARF8A* and *AtARF8*. To address the role of *SlARF8A* and *SlARF8B* in tomato fruit set process, the corresponding knockout mutants were generated by CRISPR/Cas9 genome editing system respectively. The early stop codon of SlARF8A and SlARF8B amino acid sequences at the position of nucleotide 13 and 14, respectively, both led to dramatic reduced fruit size with no seeds, which occurs independently of pollination and fertilization. Goetz *et al.,* first proved that *ARF8* is a negative regulator of fruit initiation in Arabidopsis, since the *ARF8::GUS* lines displayed the dehiscence zone formation in the absence of fertilization.

As a fleshy fruit, tomato is able to provide different clues in regulating fruit set. In our study, the histological analysis of the single mutant *slarf8a-cr*, *slarf8b-cr* and the double mutant *slarf8a&b-cr* fruits revealed the altered placenta and ovule development, which are important traits for fleshy fruits production. The *slarf8a-cr* and *slarf8b* mutants displayed a similar increased pericarp that also showed in the parthenorcarpic fruits of *SlARF7* RNAi lines (de Jong *et al.*, 2009b). While in *SlARF7* RNAi lines the reduced locular gel and placenta area are comparable in *slarf8b*, and the expression level of *SlARF7* is similar than that of *SlARF8B* with decreased transcript level after pollination and fertilization. Besides, *SlARF7* and *SlARF8B* belong to the same subclade of ARFs (Zouine *et al.*, 2014), the redundant function of these two genes in regulating fruit set was suggested. Noteworthy, the phenotype displayed in *slarf8a-cr* is reminiscent of the down-regulation lines of *SlIAA9* in tomato (Wang *et al.*, 2005a), suggesting that *SlARF8A* might interact with *SlIAA9* to mediate the auxin-dependent regulation processes in tomato. Recently, a study confirmed this hypothesis by displaying the evidence that ARFs of the same subclade including SlARF5, SlARF7, SlARF8A, SlARF8B, SlARF19A, and SlARF19B all interacted with SlDELLA and SlIAA9 by Y2H assays (Hu *et al.*, 2018). Moreover, a molecular model of SlDELLA and SlARF7/SlIAA9 regulating downstream fruit development related genes was proposed, SlDELLA and SlIAA9 inhibit SlARF7 through direct protein-protein interactions, and fruit set and growth-related genes were repressed by this inhibition. The increased auxin and GA in ovule after pollination and fertilization can induce the degradation of SlDELLA and SlIAA9 respectively, which releases SlARF7 to activate the transcription of downstream genes related with fruit set (Hu *et al.*, 2018). Since SlARF8A and

SlARF8B also displayed interaction with SlDELLA and SlIAA9, the similar regulation schema can be proposed in the fruit set process.

Molecular regulation of feedback-regulated genes by SlDELLA and SlARF8B/SlIAA9

Our data displayed that many DEGs are phytohormones related genes, such as GA, auxin and cytokinin related genes are enriched at 0DPA and 4DPA *slarf8b* versus WT, including the GA biosynthesis gene *GA20OXs* and *GA3OXs*, the auxin biosynthesis gene YUCs and transport genes PINs, the bioactive cytokinin synthesis enzyme LOGs, and the cytokinin oxidase CKXs. It has been confirmed in recent research that SlARF7/SlIAA9 and SlDELLA adversely regulate the expression of GA and auxin feedback-regulated genes, including *GA20ox1* and *GA3ox1* and auxin deactivation gene *GH3.2* (Serrani *et al.*, 2008; Kumar *et al.*, 2012). Those GA and auxin metabolism feedback-regulated genes are supposed to be repressed by the SlARF7/SlIAA9 protein complex but are induced by SlDELLA. The binding of SlDELLA to SlARF7 may remove the inhibition of the SlARF7/SlIAA9 protein complex, activating the transcription of these genes (Hu *et al.*, 2018). This regulation model was supposed to be suitable for *SlARF8B*, since the data displayed that the up-regulation of GA and auxin feedback-regulated genes *GA20OXs*, *GA3OXs*, and *GH3.4* in *slarf8b-cr*. The up-regulation of these hormone-related genes may correspond with the increased GA or auxin level, which leads to the parthenorcapic fruits. By measuring the GA, auxin, and cytokinin accumulation level in *slarf8a-cr* and *slarf8b-cr* in the future will help to confirm the explaination. Besides, the SlARF8B interacts with SlIAA9, and SlDELLA involves with SlARF8B have been confirmed by Y2H assay (Hu *et al.*, 2018). Therefore, the emerging question here is to know whether SlARF8B could involve with SlIAA9 and SlDELLA, to regulate GA and auxin feedback-regulated genes, which may elucidate the parthenocarpic fruit formation mechanism in ARFs mutants. The next step about this thesis will be to assess and confirm the protein interaction between SlARF8B, SlIAA9, and SlDELLA. A proposed model was described in Figure 18(a,b).

Molecular regulation of downstream fruit development-related genes by *SlARF8*

The recent study in Arabidopsis displayed that 5 SAURs from a single clade, the sister genes of which *IAA3* and *IAA4* have the strong probability to be direct downstream target genes

of *AtARF6* and *AtARF8*; and the anther dehiscence without pollination and fertilization in *AtARF6* and *AtARF8* double mutants was suggested to be induced by increased JA production (Nagpal *et al.*, 2005). By comparison, several SAURs and IAAs genes were down-regulated in *slarf8b-cr* (Table 3). This similar expression pattern suggests that the future chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) could be performed to determine whether SlARF8B binds directly to the promoters of these genes. Moreover, the JA related genes in *slarf8b-cr* did not display the similar induced JA production in Arabidopsis, suggesting the difference between Arabidopsis and fleshy fruit tomato.

Combined with our published review, ethylene related genes also regulate the fruit set process. Accordantly, several of ACSs and ACOs genes were up-regulated while only ACS2, ACO2 and ACO4 were down-regulated in *slarf8b-cr*. Besides tomato, it has been observed in tobacco that ACOs are expressed in the early stages of ovule development, and the silencing of which leads to the arrest of ovule development, interfering with the fruit set process (De Martinis & Mariani, 1999). *ACO4* is an ethylene biosynthesis gene that may inhibit the fruit set process, for ethylene production represses the fruit set, and *ACO4* has been proved to be the direct target gene of *SlARF7* (Vriezen *et al.*, 2008; Hu *et al.*, 2018; Shinozaki *et al.*, 2018). Besides, in order to identify the direct target genes of *SlARF8A* and *SlARF8B*, the constructs of SlARF8A and SlARF8B fused with GFP protein tag have been generated and transformed successfully in tomato in GBF lab. The plant material obtained is being used to perform the western-blot assay and then a ChIP-seq approach. After obtain the direct target genes of *SlARF8A* and *SlARF8B*, the reverse genetic will be used to unravel the function of these genes. Therefore, with more data obtained in the future, it will be interesting to expand the hormone crosstalk studies of GA and auxin to GA, auxin, and ethylene in regulating the fruit set process. This can be realized by checking the expression level of *SlARF8A* and *SlARF8B* in the hormone-related mutants existed in GBF lab, including *sldella, sletr,* and *sletr.* Besides, the accumulation level of hormones like GA, ethylene and cytokinin will be measured in *slarf8a-cr* and *slarf8b-cr* to check if there exist crosstalks between them and auxin. A proposed model was described in Figure 19(c,d).

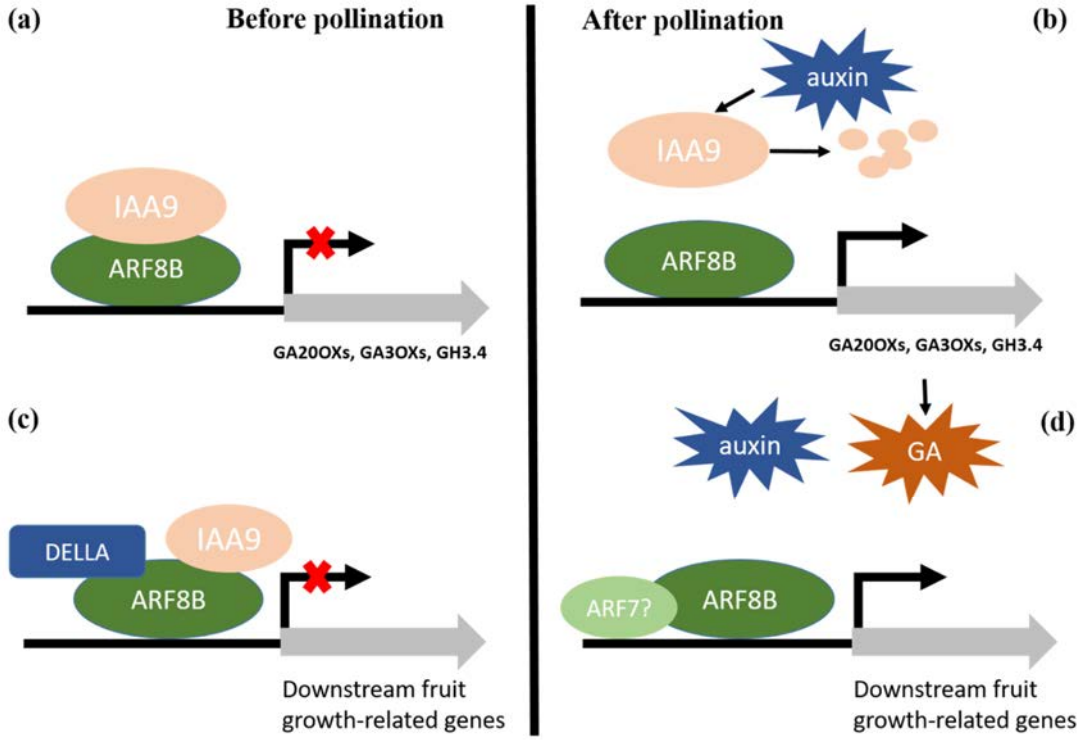

Figure18. Model of SlARF8B regulate tomato fruit initiation.

(a) (b) Model for the regulation of feedback-regulated genes by *SlIAA9* and *SlARF8B*. Feedback-regulated genes involved in GA biosynthesis genes (*GA20OXs, GA3OXS*) and auxin metabolism (GH3.4) are repressed by the SlARF8B/SlIAA9 complex. After pollination, the increased auxin level induces the degradation of SlIAA9 protein, activating the transcription of GA biosynthesis and auxin metabolism genes. The activation of GA biosynthesis genes increases the GA level. (c)(d) Model for the regulation of downstream fruit growth-related genes by SlIAA9, SlARF8B, and SlDELLA. Fruit set and growth-related genes are repressed by the SlIAA9/SlARF8B/SlDELLA interaction. After pollination, the increased auxin and GA levels remove the depression of SlIAA9 and SlDELLA, the SlARF8B and other possible ARFs, like ARF7, activate the transcription of fruit set and growth-related genes.

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